THE INFLUENCE OF DIETARY AND OTHER ENVIRONMENTAL CHANGES ON VASCULAR RISK MARKERS IN TYPE 2 DIABETES

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A thesis submitted for the degree of Doctorate in Medicine
To the Faculty of Clinical Medicine

The University of Hull and the University of York
Hull York Medical School
October 2014
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Dedication

To my family
Abstract

Introduction
Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance, impaired glycaemia, endothelial, clotting and platelet dysfunction, resulting in increased cardiovascular risk. Cardiovascular parameters are influenced by various environmental and dietary factors.

Methods
Two separate studies were undertaken to explore the effect of different soy and/or cocoa dietary interventions on cardiovascular risk makers in type 2 diabetes, and to determine the underlying mechanism. A third study was organized to explore the effect of hypoxia and low humidity on platelet function and clotting indices in addition to microparticle concentration in patients with type 2 diabetes compared to healthy volunteers.

Results
We have shown that the addition of soy with isoflavones improves overall glycaemia, while soy protein alone or either of these two preparations in combination with cocoa are ineffective. The underlying mechanism could be the improvement in postprandial glycaemia as fasting insulin and glucose remained unchanged. Endothelial function did not change as a result of the dietary interventions.

We have found that a simulated flight environment increased basal platelet activity in type 2 diabetes patients compared to healthy volunteers, while there was no difference in endothelial function.

Discussion
These studies showed that dietary soy might modulate glycaemic control through a mechanism, which alters postprandial hyperglycaemia. The effects were dependent on a combination of soy protein with isoflavones as soy protein alone was ineffective.

It was shown that mimicking the parameters of a commercial flight affected T2DM with an increase in platelet reactivity that may theoretically increase the risk of a venous thromboembolic episode.
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<td>AACE</td>
<td>American Association of Clinical Endocrinologists</td>
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<tr>
<td>Ach</td>
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<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<td>ACS</td>
<td>Acute Coronary Syndrome</td>
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<td>American Diabetes Association</td>
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<td>ADMA</td>
<td>Asymmetric Dimethylarginine</td>
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<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>cIMT</td>
<td>carotid Intima Media Thickness</td>
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<td>Dipeptidyl Peptidase IV</td>
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<tr>
<td>EMP</td>
<td>Endothelial Microparticle</td>
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<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<td>FDA</td>
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<tr>
<td>FFA</td>
<td>Free Fatty Acids</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>FMD</td>
<td>Flow Mediated Vasodilatation</td>
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<tr>
<td>FPI</td>
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<td>GCP</td>
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<td>Glucagonlike Peptide-1</td>
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<td>Guanosine Monophosphate</td>
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<td>GTN</td>
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<td>HbA1c</td>
<td>Haemoglobin A1c</td>
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<td>hCRP</td>
<td>high sensitivity C Reactive Protein</td>
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<td>HDL</td>
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<td>HEPES</td>
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<td>HIV</td>
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<td>HOMA-IR</td>
<td>Homeostatic Model Assessment of Insulin Resistance</td>
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<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
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<td>ICH</td>
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<td>IDF</td>
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</tr>
<tr>
<td>IFG</td>
<td>Impaired Fasting Glucose</td>
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<td>IGF-1</td>
<td>Insulin-Like Growth Factor</td>
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<td>IR</td>
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ISTH  International Society on Thrombosis and Haemostasis
IUGR  Intrauterine Growth Retardation
KCl  Kalium Chloride
LA  Lysis Area
LAM  Leukocyte Adhesion Molecule
LCCA  Left Common Carotid Artery
LDF  Laser Doppler flowmetry
LMP  Leukocyte derived Microparticle
LPS  Lipopolysaccharide
LT  Lysis time
MA  Maximum Absorbance
MFI  Median Fluorescence Intensity
MgCl₂  Magnesium Chloride
MI  Myocardial Infarct
mmHg  Millimeters of Mercury
MODY  Maturity Onset Diabetes of the Young
MONICA  Monitoring of Trends and Determinants in Cardiovascular Disease
MP  Microparticle
MRI  Magnetic Resonance Imaging
NaCl  Natrium Chloride
NaHCO₃  Natrium Hydrogen Carbonate
NaH₂PO₄  Natrium Hydrogen Phosphate
NATS  National Air Traffic Services
NHANES  National Health and Nutrition Examination Survey
NO  Nitric Oxide
OGTT  Oral Glucose Tolerance Test
P  Placebo
PAI-1  Plasminogen Activator Inhibitor-1
PAT  Peripheral Arterial Tonometry
PE  Phosphatidylethanolamine
Pulmonary Embolism
R-Phycoerythin
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<td>PECAM-1</td>
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<td>Platelet Free Plasma</td>
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<tr>
<td>PaO₂</td>
<td>partial arterial O₂ pressure</td>
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<td>Prostaglandin H₂</td>
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<td>PMP</td>
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<tr>
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<td>Phosphatidylserine</td>
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<td>Tissue Factor</td>
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<td>Tissue Factor Pathway Inhibitor</td>
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<td>TNF-alpha</td>
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<td>tPA</td>
<td>tissue Plasminogen Activator</td>
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<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
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<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
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<tr>
<td>US</td>
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<td>VSM</td>
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<td>von Willebrand Factor</td>
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<td>World Health Organisation</td>
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<td>WRIGHT</td>
<td>WHO Research Into Global Hazards of Travel</td>
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Acknowledgements

I would like to express sincere gratitude to Professor Stephen Atkin, Dr Thozhukat Sathyapalan and Professor Eric Kilpatrick for their continued guidance and support throughout my projects, including assistance with the concepts and design of the studies, data management, interpretation and reviewing the manuscripts of this thesis.

I would like to show my gratitude to Dr Leigh Madden, Professor Sean Carroll, Dr Andrew Garrett, Professor Khalid Naseem, Dr Ramzi Ajjan and Philip Pemberton for their collaboration in order to complete this work.

I would also like to thank my colleagues Dr Myint Aye and Dr Hassan Kahal for their support and encouragement with my studies. I am grateful for the nurses’ support from the Diabetes Centre, who helped me to finish my studies.

Not least, I am indebted to the participants, who offered their courage and time to participate in my research projects and coped with the study bars and environmental conditions.

I would like to thank my husband and my children for their incredible patience throughout the years while doing my research and writing up this thesis.
Author’s declaration

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1. CHAPTER Introduction

1.1. Definition and diagnosis of diabetes mellitus

Diabetes mellitus (DM) is the most common metabolic disorder worldwide. The prevalence of the condition in adults has shown a steady rise in the last decades (1, 2). In 2013 there were 382 million people with diabetes, this is projected to increase to 592 million by 2035 (3). 80% of patients with diabetes live in low- and middle-income countries.

The prevalence in the United Kingdom is 6.6%, and this is predicted to rise to 7.4% by 2035. This prevalence, which is highest between 40 and 59 years of age is believed to rise due to increasing rate of obesity and sedentary lifestyle (4).

Ninety-five percent of patients with diabetes have type 2 diabetes. Type 2 diabetes is usually diagnosed later in life (age>40) and patients are at increased risk of developing microvascular and macrovascular complications (5, 6).

Genetic factors certainly play a role in the development of type 2 diabetes. There is a very high concordance rate in identical twins; such that if one of the pair of twins has type 2 diabetes, the other has a high probability to also develop the condition even if they grow up in socially completely different environments (7).

Diabetes mellitus is a heterogenous condition that has long been known to exist and whose main feature is hyperglycaemia. The most common types are type 1 and type 2 diabetes. In the background of type 1 diabetes there is β-cell destruction, usually leading to absolute insulin deficiency. Type 2 diabetes is characterized by the range, from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance. The etiologic classification of diabetes mellitus is presented in Table 1.
<table>
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<td>2. Trauma/pancreatectomy</td>
<td>1. “Stiff-man” syndrome</td>
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<td>3. Neoplasia</td>
<td>2. Anti-insulin receptor antibodies</td>
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<td>4. Cystic fibrosis</td>
<td>3. Others</td>
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<td>5. Hemochromatosis</td>
<td>H. Other genetic syndromes sometimes associated with diabetes</td>
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<td>6. Fibrocalculous pancreatopathy</td>
<td>1. Down syndrome</td>
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<tr>
<td>7. Others</td>
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<td>D. Endocrinopathies</td>
<td>3. Turner syndrome</td>
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<td>1. Acromegaly</td>
<td>4. Wolfram syndrome</td>
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<tr>
<td>2. Cushing’s syndrome</td>
<td>5. Friedreich ataxia</td>
<td></td>
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<tr>
<td>3. Glucagonoma</td>
<td>6. Huntington chorea</td>
<td></td>
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<tr>
<td>5. Hyperthyroidism</td>
<td>8. Myotonic dystrophy</td>
<td></td>
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<tr>
<td>8. Others</td>
<td>11. Others</td>
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<tr>
<td>IV. Gestational diabetes mellitus</td>
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</tbody>
</table>

Table 1. Etiologic classification of diabetes mellitus – adapted from (8).

Until the 1920s the diagnosis was based on the presence of classical symptoms (polyuria, polydipsia and weight loss) and the examination of the urine (9). The oral glucose tolerance test (OGTT) was then introduced, however first only as a research method and later as a diagnostic procedure (10).
The diagnostic criteria for diabetes have changed over time and today different organisations provide different cut-off values for hyperglycaemic states. Today, the diagnosis is based on the level of fasting plasma glucose (FPG), the 2-hour glucose level following OGTT with 75 g glucose, random glucose concentration and the level of Haemoglobin A1c (HbA1c).

The diagnosis was first revised in 1997, by the first Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Their recommendations were based on the association between the FPG levels and any retinopathy present (8).

The followings are associated with the increased prevalence of retinopathy:
- fasting plasma glucose ≥ 7 mmol/l
- OGTT 2-hour glucose level ≥ 11.1 mmol/l
- HbA1c ≥ 48 mmol/mol (6.5%)

The American Diabetes Association (ADA) currently uses the following diagnostic criteria for diabetes (8):
- fasting plasma glucose ≥ 7 mmol/l
- HbA1c ≥ 48 mmol/mol (6.5%)
- OGTT 2-hour glucose level ≥ 11.1 mmol/l
- Random plasma glucose ≥ 11.1 mmol/l in the presence of classical symptoms

The World Health Organisation (WHO) currently uses the following criteria for diagnosing diabetes mellitus (11, 12):
- fasting plasma glucose ≥ 7 mmol/l
- 2 hour postprandial glucose ≥ 11.1 mmol/l
- HbA1c ≥ 48 mmol/mol (6.5%)

The original WHO criteria used the definition for diabetes as a fasting plasma glucose of ≥ 7.8 mmol/l or a two random plasma glucose of ≥ 11.1 mmol/l. In 1998 this was modified to reflect the ADA criteria. The HbA1c criteria were later added in 2011.
The diagnosis of impaired fasting glucose (IFG) is different between the ADA and WHO criteria. ADA defines IFG as a fasting plasma glucose 5.6-6.9 mmol/l, while WHO defines as 6.1-6.9 mmol/l.

The diagnostic criteria for impaired glucose tolerance (IGT) are the same for WHO and ADA. Both use the OGTT 2-hour plasma glucose level of 7.8-11.1 mmol/l.

Impaired fasting glucose and impaired glucose tolerance reflect conditions with impaired glycemic control and both increase the risk of the development in diabetes in a later stage.

The advantage of measuring HbA1c is that it reflects glycaemia over a longer period, and the error arises from the high biological variability in measuring fasting blood glucose or glucose response to OGTT, is limited. However HbA1c is not reliable when hyperglycaemia develops rapidly. Further precautions should be taken or the use of HbA1c for diagnostic purposes should be avoided all together in patients with altered red cell survival such as iron deficiency or haemolytic anaemia, chronic kidney disease, children and young people, pregnancy, suspected type 1 diabetes, acutely ill patients, patients on medication that may cause rapidly rising glucose, acute pancreatic disease, patients treated for Human Immunodeficiency Virus (HIV) or received blood transfusion prior to the HbA1c measurement (13, 14).

1.2. Type 2 diabetes

1.2.1. Features of Type 2 diabetes

Type 2 diabetes is associated with reduced insulin sensitivity or insulin resistance, pancreatic β-cell dysfunction with relative insulin deficiency, and increased hepatic glucose production – these factors ultimately lead to hyperglycaemia. The increased hepatic glucose production is a relatively late phenomenon in type 2 diabetes – and is most likely caused by an altered ratio of hepatic insulin to glucagon action.
Type 2 diabetes can be genetically determined, and may be affected by various intrauterine exposures and later lifestyle and environmental factors contribute to the development of the condition (15-17). Many patients with type 2 diabetes are obese and obesity itself is a cause of insulin resistance (18). The development of type 2 diabetes usually follows the presence of impaired fasting glucose and/or impaired glucose tolerance with a concomitant reduction in insulin secretion stimulated by glucose and glucose disposal stimulated by insulin. Increase in weight is thought to be the reason for the continuous marked worsening of glucose metabolism (19) and this reflects that majority of patients with type 2 diabetes are obese. A comparison of glycaemic disorders is shown in Figure 1.

![Diagram of glycaemic disorders]

*These patients can briefly return to normoglycemia without requiring continuous therapy (i.e., "honeymoon" remission)

**In rare cases, patients may require insulin

**Figure 1. Disorders of glycaemia: etiologic types and stages. Adapted from (8). Type 1 diabetes is characterized by β-cell destruction and in most cases absolute insulin deficiency presents. Type 2 diabetes is a range of glycaemic disorders from insulin resistance to insulin secretory defect with some degree of insulin resistance. Other specific types include genetic defect of the β-cells, insulin action, or acquired forms including exocrine pancreas disorder, endocrinopathy, infection, or drug-induced insulin deficiencies. Most cases of gestational diabetes are resolved after delivery.

The glucose-stimulated insulin secretion by the pancreatic β-cells has a pulsatile fashion and is characterized by two phases: a rapid insulin release that is followed by a slow continuous insulin secretion. In the development of type 2 diabetes, early signs are the absence of the first phase of insulin release and the alteration of the
pulsatile character. These can be detected prior to the clinical manifestation of type 2 diabetes.

Martin et al. conducted a study involving children whose both parents had type 2 diabetes. They showed that reduced insulin sensitivity was already present in the children 20 years prior to the manifestation of type 2 diabetes. Later, 3-5 years prior to the clinical diagnosis, alterations in the secretion of insulin was detectable (20).

1.2.2. Management of type 2 diabetes

The medical management of type 2 diabetes is ideally lead by a multidisciplinary team. The main goals are to reduce symptoms and prevent or slow the development of complications. Microvascular complications of diabetes include nephropathy, ophthalmopathy and neuropathy, while macrovascular complications include coronary, cerebrovascular and peripheral vascular disease due to advanced atherosclerosis. Not only hyperglycaemia itself requires control, but also the frequently present hypertension and dyslipidaemia, along with smoking cessation. Ideally the management plan should consider the family members too (21).

Pharmacological therapeutic agents that are used in the management of hyperglycaemia in type 2 diabetes:

- Biguanides
- Sulfonylureas
- Meglitinide derivatives
- Alpha-glucosidase inhibitors
- Thiazolidinediones (TZDs)
- Glucagonlike peptide−1 (GLP-1) agonists
- Dipeptidyl peptidase IV (DPP-4) inhibitors
- Selective sodium-glucose transporter-2 (SGLT-2) inhibitors
- Insulin
1.2.3. Type 2 diabetes and cardiovascular risk

Patients with type 2 diabetes are at high risk to develop cardiovascular diseases that are accountable for 70-75% of the deaths in these patients. The overall mortality rate due to cardiovascular disease is 2-4 times higher in this patient group compared to the non-diabetic population (22-24). From the Bedford Survey (25) this can be clearly seen (Figure 2.). The Bedford Survey reported on a follow-up study of three different patient populations: newly diagnosed diabetics, patients with impaired glucose tolerance (IGT) (borderline diabetics) and patients with normal glucose tolerance. Mortality rates from all causes were highest in the diabetic group, while intermediate in the IGT group. They identified coronary heart disease as the main contributor to the excess deaths. Other large population based studies also confirmed this (25-27). Female diabetic patients have four-times higher risk to die from myocardial infarction than male diabetic patients (28). The higher risk of cardiovascular disease seen in women further increases with advanced age, especially after the menopause, just as the prevalence of type 2 diabetes itself increases (29). The underlying cause is likely to be the loss of protective role of endogenous oestrogen (30).

![Figure 2. Cardiovascular survival. Adapted from (25). The Bedford Survey showed that 10 year mortality is approximately 40% in the group with newly diagnosed diabetes, while appr. 25 years in the group with impaired glucose tolerance, while approximately 15% in the control group with individuals without IGT or diabetes.](image-url)
There are several factors identified accountable for this increase cardiovascular disease rate seen in type 2 diabetes.

Type 2 diabetes is associated with dyslipidaemia, with elevated total and LDL cholesterol, triglyceride and lipoprotein(a) levels with reduced HDL levels compared to non-diabetic individuals (31-33). This adverse lipid profile is also seen in type 1 diabetes; however, in type 1 diabetic patients the lipid abnormalities normalise after establishing optimal glycaemic control, while this is not seen in type 2 diabetes (34).

In type 2 diabetes the prevalence of hypertension is twice as much as seen in the non-diabetic population. The United Kingdom Prospective Diabetes Study (UKPDS) showed that hypertension significantly worsened the incidence of clinical complications such as angina, myocardial infarct (MI), stroke, peripheral vascular disease, vitreous haemorrhage and the need for retinal photocoagulation (35). It was concluded that the lowest risk to develop these complications was seen if systolic blood pressure was below 120 mmHg.

Diabetes is also associated with platelet and endothelial dysfunction that contribute to the increased cardiovascular risk. Hyperglycaemia causes reduced endothelium-dependent arterial vasodilatation (36), and this leads to the impairment of coronary artery autoregulation through the dysfunction of nitric oxide (NO), causing vasoconstriction (37).

Poor glycaemic control itself further increases the risk of coronary heart disease (36, 38).

### 1.3. Insulin resistance

#### 1.3.1. Definition of insulin resistance

Insulin is a peptide hormone, produced by the beta cells of the pancreas (Figure 3.). Insulin is the main regulator of glucose metabolism. Insulin's main actions are:
increasing the glucose uptake in the skeletal myocytes and the fat tissue adipocytes, while inhibiting the gluconeogenesis in the liver. As a net effect, blood glucose levels decrease.

Figure 3. Insulin. Adapted from (39). The human insulin is a peptide hormone that is produced by the β-cells of the pancreas. It is composed of fifty-one amino acids, with a molecular weight of 5808 Dalton. Insulin is a dimer of an α- and a β-chain. These are linked by disulfide bonds.

Insulin resistance is the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population (40).

Patients with diabetes mellitus have absolute or relative loss of insulin. In type 1 diabetes, the pancreas does not produce insulin due to destruction of the beta cells. Type 2 diabetes is more associated with insulin resistance, where the target cells are less responsive to insulin due to their resistance (41). In fact, the difference between type 1 and type 2 diabetes was first noted in 1936, identifying that patients with diabetes are either “insulin sensitive” or “insulin insensitive” (42). In 1968 higher concentration of insulin was shown in patients with type 2 diabetes (43). Insulin resistance (IR) is not only characteristic to type 2 diabetes, it can be
found in various other conditions, as seen in Table 2. (44). Factors that contribute to IR are genetics, obesity, physical inactivity and advancing age (45).

<table>
<thead>
<tr>
<th>Type of insulin resistance</th>
<th>Condition</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary insulin resistance</td>
<td>Type 2 diabetes</td>
<td>Degree of resistance related to BMI</td>
</tr>
<tr>
<td>Secondary insulin resistance</td>
<td>Illness</td>
<td></td>
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<tr>
<td></td>
<td>Starvation, DKA</td>
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<td></td>
<td>Uraemia</td>
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<td></td>
<td>Cirrhosis</td>
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<tr>
<td></td>
<td>Acromegaly</td>
<td>Excess counter-regulatory hormone production</td>
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<td>Cushing’s disease and syndrome</td>
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<td></td>
<td>Phaeochromocytoma</td>
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<td></td>
<td>Glucagonoma</td>
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<tr>
<td></td>
<td>Polycystic Ovary Syndrome</td>
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<tr>
<td>Physiological</td>
<td>Pregnancy</td>
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<tr>
<td></td>
<td>Puberty</td>
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<td></td>
<td>Old age</td>
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<tr>
<td></td>
<td>Stress</td>
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<td></td>
<td>Increasing body weight/obesity</td>
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</tr>
<tr>
<td>Insulin receptor mutations</td>
<td>Leprechaunism</td>
<td>IUGR, dysmorphic facies, acanthosis nigricans, lipoatrophy</td>
</tr>
<tr>
<td></td>
<td>Rabson–Mendenhall syndrome</td>
<td>Pineal hyperplasia, dental dysplasia</td>
</tr>
<tr>
<td>Autoantibodies against insulin receptors</td>
<td>Ataxia telangiectasia</td>
<td>IgM antibodies to the insulin receptor. Autosomal recessive.</td>
</tr>
<tr>
<td></td>
<td>Systematic autoimmune disease</td>
<td>IgG antibodies to the insulin</td>
</tr>
<tr>
<td>Type of insulin resistance</td>
<td>Condition</td>
<td>Features</td>
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<td>-----------------------------</td>
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</tr>
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<td>Other</td>
<td>Alstrom's</td>
<td>Retinal pigment degeneration, obesity, hypogonadism, sensorineural deafness. Autosomal recessive</td>
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<td></td>
<td>Pseudo-acromegaly</td>
<td>Normal IGF-1 and GH levels, high insulin levels, physical features of acromegaly</td>
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<td></td>
<td>Laron dwarfism</td>
<td>High growth hormone, low IGF-1 levels. Autosomal recessive</td>
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<td>Lawrence Moon Biedl</td>
<td>Retinitis pigmentosa, mental retardation, hypogonadotrophic hypogonadism</td>
</tr>
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<td>Prader–Willi syndrome</td>
<td>Hyperphagia, central obesity, dysmorphic facies, mental retardation/developmental delay, hypogonadism.</td>
</tr>
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<td></td>
<td>Myotonic dystrophy</td>
<td>Autosomal dominant</td>
</tr>
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<td>Lipodystrophies</td>
<td>Berardinelli–Seip syndrome (Generalized lipoatrophy)</td>
<td>Hepatomegaly, muscle hypertrophy and hypertriglyceridaemia. Autosomal recessive</td>
</tr>
<tr>
<td>Kobberling–Dunnigan syndrome (partial lipodystrophy)</td>
<td>Facial sparing, X-linked dominant or autosomal dominant</td>
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<td>-----------------------------------------------</td>
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<tr>
<td>Mandibuloacral dysplasia</td>
<td>Skeletal abnormalities</td>
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<tr>
<td>Acquired</td>
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<tr>
<td>Lawrence syndrome (generalized lipodystrophy)</td>
<td>Hyperlipidaemia, hepatomegaly. Antecedent viral infection in 50% of cases.</td>
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<td>Sporadic, female preponderance, C3 nephritic factor and mesangiocapillary glomerulonephritis</td>
<td></td>
</tr>
<tr>
<td>Partial lipodystrophy</td>
<td>Treatment with HIV-1 protease inhibitors</td>
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</tr>
</tbody>
</table>

BMI, Body mass index; DKA, diabetic ketoacidosis; IUGR, intrauterine growth retardation; IgM, Immunoglobulin M; IgG, Immunoglobulin G; IGF-1, insulin-like growth factor; GH, growth hormone

**Table 2. Physiological and pathological states associated with insulin resistance. Adapted from (44).**

The definition of insulin resistance is suggested if an insulin dose of 1.5 units/kg/day or greater is required in a patient with type 2 diabetes (46) however several factors should be considered when measuring insulin resistance, as seen in Table 3 (44).
Descriptor | **Mechanism by which insulin resistance is affected** | **Effect on insulin resistance**
---|---|---
Fasting or stimulated | Following stimulation, insulin response augments with time. Basal and stimulated insulin resistance are not synonymous. | Estimates of stimulated insulin resistance may be lower than estimates of basal insulin resistance.
Hyperglycaemia | Increased glucose-mediated glucose uptake. Increased β-cell stimulation. | Estimates of insulin resistance derived from a hyperglycaemic clamp may be lower than estimates obtained at normoglycaemia.
Exercise exposure | Increased glucose-mediated glucose disposal. Increase insulin-mediated glucose uptake. | Insulin resistance is decreased following exercise.
Time of day | Increased cortisol and FFA levels at 8–9 a.m. leading to increased HGO. | Insulin resistance is increased in the morning.
Exposure | Increased adrenaline and cortisol levels leading to increased HGO. | Insulin resistance is increased by stress.

FFA, Free fatty acids; HGO, hepatic glucose output

Table 3. Necessary descriptors for interpretation of quantitative estimates of insulin resistance. Adapted from (44).

1.3.2. Insulin resistance and cardiovascular risk factors

1.3.2.1. Insulin resistance and dyslipidaemia

Hyperinsulinaemia as a measure of insulin resistance is associated with the
features of atherogenic lipoprotein phenotype (45), such as reduced high density lipoprotein cholesterol (HDL) level (47) and raised triglyceride (TG) level (48).

Insulin resistance causes elevated very low density lipoprotein (VLDL) levels through the increased level of free fatty acids (FFA) in the circulation. This is due to the increased intracellular hydrolysis of triglycerides and the decreased uptake and increased release of FFA by the adipocytes (49) that also contributes to the low HDL levels.

1.3.2.2. Insulin resistance and hypertension

It has long been suggested that insulin resistance is a contributing factor towards the appearance of hypertension, a well-established risk factor for macrovascular events: patients with hypertension tend to be relatively more insulin resistant than patients with normotension (50), and a correlation was shown between systolic/diastolic blood pressure and fasting serum insulin concentration (51).

In the Framingham Offspring Study, 2458 non-diabetic participants’ data was analysed and supported that hypertension was linked to the metabolic syndrome through shared correlations with hyperinsulinaemia (as a measure of insulin resistance) (52). Later a complex relationship between insulin resistance and hypertension was indicated after reviewing the available clinical and epidemiologic evidence (53) suggesting that it is not clear whether insulin resistance precedes the development of hypertension or vice versa. Insulin resistance increases noradrenalin secretion leading increased systolic blood pressure (54) while in addition insulin resistance may enhance sodium retention by the renin-angiotensin system activation (55).

1.3.2.3. Insulin resistance and carotid intima media thickness

The Insulin Resistance Atherosclerosis Study involved more than 1600 participants aged 40-69 years (56). The aim of the study was to assess the relationship between cardiovascular risk factors and insulin resistance, hyperinsulinaemia and glycaemia. Participants were recruited to represent different ethnicity groups (Hispanic, non-Hispanic white and African-American) with different glucose tolerance, classified as normal, impaired, diabetes. Intima-
media carotid thickness (cIMT) was used to assess atherosclerosis while frequently sampled intravenous glucose tolerance tests was used to evaluate insulin resistance. From the results it was concluded that both in Hispanics and non-Hispanic whites there was a significant negative association between cIMT and insulin sensitivity (57).

cIMT is a good predictor of cardiovascular disease that can detect early abnormalities of atherosclerosis using brightness mode ultrasonography (58). In a recent meta-analysis higher mean cIMT has been associated with increased risk of myocardial infarction, stroke, vascular death, and total mortality (59).

1.3.2.4. Insulin resistance and endothelial dysfunction

Endothelial dysfunction is paradoxical or inadequate endothelial-mediated vasodilation. The combined presence of insulin resistance and endothelial dysfunction is an early event in individuals at high risk for developing cardiovascular disease in a later phase (60). Insulin resistance is associated with impaired endothelial function (61). It’s been shown that obesity and insulin resistance in combination result in endothelial dysfunction compared to lean subjects.

1.3.2.5. Insulin resistance and prothrombotic state

Insulin resistance has been shown to be associated with impaired fibrinolysis and altered clotting factors such as fibrinogen (49, 62), factor VII, Plasminogen Activator Inhibitor (PAI-1), and this increases cardiovascular risk (49). Increased coagulability may be a result of the reduced levels of the inhibitors of clot formation (63) such as factor C, S, and antithrombin III (62).

Figure 4. summarizes the potential links between insulin resistance and cardiovascular disease.
Figure 4. Potential links between insulin resistance and cardiovascular disease. Adapted from (64). Genetic and environmental factors along with obesity have an adverse effect on endothelial function and insulin sensitivity, leading to dyslipidaemia, abnormal glucose tolerance and hemostasis with hypertension. The result is cardiovascular disease.

1.3.3. Insulin resistance syndrome

Berson and Yalow reported in 1960 that patients with adult onset diabetes had increased insulin response following an oral glucose tolerance test (65). Insulin resistance then was described by Gerald M. Reaven who developed the first quantitative method to measure insulin-mediated glucose uptake in humans (66). He established the importance of insulin resistance in humans, especially in patients with type 2 diabetes (67, 68). He demonstrated the role of insulin resistance in the development of unfavorable changes in cardiovascular risk markers in non-diabetic individuals. These cluster of factors are referred as insulin resistance syndrome. Insulin resistance and insulin resistance syndrome are two different entities however resistance to insulin-mediated glucose disposal increases the risk to develop insulin resistance syndrome (69).
The insulin resistance syndrome is also known as metabolic syndrome. It represents a cluster of cardiovascular risk factors such as dyslipidaemia including raised triglyceride and decreased HDL levels, elevated blood pressure, elevated fasting plasma glucose. Metabolic syndrome is associated with obesity in most cases. Several bodies such as National Cholesterol Education Program’s Adult Treatment Panel III report (ATP III), WHO, American Association of Clinical Endocrinologists (AACE) use different diagnostic criteria for metabolic syndrome (70). In 2006, the International Diabetes Federation (IDF) developed their own “platinum standard” diagnostic criteria, based on the previous criteria of other bodies, during a workshop in London (71). Below there are the ATP III and IDF diagnostic criteria for Metabolic Syndrome (Table 4. and Table 5.).

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Value</th>
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<tbody>
<tr>
<td>Waist circumference</td>
<td>&gt; 102 cm in men, &gt; 88 cm in women</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>≥ 150 mg/dl</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol</td>
<td>&lt; 40 mg/dl in men, &lt; 50 mg/dl in women</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>≥ 130/85 mmHg</td>
</tr>
<tr>
<td>Fasting blood glucose</td>
<td>≥ 110 mg/dl</td>
</tr>
</tbody>
</table>

**Table 4.** ATP III diagnostic criteria of metabolic syndrome. Diagnosis can be made of three or more criteria are present. Adapted from (72).
Central obesity (waist circumference ≥ 94 cm for Europid men, > 80 cm for Europid women; ethnicity specific values for other groups)

And any two of the following four factors:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raised triglycerides</td>
<td>&gt; 150 mg/dl (1.7 mmol/l) or specific treatment for raised triglycerides</td>
</tr>
<tr>
<td>Reduced HDL cholesterol</td>
<td>&lt; 40 mg/dl (1.03 mmol/l) in males and &lt; 50 mg/dl (1.29 mmol/l) in females, or specific treatment for reduced HDL</td>
</tr>
<tr>
<td>Raised BP</td>
<td>Systolic BP &gt; 130 or diastolic BP &gt; 85 mmHg, or treatment of previously diagnosed hypertension</td>
</tr>
<tr>
<td>Raised FPG</td>
<td>100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes</td>
</tr>
</tbody>
</table>

(If above 5.6 mmol/L or 100 mg/dL, OGTT is strongly recommended but is not necessary to define presence of the syndrome.)

Table 5. IDF diagnostic criteria of metabolic syndrome. Adapted from (73).

Decreased insulin sensitivity is detected clinically as reduced insulin-stimulated glucose uptake however on the cellular level it means reduced insulin action among other cellular responses that affect glucose uptake. The heterogeneous clinical phenotype of patients with insulin resistance is a result of the different effects and disorders in various signaling pathways (74, 75).

1.3.4. Methods of measuring insulin sensitivity/resistance

Epidemiological studies utilize hyperinsulinaemia to define insulin resistance, however, it is unreliable considering that plasma insulin concentration is a reflection of ambient glucose and pancreatic β-cell function. In addition, plasma insulin concentration interpretation is complicated due to the lack of standardization in insulin assays.

Research studies use complex experimental techniques to assess insulin sensitivity/resistance. The ultimate gold standard is the invasive
hyperinsulinemic-euglycemic clamp. Formulae have been proposed to validate fasting insulin and insulin/glucose ratio, intravenous glucose tolerance test (plasma glucose and insulin both measured) against the hyperinsulinaemic-euglycaemic clamp. Constant infusion of glucose with model assessment is also used, as well as the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) that is a formula, and can be easily calculated using fasting plasma glucose and insulin or C-peptide concentration (64). The original HOMA1 model was developed by Matthews et al. (76). HOMA1-IR = (FPIxFPG)/22.5, where FPI is fasting plasma insulin (mU/l) and FPG is fasting plasma glucose (mmol/l). Insulin resistance and β-cell function is calculated, respectively, as follows: HOMA1-%B= (20xFPI)/(FPG-3.5). The updated model is HOMA2-IR and it takes into consideration the variations in hepatic and peripheral glucose resistance and the reduction of peripheral glucose-stimulated glucose uptake (77, 78). The HOMA2-IR model can also be used to determine insulin sensitivity (%S) and β-cell function (%).

The quantitative insulin sensitivity check index (QUICKI) is calculated from fasting plasma and insulin measurements and uses the following formula: 1 /[log(fasting glucose) + log(fasting insulin)] (79). A value of less than 0.339 indicated insulin resistance. Both HOMA-IR and QUICKI compensate for fasting hyperglycemia, and the results correlate with the euglycaemic clamp technique. QUICKI is identical to the to the HOMA-IR1 model, except it uses a log transform of the insulin glucose product (80).

1.4. Endothelial dysfunction and diabetes mellitus

1.4.1. The endothelium

Endothelial cells (ECs) form the inner layer of any blood and lymphatic vessel of the vascular system. In the adult human body there are approximately one trillion endothelial cells with a total weight of more than 100g and a total surface area of more than 3000 square meters (81, 82) thus the endothelium is considered as a distributed organ forming a dynamic interface with the other internal organs. The single layer of intact endothelial cells form a continuous thrombo-resistant surface between the blood and the potentially thrombogenic subendothelial matrix and
vascular smooth muscle cells (VSMC) (83) and can be considered as a first line physiological defense against atherosclerosis. The endothelial cells undergo programmed cell death.

As endothelial cells communicate with various tissues and cells, individual endothelial cells possess properties that are specific to the local environment therefore endothelial cells form a heterogeneous cell group reflecting the various differences in morphology, behavior and biosynthetic repertoire (84). The main functions of the endothelium: maintaining vasomotor tone and the balance of vasodilatation and vasoconstriction. The main vasodilatator produced by ECs is nitric oxide (NO) while the main vasoconstrictors are: endothelin (85-87), prostaglandins (88, 89), angiotensin II (ANG-II) (90-93). The endothelium also maintains the balance between pro- and anti-inflammatory mediators and blood fluidity, regulating cellular and nutrient flow, preventing bleeding, preventing platelets and leukocytes from adhesion to the vascular surface (94). The endothelial cells have crucial role in the formation of new blood vessels while interacting with the existing, circulating blood cells (95) and also influence the growth and/or changes in the phenotypic characteristics of VSMC (96) therefore has an impact on vascular remodeling (97).

The endothelium, as the active biological interface, activity or integrity is dysfunctional (endothelial dysfunction) in various pathological conditions such as cardiovascular, pulmonary, obstetrical, infective, haematological, neurological and gastrointestinal diseases. The endothelial dysfunction is also present in malignancy and diabetes.

Common patterns in change of the endothelial function include endothelial cell activation, endothelial cell dysfunction, endothelial cell apoptosis. EC activation as a term originally was based on a laboratory observation that cultured endothelial cells showed increased leukocyte adhesion after exposure to inflammatory mediators. From our present understanding, the activated endothelium reflects a procoagulant, pro-adhesive state with increased vasoconstriction. EC dysfunction originally referred to structural changes in the endothelial cells, such as loss of integrity or platelet hyperadhesiveness (ie. as seen in atherosclerosis) (98, 99).
Recently endothelial dysfunction refers to the loss of the ability to regulate vascular resistance.

Under normal circumstances, less than 0.1 percent of the endothelial cells are apoptotic. In the presence of abnormal Tumor Necrosis Factor-alpha (TNF-alpha), interleukin-1 (IL-1), interferons, oxidative stress or hypoxia, the rate of endothelial cell apoptosis increases, as well as in the presence of lipopolysaccharide- (LPS) activated monocytes (100). Endothelial cell apoptosis triggers a pro-inflammatory response in vitro and in vivo. Under laboratory conditions, apoptotic endothelial cells mediate increased expression and release of Intercellular Adhesion Molecule-1 (ICAM-1) and VCAM-1 through an IL-1-dependent mechanism, along with increased production of reactive oxygen radicals, increased procoagulant properties, decreased production of prostacyclin and compliment activation, causing increased binding of yet non-activated platelets (101). These indicate a dynamic interaction between inflammation and programmed endothelial cell death.

Under physiological conditions, the endothelium maintains a relatively dilated state in the blood vessels and responds to various physical stimuli. In response to shear stress, blood vessels dilate through a process called flow-mediated vasodilatation (FMD). FMD is regulated by the release of nitric oxide (NO) by the endothelial cells. Nitric oxide is synthesized by the endothelial nitric oxide synthase (eNOS) enzyme (102) proved by the impaired FMD response caused by asymmetric dimethylarginine (ADMA) that is an endogenous eNOS inhibitor (103, 104). eNOS produces picomolar levels of NO and only a small portion has a biological effect. eNOS produced NO diffuses to the vascular smooth muscle (VSM) and activates the guanylate cyclase enzyme. The increased level of guanosine monophosphate (GMP) relaxes the VSM and results in vasodilatation. eNOS is regulated by local bradykinins (105): bradykinin increases the level of NO by activating the eNOS after a signal pathway through the b2 receptors on the endothelial cell surface.
Endothelium-dependent vasodilatation and vasoconstriction is a complex, controlled mechanism. To explain, here is an example of the regulation of the EC-derived vasoconstrictor ANG-II and vasodilator NO. ANG-II is produced locally by the endothelial cells (106) and induces vasoconstriction while also regulate other functions of the VSMC such as growth, proliferation and differentiation. The eNOS activating bradykinin is inactivated by the angiotensin converting enzyme (ACE) (107, 108) that also turns the inactive angiotensin I into the active vasoconstrictor angiotensin II.

The endothelium produces several other molecules, such as leukocyte adhesion molecule (LAM), intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), VE-cadherin. These are adhesion molecules and attract cells that are involved in the inflammatory process and their levels are useful in research settings to determine the degree of endothelial dysfunction.

1.4.2. Endothelial dysfunction in diabetes

Endothelial dysfunction was first described in 1986 in epicardial coronary arteries with advanced atherosclerosis (109). The balance between the endothelial-derived blood vessel-relaxing and –contracting factors is impaired in diabetes and atherosclerosis (110). Endothelial dysfunction as proved by impaired insulin-induced vasodilatation, is present in non-diabetic obese insulin resistant individuals (111, 112). Endothelial dysfunction and impaired vascular reactivity is an early stage in large vessel disease and is seen in type 1 and 2 diabetes and in first degree relatives of patients with type 2 diabetes (113-116) due to the autonomic dysfunction and altered neurotransmission (117). The degree of endothelial dysfunction reflects the duration of diabetes and the degree of glycaemic control: endothelial dysfunction in poor glycaemic control improves after introducing insulin (118).

Insulin resistance itself is often associated with hypertension, obesity, hyperlipidaemia, increased fibrinolysis and if progressing, then hyperglycaemia. As explained elsewhere in this thesis, diabetes is also associated with increased
cardiovascular risk, on the basis of accelerated atherosclerosis. In early atherosclerosis, endothelial dysfunction can be detected. Endothelial dysfunction is also associated with dyslipidaemia, hypertension, increased oxidative stress, hypercoagulation as proved by such as increased PAI-1 level, hyperuricaemia (119-130). As these are all features of type 2 diabetes, the dynamic relationship between insulin resistance, metabolic syndrome, type 2 diabetes and endothelial dysfunction is complex.

Hyperinsulinaemia seems to directly effect endothelial function in a dose-dependent manner, causing increased neutrophil migration through the endothelium with elevated platelet endothelial cell adhesion molecule-1 (PECAM-1) expression, without increased expression of ICAM-1, P-selectin or E-selectin (131). Physiological hyperinsulinaemia does not affect endothelium-independent vasodilatation, while abolishes endothelium-dependent vasodilatation (132). Several studies confirmed that insulin therapy is beneficial for vascular function in diabetic patients (133-135), possibly due to the restoration of insulin-stimulated endothelial function, due to a yet not understood mechanism.

Endothelial dysfunction can be assessed by soluble endothelial marker levels. In the Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) Cooperative Research, elevated soluble E-selectin and ICAM-1 levels were associated with increased risk to develop type 2 diabetes in a later phase. In the case of sICAM-1, the effect was not independent of the level of soluble E-selectin. Impaired von Willebrand Factor (vWF) levels did not affect the risk of diabetes development (136). Others found that elevated soluble E-selectin and ICAM-1 levels both were predictors of the development of type 2 diabetes, after multifactorial adjustments (137).

Causes and effects of endothelial dysfunction are summarized in Figure 5.
Figure 5. Causes and effects of endothelial dysfunction. Adapted from (138). Various factors including diabetes, hypertension, smoking, oxidative stress, dyslipidaemia, altered levels of homocysteine and ADMA along with yet not clearly identified uraemic factors affect the endothelial function. Endothelial dysfunction results in peripheral vasoconstriction, lipid accumulation in the blood vessel wall, and increased platelet, leukocyte and vascular smooth muscle cell proliferation.

1.4.3. Methods for evaluating endothelial function

Endothelial function can be assessed using different approaches (139).

1.4.3.1. Evaluation of coronary endothelial function

Coronary endothelial function is assessed using an invasive approach. Acetylcoline (Ach), metacholine or papaverine is used as an intracoronary infusion to assess epicardial coronary vasodilatation. Ach is used most widely. Ach causes paradoxical vasoconstriction in endothelium-denuded arteries in vitro, and in atherosclerotic coronary arteries in vivo (109). Recently, a noninvasive method using computed tomography (CT) or magnetic resonance imaging (MRI) has been used as a substitute of quantitative coronary angiography (QCA), which is another method to assess intracoronary endothelial function (140, 141).
1.4.3.2. **Venous occlusion plethysmography**

Venous occlusion plethysmography (VOP) was established more than 100 years ago (142). It is based on usually muscular blood flow measurement by assessing the volume change of the tissue induced by an inflated cuff placed proximally to the evaluated tissue.

1.4.3.3. **Flow-mediated vasodilatation**

Flow-mediated vasodilatation (FMD) is mainly investigated by imaging of a straight segment of the brachial artery using an ultrasound (US) probe. The principle behind this methodology is that when a cuff is inflated to supra-systolic pressure distally to the imaged area, following the release of the cuff, reactive hyperaemia can be detected and quantified by using Doppler US technique, after recording end-diastolic arterial diameter (143).

1.4.3.4. **Pulse wave analysis**

Pulse wave analysis is based on the fact that the arterial waveform carries important information about the arterial stiffness and the wave reflection in the arterial blood vessels (144). Wave reflection is quantified by the augmentation index (Aix), the difference between the first and second systolic peaks (145). Impedance in smaller arteries and arterioles are more dynamic than in large arteries and they are more sensitive to glycercyl trinitrate (GTN). Endothelial function can be assessed by recording the shape of the arterial waveform before and after administering the NO donor GTN, causing vasodilatation without systemic effect on the blood pressure.

1.4.3.5. **Laser Doppler flowmetry**

Laser Doppler flowmetry (LDF) monitors the microvascular blood flow of the skin that is assumed to reflect other vasculature (146, 147).
1.4.3.6. **Biochemical markers**

1.4.3.6.1. **Soluble markers**

Soluble biochemical markers, such as sICAM, sVCAM, sE-selectin, vWF are also used to assess endothelial function, as well as endothelial progenitor cells. The latter were proposed to have a role in endothelial repair, and the impaired mobilization or depletion of these cells contribute to the development of cardiovascular disease (148, 149).

Adhesion molecules play central role in endothelial function. They are classified as selectins, integrins and immunoglobulins. Many of them play crucial role in the leukocyte-endothelial adhesion in the pathogenesis of inflammation, which thought to be a major factor in the development of atherosclerosis (150).

Selectins are type I transmembrane glycoproteins expressed on leukocytes and endothelial cells. P-selectin is constitutively expressed and stored in secretory granules within the endothelial cells. P-selectin expression is increased on the surface of the endothelial cells upon stimulation. E-selectin is synthesized de novo and expressed on endothelial cells after stimulation. Among the immunoglobulins, ICAM-1 is constitutively expressed on endothelial cells, at low levels, and expression increases after EC stimulation. VCAM-1 is only synthesized and expressed after EC activation.

1.4.3.6.2. **Microparticles**

Microparticles (MPs) are small membrane sheds derived from vascular endothelium or circulating blood cells. They were first described in 1967 as plasma membrane fragments and were called as “platelet dust” (151). It was recognized that this “dust” consisted of small vesicles with procoagulant potential. Later, the small particles with a diameter of 1-100 nm were called microparticles. In vitro microparticles have been released from endothelial cells, platelets, vascular smooth muscle cells, white blood cells and erythrocytes. MPs can be found in
healthy individuals while their numbers are elevated in several pathological states. MPs arise from activated or apoptotic cells.

Now their active physiological and pathophysiological role is evident. MPs now are recognized as emerging markers of cardiovascular disease (152). In a 6-months follow-up study, circulating annexin V+ MPs were robust predictors of the occurrence of death or second myocardial infarction in patients with acute coronary syndrome (ACS) (153). In a different study involving more than 200 asymptomatic patients, leukocyte derived microparticles (LMPs) were shown to be a predictor of subclinical atherosclerotic burden characterized by the number of plaques in the carotid, abdominal and femoral arteries (154).

MPs consist of lipids and proteins. The composition is determined by the cell of origin and the mechanism that lead to the formation of MPs. The outer layer of the MPs is a phospholipid bilayer while little is known about the intravesicular content of MPs. While cell membranes in resting cells is asymmetrical, this structure is altered during MP formation and results in the exposure of negatively charged phospholipids on the surface, such as phosphatidylserine (PS). The exposure of PS is believed to be a key factor in their procoagulant activity as PS binds various coagulation factors (155, 156). Endothelial microparticles (EMPs) also bind vWF (157). The phospholipid bilayer is different from the originating cells and varies between the MPs, depending on their cell of origin.

The presence of PS on the MP surface is believed to be closely linked to the apoptotic process and was suggested to be a defense mechanism (158). After an apoptotic stimulus, membrane fragments that express PS are removed by macrophages (159).

MPs display different surface proteins that are characterized by the cell of origin. These proteins can be used to qualify and quantify the different MPs. CD42b is expressed on PMPs (157).
MPs have several effects on cell function and communication (160). Endothelial microparticles (EMPs) promote monocyte-endothelial cell binding (161). Platelet derived microparticles (PMPs) up-regulate ICAM-1 expression on endothelial cells, resulting in increased cellular interaction between monocytes and endothelial cells (162) while PMP derived CD11b up-regulation on leukocytes causes increase in phagocytosis and leukocyte-leukocyte interaction (163, 164). EMPs LMPs increase neutrophil activation, and the attraction of leukocytes by chemokines (165-167). MPs isolated from patients with acute myocardial infarction diminished endothelium-dependent relaxation in isolated arteries while MPs isolated from patients with non-cardiac chest pain, did not demonstrate that effect (168).

Several diseases are associated with elevated number of MPs. In diabetes, the level of total MP count and PMPs are elevated (169, 170). Elevated levels of CD144 (VE-cadherin) positive EMPs, that are believed to play a role on angiogenesis, were shown in patients with type 2 diabetes, and this was a predictor for further cardiovascular events in this group of patients in a separate study (171, 172).

In acute artery coronary syndrome, total MP count, EMPs and PMPs are elevated (173). In a study involving 84 patients, CD31+/CD42- EMP level was found to be elevated and the EMP number showed a direct correlation with the morphology and degree of coronary artery stenosis (174). In a different study, higher CD31+/annexin V+ EMP number was associated with endothelial dysfunction, showed by loss of vaso-relaxation after acetylcholine infusion during angiography (175). Elevated number of CD61+/CD42b+ PMPs was reported in a study involving 54 stable coronary artery disease (CAD) patients, suggesting that PMPs can be valid marker of prothrombotic state in this patient group (176).

Independently of age and blood pressure, CD144+ EMPs inversely correlate with the amplitude of flow-mediated vasodilatation of the brachial artery in patients with end-stage renal disease, while there was no correlation for PMPs or annexin V+ MPs (177) and similar findings were reported investigating patients with ACS (175).
Elevated CD42+ PMP level was shown in patients with venous thrombo-embolism (178). D-dimer and P-selectin levels combined with PMP levels was shown to significantly correlate with the diagnosis of deep vein thrombosis (DVT) (179). It has also been shown that microparticles are involved in thrombus generation and reorganization after venous thrombosis (180).

To date, it is not clear whether elevated number of MPs can be identified as a cause or consequence of cardiovascular disease (181).

1.4.3.7. Peripheral artery tonometry

Fingertip peripheral artery tonometry (PAT) has been recently developed and used for assessing endothelial function (182). The test is non-invasive, and the methodology is based on the assessment of digital reactive hyperaemia. It is not yet used as routine screening tool for functional endothelial impairment, it is currently only appropriate to be used in a research setting.

The sensitivity and specificity of the test has been evaluated and compared to the gold standard intra-coronary Ach challenge method (as described earlier in this chapter). A study involving 111 patients revealed a 82% sensitivity and 77% specificity in the diagnosis of endothelial dysfunction (183).

The test is carried out on fasting patients, after resting for 10 minutes, in a relaxed, calm environment. Two probes with rigid external case and inflatable inner membranes are placed on the patient’s index fingers, after placing a blood pressure cuff on one of the patient’s forearms (study arm). Gender, age, baseline blood pressure are recorded prior to initialing the test. PAT signal is recorded for five minutes in a sitting or lying position on both of the study and control arms. After five minutes, the blood pressure cuff is rapidly inflated, and kept on a pressure around 220-220 Hg mm, to result in a flat PAT line. After five minutes the cuff is rapidly deflated, and the reactive hyperaemic signal is recorded for further five minutes. In response to hyperaemic flow, there is an increase in digital pulse amplitude therefore PAT signal amplitude also increases. This response is
dependent on NO synthesis (184) therefore a good indicator of endothelial function. The difference in terms of PAT signal between normal and impaired endothelial function is demonstrated in Figure 6.

![Figure 6. Reactive hyperaemia. In healthy individuals, reactive hyperaemia triggers compensatory increase in the PAT signal amplitude, while it is diminished in pathological states. Adapted from (185).](image)

EndoScore [Reactive Hyperaemia Index, (RHI)] is calculated using the post-to-pre occlusion PAT signal ration on the study arm, after normalization to the control arm and further corrected for vascular baseline tone. RHI > 1.67 is considered normal, while RHI ≤ 1.67 indicates endothelial dysfunction.

Children with type 1 diabetes have lower PAT scores compared to children without diabetes indicating the presence of endothelial dysfunction (186). It was also demonstrated that overweight adolescents present impaired PAT scores compared to adolescents with normal body weight (187). Patients with coronary endothelial dysfunction were shown to have lower PAT scores (188). In the Framingham Heart Study, lower EndoScores were associated with obesity and diabetes mellitus, dyslipidaemia and insulin resistance (189). In other studies, impaired EndoScore was also linked to hypertension, CAD, diabetes, hyperlipidaemia, glucose intolerance or smoking (182, 190-193). In a study involving 89 patients with chest pain, there was a positive correlation between FMD and PAT score (182). Prolonged pharmaceutical intervention, smoking cessation and dietary alterations with isoflavone or polyphenol supplementation,
proved to improve EndoScore, indicating improved endothelial function (194-199).

1.5. Type 2 diabetes and coagulation

1.5.1. Overview

Type 2 diabetes is associated with increased fibrinogen levels, PAI-1 levels besides increased platelet activation (200), endothelial dysfunction (116) and increased growth factor stimulation (201).

Several studies demonstrated that diabetes is a risk factor to develop venous thromboembolism and atherothrombosis. The underlying cause is the altered coagulation system present in diabetes.

Hemostasis is the process of blood clot formation at the site of a blood vessel injury. The key structures in the blood clot formation are the platelets and coagulation factors and their function is compromised in patients with diabetes.

Patients with diabetes have high risk to develop atherothrombosis and deep vein thrombosis (202, 203). The underlying reasons are the presence of premature atherosclerosis that predisposes to plaque rupture and thrombus formation (202). The presence of platelet disturbances discussed elsewhere in this thesis. Patients with diabetes also have increased activation of pro-thrombotic coagulation factors and decreased fibrinolysis (204).

The common risk factors for venous thromboembolism and atherosclerotic cardiovascular disease are the same, such as obesity, hypertension, dyslipidaemia, smoking and diabetes (205). As patients with type 2 diabetes often have associated obesity, dyslipidaemia and hypertension, it is evident, that the risk is multiplied in this patient group.
1.5.2. Normal haemostasis

The thrombus formation is a complex, multi-step process that ends with fibrin formation and consequent vessel occlusion. The haemostatic cascade is presented in Figure 7.

![Haemostatic cascade diagram](image)

*Figure 7. Haemostatic cascade. Plaque rupture activates the haemostatic cascade and results in the formation of a blood clot. Fibrinolysis develops parallel. Adapted from (206).*

After thrombogen surface exposure, it contacts platelets and coagulation factors. FVII binds to exposed tissue factor (TF), and the formed complex activates further coagulation factors. Activated FX and FV facilitate limited prothrombin-thrombin conversion. The formed thrombin and exposed collagen at the site of endothelial injury fully activates platelets, resulting in release of the granules and FV, FVIII, FIX, and activation. When sufficient thrombin is generated, it converts fibrinogen into fibrin fibre network. Then FXIII-cross-linked mature fibrin clot forms (207). During fibrinolysis, the mature clot is lysed, and fibrin degradation products remain. Tissue Plasminogen Activator (tPA) is released by endothelial cells and facilitates the plasminogen – plasmin conversion. PAI-1 is a potent inhibitor of the
fibrinolytic process, by forming an inactive complex with tPA (206). Natural anti-coagulants such as protein C and tissue factor pathway inhibitor (TFPI) provide defense mechanisms preventing inappropriate thrombus formation (208).

1.5.3. Coagulation in type 2 diabetes

Type 2 diabetes represents a prothrombotic state with a pattern of coagulation factors that promote thrombosis or retard thrombolysis (45). Coagulation proteins involved in clot formation, fibrinolysis, clot structure are all impaired in diabetes (206). This prothrombotic state is characterized by abnormalities in platelet function (209), increased fibrinogen (210) (211) and PAI-1 levels (212). The European Concerted Action on Thrombosis Study confirmed the link between hyperinsulinaemia and other components of insulin resistance to the progress of coronary heart disease (213). After investigating 1500 patients with angina pectoris, they found that higher circulating insulin levels correlated with increased level of fibrinogen, PAI-1, vWF and tPA. The strongest relationship was confirmed between insulin and PAI-1 levels. In the Insulin Resistance Atherosclerosis Study, a strong independent association was found between PAI-1 and proinsulin levels (214). Therefore fibrinogen and PAI-1 levels, two crucial components of the pathogenesis of plaque formation, are related to the insulin or proinsulin concentration.

An association between fibrinogen, insulin and HDL has been described (215-217). The main mechanisms leading to increased fibrinogen level in T2DM are linked to insulin resistance and postprandial alterations. Insulin resistance is associated with increased insulin-mediated hepatic fibrinogen synthesis in T2DM, however this has not been demonstrated in healthy individuals or patients with type 1 diabetes (218, 219). In postprandial states, it has also been shown that increased fibrinogen production is present in T2DM, in contrast to healthy individuals (219).

Among coagulation proteins, TF expression on endothelial cells and the level of circulation TF are elevated in T2DM (220, 221). The latter is directly modulated by
glucose and insulin, thus it is not surprising to appear in T2DM providing that the main features are hyperglycaemia and hyperinsulinaemia.

FVII coagulant activity is elevated in T2DM (222) and is also influenced by elevated triglyceride levels (223). Thrombin generation is increased in T2DM (221). Hyperglycaemia control reduces thrombin generation (224). Elevated thrombin levels enhance the formation of more stable and dense blood clots (225).

Hyperglycaemia affects PAI-1 levels as showed by a 18-years long-term study, indicating that HbA1c positively correlates with PAI-1 levels and correlates negatively with tPA (226) while hyperinsulinaemia itself appears also to enhance PAI-1 level (227). The rate of coagulation in diabetes compared to healthy controls is altered too, meaning the clot lysis is slower in diabetes (228). PAI-1 release is affected by all of the following: plasma insulin, proinsulin, cytokines, glucose, modified lipid levels (213, 214).

Further to these, clot structure is also altered in diabetes. Recently it was shown that clots formed in vitro had more compact structure with smaller pore size, increased fibre thickness, and more number of branch points in T2DM compared to healthy controls. HbA1c negatively correlates with pore size and positively with the number of branch points (229). However other studies failed to show the same conclusion (230).

1.6. Diabetes and platelet dysfunction

1.6.1. Platelets and primary haemostasis

1.6.1.1. Overview

Platelets have a crucial role in haemostasis. Primary haemostasis is the early stage when coagulation is still yet to develop. Platelets’ role in primary haemostasis include two crucial steps: first, due to age and/or high local shear stress, individual
endothelial cells are lost and platelets repair the exposed subendothelium and second, activated platelets capture endothelial precursor cells to fill these gaps (231).

1.6.1.2. **Primary and secondary adhesion, stabilization**

The first step in primary haemosatsis is that platelets interact with the exposed subendothelial matrix, mainly through vWF and the GPIb complex on the platelet surface membrane (232). vWF initially originates from the plasma, later from activated platelets and/or endothelial cells. vWF binds to the sub-endothelial exposed collagen on the site of the vascular injury, then the binding site of GPIb binds to vWF hence platelets adhere to the subendothelium.

1.6.1.3. **Platelet activation**

After platelet adhesion, platelets are activated. The process is mainly derived by the GPVI complex, that binds to exposed collagen filaments and laminin. The degree of activation depends on the extent of the endothelial injury. After collagen – GPVI interaction, platelets release the content of their α-granules and dense granules (Table 6).

<table>
<thead>
<tr>
<th>α granules</th>
<th>Dense granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>Adenosine diphosphate (ADP)</td>
</tr>
<tr>
<td>Factor V</td>
<td>Adenosine triphosphate (ATP)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Serotonin</td>
</tr>
<tr>
<td>vWF</td>
<td>Calcium</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. The main granular constituents secreted by platelets upon activation.
The release of the granules further escalates the activation of the platelets through activation of other integrins, such as α2β1. After the activation of these integrins, they are bound to collagen and fibrinogen/fibrin, then further activation occurs mainly driven by the intracellular calcium cascade. As a consequence, negatively charged phospholipids such as PS are exposed on the surface of the activated platelets, that plays a critical role for the later step of prothrombin-thrombin conversion.

During primary haemostasis, platelets do not only interact with the subendothelial matrix components, but a degree of platelet-platelet interaction occurs, mainly through the αIIbβ3 receptor that binds various plasma and platelet proteins and fibrinogen in particular (233). Fibrinogen is a bivalent ligand hence it can bind to two separate platelets causing platelet aggregation. VWF also contributes to platelet aggregation by binding both to αIIbβ3 and GPIb.

### 1.6.1.4. Spreading

After integrins bind to the endothelial matrix proteins such as collagen, platelets spread out first by extending pseudopods and then once these are fixed, the remaining gaps become filled (234). Spread platelets provide tight junctions that prevent blood loss.

After forming an initial layer of activated platelets, more platelets attach. In this step, the most likely activation mechanism is thought to be the release of activators by the first layer of platelets, for example ADP (234). In this stage, the role of COAT platelets, that are the most activated platelets and formed through activation by thrombin- and collagen activated platelets, should also be considered (235).

The schematic process of platelet function is visualized in Figure 8.
Figure 8. Platelet function. The main stages of platelet activation are primary adhesion, secondary adhesion and stabilization, followed by activation with shape change, and finally spreading. In all stages different adhesion molecules such as GPIb-IX-V, GPIIb-IIIa play crucial role. Adapted from (236).

1.6.1.5. Platelet-derived microparticles

Microparticles are small membrane sheds or cell fragments that are present in the circulation. They originate from activated or apoptotic cells and their number is increased in certain pathological conditions especially cardiovascular disease, cancer, sepsis or diabetes (237). The majority of MPs are normally derived from platelets and they are believed to be highly procoagulant as noted above.
1.6.2. Platelet dysfunction in type 2 diabetes

The hypercoagulant state, that is present in diabetes and represented by increased level of platelet activators, is one of the factors that contribute to the increased platelet activation however there are others that also play a role such as the loss of the anti-aggregatory factors.

The platelets’ main function is to cover blood vessel wall discontinuity at sites of vascular injury. After activation, their shape changes, they adhere to subendothelial matrix proteins, they play a role in clot formation by releasing contents of their intracellular granules, and they form a plug by aggregation, as all described previously. Pro-aggregators that facilitates the thrombus formation by activating platelets are thrombin, collagen, epinephrine, ADP and thromboxane A2 (TxA₂) (238). There seems to be increased platelet sensitivity to various aggregants, such as epinephrine, ADP, thrombin and collagen (239-242).

The surface glycoproteins, as described above, are also receptors for platelet agonists and antagonists (238). The main anti-aggregants are Prostaglandin Inhibitor₂ (PGI₂) and NO that are continually released by the healthy endothelium (243). Several studies reported decreased synthesis of PGI₂ and NO in patients with diabetes (240, 244-247). PGI₂ acts through a G-protein receptor that is expressed on the platelet surface, while NO directly activates the guanylate cyclase after diffusing through the platelet membrane. Beyond the lower level of PGI₂ and NO in diabetes, the response to these platelet inhibitors is reduced in diabetes (248, 249).

Insulin has various metabolic effects and these are not restricted only to the glucose and lipids. Vinik et al recommended that the definition of insulin resistance should be widened (“the metabolic state in which the measured tissue response to insulin is less than that expected for the apparently available insulin”) to reflect the haemostatic effects of insulin, on such as endothelial cells, platelets, monocytes and erythrocytes as represented by figure 9 (239).
Figure 9. Effects of insulin on blood cells and endothelial cells. On monocytes, insulin enhances Prostaglandin E\(_2\) binding therefore facilitates immune response. On platelets, it up-regulates the prostaglandin E\(_1\) and prostaglandin I\(_2\) receptors, therefore enhances platelet anti-aggregation, while inhibits the ADP, collagen, thrombin and epinephrine derived aggregation. On the endothelial cells insulin stimulates nitrogen-oxide and prostaglandin I\(_2\) release, and regulates the expression of Plasminogen-Activator Inhibitor-1, causing peripheral vasodilatation. Insulin affects the erythrocyte membrane fluidity. Adapted from (239).

Insulin may have direct (through the insulin receptor on the platelet surface) and indirect actions on platelets. Insulin reduces platelets responses to agonists such as ADP, collagen, thrombin (209). Decreased platelet insulin receptor expression and affinity was shown in patients with type 2 diabetes, indicating that reduced insulin sensitivity may contribute to the platelet hyperactivity in this group of patients (250).

1.7. Type 2 diabetes mellitus and thyroid disease

1.7.1. Overview

Hyper- and hypothyroidism have both been associated with insulin resistance that is the major underlying factor leading to hyperglycaemia in type 2 diabetes.

Thyroid dysfunction is a common endocrine disorder. The prevalence in male adults in England is 6.6% (251). In the National Health and Nutrition Examination Survey (NHANES III Study), 4.6% of the participants were reported to have hypothyroidism, while the prevalence of hyperthyroidism was 1.3%, respectively
Worldwide, the prevalence increases with age and it is more frequent in females than in males. The prevalence of subclinical hypothyroidism is between 3% to 12% (253, 254), while 1% to 6% in subclinical hyperthyroidism (254, 255).

In patients with type 2 diabetes, the prevalence of thyroid disorders is 13.4% (31.4% in women, 6.9% in men) (256).

Thyroid hormones stimulate various metabolic activities, leading to increase in basal metabolic rate.

Thyroid hormones have a direct controlling effect on insulin secretion. Insulin imbalance is closely related to thyroid dysfunction and this is mediated by β-cell dysfunction (257). Glucose-induced insulin secretion in the pancreas β-cells is reduced in hypothyroidism, while in hyperthyroidism the glucose- or catecholamine- mediated response of beta cells is increased. Interestingly, insulin resistance occurs both in hypo- and hyperthyroidism (258).

In euthyroid adults, serum thyroid stimulating hormone (TSH) level is positively associated with fasting and postprandial insulin concentrations and negatively associated with insulin sensitivity and β-cell function (259).

Figure 10. shows the relationship between altered thyroid function and impairment in glycaemia.
1.7.2. The effect of thyroid hormones on insulin secretion and insulin sensitivity

1.7.2.1. Hypothyroidism

Hypothyroidism causes reduced glucose absorption from the gastrointestinal tract, prolonged peripheral glucose accumulation, reduced gluconeogenesis, diminished hepatic glucose output, reduced disposal of glucose (261). Insulin resistance in hypothyroidism leads to decreased glucose-stimulated insulin secretion (262). In subclinical hypothyroidism, insulin resistance may be caused by the altered expression of glucose transporter type 2 gene (GLUT 2) leading to decreased insulin stimulated glucose transport.

1.7.2.2. Hyperthyroidism

The association between worsening glycaemic control in type 2 diabetes and hyperthyroidism was first reported in 1927 (263).

Hyperthyroidism causes glucose intolerance through increased hepatic glucose output and up-regulated glycogenolysis (262). This might cause worsening of
impaired glucose tolerance and hyperglycaemia in patients with type 2 diabetes. Common features both in type 2 diabetes and in hyperthyroidism are: increased intestinal glucose absorption, reduced insulin secretion, altered β-cell mass, increased insulin degradation, increased glucagon secretion, increased hepatic glucose production, insulin resistance (264).

In patients with type 2 diabetes, thyroid function is monitored more closely, and routine screening for thyroid disease is recommended (265-267).

1.8. Diabetes and diet

1.8.1. Overview

In the management of type 2 diabetes, the first and most important step is lifestyle modification consisting of implementation of change in diet and increase physical activity.

The Diabetes UK Nutrition Working Group recently reviewed the dietary and nutritional guidelines in diabetes (268). The new recommendations are in line with the ADA guidelines (269) and resulted in the up-date of the previous UK recommendations (270). The main focus of the up-dated guideline is weight management as primary aim to improve glycaemic control and cardiovascular risk (271). It's been proved that in high-risk individuals, weight loss is the strongest predictor of decreasing the risk to develop type 2 diabetes, in various nations (272-275). Weight loss was more effective in the prevention of type 2 diabetes when compared to metformin: 5-7% of weight loss reduced the risk of progression from impaired glucose tolerance to T2DM by 57%, while metformin only reduced the risk by 31% (276).

Low calorie diet is proved to normalize beta cell function, improve insulin resistance and pancreas and liver triacylglycerol in patients with type 2 diabetes (277). Bariatric surgery is also proved to improve or reverse type 2 diabetes (278, 279). Plasma glucose level returns to normal within days after surgery, prior to the
excessive degree of weight loss, likely due to the altered secretion of incretins (279, 280). Both interventions are effective also due to the acute negative energy balance (281).

Beyond weight loss achieved by daily calorie intake restriction, the change of the quality of the diet is also an important factor in patients with type 2 diabetes to improve glycaemic control and cardiovascular risk.

1.8.2. Polyphenols

Polyphenols have been identified to carry potential benefit for this patient group, especially isoflavones that present in soy, and flavanols, that can be found in high concentration in cocoa. A recent study involved nearly 100 postmenopausal women on established lipid-lowering therapy with type 2 diabetes found that glycaemic control and cardiovascular risk significantly improved in the patient group who consumed combined flavan-3-ol and isoflavone food supplementation for 12 months, compared to placebo, however HbA1c worsened in both groups while the difference was not found to be significant (282). The intervention attenuated insulin resistance shown by HOMA-IR, while fasting glucose and insulin improved. Total cholesterol:HDL-cholesterol ratio, HDL:LDL ratio and LDL level improved. There was an improvement in total cholesterol and HDL, however these were not statistically significant. Coronary heart disease (CHD) and fatal CHD risk improved, while there was no difference in stroke and fatal stroke risk between the two groups.

The classification of polyphenols based on their chemical structure (283):

1. Phenolic acids – fruits, vegetables, grains, seeds
2. Flavonoids
   2.1 Isoflavones, neoflavonoids, chalcones
      Isoflavones – soy (genistein, daidzein, glycetein, biochanin A, formononetin)
   2.2 Flavones, flavonols, flavanones, flavanonols
2.3 Flavanols (flavan-3-ols), proanthocyanidins
   Catechin, epicatechin – cocoa
2.4 Anthocyanidins – flower petals, fruits, vegetables
3. Polyphenolic amides
4. Other polyphenols

The main flavanols in cocoa are catechin and epicatechin, while the main isoflavones in soy are genistein and daidzein (Figure 11).

![Chemical structure of soy isoflavones and cocoa flavanols](image)

Figure 11. Chemical structure of the main soy isoflavones daidzein and genistein, and cocoa flavanols catechin and epicatechin.

1.8.2.1. Soy isoflavones

Isoflavones present in soybean in glycosylated form and this is biologically inactive. The intestinal flora activates the isoflavones by transforming them into aglycone form. The biologically active form then absorbed by the intestinal epithelium and 50% is bound to proteins whilst circulating, and the other 50% is
free to competitively bind to oestrogen receptors. Their activity as oestrogen agonist (284) just one of the features that may be an underlying cause of their beneficial biological role in protecting against cardiovascular disease. Soy isoflavones have antioxidant features (285) and inhibit angiogenesis in vitro (286). Isoflavones have protein kinase C activity (287), possibly calcium antagonistic action (288) and they facilitate LDL oxidation (289). Phytoestrogens were shown to enhance vascular reactivity (290, 291), inhibit atherosclerotic plaque development (292), reduce systolic and diastolic blood pressure (293) and improve biomarkers of lipid peroxidation (294).

Isoflavones were long proposed to protect against cardiovascular disease and various study results support this theory. Soy consumption showed an inverse relationship with cardiovascular disease mortality. Soy is a main food component in Japan. Epidemiological data suggest that Japanese people who live in Seattle in the USA, have four times higher prevalence of type 2 diabetes than Japanese people who live in Tokyo (295, 296). Japanese Americans with type 2 diabetes also showed significantly higher levels of insulin, following an oral glucose tolerance test than Japanese with type 2 diabetes (295, 297). These suggest greater degree of insulin resistance among Japanese Americans and it prompts to the lifestyle and dietary differences between the two populations.

Isoflavone-rich soy based diets improved insulin resistance in cynomologus monkeys (298) and reduced fasting insulin levels in postmenopausal women (299, 300). Other previous studies also suggested beneficial effects in patients with type 2 diabetes (301-304).

Soy protein supplementation lowered total and LDL cholesterol in various studies, however to date available data are still conflicting (305).

In 1910 the therapeutic potential of soy food supplementation was first suggested in (306). More recently various studies were conducted on various populations using different soy preparations. Serum lipid levels improved in patients with type 2 diabetes whom consumed soy protein and/or soy fibre preparations while not in
healthy subjects (301). Some studies showed improvement in fasting and postprandial plasma glucose (302, 307) while others did not (301, 308) while insulin levels did not change significantly in most of these studies.

Animal data suggest that isoflavones improve vascular reactivity (291, 309, 310) while studies involving humans resulted in conflicting outcomes (290, 311-313). Several studies demonstrated decrease in the level of adhesion molecules, indicating improvement of the endothelial function (314-316).

1.8.2.2. Cocoa flavanols

Cocoa is a widely consumed food ingredient. In 2011-2012, the worldwide production of cocoa was 3.98 million tonnes according to the World Cocoa Foundation. Cocoa is a rich source of polyphenols – 12-18% by dry weight.

The bioavailability of flavanols is limited – cocoa epicatechins show the greatest bioavailability, with a maximal plasma concentration two hours after consumption (317). 20% of the absorbed epicatechins is excreted in the urine. Cocoa consumed with milk or as milk chocolate seems to be less effective in terms of antioxidant capacity (318).

Cocoa consumption has been proved to improve blood pressure, dyslipidaemia, insulin sensitivity, vascular endothelial function and cardiovascular risk (195, 319-331).

Cocoa lowers the activity of vascular arginase enzyme in human endothelial cells in vitro, thus augmenting the local levels of L-arginine, while induce the enzyme NOS (332, 333). In vivo cocoa enhances the circulation pool of available NO and augments flow-mediated vasodilatation (334, 335).

Cocoa has antioxidant properties, shown by delayed LDL oxidation (336) and reduced production of reactive oxygen species in activated leukocytes (337). Various studies also proved platelet inhibitory potential of cocoa (338, 339).
Epidemiological studies support the blood pressure-lowering effect of cocoa. In the Zutphen Elderly Study, cocoa intake was inversely related to blood pressure (340). In a study involving pre-hypertensive or phase I hypertension patients, long-term cocoa ingestion significantly reduced systolic and diastolic blood pressure (341). Cocoa seems to lower the level of angiotensin-converting enzyme activity in vitro (342). A recent meta-analysis also confirmed the blood pressure-lowering effect of cocoa flavanols (343).

Considering the link between insulin resistance and endothelial dysfunction (see in previous chapter) it is not surprising that cocoa flavanols may have an effect on insulin resistance simply by improving endothelial function. Various studies reported improvement in insulin sensitivity after chronic or acute cocoa ingestion (326, 344). In hypertensive patients with impaired glucose tolerance, cocoa improved insulin sensitivity and β-cell function, as well as blood pressure and endothelial function (325). In animal experiments, cocoa dose-dependently prevents hyperglycaemia in obese diabetic mice (345).

Cocoa also improves dyslipidaemia. In hypertensive patients, 100g flavonoid-rich chocolate over 2 weeks significantly reduced serum total and LDL cholesterol levels (344). Another study showed that flavanol-rich cocoa reduced plasma level of LDL and oxidized LDL cholesterol, and increased HDL cholesterol levels (346).

1.9. Summary

Type 2 diabetes is increasingly common and it imposes an economic healthcare burden. Patients with type 2 diabetes are more frequently hospitalized, mostly due to the elevated cardiovascular risk that is a consequence of the underlying insulin resistance and impaired glucose control, as well as the dyslipidaemia and hypertension that are frequently presented with diabetes. Type 2 diabetes is also associated with endothelial and platelet dysfunction along with clotting disturbances, while thyroid function is more frequently altered in this patient population than in healthy individuals.
As patients with type 2 diabetes are frequently obese, the first step in treatment is lifestyle modification including dietary changes and increase in physical activity. The next step in treatment is by oral glucose lowering agents, or insulin.

Several dietary intervention is beneficial for patients with type 2 diabetes, for example the addition of soy and cocoa. These were shown to improve lipid profile, blood pressure, insulin resistance. Some studies also suggest that they improve endothelial and platelet function, along with clotting indices. However the exact mechanism is yet to be identified.

Patients with type 2 diabetes have endothelial dysfunction. These features are both associated with atherosclerosis and elevated risk to develop Venous Thromboembolism Events (VTE). A commercial flight is also associated with elevated risk to develop VTE. It has not yet been studied whether patients with type 2 diabetes have higher risk to develop VTE during air travel.

1.10. Aims and Objectives

The aims of this thesis are to explore:

- if dietary interventions with different soy preparations with or without cocoa modify glycaemia, insulin resistance, lipid parameters, blood pressure and endothelial function

- the possible mechanism that explains the beneficial effect of soy protein and isoflavones on glycaemic control

- if patients with type 2 diabetes have elevated risk to develop VTE during air travel compared to healthy individuals by assessing endothelial function, platelet activity and clotting indices.
2. CHAPTER Methods

2.1. Purpose

The purpose of this chapter is to provide patient details, study documentation, along with methodological descriptions, technical data and protocols. Study protocols, ethics approvals, patient information leaflets are provided in Appendix II.

2.2. Participants

Participants were recruited through the Diabetes Research Centre in Hull, local diabetes clinics and media advertisements.

The main inclusion/exclusion criteria were similar in all studies.

2.2.1. Dietary intervention experiments:

- Inclusion criteria:
  o Diagnosis of type 2 diabetes, based on the WHO guidelines.
  o Patients on stable medication (treatment only metformin) for their diabetes, hypertension, lipids and gout (if appropriate) for 3 months prior to entry into the study.
  o Age between 45-80 years at the start of the study.

- Exclusion Criteria
  o Patients with concurrent illness or any medication in the last 3 months.
  o Patients not wishing to allow disclosure to their GPs.
  o Patients on hormone replacement therapy.
  o Patients who are on or have taken antibiotics in the preceeding 3 months prior to entry to the study.
  o HbA1c at recruiting stage of >9%.
  o Patients with known food allergies.
- Smokers.
- Vegans and vegetarians.
- Patients not willing to consume snack bars.
- Pre-menopausal women.

2.2.2. Simulated flight study

- Inclusion criteria:

Diabetes group:
- Diagnosis of type 2 diabetes, based on the WHO guidelines.
- Patients on stable medication (for their diabetes metformin being the only treatment if any) for 3 months prior to entry into the study.
- Age between 45-75 years at the start of the study; women need to be postmenopausal and not undergoing hormone replacement therapy (HRT).
- No diabetes related hospital admission or any episodes of hypoglycaemia or hyperglycaemia that required medical intervention/treatment in the preceding 12 months before entry into the study.

Healthy volunteers group:
- No co-morbidity, which would have affected the safety of the subjects or the study results.
- No medication that would have interfered with the study results.
- Age between 45-75 years at the start of the study; women postmenopausal and not on HRT.

- Exclusion criteria for both groups:
- Patients with concurrent illness (i.e. Acute coronary syndrome or major clinical event) or any medication in the preceding 3 months that would have interfered with the study results based on the investigator’s judgment.
- Patients not wishing to allow disclosure to their general practitioners.
- HbA1c >9%.
- Smokers.
- Pre-menopausal women or post-menopausal women on HRT.
• Claustrophobia, panic attacks in the anamnesis.
• Individuals with known food allergies.
• Vegans and vegetarians.
• Individuals not willing to consume snack bar.
• Subjects who travelled by airplane 4 weeks prior to the study.

2.3. Methods, materials and techniques

2.4. Flow cytometry

2.4.1. Overview

The principle behind flow cytometry is the identification of various characteristics on single cells within a hydro dynamically focused fluid suspension (347). These suspended individual cells rapidly pass through on a focused laser beam or light source as a single stream. In order to produce the single stream, sheath fluid is pumped through a laminar flow chamber, creating a flow system. The sample is added to the centre of this flow system and the cells included pass through one-by-one several laser beams at the interrogation point (Figure 12).
Figure 12. Schematic structure of a flow cytometer. Individually suspended cells rapidly pass through on a focused laser beam or light source as a single stream with a sheath fluid that is pumped through a laminar flow chamber. The laser or light source hits the cells and the reflected light is captured by photodiodes. Adapted from (348).

When the laser hits the cells, light is reflected or refracted and this is detected by photodiodes/photomultiplier tubes. Reflected light is translated into electronic signals and is a measure of cell size or granularity. If cells were incubated with fluorescently labeled antibodies that recognize antigens on the cell surface, the fluorophore gets excited and emits fluorescence at a characteristic wavelength. This is detected by the flow cytometer and then transformed to an electric signal that corresponds with the number of antigens on the cell surface.

Results can be expressed as percentage of positive cells above a pre-set threshold that is identified with an appropriate negative control. Results can also be expressed as fluorescence intensity: either mean or median.

Sample preparation usually consists of six steps, as detailed in Figure 13.
Figure 13. Common steps in sample preparation prior to flow cytometry in platelet studies. Adding anticoagulant to whole blood prevents clotting therefore inhibits platelet aggregation. Dilution ensures that individual platelets can be studied. Fluorescent antibodies provide detectable signal. Fine details of platelet function, i.e. response to agonists or antagonists can be expressed numerically after fixing the sample.

For antigens that are present on the cell surface constitutively, mean or median fluorescence intensity (MFI) is the appropriate data to express results, while for an activation antigen that is not present continuously on the surface of the cells but only when they are activated, the percentage positive data is more appropriate.

2.4.2. Platelet function testing using flow cytometry

2.4.2.1. Platelet biology

Platelets are the smallest blood cells, lacking nucleus that are originated from the cytoplasm of the highly specialized precursor cells called megakaryocytes, in the bone marrow (349, 350). In quiescent state, they are in a discoid shape and their surface is smooth and rippled. The normal platelet size is 2.0 to 5.0 μm in diameter with a thickness of 0.5 μm. Mean cell volume is averaging between 6-10 femtoliters (351).
Individual platelets circulate in the blood flow close to the wall of arteries and veins during their 7-10 days of lifespan and survey the integrity of the inner layer of the blood vessels (352).

Platelets have a complex structured plasma membrane including a layer of lipids, proteins and sugars. In an inactivated state, on the outer side of the cell membrane, mainly uncharged phospholipids i.e. phosphatidylcholine and sphingomyelin are present, while on the inner surface of the plasma membrane negatively charged aminophospholipids ie PS and phosphatidylethanolamine (PE) are found (353). Upon activation the latters become exposed on the outer surface (354) and this redistribution facilitates the activation of the coagulation cascade (355).

The major function of platelets is to arrest blood loss after vascular damage. This is crucial in the haemostatis however if platelet function is altered, they might be more prone to activation. Unnecessary activation is not desirable.

### 2.4.2.2. Flow cytometry as a method to study platelet biology

Platelets were identified in 1881 (356). Platelet function since then has been studied by using various methods. Flow cytometry is one of the most widely used method that enables the platelet function to be evaluated using washed platelets or platelet-rich plasma (357, 358) however using these assays, the possibility of artifactual in vitro platelet activation due to the separation procedures should be taken into consideration while interpreting results. Shattil et al (359) introduced whole blood flow cytometry to assess platelet function and this meant a major important step towards most accountable tests of platelet function.

Flow-cytometric measurement of platelets using whole blood is a sensitive and quantitative method that is based on certain surface antigens of the platelets and is a reliable method (360, 361).

Flow cytometry is currently used for various purposes in order to study platelet features as seen in Table 7 (362).
Measurement of Platelet Activation  
- Activation-dependent monoclonal antibodies  
- Leukocyte–platelet aggregates  
- Platelet-derived microparticles  
- Platelet–platelet aggregates  
- Shed blood  

Platelet-Associated IgG  
- Immune thrombocytopenias  
- Alloimmunization  

Blood Bank Applications  
- Quality control of platelet concentrates  
- Identification of leukocyte contamination in platelet concentrates  
- Immunophenotyping of platelet HPA-1a  
- Detection of maternal and fetal anti-HPA-1a antibodies  
- Platelet cross-matching  

Monitoring of Thrombopoiesis  
- Reticulated platelets  

Diagnosis of Specific Disorders  
- Bernard-Soulier syndrome  
- Glanzmann thrombasthenia  
- Storage pool disease  
- Heparin-induced thrombocytopenia  

Monitoring of Antiplatelet Agents  
- Thienopyridines  
- GPIIb-IIIa antagonists  
- Aspirin  

Platelet Counting  

Other Research Applications  
- Platelet survival, tracking, and function \textit{in vivo}  
- Calcium flux  
- F-actin  
- Signal transduction  
- Fluorescence resonance energy transfer  
- Platelet recruitment  
  - Bacteria–platelet interactions  

| Table 7. Purpose of flow cytometry in platelet studies. Adapted from (362).  

Advantages of flow-cytometry for platelet studies (348, 361):  
- Whole blood can be used – therefore sample preparation is rapid, simple and the artificial activation of platelets can be avoided. Whole blood environment also means physiological environment so platelets can be studied in autologous plasma and in the presence of other blood cells and soluble mediators  
- Multiple aspects of platelet biology can be studies within a short time on large number of single platelets
- All platelet populations can be studied – megakaryocytes, platelets, platelet-leukocyte aggregates, platelet-derived microparticles
- Platelet response to agonist stimulation and antagonist inhibition can be studied simultaneously
- Not radioactive

Disadvantages of flow-cytometry (361):
- Flow cytometers are expensive to purchase and maintain
- Sample preparation can be complicated and technician-dependent
- Blood samples should be processed quickly, in real-time
- Only measures the function of circulating platelets, while does not reflect the activation at the endothelium or cleared platelets

2.4.2.3. Platelet activation/reactivity/inhibition

ADP is a platelet activator that is produced in the platelets and stored in the dense granules from where it is released upon activation of the platelets. This soluble agonist is secreted by exocytosis (363) and is essential in recruiting further platelets to the site of the vascular injury.

PGI₂ is synthesized from prostaglandin H₂ (PGH₂) by PGI₂ synthase upon various enzymatic reactions. PGI₂ is a platelet inhibitor (364, 365) that is produced by the endothelial cells in order to prevent unnecessary platelet activation (366).

P-selectin is stored in the α granules of the platelets and is released upon activation. P-selectin therefore only present on the surface of activated platelets and is a good indicator of the degranulation process.

Fibrinogen expression is another marker of platelet activation. When platelets are activated, a conformational change in the GPIIb/IIIa complex happens that exposes the receptor site for fibrinogen binding. Fibrinogen then binds to the receptor and this can be recognised by fluorescent-labelled fibrinogen antibodies (Figure 14) (359, 367, 368).
a) Fibrinogen binding in the presence of an agonist (ADP) or antagonist (PGI₂). NB. ADP 10 µM was added to the tubes containing PGI₂.
b) P-selectin expression in the presence of an agonist (ADP) or antagonist (PGI₂). NB. ADP 10 µM was added to the tubes containing PGI₂.
Figure 14. Fibrinogen binding and P-selection expression. Higher concentration of ADP increases platelet activity (fibrinogen binding and P-selectin expression), expressed by the number of detectable platelets, after conjugating FITC fibrinogen and PE P-selectin conjugated monoclonal antibodies.

2.4.2.4. Sample preparation

Samples were prepared as previously described (348, 361, 368). To prevent artifactual in vitro platelet activation and aggregation, blood samples were taken by clean venepuncture and were drawn without statis using 21 gauge butterfly needles, Polypropylene syringes were used to collect blood sample. The first 2 ml was discarded, then 4.5 ml blood was drawn gently without stopping into a 5ml syringe that already contained 0.5ml 3.8% trisodium citrate, to prevent coagulation. Sodium citrate was gently mixed with whole blood and samples were processed immediately. Flow cytometry tubes were prepared no longer than 15 minutes before sample preparation.

FACS tubes were prepared by adding 50 µl of modified Tyrode’s buffer (150mM NaCl, 5mM HEPES, 0.55mM NaH₂PO₄, 7mM NaHCO₃, 2.7mM KCl, 0.5mM MgCl₂, 5.6mM D-glucose, pH7.4) to the appropriate antibodies (CD42b FITC, fibrinogen FITC, CD62P) or the negative controls (FITC, PE, Fibrinogen + EDTA). For the fibrinogen antibody (see material list at the end) there was no appropriate negative control so in order to assess non-specific binding, a sample containing the fibrinogen antibody was used with 6mM Ethylenediaminetetraacetic Acid (EDTA) as described previously (348), providing that EDTA prevents fibrinogen binding by inhibiting integrin activity.

After pre-preparing the FACS tubes, 5 µl of whole blood was added to the tubes. Mixing was done by gentle flicking to prevent artifactual activation/aggregation of the platelets. For the assessment of activation with ADP and inhibition with PGI₂, 5µl of various concentrations of ADP and/or PGI₂ were added. Where inhibition with PGI₂ was evaluated, after adding whole blood, 5µl of PGI₂ 0.1nM, 1nM, 10nM was added subsequently, followed by 5µl of 10µM of ADP. In tubes where stimulation with ADP was measured, 5µl of ADP 0.1µM, 1µM and 10µM was added. Timing of pipetting was strictly controlled to ensure that samples were incubated
with PGI2 for 2 minutes, and any reaction to ADP was incubated for 10 minutes before fixation. Samples were then left at room temperature to incubate for 10 minutes before fixation was done by adding 500µl of formyl saline (0.2%, v/v). Samples were run in duplicates for better accuracy (368).

Fresh stock of ADP 10mM and PGI2 2mM was used for each set of samples. ADP and PGI2 were diluted and concentration titrated in order to obtain the required concentrations (0.1µM, 1µM and 10µM of ADP and 0.1nM, 1nM, 10nM of PGI2) prior to blood drawing.

### 2.4.2.5. Sample analysis

Samples were analysed on BD FACSCalibur (BD Biosciences). All antibodies were optimised for maximum fluorescence with minimal non-specific binding (369).

The platelet population was identified prior to running each set of samples by incubating whole blood with FITC-conjugated mouse anti-human CD42b antibody (Figure 15). CD42b is also known as platelet glycoprotein Ib alpha chain that is coded by the GP1BA gene and is part of the GPIb/IX/V complex. CD42b is bonded by a disulfide bridge to CD42c – they form a 170 kDa heterodimer, GPIb. GPIb is a part of the noncovalent CD42 complex which also includes CD42a and CD42d and is presented on the surface of megakaryocytes and platelets. CD42 complex is the surface receptor of vWF and it is involved in the adhesion of platelets to the sub-endothelium in the sites of vascular injury. Cells that were expressing CD42b were examined only to make sure that all analysed cells were platelets. Samples were run on the flow cytometer in ascending order of agonist concentration. For each sample 10,000 events were recorded.

Color compensation was done prior to the experiments to prevent the effects of spill-overs of different fluorochromes during the sample analysis (370, 371).
Figure 15. The population of platelets identified using FITC CD42b antibody prior to running of the samples. Specific cells can easily be identified by binding the to specific receptors, and by their size (forward scatter, FSC) and granularity (side scatter, SSC).

2.4.3. Determining microparticle numbers using flow cytometry

2.4.3.1. Microparticles biology

Microparticles are membrane sheds with a diameter of 0.1-1 μm that are present in the circulation under normal circumstances and originates from healthy, apoptotic and activated cells (372). Their levels are elevated in case of increased cell activation and apoptosis therefore in several disease and pathological conditions just as in diabetes (373). MPs can be identified by their size and characterised by their cell of origin.

2.4.3.2. Standards for microparticle characterization using flow cytometry

Microparticles are hard to analyse and no standardised method exists for their numeration. The most widely used methods involve characterization using flow cytometry (362, 374-376).
In 2007, the International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee (SSC) organised a workshop to address the issue regarding the optimization of microparticle numeration. In their study, 40 laboratories from 14 countries were included. One of them was the Centre for Biomedical Research Postgraduate Medical Institute of the University of Hull, where a BD FACSCalibur was used for the purpose of the standardization. During Stage A and Stage B, the standardized MP analysis protocol was set up on the flow cytometer and since then was used to measure MPs according to the recommendations of the ISTH (377).

### 2.4.3.3. Sample preparation

For MP analysis, blood samples were collected in 2.7 ml tubes containing 3.2% 0.105 mol/litre (M) sodium citrate (BD Vacutainer). Platelet Free Plasma (PFP) was prepared using 1,500 g centrifugation for 10 minutes, followed by the separation of Platelet Poor Plasma (PPP). PPP then was micro centrifuged for another 10 minutes on 12,000 g. Then supernatant PFP was carefully removed by pipetting.

Aliquots (50 μl) of PFP in polypropylene tubes were mixed with target-specific antibodies and annexin V (1 μg/ml, 5 μl) as per Table x, then incubated in the dark at room temperature for 30 minutes. 300 μl of annexin V binding buffer (diluted with distilled water according to the manufacturer's recommendations with a ratio of 1:9 = annexin V binding buffer concentrate : distilled water) was then added to the tubes prior to adding 50 μl AccuCheck counting beads. Negative control FITC (mouse IgG, 1 μg/ml, 5 μl) mixed with 300 μl filtered (0.1 μm filter) phosphate buffered saline (PBS) without counting beads was used to identify the positive expression of the surface-specific antibodies.

### 2.4.3.4. Sample analysis

Megamix beads (20 μl diluted with 180 μl distilled water) were used to determine the appropriate gate for MPs, based on their size. Megamix beads are a mixture of
fluorescent beads designed to cover the size range of MPs (0.5 µm and 0.9 µm) and platelets (0.9-3 µm). Megamix beads are essential for the appropriate setting of the flow cytometer in order to measure MPs within a fixed size region and to get reproducible MP counts (374, 378), as seen on Figure 16.

![Figure 16](image)

**Figure 16.** A dot plot of Side Scatter (Y axis) and forward Scatter (X axis) for megamix beads. 0.5 µm and 0.9 µm diameter beads are presented. MPs are expected within the quadrant box, and this gate was subsequently used for the data acquisition and analysis.

MPs were identified according to their scatter properties and further characterized by specific cell surface-receptor expression (platelet-derived MP [PMP], leukocyte-derived MP [LMP], endothelial-derived MP [EMP]: Vascular Cell Adhesion Molecule-1 [VCAM-1] positive and Vascular Endothelial cadherin [VE-cadherin]) by using FITC-conjugated monoclonal antibodies reflecting the common cell surface antigens, as shown in Table 8. To further differentiate the MPs that are originated from apoptotic cells, PE-conjugated annexin V was added to the tubes (Figure 17).

<table>
<thead>
<tr>
<th></th>
<th>PMP</th>
<th>LMP</th>
<th>EMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody (FITC)</td>
<td>CD42b</td>
<td>CD45</td>
<td>CD106</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD144</td>
</tr>
</tbody>
</table>

**Table 8.** MPs from different cell types are characterized by their cell surface receptors.
Figure 17. A dot plot of Side Scatter (Y axis) and Forward Scatter (X axis) was determined to represent the microparticles within the pre-defined gates and the counting beds.

Within the Microparticles gate, MPs were shown as FITC-positive, FITC/PE positive and PE-positive MPs (Figure 18).
Figure 18. A representative of PMP acquisition panel is shown, acquired within the MP gate, as per Figure 17. A dot plot within the MP gate was determined. The upper left quadrant represents the annexin V-positive MPs, the upper right quadrant represents the annexin V/CD42b double-positive PMPs, while the lower right quadrant represents the annexin V-negative PMPs.

MP numbers were calculated based on the concentration of the AccuCheck counting beads, which was provided by the manufacturer. The same volume of PFP and counting beads was mixed. As the MP concentration was not known, the number of MPs (MP/μl) was calculated using the following formula:

\[
\text{Number of MPs counted} = \frac{\text{Absolute MP count (MP/μl)}}{\text{Total number of beads counted}} \times \text{Absolute bead count (beads/μl)}
\]
AccuCheck beads are a mixture of two different bead populations (beads A and B). Beads A and B have been designed to allow the determination of whether they have been homogenously sampled based on their unique properties.

Therefore the total number of beads counted is the sum of Bead A + Bead B (Figure 19).

![Figure 19. Accucheck beads A and B acquisition panel, acquired within the counting beads gate, as per Figure 17. Total number of beads are the sum of number of Beads A + number of Beads B.](image)

Therefore an example of MP calculation:

Number of MPs in the lower right quadrant (CD42b-positive MPs) is 6202.
Number of Bead A is 742, number of Bead B is 704. Total number of beads 1446.

Absolute bead count/μl (supplied by manufacturer) is 1049.

\[
\frac{6202}{1446} \times 1049 = 4499
\]

Absolute MP count (MP/μl) = \______ x 1049 = 4499

\[
\frac{1446}{1446}
\]
2.5. Carotid Intima Media Thickness measurement

For the purpose of the cIMT measurement, a Toshiba Xario 15 US machine (Toshiba Medical systems, Tokyo, Japan) was used. The machine was equipped with an 11-MHz linear probe. In order to be consistent, all scans were performed by the same trained operator, according to the Mannheim consensus (58).

There are two common carotid arteries: the left common carotid artery (LCCA) and the right carotid artery (RCCA). Both were scanned, from three different angles (RCCA 90°, 120°, 150°; LCCA 210°, 240°, 270°), 1cm proximal from the carotid bifurcation where the common carotid arteries split into external and internal carotid arteries. The images were analysed with the SonoCalc IMT 4.1 (SonoSite Ltd, UK) software, and the average thickness was used for data analysis. Reproducibility were assessed by using 5 volunteers outside of study participation.

2.6. Endothelial function

Endo-PAT 2000 (Itamar Medical Ltd, Israel) was used to assess cardiovascular endothelial function by evaluating fingertip peripheral arterial tonometry. Endo-PAT has been validated (188). RHI lower than a cut-off value of 1.67 was proved to provide 82% sensitivity and 77% specificity in the diagnosis of endothelial dysfunction (188). Reproducibility was proved good with an interclass correlation of 0.74 (379).

2.7. Clot structure analysis

Clot structure was assessed using turbidimetry assays according to the EuroCLOT study methods (380). Three parameters were assessed. The Lysis Area (LA) is a complex measurement of clot formation, density and lysis potential. Maximum absorbance (MA) is a measure of fibrin fibre thickness and fibrin network density. Lysis Time (LT) is a representative of the fibrinolytic potential, and it is the time period needed between full clot formation to 50% lysis.
2.8. Biochemical markers and measures of glycaemia and metabolism

Plasma glucose was measured using a Synchron LX 20 analyser (Beckman-Coulter) according to the manufacturer's protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 94.6 mg/dl (5.3 mmol/l).

Total cholesterol, triglycerides and HDL-C were measured enzymatically using a Synchron LX20 analyser (Beckman-Coulter, High Wycombe, UK). LDL-C was calculated using the Friedewald equation (381).

All HbA1c measurements were made using ion-exchange chromatography via the Menarini HA-8160 HbA1c analyser (A. Menarini, Berkshire, United Kingdom).

High sensitivity C reactive protein (hCRP) was measured using Synchron systems CRPH reagent kit (Beckman-Coulter, UK).

Samples for insulin, vWF, E-selectin, P-selectin, VCAM, ICAM and turbidity assays were stored at -80°C until analysis to avoid batch-to-batch variability. Serum insulin was measured using a competitive chemiluminescent immunoassay and was performed on the manufacturer's DPC Immulite 2000 analyser (Euro/DPC, LLanberis, UK). The analytical sensitivity of the assay was 2 μU/ml, the coefficient variation was 6%; there was no stated cross-reactivity with pro-insulin. vWF, E-selectin, P-selectin, VCAM, ICAM were measured using serum ELISA assays (Bender MedSystems, Vienna, Austria).

2.9 Environmental chamber

The simulated flight study visits took place in an environmental chamber, on the premises of the University of Hull. On the control days, temperature was set to 23°C, humidity 45%, oxygen concentration 21% - as on sea level. The simulated flight settings were 23°C, 15% humidity and 15% oxygen concentration. The technical specifications of the chamber are included in Appendix II.
3. CHAPTER The effects of soy protein with or without isoflavones on glycaemic control in type 2 diabetes. A parallel randomized, double-blind, placebo-controlled pilot study.

3.1. Overview

The prevalence of diabetes is increasing and is presently 8.3%. According to the IDF there are 387 million people with diabetes and 46.3% of these patients are undiagnosed (382). The number of patients with diabetes is projected to rise to 592 million in the next 25 years (383). Diabetes imposes an increasing economic healthcare burden (384-386) and patients with type 2 diabetes are more frequently hospitalised compared to the non-diabetic population (387). The prevalence of cardiovascular disease is three times higher in patients with type 2 diabetes (388) with a four-to six fold higher mortality (389).

Lifestyle and diet modification remains the first step in improving glycaemic control and soy has been shown to improve insulin resistance in both primate studies and postmenopausal women (301, 302, 390, 391). Other animal studies have suggested that diets containing soy result in an improvement in insulin sensitivity, glycaemic control and a decrease in fasting insulin levels (392-395). In vitro data suggests that the glycaemic action of soy phytoestrogens may be due to their α-glucosidase inhibitory effects, inhibition of the glucose uptake at the intestinal brush border, and a tyrosine kinase inhibitory action (396-398).

Consumption of soy has been suggested to have an inverse relationship with mortality from CVD perhaps through a favourable effect on lipid levels and glycaemic control. However, a meta-analysis of various soy preparations with a wide range of soy protein and isoflavone intake, did not support this conclusion (399), and isoflavones given alone also appeared to be ineffective (400).

A recent meta-analysis (401) and a further report (402) have suggested that the soy protein may have an intrinsic effect to reduce serum LDL-cholesterol levels.
However, a major confounding issue is that all of the studies to date have been undertaken with soy protein that will also contain isoflavones and there are no studies with soy protein alone that is confirmed to be free from isoflavones.

Cocoa, with high polyphenol content, is potentially beneficial for patients with type 2 diabetes, due to its favorable effects to lower cholesterol level, blood pressure and overall cardiovascular risk. In studies the acute beneficial effect of high-flavanol content food products improved endothelial function in patients with cardiovascular risk factors and diabetes (334, 335, 403). In patients with essential hypertension, insulin resistance improved after 15 days with a diet including 100g high polyphenol content chocolate (326, 344). In another study, insulin sensitivity and beta-cell function improved along with blood pressure and endothelial function in hypertensive patients with impaired glucose tolerance (325). In our centre a recent study showed improved serum HDL cholesterol in patients with type 2 diabetes after consuming high polyphenol chocolate for 8 weeks in a crossover design (327).

A recent study showed improvement in multiple cardiovascular risk factors in postmenopausal women who consumed a food product with isoflavones and flavonols over a one-year period, compared to placebo (334).

Diabetes is also associated with thyroid dysfunction and soy isoflavones have been suggested to alter thyroid function (404, 405). The effect is hypothesized to be due to the thyroid peroxidase enzyme inhibition by the isoflavones, resulting in reduced synthesis of Triiodothyronine (T3) and Thyroxine (T4) (406, 407). Subclinical hypothyroidism that is more common in patients with type 2 diabetes than in non-diabetic individuals (256) is associated with increased cardiovascular risk (408).

Type 2 diabetes is associated with endothelial dysfunction (110, 111) contributing to the increased cardiovascular risk. Soy isoflavones and cocoa polyphenols were shown to improve endothelial function (313, 314, 332, 334).
The aim of our study was to identify which of the soy components of protein, isoflavones or both had an effect on glycaemic control and whether this can be further enhanced with the addition of cocoa. Endothelial and thyroid function were also assessed before and after the intervention period.

3.2. Research design and methods

3.2.1. Patients

Eighty-four patients with diet- or metformin controlled type 2 diabetes aged 45-80 entered the study after recruitment through routine diabetes clinics and local media advertisement. Seventy patients were randomised and sixty patients completed the study (Figure 20).

Figure 20. Recruitment and patient retention during the study period. 84 patients were assessed for eligibility. 70 patients were randomized, and 60 patients completed the study.
The diagnosis of diabetes was made according to the WHO guidelines (409). Patients were either diet controlled (n= 24) or on stable metformin therapy (n= 36) for at least three months before study commencement, and medication was not altered over the study period. Antibiotic treatment within the previous 3 months, or during the study was an exclusion criterion as antibiotics have been shown to alter isoflavone metabolism and absorption through interference with gut flora (410).

All women were postmenopausal and had not received hormonal replacement therapy over the preceding 6 months. Smokers, vegans, vegetarians, patients with regular soy consumption and patients with allergy to any nutritional component of the study bars were excluded during the screening process. Concomitant participation in any other interventional medical trial was not allowed.

The study was approved by the Humber Bridge Regional Ethics Committee (11/YH/0219), and all participants provided written informed consent. The conduct of the trial was in accordance with all relevant legislation, International Conference of Harmonisation Good Clinical Practice (ICH GCP) and the Declaration of Helsinki (ClinicalTrials.gov Identifier: NCT01754662).

3.2.2. Study protocol

This was a randomized, parallel, double-blind, placebo-controlled clinical trial. Following informed consent, patients met with a dietician who explained the need to avoid dietary products with a high isoflavone-content and to maintain their current diet, and when to consume the soy trial bars (midmorning and mid afternoon). Patients were asked to maintain their current level of physical activity.

Study timeline and procedures are included in Figure 21. All patients attended a single site (Diabetes Research Centre in Kingston upon Hull, UK) on four occasions between February 2012 and June 2013.
During the first study visit the study procedures were discussed in great detail with the participants. After confirming eligibility, a dietitian explained the necessary dietary restrictions. 2 weeks later the participants returned for the baseline visit, where baseline fasting bloods were taken, height, weight were measured. 4 weeks supply of study bars were dispensed. 4 weeks later participants returned for another visit, where compliance was checked and they have received the second four weeks supply. The final visit was due at week 10, when the last compliance check was carried out along with fasting blood sampling, and weight measurement.

The randomisation was performed by Essential Nutrition Ltd, UK. A computer generated randomisation list was used to provide balanced blocks of patient numbers for each of the groups. A 1:1:1:1:1 treatment allocation was used without revealing the block size. Patients were randomised during Visit 2 by the study staff, when the next available randomisation number was assigned.

3.2.3. Intervention

Patients were randomised to placebo (P) (n=11), soy protein (SP) (n=15), soy protein+isoflavones (SPI) (n=16), soy protein +cocoa (SPC) (n=13) or soy protein+isoflavones+cocoa (SPIC) (n=15) twice daily for two months.
Two bars containing a base of 7.5g (15g daily) of 70% isolated soy protein powder (Solcon F; CHS Ashdod, Israel) with or without added isoflavones (Solgen 4016mg per bar, 32mg in total daily; CHS Ashdod, Israel, Israel) with or without 400mg of cocoa polyphenols in 1.6g of cocoa powder (CocoaNoX 12%, Natraceutical S. A., Barcelone, Spain) were given for 8 weeks following a 2 week run-in period. The isolated soy powder had the isoflavones removed by repeat 95% alcohol extraction (Dishman Ltd, UK) to give an isoflavone content of less than 300 parts per billion (Assayed by FERA, Sand Hutton, UK). The placebo protein bar contained casein alone (Halo Foods Ltd, Swansea, UK). All the bars used in the study were matched for taste and macronutrient content (Table 9.) (Halo Foods Ltd, Swansea, UK). Randomisation and labelling of the trial supplies were done by Essential Nutrition, Brough, UK.

<table>
<thead>
<tr>
<th>Nutrient analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kj</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td>337.0</td>
</tr>
<tr>
<td>1246.8</td>
</tr>
</tbody>
</table>

Ingredients: Casein or Soya Flour with or without isoflavones or cocoa, Sultanas, Dates, Apple juice concentrate, Apricots, Apple Paste, Glucose Syrup, Palm Oil, Glycerine, Strawberry Juice Concentrate, Natural Strawberry Flavour

kJ – kilojoule, kcal – kilocalorie, carbs - carbohydrates

Table 9. Macronutrient content of the study bars. Study bars were matched for taste and macronutrient content. The energy content was approximately similar to the Kcal content of two biscuits. Participants were advised to consume the study bars mid-morning and mid-afternoon, as a snack.

3.2.4. Study measurements

Following informed consent, physical examination, and an interview with the dietitian and venesection were undertaken. Additional blood samples were taken after an overnight fast during Visit 2 (week 2) and Visit 4 (week 10). Weight and blood pressure were measured at baseline, week 2, week 6 and week 10 (Figure 3.2.). Compliance was checked by collecting and counting empty wrappers and uneaten bars. Fasting venous blood samples were collected into serum gel, EDTA, and fluoride oxalate Vacutainer tubes. Samples for insulin, thyroid hormones and endothelial function markers were spun down at 3500g for 15 min at 4°C, within
30 minutes after drawing the samples. Glucose, total cholesterol, triglyceride, HDL cholesterol levels were analysed using an enzymatic method (Synchron LX20 analyzer, Beckman-Coulter, High Wycombe, UK). Friedewald equation was used to determine LDL cholesterol levels ([LDL-chol] = [Total chol] - [HDL-chol] - [TG]/2.2). Serum insulin was measured by competitive chemiluminescent immunoassay (DPC Immulite 2000 analyzer, Euro/DPC, Lanberis, UK) where coefficient of variation was 8% with an analytical sensitivity of 2μU/ml without cross-reactivity with proinsulin. Insulin resistance (IR) was calculated using the Homeostasis Model Assessment (HOMA) calculation (HOMA-IR=[insulin x glucose] / 22.5) (76). HbA1c was measured using Menarini HA-8160 analysers (Menarini Diagnostics Limited). TSH assays were performed on AxSYM ultrasensitive hTSH11 Assay (normal 0.49 – 4.67 mIU/l), fT4 assays were performed on AxSYM FT4 Assay (normal 9-24 pmol/l; Abbott Diagnostics Division, U.K.). Soluble endothelial function markers were measured by commercially available quantitative ELISA (R&D Systems, USA) assays according to the manufacturer's instructions.

3.2.5. Statistical analysis

The sample size calculation was based on the effect of soy phytoestrogen in postmenopausal women with diabetes (411) and was performed using N-Query Advisor 5.0 (Statistical Solutions, Cork, Ireland). Powered specifically for HOMA, the minimum difference worth detecting/observed difference was 1.0, estimated within group SD was 0.9; therefore, for 80% power and a significance level of 5%, a sample size of 10 per group was calculated.

Results are expressed as mean ± SD where applicable. Differences between baseline and corresponding 2-months data were assessed using Student’s paired t-test if they followed the normal distribution. The Wilcoxon’s signed-rank test was used if the data violated from the Gaussian distribution. Distribution histograms and results of the Kolmogorov-Smirnov test are attached in Appendix I. Correlation was calculated using Pearson’s correlation. Statistical analysis was performed.
using SPSS 19.0.0 (IBM Corp., New York, NY) and statistical significance was defined as (p<0.05).

### 3.3. Results

#### 3.3.1. Baseline characteristics

Sixty of the seventy patients who were randomised completed the trial (Figure 20). Baseline patient characteristics were comparable between the groups (Table 10).

<table>
<thead>
<tr>
<th></th>
<th>SPI (n=13)</th>
<th>SP (n=13)</th>
<th>SPC (n=12)</th>
<th>SPIC (n=11)</th>
<th>P (n=11)</th>
<th>Overall (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>11,2</td>
<td>7,6</td>
<td>10,2</td>
<td>10,1</td>
<td>9,2</td>
<td>47, 13</td>
</tr>
<tr>
<td>(male, female)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td>63.46±6.33</td>
<td>66.77±8.35</td>
<td>64.42±9.53</td>
<td>66.91±10.28</td>
<td>61.82±7.93</td>
<td>64.7±8.46</td>
</tr>
<tr>
<td>Diabetes control</td>
<td>4, 9</td>
<td>4, 9</td>
<td>5, 7</td>
<td>7, 4</td>
<td>4, 7</td>
<td>24, 36</td>
</tr>
<tr>
<td>(diet, metformin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid treatment</td>
<td>8, 5</td>
<td>11, 2</td>
<td>10,2</td>
<td>5, 6</td>
<td>9, 2</td>
<td>43, 17</td>
</tr>
<tr>
<td>(statin, no statin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>4.96±4.2</td>
<td>3.81±3.16</td>
<td>4.98±4.30</td>
<td>5.14±4.48</td>
<td>3.99±3.96</td>
<td>4.57±3.93</td>
</tr>
</tbody>
</table>


**Table 10. Patient groups characteristics**

#### 3.3.2. Cardiovascular markers, lipids and glycaemic control

HbA1c fell in the SPI group [6.42 ± 0.53% (46.77±5.90 mmol/mol) vs. 6.23 ± 0.58% (45.38±6.21 mmol/mol), p=0.02] while there was no significant change in fasting glucose, homeostatic model assessment of insulin resistance (HOMA-IR),
lipids or blood pressure for any group. There was a trend towards decrease in cholesterol and LDL levels in the SPI group (cholesterol 4.21 [3.65, 5.12] mmol/l vs. 3.80 [3.40, 4.90] mmol/l, p=0.10; LDL 2.38 ± 1.19 mmol/l vs. 2.23 ± 1.00 mmol/l, p=0.09). There was a significant improvement in fasting insulin level in the SP group (19.20 ± 9.42 mIU/l vs. 15.75 ± 7.30 mIU/l, p=0.05). BMI changes that were small but statistically significantly changed in the P and SP groups (SP group 30.37 [26.91, 35.30] kg/m2 vs. 30.51 [26.59, 35.24] kg/m2, p=0.03; P group 31.31 [28.83, 43.21] kg/m2 vs. 30.45 [28.43, 42.67] kg/m2, p=0.01) during the study period (Table 11, 12).

3.3.3. Endothelial function

There was no significant change in endothelial function, measured by the levels of soluble endothelial markers vWF, E-selectin, P-selectin, ICAM-1 and VCAM-1 (Table 13) in any of the groups.

3.3.4. Thyroid function

There was no change in thyroid function during the two months study period in any of the groups (Table 14).
### Table 11. Study results. Anthropometric data and lipids. Results are presented as mean ± SD or median (25th, 75th centiles).

*: paired t-test, otherwise Wilcoxon-signed rank test

** (CI) Confidence interval for parametric tests; (N) negative rank for non-parametric tests

<table>
<thead>
<tr>
<th></th>
<th>Soy + isoflavones</th>
<th>Soy protein (no isoflavones)</th>
<th>Cocoa + soy protein (no isoflavones)</th>
<th>Cocoa + soy + isoflavones</th>
<th>Placebo (casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 months</td>
<td>2 months</td>
<td>p</td>
<td>CI (N(Z)**</td>
<td>0 months</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>93.56 ± 1.90</td>
<td>93.38 ± 1.90</td>
<td>0.74</td>
<td>-0.56 to 1.30</td>
<td>89.26 ± 1.26</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.72 (27.38, 28.66)</td>
<td>28.76 (27.45, 33.94)</td>
<td>0.51</td>
<td>30.37 (26.91, 35.30)</td>
<td>30.51 (26.59, 35.24)</td>
</tr>
<tr>
<td>Waist/Hip Ratio*</td>
<td>0.96 ± 0.05</td>
<td>0.93 ± 0.12</td>
<td>0.17</td>
<td>-0.04 to 0.11</td>
<td>0.97 ± 0.11</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>144.00 (122.50, 149.50)</td>
<td>138.00 (115.00, 151.50)</td>
<td>0.91</td>
<td>137.00 (118.00, 160.00)</td>
<td>134.00 (128.00, 148.00)</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>77.54 ± 10.14</td>
<td>81.69 ± 10.50</td>
<td>0.10</td>
<td>-9.26 to 9.95</td>
<td>76.77 ± 4.11</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.21 (3.65, 5.12)</td>
<td>3.80 (3.40, 4.90)</td>
<td>0.10</td>
<td>4.18 (3.35, 4.35)</td>
<td>4.30 (3.65, 4.78)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.30 (1.00, 1.80)</td>
<td>1.30 (0.85, 1.75)</td>
<td>0.91</td>
<td>1.50 (1.00, 1.50)</td>
<td>1.10 (1.00, 1.70)</td>
</tr>
<tr>
<td>HDL* (mmol/L)</td>
<td>1.24 ± 0.31</td>
<td>1.18 ± 0.31</td>
<td>0.19</td>
<td>-0.04 to 0.17</td>
<td>1.2 ± 0.34</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.30 ± 2.19</td>
<td>2.23 ± 1.00</td>
<td>0.09</td>
<td>-0.05 to 0.58</td>
<td>3.93 ± 0.56</td>
</tr>
<tr>
<td>Chol/HDL Ratio*</td>
<td>1.74 ± 0.62</td>
<td>1.09 ± 0.99</td>
<td>0.37</td>
<td>0.17 to 0.42</td>
<td>1.41 ± 0.90</td>
</tr>
</tbody>
</table>

BMI – body mass index, BP – blood pressure, HDL – high density lipoprotein, LDL – low density lipoprotein, Chol/HDL ratio – cholesterol/HDL ratio

*pairwise t-test, otherwise Wilcoxon-signed rank test

** CI (N) Confidence interval for parametric tests; (N) negative rank for non-parametric tests
<table>
<thead>
<tr>
<th></th>
<th>Soy + isoflavones</th>
<th>Soy protein (no isoflavones)</th>
<th>Cocoa + soy protein (no isoflavones)</th>
<th>Cocoa + soy + isoflavones</th>
<th>Placebo (casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 months</td>
<td>2 months</td>
<td>p</td>
<td>0 months</td>
<td>2 months</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.42 ± 0.53</td>
<td>6.23 ± 0.58</td>
<td>0.03</td>
<td>0.02 to 0.23</td>
<td>0.62 ± 0.57</td>
</tr>
<tr>
<td>(mmol/mol)</td>
<td>46.77 ± 5.90</td>
<td>45.38 ± 6.21</td>
<td>0.02</td>
<td>0.24 to 2,53</td>
<td>44.69 ± 6.17</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>5.60 (5.90, 6.0)</td>
<td>5.80 (6.25, 7.55)</td>
<td>0.09</td>
<td>3 (-1.69)</td>
<td>5.15 (5.43, 6.90)</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>14.20 (11.75, 21.10)</td>
<td>18.60 (13.15, 32.30)</td>
<td>0.06</td>
<td>5 (-1.95)</td>
<td>17.50 (12.85, 23.60)</td>
</tr>
<tr>
<td>Insulin (mIU/l)</td>
<td>5.46 (2.91, 7.36)</td>
<td>5.64 (3.63, 9.26)</td>
<td>0.06</td>
<td>4 (-1.92)</td>
<td>4.70 (3.33, 6.40)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.77 (0.50, 0.96)</td>
<td>0.87 (0.73, 1.10)</td>
<td>0.12</td>
<td>-0.01 to 0.04</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.00 (0.00, 0.00)</td>
<td>0.00 (0.00, 0.00)</td>
<td>0.00</td>
<td>-0.01 to 0.01</td>
<td>0.00 ± 0.01</td>
</tr>
</tbody>
</table>

HbA1c – Haemoglobin A1c, HOMA-IR – Homeostatic model assessment of insulin resistance, QUICKI – Quantitative insulin sensitivity check index

Table 12. Study results. Glycaemic control and insulin resistance. Results are presented as mean ± SD or median (25th, 75th centiles).

*: paired t-test, otherwise Wilcoxon-signed rank test

** (CI) Confidence interval for parametric tests; (N) negative rank for non-parametric tests
<table>
<thead>
<tr>
<th></th>
<th>Soy + isoflavones</th>
<th>Soy protein (no isoflavones)</th>
<th>Cocoa + soy protein (no isoflavones)</th>
<th>Cocoa + soy + isoflavones</th>
<th>Placebo (casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>CI/N(z)**</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>vWF* (μg/ml)</td>
<td>12.73 ± 5.73</td>
<td>11.90 ± 3.76</td>
<td>0.31</td>
<td>0.67 to 2.54</td>
<td>13.98 ± 4.96</td>
</tr>
<tr>
<td>E-selectin (ng/ml)</td>
<td>6.98 (4.66, 8.65)</td>
<td>6.42 (3.39, 8.07)</td>
<td>0.17</td>
<td>-1.36</td>
<td>2.88 (1.78, 5.67)</td>
</tr>
<tr>
<td>P-selectin (ng/ml)</td>
<td>32.61 (21.94, 41.76)</td>
<td>29.68 (23.30, 34.66)</td>
<td>0.15</td>
<td>-1.43</td>
<td>24.94 (19.40, 28.22)</td>
</tr>
<tr>
<td>ICAM-1* (ng/ml)</td>
<td>197.98 ± 60.19</td>
<td>196 ± 70.27</td>
<td>0.80</td>
<td>-1.89 to 18.04</td>
<td>156.87 ± 30.65</td>
</tr>
<tr>
<td>VCAM-1-1 (ng/ml)</td>
<td>389.80 (319.60, 459.15)</td>
<td>371.90 (340.90, 417.70)</td>
<td>0.13</td>
<td>-1.50</td>
<td>383.500 (307.05, 481.50)</td>
</tr>
</tbody>
</table>

vWF – von Willebrand Factor, ICAM-1 – Intercellular Adhesion Molecule-1, VCAM-1 – Vascular Cellular Adhesion Molecule – 1

Table 13. Endothelial markers. Results are presented as mean ± SD or median (25th, 75th centiles).

*: paired t-test, otherwise Wilcoxon-signed rank test

** (CI) Confidence interval for parametric tests; (N) negative rank for non-parametric tests
### Soy + isoflavones

<table>
<thead>
<tr>
<th></th>
<th>Soy protein (no isoflavones)</th>
<th>Cocoa + soy protein (no isoflavones)</th>
<th>Cocoa + soy + isoflavones</th>
<th>Placebo (casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before</strong></td>
<td>1.66 (1.48, 2.31)</td>
<td>1.67 (1.21, 2.05)</td>
<td>1.67 (1.38, 2.41)</td>
<td>1.55 (1.32, 3.20)</td>
</tr>
<tr>
<td><strong>After</strong></td>
<td>1.08 (-1.78)</td>
<td>1.72 (1.30, 2.29)</td>
<td>1.87 (1.38, 2.41)</td>
<td>1.72 (1.33, 3.30)</td>
</tr>
<tr>
<td><strong>p</strong></td>
<td>0.08</td>
<td>0.08</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>N(2)</strong></td>
<td>9</td>
<td>10</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

### TSH

<table>
<thead>
<tr>
<th></th>
<th><strong>Before</strong></th>
<th><strong>After</strong></th>
<th><strong>p</strong></th>
<th><strong>N(2)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH (mU/l)</td>
<td>2.42 (1.70, 2.72)</td>
<td>1.97 (1.66, 2.76)</td>
<td>0.31</td>
<td>9</td>
</tr>
<tr>
<td>T3 (pmol/l)</td>
<td>4.40 (4.05, 4.60)</td>
<td>4.00 (4.13, 4.50)</td>
<td>0.08</td>
<td>4</td>
</tr>
<tr>
<td>T4 (pmol/l)</td>
<td>12.00 (11.50, 13.50)</td>
<td>12.50 (12.00, 13.00)</td>
<td>0.76</td>
<td>4</td>
</tr>
</tbody>
</table>

**Wilcoxon-signed rank test**

### 3.3.5. Confounding factors

There was no correlation between baseline weight and percentage change in HbA1c in any of the groups (p= 0.11 to 0.90, z= -0.61 to 0.039), or cholesterol (p= 0.21 to 0.95, z= -0.38 to 0.16) or fasting glucose (p= 0.39 to 0.83, z= -0.12 to 0.26). The duration of diabetes did not affect the percentage change in HbA1c (p= 0.06 to 0.99, z= -0.31 to 0.7), cholesterol level (p= 0.29 to 0.81, z= -0.35 to 0.18) or fasting glucose (p= 0.40 to 0.72, z= -0.28 to 0.16) in any of the groups.
3.4. Discussion

In this short term study there was a small, but statistically significant improvement in HbA1c amongst type 2 diabetes patients taking a soy-isoflavone combination while there was no change observed in those consuming either soy protein alone or placebo. The improvement in HbA1c can also be considered as clinically significant, as HbA1c is a measure in long-term glycaemia. The reason for this improvement is unclear since the fasting glucose, insulin and insulin resistance as expressed by HOMA-IR or QUICKI did not change. It implies that the soy-isoflavone combination may be reducing postprandial hyperglycaemia when consuming over longer time periods and indeed others have reported that postprandial hyperglycaemia can be improved with soy consumption (302, 307) although the authors of these studies speculated that the mechanism was through the fibre content in the soy delaying gastric emptying. This is the first study to compare soy protein alone to a soy-isoflavone preparation and suggests that, for whatever reason, the latter combination is required to exert a hypoglycaemic effect.

It has been reported that two-hour postprandial glucose concentrations are more strongly associated with CVD risk factors than fasting glucose levels in a soy consuming group (412) but not in another study using a different soy preparation and isoflavone concentration (31). This highlights the major limitation in comparing different studies that have been done in differing populations, with differing soy preparations and isoflavone content and bioavailability. For this reason a defined preparation was used for each arm of this study to ensure reproducibility.

We have previously shown that 132mg of isoflavones alone for 3 months in a crossover study in postmenopausal women with type 2 diabetic had no effect on glycaemic control or any cardiovascular parameter measured (22). However, when that same dose of isoflavone was given in combination with 30g of soy resulted in significant improvement in HbA1c, fasting insulin, HOMA-IR, cholesterol and LDL levels (411). Together with the data in this study, this suggests that the soy protein and the isoflavone component are both inactive/ineffective when given in isolation.
and that the protein/isoflavones combination is critical with the isoflavones embedded in the soy protein matrix.

In a randomized parallel study with obese patients with type 2 diabetes, it was shown that a soy-based diet improved weight and glycaemic control more significantly than a control diet (413). In our short-term study weight changed minimally, though significantly in the P and SP groups, but the study was not powered to look at these endpoints.

This study was of short duration to focus on glycaemic control changes and therefore not designed to look specifically at cardiovascular risk indices. No changes in serum lipid levels were seen, in accord with a meta-analysis that suggested that the magnitude of the favourable change in serum cholesterol and LDL levels may be affected by the baseline degree of hypercholesterolemia (305). In the present study the participants had well controlled lipid levels at the time of their enrolment into the study and most of them were on established lipid-lowering treatment that may have precluded any lipid changes. In addition, we used a low isoflavone content (32 mg daily) while others report higher isoflavones doses used (50mg – 192 mg daily) that may have contributed to this finding.

A recent study showed improvement in insulin resistance measured by HOMA-IR and QUICKI, fasting glucose and insulin, fasting triglycerides, LDL cholesterol, HDL/LDL and total cholesterol:HDL ratios in postmenopausal women who consumed a food product with isoflavones and flavanols over a one-year period, compared to placebo (334). However the study used soy protein, isoflavone and polyphenol free placebo so the degree to which the isoflavones or polyphenols contributed to the overall beneficial effects is not known. In our shorter duration study period we did not find these effects, therefore the study by Curtis et al. may have reported decreased effectiveness as our study suggests that when cocoa is added to soy isoflavones, the favourable effect in glycaemic control may be lost.

A report indicated that in the United Kingdom, the geometric mean TSH concentration adjusted for age and BMI, is higher in vegans than in omnivores
(414) however other studies did not support this finding (415). In our centre a recent study showed that females with subclinical hypothyroidism are at higher risk to develop overt hypothyroidism when consuming high isoflavone content food supplement (416) while cardiovascular risk markers improved. We are not aware of studies investigating the effect of cocoa polyphenols on thyroid function. In our study we did not find change in thyroid hormone levels and this is in accordance with previous results i.e. thyroid function may be altered by soy isoflavones on the basis of subclinical biochemical changes in the thyroid hormone levels.

In our study, endothelial function measured by soluble endothelial markers did not change significantly however there was a trend in the SI group toward decrease in the level of vWF, E-selectin, P-selectin and VCAM-1 when compared to placebo.

In conclusion, a combination of isoflavones with soy protein improved glycaemic control in comparison to isoflavone free soy or placebo, suggesting that it is the combination of isoflavones with soy protein that is required to be biologically effective, although the mechanism of action is no clear.
4. CHAPTER Effects of isolated soy protein versus combined soy protein and isoflavones on postprandial glycaemia and insulin resistance in patients with type 2 diabetes.

4.1. Overview

Soy food products have been shown to improve endothelial dysfunction, lipid profile and hyperglycaemia in type 2 diabetes, as detailed in previous chapters. In a few studies, there was an improvement in the lipid profile (301) and a reduction in HbA1c in patients with type 2 diabetes, through improvement in insulin resistance and either fasting glucose or postprandial hyperglycaemia (307, 417).

Postprandial hyperglycaemia is also a cardiovascular risk (CVR) marker and has a linear relationship with cardiovascular mortality. Improvement in postprandial hyperglycaemia reduces the progression of atherosclerosis and the occurrence of cardiovascular events (418).

We found a significant reduction in HbA1c levels in patients with type 2 diabetes after two months dietary intervention with soy products, as detailed in chapter 3. However, there were no changes in fasting glucose or insulin resistance. This suggests that an improvement in the postprandial hyperglycaemia may be involved in the effect of soy products.

There is very limited literature assessing the postprandial metabolic effects of acute soy ingestion in humans (419). In a study conducted by Mahalko et al, patients with type 2 diabetes consumed a diet with additional fiber intake for 4 weeks (307). Patients were divided into 4 groups based on the source of the additional fiber intake: control low fiber content bread, soy hulls, corn bran or dehydrated powdered apple. After the 4 weeks intervention period, there was a significant improvement in the area under the curve for glucose during a meal test after 52g soy hull fibre intake daily, which was not shown when fiber intake was only 26g daily. The isoflavone content of the soy hull was not reported.
Animal studies seem to suggest a beneficial effect of soy isoflavones on postprandial glycaemia (420). In a study with non-diabetic mice dietary isoflavone intake prolonged lifespan and improved glucose tolerance (394).

A study conducted by Jee-Youn Shim et al. demonstrated a favorable effect of soy isoflavones on glucose tolerance in diabetic rats (420). In their experiment diabetic rats were fed with four different soy isoflavone content diets. The first group was the diabetic control group, consuming 0 mg/body weight kg isoflavones for 7 weeks. Other groups were fed with diets containing 0.5-3mg-30mg/body weight kg isoflavones that was equivalent to the average and maximum daily isoflavone intake of an adult Korean woman, while the highest concentration of isoflavones represented a ten-time fold higher daily intake than the maximum daily isoflavone consumption of an adult Korean woman. Glucose tolerance was measured following a glucose load at baseline and at the end of the study. The group with 3mg/body weight kg daily isoflavone intake showed significantly lower fasting and postprandial blood glucose levels at all time points measured (30, 60, 120 and 180 minutes after a single glucose load). In the group with the highest daily isoflavone intake the maximum glucose levels were observed at 180 minutes, while in the other two groups on isoflavone diets the peak glucose concentrations were observed 120 minutes after the glucose load suggesting prolonged glucose absorption after dietary intervention with high isoflavone content.

Hsu et al did not find significant changes in plasma glucose or insulin levels after feeding different isoflavone concentration diets to diabetic animals (421). Lee et al found significantly decreased glucose and insulin levels during an oral glucose tolerance test in diabetic rats after a period of dietary intervention with soy protein and isoflavones (422). The benefits of soy protein on glucose tolerance and insulin sensitivity in normal rats was also reported (395).

It seems that soy consumption may carry beneficial effect to improve glycaemic control however the underlying mechanism is not yet clear. It is also not understood that if the beneficial effect is present, whether it is due to the soy
protein content or the isoflavones have an added role in improving glycaemia in the postprandial period.

The aim of our cross-over study was to compare the glycaemic response to an oral glucose tolerance test after consuming different soy bars containing either soy protein alone or soy protein and isoflavones. The study was undertaken to define the potential mechanism behind the improvement in HbA1c following dietary intervention with soy and isoflavones in humans, as reported in Chapter 3.

4.2. Research design and methods

4.2.1. Patients

Six patients with type 2 diabetes on metformin aged 65-73 years entered the study after recruitment through local media advertisement. All six patients were eligible to participate in the study and completed the study visits between April 2013 and December 2013.

The main inclusion criteria were diagnosis of type 2 diabetes based on the WHO guidelines, stable medication for 3 months prior to entry into the study and age between 45-75 years. Patients with food allergy or significant medical problems were not recruited. Smokers, vegans or vegetarians were also excluded from the study. Antibiotic treatment within the previous 3 months was an exclusion criteria and medication was not changed during the study period. Women were postmenopausal and none were on oestrogen replacement therapy.

The study was approved by the Humber Bridge Regional Ethics Committee (12/YH/0016), and all participants provided written informed consent. The study was conducted according to the relevant legislation, ICH GCP, and the Declaration of Helsinki.
4.2.2. Study protocol

This was a placebo-controlled, crossover study. On the first visit patients consumed a soy bar containing soy protein (7.5g) only (SP), while on the second occasion they consumed a soy bar containing soy protein (7.5g) + isoflavones (66mg) (SPI). 30 minutes after the consumption of the study bars, an oral glucose tolerance test was performed with 75g glucose loading. Bloods were taken for glucose and insulin as per Figure 22.

At the beginning of each study visit, an intravenous cannula was inserted to make frequent blood sampling easier and more convenient for the participants. Study procedures then followed the sampling protocol detailed in Figure 22.

Figure 22. Study visit procedures. 0 and 30 minutes data was used to show that 30 minutes can be counted as baseline, i.e. the start of the Oral Glucose Tolerance Test. Blood sampling was taken place at 0, 30, 60, 90, 150, 270 minutes into the experiment. 150 minutes time point marked the end of the OGTT.
4.2.3. Intervention

One snack bar containing a base of 7.5g of 70% isolated soy protein powder (Solcon F; CHS Ashdod, Israel) with or without added isoflavones (Solgen 4016mg per bar, 32mg in total daily; CHS Ashdod, Israel, Israel) was provided after taking the first blood sample. The isolated soy powder had the isoflavones removed by repeat 95% alcohol extraction (Dishman Ltd, UK) to give an isoflavone content of less than 300 parts per billion (Assayed by FERA, Sand Hutton, UK). The active bar contained 66mg of added isoflavones. The bars were matched for taste and macronutrient content (Table 4.1.) (Halo Foods Ltd, Swansea, UK). Labelling of the trial supplies were done by Essential Nutrition, Brough, UK. The comparator bar was chosen to be the bar including soy protein only, as we have shown no improvement in glycaemia over a two months dietary intervention, as detailed in Chapter 3.

<table>
<thead>
<tr>
<th>Nutrient analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>KJ</td>
</tr>
<tr>
<td>337.0</td>
</tr>
<tr>
<td>1246.8</td>
</tr>
</tbody>
</table>

Ingredients: Soya Powder with or without isoflavones, Sultanas, Dates, Apple juice concentrate, Apricots, Apple Paste, Glucose Syrup, Palm Oil, Glycerine, Strawberry Juice Concentrate, Natural Strawberry Flavour

kJ – kilojoule, kcal – kilocalorie, carbs - carbohydrates

Table 15. Macronutrient content of the study bars. Bars were matched for taste and macronutrient content.

4.2.4. Study measurements

Biochemical analysis of the blood samples for glucose and insulin were performed as detailed in other chapters.

Area Under the Curves (AUC) were calculated for glucose, insulin and HOMA-IR using Tai’s formula (423). HOMA-IR was calculated using HOMA-IR=insulin*glucose/22.5 formula (76). HOMA-IR is just for illustration only, as
HOMA-IR was only applicable at time point 0 and 30, due to the non-fasting state at the other time points.

HOMA-IR does not provide information about beta cell function. We used early insulin response to glucose (AIRg) and Oral Disposition Index (ODI) to measure the ability of beta cells to respond to rising glucose concentrations following a glucose load (76). To evaluate beta-cell function, the oral disposition index was calculated from early insulin response to glucose (AIRg) and Insulin Sensitivity (IS), using the following formula: $\text{ODI} = \text{AIRg} \times \text{IS}$, where $\text{IS} = 1/\text{fasting insulin concentration}$ (424). AIRg was determined using the 30-minute time point as baseline, providing that the OGTT started at this specified time point. Subsequently, 30 to 60, 30 to 90, 30 to 150 and 30 to 270 minutes AIRg values were calculated as the ratio of the mean increment in insulin and glucose. For example, regarding the time period between 30 and 60 minutes then $\text{AIRg}_{\text{30-60 minutes}} = (\Delta \text{Insulin}_{\text{30-60}}/\Delta \text{Glucose}_{\text{30-60}})$. The ODI was used as a measure of compensatory beta cell function in response to insulin resistance (425).

**4.3. Results**

Results are expressed as mean ± SD (p, CI) or medians (25th, 75th percentiles) [z (N)] where N is Negative Rank. Differences were assessed using Student’s Paired T-test when the distribution was normal and Related-Samples Wilcoxon Signed Rank non-parametric test for variables that violated from the Gaussian distribution. Distribution histograms and normality testing results using Shapiro-Wilk test are attached in the Appendix. Statistical analysis was performed using SPSS 19.0.0 (IBM Corp., New York, NY) and statistical significance was defined as (p<0.05).

**4.3.1. Baseline patient characteristics**

Patient characteristics are summarized in Table 16. All patients were on established lipid therapy.
Gender (male, female) | 5, 1
---|---
Age (years) | 70 ± 3.22
Duration of diabetes (years) | 7.58 ± 3.26
HbA1c (mmol/mol) | 42.67 ± 3.27
Weight (kg) | 94.33 ± 15.27
BMI (kg/m²) | 31.50 ± 6.32

Table 16. Baseline patient characteristics. Data are expressed as mean ± SD where applicable.

### 4.3.2. Glycaemic control and insulin resistance Area Under the Curves

Glucose, insulin and HOMA-IR AUC results are shown in Table 17 and Figure 23.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Active</th>
<th>p</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)*</td>
<td>2764 ± 716</td>
<td>2776 ± 652.30</td>
<td>0.75</td>
<td>-311.35 to 285.60</td>
</tr>
<tr>
<td>Insulin (μIU/ml)*</td>
<td>15075.10 ± 8534.1</td>
<td>12948.50 ± 8751.62</td>
<td>0.46</td>
<td>-2672.96 to 6926.06</td>
</tr>
<tr>
<td>HOMA-IR*</td>
<td>2764 ± 716</td>
<td>2776 ± 652.30</td>
<td>0.46</td>
<td>-2030.96 to 4942.13</td>
</tr>
</tbody>
</table>

*paired t-test

Table 17. AUC for glycaemic markers and lipids.
Glucose response during the study visits [x axis – time in minutes, y axis – glucose concentration (mmol/l)]

Insulin response during the study visits [x axis – time in minutes, y axis – insulin concentration (μIU/ml)]

Change in HOMA-IR during the study visits [x axis – time in minutes, y axis – insulin resistance measured by HOMA-IR)]

Figure 23. Insulin, glucose and HOMA-IR values during the study visits. Results are shown as mean ± SD. Error bars are also displayed. Area Under the Curve values were not different between the two interventions.
There was no statistical significance in glucose, insulin or HOMA-IR AUCs between the placebo and active arms using Related-Samples Wilcoxon Signed Rank parametric test. Cholesterol and triglyceride AUC values were also measured and there was no difference between the placebo and active arm of the study (data not shown).

Peak glucose, insulin and HOMA-IR values are presented in Table 18. along with the time points of peak concentrations.

<table>
<thead>
<tr>
<th>Peak levels of glucose, insulin and HOMA-IR</th>
<th>Placebo</th>
<th>Active</th>
<th>CI/z (N)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak glucose value (mmol/l)*</td>
<td>14.62±3.61</td>
<td>14.6±3.30</td>
<td>-1.99 to 2.02</td>
<td>0.98</td>
</tr>
<tr>
<td>Peak insulin value (microlU/l)*</td>
<td>103.3±57.30</td>
<td>86.33±64.91</td>
<td>-16.37 to 50.30</td>
<td>0.25</td>
</tr>
<tr>
<td>Peak HOMA-IR value**</td>
<td>77.79 (23.51, 104.76)</td>
<td>27.80 (18.96, 101.35)</td>
<td>-1.15 (4)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

| Time points of peak levels | | |
|---------------------------|---------|--------|----------|---|
| Time of glucose peak (min)** | 90.00 (82.50, 150.00) | 90.00 (75.00, 105.00) | -1.34 (2) | 0.20 |
| Time of insulin peak (min)** | 120.00 (90.00, 150.00) | 150.00 (90.00, 150.00) | -1.00 (0) | 0.36 |
| Time of HOMA-IR peak (min)** | 120.00 (90.00, 150.00) | 150.00 (150.00, 150.00) | -1.73 (0) | 0.08 |

*paired t-test, **Wilcoxon-Signed Rank test

**Table 18. Peak levels and time points of insulin, glucose and HOMA-IR.**

There was no significant difference in peak values of glucose, insulin or HOMA-IR between the study arms however peak glucose, insulin and HOMA-IR was lower after the consumption of the soy bar enriched with isoflavones compared to the placebo bar that contain soy protein only.

The occurrence of peak HOMA-IR was significantly later during the visit involved the consumption of the soy bar enriched with isoflavones however the significance of this finding is not yet clear considering that HOMA-IR is only applicable in the fasting state.
4.3.3. Early insulin response to glucose (AIRg) and Oral Disposition Index (ODI)

AIRg and ODI results are summarized in Table 19.

<table>
<thead>
<tr>
<th></th>
<th>AIRg</th>
<th></th>
<th>ODI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CI/z (N)</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>8246.88 (-9250.00, 9678.57)</td>
<td>0.30**</td>
<td>381 ± 1207</td>
<td>-89 ± 913</td>
</tr>
<tr>
<td>Active</td>
<td>-41.37 (-9987.50, 7839.13)</td>
<td>0.94 (4)</td>
<td>-410.65 to 1349.68</td>
<td>0.17**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>0-30**</td>
<td></td>
<td></td>
<td>0.60 (0.41, 0.87)</td>
<td>0.30 (-0.02, 0.70)</td>
</tr>
<tr>
<td>30-60*</td>
<td>9206 ± 3304</td>
<td>0.03*</td>
<td>0.66 (0.42, 1.29)</td>
<td>0.40 (0.14, 0.94)</td>
</tr>
<tr>
<td>30-90*</td>
<td>10606 ± 4257</td>
<td>0.03*</td>
<td>-0.31 (3)</td>
<td>0.75**</td>
</tr>
</tbody>
</table>

*paired t-test, ** Wilcoxon-Signed Rank test

Table 19. AIRg and ODI during the OGTT following the consumption of soy protein bar only (placebo), or soy protein bar enriched with 66mg additional isoflavones (active)

0-30 minutes data are included for illustration purposes only, to prove that the consumption of the soy bar did not affect the results and that the 30 minute values were appropriate to be used as “fasting” baseline results when analyzing OGTT data.

There was a significant reduction in AIRg between time points 30-60 and 30-90 when consuming the isoflavone-enriched soy bar compared to soy protein only. This indicates that there was a relative reduction in insulin secretion during the postprandial rise in glucose concentration. AIRg was also lower between the other analysed time points after consuming the soy bar with additional isoflavone content however the difference was not clinically significant (data not shown).

There was no statistically significant difference in ODI. However, ODI values trended lower during the OGTT following the consumption of the isoflavone-enriched soy bar, suggesting that beta cells did not respond appropriately to the larger insulin requirement during the post-glucose load.
4.4. Discussion

Soy products are assumed to carry general benefits to ones health. Soy consumption has been proved to improve lipid profile and glycaemic state in type 2 diabetes (426). Conflicting data suggest that the improvement in overall glycaemic control as measured by HbA1c may be due to an improvement in postprandial glycaemia. However to date evidence from human studies are limited.

In this preliminary study, we investigated the acute effect of two different soy bars (soy protein only [placebo] vs. soy protein enriched with 66mg additional isoflavones [active]) on a postprandial period following a 75g oral glucose load (OGTT).

In a recent randomized controlled study in humans, the beneficial effect of polyphenols in postprandial lipid levels were shown after a 8-week dietary intervention in subjects with high cardiovascular risk (427). It was shown that the 8 week intervention with high polyphenol content diet reduced postprandial triglyceride and VLDL total area under the curve in plasma. They also showed that urinary 8-isoprostane, which is a reliable measure of oxidative stress, significantly decreased and lipoprotein changes significantly correlated with changes in 8-isoprostane levels.

Munoz et al (428) have also reported an improvement in oral glucose tolerance after a 30 days of dietary intervention with soy hulls. The underlying mechanism is not yet clear but has been linked to the longer GI transit time and the increased insulin binding activity of monocytes in patients with diabetes on a high carbohydrate, fiber and low fat diet (429, 430).

These effects could be explained by slower gastrointestinal (GI) transit time in both animals and humans. The addition of soy isoflavones to the normal diet may prolong the GI transit time and improve the postprandial blood glucose levels. It is not yet clear if different doses of isoflavones alter the glucose tolerance differently.
and whether this effect is attributed to the presence of soy protein or soy isoflavones.

In our study we used soy bars with similar macronutrients and fibre content, so any changes seen cannot be the result of any different GI transit times. We did not find a significant difference in glucose or insulin AUC values between the two dietary interventions. However, there was a delay in glucose absorption as indicated by the lower AIRg in the active arm while overall glucose absorption was unchanged. Our study assessed the acute effect of different soy preparations, while other trials compared postprandial response after longer period of dietary interventions with soy products. This suggests that longer periods of ingestion may be required to achieve a significant improvement in postprandial response.
5. CHAPTER The effect of simulated flight environment with hypoxia and low humidity on endothelial function in healthy volunteers and patients with type 2 diabetes

5.1. Overview

5.1.1. Cardiovascular risk in type 2 diabetes

Type 2 Diabetes Mellitus is a complex disorder that is associated with multiple metabolic and cellular alterations including atherogenic dyslipidemia, glucose intolerance and a prothrombotic state represented by altered coagulation, fibrinolysis and platelet function. These all lead to the increased risk of cardiovascular events in patients with diabetes (431-435). This triad of factors predisposes patients with T2DM to accelerated atherothrombosis, a chronic inflammatory process in which blood platelets play a central role along with the vascular endothelium, which acts as a dynamic interface between the circulation and the arterial wall (239). Atherothrombosis is characterised by endothelial dysfunction in which endothelial cell function is compromised, which in turn facilitates altered activity of platelets, neutrophils and the underlying VSMC (436). Elevated glucose and insulin levels (as seen in type 2 diabetes) increase tissue factor procoagulant activity (221). However, endothelial function is difficult to quantify in clinical practice and difficult to modulate in the research setting in order to investigate therapeutic interventions. As an overall consequence, patients with type 2 diabetes are at an increased risk of morbidity and mortality from cardiovascular events (437, 438).

5.1.2. Air travel

In recent years the number of passengers travelling by aircraft has rapidly increased. In 2010 the National Air Traffic Services (NATS) in the United Kingdom handled 2.1 million flights, involving more than 200 million passengers (439). Every year, over one billion people travel by air and that figure is predicted to
double in the next two decades according to the Civil Aviation Authority (CAA) (440).

The special environmental conditions on an aircraft have several effects on passengers. Aircraft cabins are pressurised to maintain good oxygenation and the cabin altitude is around 8000 ft (2438 m). This results in reduced barometric pressure with a concomitant decrease in the partial pressure of oxygen (PO₂). The percentage of oxygen in the air on board an aircraft is the same as at sea level, but because of the lower atmospheric pressure (equal to 8000 ft), the PO₂ is decreased. Consequently, the driving pressure of the gas exchange in the lungs is also lower, causing hypoxia. While the barometric pressure is 760 mmHg at sea level, with a corresponding partial arterial O₂ pressure (PaO₂) of 98 mmHg, the barometric pressure at 8000 ft will be 565 mmHg with a PaO₂ of about 55 mmHg. Due to the shape of the oxy-haemoglobin dissociation curve, this results in a fall of oxygen saturation to 90% (440). The humidity in an aircraft is also very low (10-20%) compared to the humidity at sea level in an average building (40-50%) (440).

5.1.3. Air travel and venous thromboembolism events

Air travel was shown to increase the risk to develop VTE when risk factors are present. The WHO Research Into Global Hazards of Travel (WRIGHT) project was set up in June 2001 in order to review the scientific evidence concerning air travel and venous thromboembolism events (VTE), to identify gaps in knowledge and develop priority areas for research. The final report was released in June 2007 and concluded that there is an elevated risk of VTE during air travel. In addition, it also concluded that pre-existing risk factors may affect this risk and it was suggested that further research was needed to identify additional factors.

5.1.4. Diabetes and venous thromboembolism events

Several studies confirmed that diabetic patients are more likely to develop venous thromboembolism than healthy individuals (203, 441-443) however other studies
did not suggest this (431, 444, 445). As diabetes is a prothrombotic disease, it may have a role in the possibility of increased risk to develop VTE during air travel.

No studies appear to have been undertaken focusing on the pathophysiological and/or clinical changes that may occur following a commercial airline flight in patients with type 2 diabetes. Indeed, there are no special precautions so far recommended for patients with type 2 diabetes undertaking a commercial airline flight (446) if the diabetes control does not require insulin therapy.

A commercial flight can be simulated in an environmental chamber, by adjusting the chamber settings to replicate the oxygen and humidity level of a cabin during a typical commercial flight (447).

### 5.1.5. Endothelial function

Vascular endothelium plays a major role in maintaining cardiovascular homeostasis as the endothelial cells provide a physical barrier between the lumen and the wall of the blood vessel. It also actively contributes to the homeostasis by a functional role with secreting different mediators that regulate the vascular tone, modify platelet function, coagulation and fibrinolytic system.

Endothelial function is thought to be a key determinant in cardiovascular risk in T2DM, but it is difficult to quantify in clinical practice and difficult to modulate in the research setting in order to investigate therapeutic interventions. A number of methodological approaches have been developed to assess EC dysfunction including endothelium-dependent vasodilatation, or circulating endothelial markers reflects the endothelial function (448).

Other functional measures of endothelial function include the use of the EndoPAT 2000. The EndoPAT 2000 is a Food and Drug Agency (FDA) cleared, reactive hyperaemia-based, reliable, operator independent device for diagnosing endothelial dysfunction (189, 449).
EMPs are vesicles shed by the endothelial cells and consist of cell membrane, cytoplasmic and nuclear elements. EMPs express surface markers reflective to their cell of origin (173). Under normal physiological conditions MPs are constantly shed into the circulation of healthy individuals (372). However, the level of EMPs is elevated in the circulation after damage, activation or apoptosis of the ECs. This phenomenon is also observed in disease states that are associated with vascular dysfunction such as acute coronary syndromes and diabetes (450). Furthermore it has been demonstrated that number of EMPs in circulation in vivo correlate with other indices of EC dysfunction, with the complications of diabetes type 2 (451), and as a result of high-fat meals (452). These observations raise the possibility that plasma EMP levels may be measurable markers of EC dysfunction (160, 173, 174, 453-455).

5.1.6. Platelet function

During a blood clot formation one of the most important steps is platelet activation that is known to be impaired in patients with type 2 diabetes. Platelet function abnormalities such as hypersensitivity of platelets to aggregant factors, hyposensitivity to antiaggregants are present in patients with type 2 diabetes contributing towards an increased platelet activity at locations of endothelial damage (209, 239).

5.1.7. Clotting indices

Fibrin clot formation is the final step in the atherothrombotic process. Platelet activation leads to activation of the coagulation factors and as a final result a fibrin clot is formed. Examining the structure of this clot gives valuable information about the clotting process, it can even predict the predisposition to cardiovascular disease (456). Patients with type 2 diabetes have been shown to have altered clot structure as the clot is more compact, the pore size is smaller, fibre thickness increased along with the number of branch points (229). Clot structure can be assessed by turbidimetric assay in a laboratory environment from plasma (457).
The aim of our pilot study was to determine whether a two-hour duration of hypoxic environment (simulated flight) causes impairment in the endothelial, platelet function and clotting indices, and whether this impairment is greater in patients with type 2 diabetes compared to healthy subjects.

5.2. Research design and methods

5.2.1. Patients

Twenty-seven volunteers with or without type 2 diabetes controlled by metformin or diet only aged 45-75 entered the study after recruitment through routine diabetes clinics and local media advertisement. Six patients failed screening or refused to participate on the ground of other time commitments (medication that affect platelet function [cyclooxygenase-2 (COX-2) inhibitor, n=1; aspirin, n=1], acute phase reaction at screening [n=1], angina [n=1], chronic obstructive pulmonary disease (COPD) [n=1] time commitments [n=1]). One patient finished her participation in the study early due to intolerance of the chamber conditions. Twenty patients completed the study.

The diagnosis of diabetes was made according to the WHO guidelines (409). Diabetic patients were either diet controlled (n=1) or on stable metformin therapy (n=9) for at least three months before study commencement, and no medication was altered during the study period. Women were postmenopausal and not on oestrogen therapy in the preceding 6 months or during study period. Smokers or individuals with claustrophobia were not recruited. Patients were not subjected to a commercial flight within one month prior to the study or during the study period. Concomitant participation in any other interventional medical trial was not allowed.

The study was approved by the Humber Bridge Regional Ethics Committee (12/YH/0016), and all participants provided written informed consent. The conduct of the trial was in accordance with all relevant legislation, ICH GCP and the Declaration of Helsinki.
5.2.2. Study protocol

This was a placebo-controlled clinical trial, where the active intervention was the hypoxic visit to the environmental chamber, while the placebo intervention was the control day. Following informed consent, patients went through a screening process to evaluate their eligibility. Patients were asked to maintain their current level of physical activity.

Patients participated in two visits in the environmental chamber between April 2013 and December 2013, excluding the screening visits.

5.2.3. Intervention

After the screening visit, patients attended the environmental chamber (Design and Manufacture of Environmental Test Chambers, A3897 - SSR60-20H) on two separate occasions, on the premises of the University of Hull, in the Department of Sports, Health and Exercise Science. On the first visit they participated in a control day, when volunteers were asked to spend 2 hours in the environmental chamber at sea level conditions (temperature: 23°C, oxygen concentration 21%, humidity 45%). On the second visit they spent 2 hours at the same time of the day in the chamber in hypoxic conditions, similar to a commercial flight air cabin conditions (temperature: 23°C, oxygen concentration 15%, humidity 15%).

5.2.4. Study measurements

Following informed consent, a physical examination, and blood sampling were undertaken during the screening visit and vital signs were recorded along with anthropometric measurements.

During the visits in the environmental chamber, a baseline and 2-hour EndoPAT and blood sampling were undertaken. EndoPAT and blood sampling was done in a temperature-controlled room prior to entering the chamber, while the 2-hour measurements took place in the chamber to maintain the hypoxic conditions, where applicable.

Blood sampling followed after the EndoPAT test, the control arm was used for
venesection. On each occasion a clean venepuncture was performed, without stasis. Blood samples for platelet function and microparticles were processed immediately, as detailed in Chapter 2. Samples for soluble endothelial markers and clotting experiments were spun down at 3500g for 15 minutes immediately after venepuncture, and then were frozen at -80°C until batch analysis at the end of the study.

Patients arrived fasting to eliminate the postprandial effects, and were provided lunch after the end of the visits in the environmental chamber.

5.2.5. **Statistical analysis**

Results are expressed as mean ± SD or median (25th, 75th centiles) where applicable. Differences between baseline and corresponding 2-hours data were assessed using Student’s paired t-test for variables that showed normal distribution, or Wilcoxon signed rank test for variables that violated from the Gaussian distribution. Between group differences were calculated using unpaired t-test where the data showed normal distribution, or Mann-Whitney U test where the distribution violated from the Gaussian distribution. Normality testing was performed using the Shapiro-Wilk test. Correlation was calculated using Pearson’s correlation test. Statistical analysis was performed using SPSS 19.0.0 (IBM Corp., New York, NY) and statistical significance was defined as (p<0.05). As this was a pilot study, no power analysis was applicable.

5.3. **Methods**

5.3.1. **Clotting indices**

Fibrin formation and clot structure analysis was undertaken using turbidimetric assays as described previously (380). Initial fibrin formation occurs during the “lag” phase, when half-staggered, double-stranded protofibrils are formed (458), and is equivalent to the clotting time. Maximum absorbance is the maximum OD of
the clot, and this is a measure of fibrin network density. Lysis time is the time from full blood clot formation to 50% lysis. This is an indicator of fibrinolytic potential. Lysis area is area under the curve. This is a complex measure of clot formation time, clot density and lysis potential. Higher maximum absorbance, longer lysis time and larger lysis area are associated with increased cardiovascular risk (380, 459-462).

5.3.2. Platelet function

Upon activation of platelets various surface receptors are expressed and degranulation happens. These events can be identified by flow cytometry that is a very sensitive method that can readily detect even small changes in activation of platelets. Platelets can be identified by a pan-platelet marker CD42b from whole blood taken by careful venesection (463). Samples were labelled with antibodies against the exposed fibrinogen-binding surface receptor (367) to assess activation as this only binds to activated platelets. Samples were labelled with P-selectin to assess degranulation (464). P-selectin is an antigen of the α-granules. Activation and degranulation were assessed in the presence of different concentrations of ADP that is an agonist of platelet activation but causes minimal degranulation (465).

5.3.3. EndoPAT

EndoPAT was performed before entering the environmental chamber and at the end of the study visit, while participants were still in the chamber. Two disposable, inflatable probes were placed on their index fingers in a sitting position while resting. After 10 minutes of baseline signal registration, a BP cuff was inflated for 3 minutes and after releasing the test was continued for further 5 minutes. EndoPAT 2000 has been validated to assess endothelial function (188). A reactive hyperaemia index lower than a cut-off value of 1.67 has 82% sensitivity and 77% specificity to diagnose coronary endothelial dysfunction (188). Reproducibility is good with an interclass correlation of 0.74 (379).
5.3.4. Microparticles

Microparticles are membrane shreds and their surface receptors are characteristic of their cells of origin. These proteins can be used to qualify and quantify the different MPs using flow cytometry. MPs are thought to carry prothrombotic potential.

Microparticles were characterised by their cell of origin. CD42b positive MPs are platelet-derived, CD45 positive MPs are leukocyte-derived, CD106 and CD144 positive MPs are EMPs. CD106 positive EMPs express VCAM-1 on their surface, while CD144 positive EMPs express VE-Cadherin on their surface. MPs were further characterised by their ability to bind annexin V on their surface. Total MPs were calculated using the following formula:

\[
\text{Total MPs} = \text{MPs}_{\text{annexin V positive}} + \text{MPs}_{\text{annexin V negative}}
\]

Flow cytometry measurements are further detailed in Chapter 2.

5.3.5. Carotid Intima Media Thickness

As an addition, a cIMT measurement was undertaken during the screening visit as multiple studies have shown that cIMT is a surrogate marker of cardiovascular disease (466) and relates to endothelial dysfunction (467).
5.4. Results

5.4.1. Baseline characteristics

Patient baseline characteristics are presented in Table 20.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>T2DM</th>
<th>Overall</th>
<th>P (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Gender (male, female)</td>
<td>7,3</td>
<td>3, 7</td>
<td>10, 10</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Age (year)</td>
<td>57.30 ± 10.48</td>
<td>66 ± 7.30</td>
<td>61.65 ± 9.86</td>
<td>0.07 (-17.19 to -0.21)</td>
</tr>
<tr>
<td>Diabetes control</td>
<td>Not applicable</td>
<td>1, 9</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>(diet, metformin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid treatment</td>
<td>1, 9</td>
<td>10</td>
<td>11, 9</td>
<td>Not applicable</td>
</tr>
<tr>
<td>(statin, no statin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes duration (year)</td>
<td>Not applicable</td>
<td>5.75 ± 3.58</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>34.20 ± 3.08</td>
<td>41.70 ± 6.38</td>
<td>37.95 ± 6.21</td>
<td>0.05 (-12.21 to -2.79)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>73.64 ± 11.57</td>
<td>91.08 ± 15.14</td>
<td>82.36 ± 15.88</td>
<td>0.01 (-30.10 to -4.79)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.34 ± 3.88</td>
<td>31.34 ± 5.17</td>
<td>29.34 ± 4.90</td>
<td>0.07 (-8.29 to 0.30)</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.87 ± 0.08</td>
<td>0.94 ± 0.09</td>
<td>0.91 ± 0.09</td>
<td>0.15 (-0.14 to 0.02)</td>
</tr>
<tr>
<td>cIMT (mm)</td>
<td>0.64 ± 0.08</td>
<td>0.73 ± 0.16</td>
<td>0.70 ± 0.14</td>
<td>0.20 (-0.24 to 0.07)</td>
</tr>
</tbody>
</table>

BMI – body mass index, cIMT – carotid intima medial thickness

Table 20. Baseline patient characteristics. Data are expressed as mean ± SD where applicable. Results were compared with unpaired t-test, where applicable. Weight and HbA1c differed significantly between the two patient groups.
5.4.2. Calculations

Baseline clotting parameters, platelet activation, RHI, and MP numbers were compared between healthy controls and patients with type 2 diabetes, using unpaired t-test, applying the following formula: 

$$\text{Value}_{\text{baseline}} = \frac{\text{value}_{\text{before hypoxia}} + \text{value}_{\text{before normal day}}}{2}.$$ 

This indicated if there was any difference in the above markers between healthy controls and diabetes patients.

Absolute results before and after the hypoxic and control intervention of clotting parameters, platelet function, RHI and MP numbers were compared for each group using paired t-test.

Percentage change in clotting indices, platelet function, RHI and MP numbers were calculated for healthy controls and diabetes patients during the normal day and hypoxic intervention, using unpaired t-test, applying the following formula: 

$$\left(\frac{\text{value}_{\text{after experiment}}}{\text{value}_{\text{before experiment}}} \times 100\right) - 100.$$ 

This showed that how many percentage decrease or increase occurred during the different experiments in the different markers.

Total % changes then were compared between healthy controls and T2DM patients, using unpaired t-test, applying the following formula: 

$$\% \text{ change}_{\text{hypoxia}} - \% \text{ change}_{\text{normal}}.$$ 

This indicated the total % change during hypoxia compared to the % change during the normal day experiment.
5.4.3. Baseline clotting indices, platelet end endothelial function

There was no significant difference in baseline clotting parameters between healthy controls and patients with type 2 diabetes. Results are summarized in Table 21.

<table>
<thead>
<tr>
<th>Clotting parameters</th>
<th>Controls</th>
<th>T2DM</th>
<th>p</th>
<th>CI/U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time*</td>
<td>450 ± 44.26</td>
<td>456.70 ± 79.31</td>
<td>0.82</td>
<td>-67.04 to</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>53.64</td>
</tr>
<tr>
<td>Maximum absorption*</td>
<td>0.386 ± 0.056</td>
<td>0.389 ± 0.073</td>
<td>0.93</td>
<td>-0.06 to</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Lysis time**</td>
<td>1500.17</td>
<td>1423.83</td>
<td>0.24</td>
<td>44.00</td>
</tr>
<tr>
<td>(1275.00, 1957.67)</td>
<td>(1340.00, 1521.67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysis area*</td>
<td>706.18 ± 349.27</td>
<td>571.59 ± 135.98</td>
<td>0.27</td>
<td>-114.43 to</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>383.60</td>
</tr>
</tbody>
</table>

*unpaired t-test, **Mann-Whitney U test

Table 21. Baseline clotting parameters.
There was no significant difference in baseline platelet function between healthy controls and patients with type 2 diabetes. Results are summarized in Table 22 and 23.

<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen binding</th>
<th>P-selectin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% of platelets)</td>
<td>(% of platelets)</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>T2DM</td>
</tr>
<tr>
<td>Basal**</td>
<td>2.11 (1.93, 2.63)</td>
<td>2.08 (1.80, 2.18)</td>
</tr>
<tr>
<td>ADP 0.1 μm**</td>
<td>4.26 (3.08, 7.38)</td>
<td>5.68 (3.13, 7.80)</td>
</tr>
<tr>
<td>ADP 1 μm *</td>
<td>44.21 ± 12.34</td>
<td>48.45 ± 15.89</td>
</tr>
<tr>
<td>ADP 10 μm*</td>
<td>63.64 ± 12.06</td>
<td>67.12 ± 15.85</td>
</tr>
</tbody>
</table>

*unpaired t-test, **Mann-Whitney U test

Table 22. Baseline platelet activation and response to ADP.

<table>
<thead>
<tr>
<th></th>
<th>% inhibition of fibrinogen binding</th>
<th>% inhibition of P-selectin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>T2DM</td>
</tr>
<tr>
<td>PGI2 1nM + ADP**</td>
<td>43.2 ± 10.91</td>
<td>37.86 ± 9.79</td>
</tr>
<tr>
<td>PGI2 10nM + ADP*</td>
<td>57.49 ± 11.15</td>
<td>51.07 ± 14.75</td>
</tr>
<tr>
<td>PGI2 100nM+ADP**</td>
<td>93.27 (90.14, 95.73)</td>
<td>92.40 (89.20, 94.52)</td>
</tr>
</tbody>
</table>

*unpaired t-test, **Mann-Whitney U test

Table 23. Baseline platelet sensitivity to PGI2.
Endothelial function was assessed measuring circulating microparticle levels and PAT arterial tonometry.

Annexin V negative and total VCAM-1 positive MPs were significantly higher in patients with type 2 diabetes, indicating impaired baseline endothelial function in patients with type 2 diabetes compared to the control group. Results are presented in Table 24.

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>Controls</th>
<th>T2DM</th>
<th>P (2-tailed)</th>
<th>CI/U</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD42b/annexin V*</td>
<td>242±188</td>
<td>356±237</td>
<td>0.25</td>
<td>-314.65 to 87.16</td>
</tr>
<tr>
<td>CD42b*</td>
<td>4074±3327</td>
<td>3911±2046</td>
<td>0.90</td>
<td>-2432.13 to 2757.75</td>
</tr>
<tr>
<td>Total CD42b*</td>
<td>4316±3455</td>
<td>4267±2251</td>
<td>0.97</td>
<td>-2690.22 to 2788.36</td>
</tr>
<tr>
<td>CD45/annexin V*</td>
<td>49±25</td>
<td>52±20</td>
<td>0.81</td>
<td>-23.62 to 18.80</td>
</tr>
<tr>
<td>CD45*</td>
<td>163±124</td>
<td>181±113</td>
<td>0.74</td>
<td>-129.41 to 93.50</td>
</tr>
<tr>
<td>Total CD45*</td>
<td>213±119</td>
<td>233±116</td>
<td>0.70</td>
<td>-130.63 to 89.91</td>
</tr>
<tr>
<td>CD106/annexin V*</td>
<td>52±25</td>
<td>66±26</td>
<td>0.25</td>
<td>-37.78 to 10.31</td>
</tr>
<tr>
<td>CD106*</td>
<td>144±68</td>
<td>248±130</td>
<td>0.04</td>
<td>-204.37 to -4.10</td>
</tr>
<tr>
<td>Total CD106*</td>
<td>196±65</td>
<td>314±130</td>
<td>0.02</td>
<td>-217.31 to -18.63</td>
</tr>
<tr>
<td>CD144/annexin V*</td>
<td>53±8</td>
<td>61±32</td>
<td>0.54</td>
<td>-34.80 to 18.84</td>
</tr>
<tr>
<td>CD144**</td>
<td>139.35 (67.85, 249.23)</td>
<td>258.56 (79.85, 444.52)</td>
<td>0.28</td>
<td>35.00</td>
</tr>
<tr>
<td>Total CD144**</td>
<td>196.26 (140.59, 295.54)</td>
<td>328.27 (110.89, 526.28)</td>
<td>0.20</td>
<td>33.00</td>
</tr>
<tr>
<td>RHI*</td>
<td>1.82 ± 0.48</td>
<td>1.49 ± 0.17</td>
<td>0.06</td>
<td>-0.04 to 0.61</td>
</tr>
</tbody>
</table>

RHI – Reactive Hyperaemia Index; *unpaired t-test, **Mann-Whitney U test

Table 24. Basal MP numbers and RHI.
5.4.4. Absolute changes in clotting parameters, platelet function and endothelial function during the normal and hypoxic experiments

5.4.4.1. Clotting parameters

Lag time that is equivalent to the clotting time significantly decreased during the control environmental experiment in both groups, while there was no statistically significant difference during the hypoxic experiment. Maximum absorption significantly increased in both groups during the hypoxic environmental intervention, while remained unchanged during the control day. When analysed the data for the whole group (n=20), maximum absorbance significantly increased during hypoxia (0.39 ± 0.07 vs. 0.38 ± 0.06, p=0.01). Lysis time and lysis area were unchanged on both occasions either in healthy individuals or patients with type 2 diabetes (Table 25).
Table 25. Clotting indices in healthy volunteers and patients with type 2 diabetes during the control day and the hypoxic experiment.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th></th>
<th></th>
<th>T2DM</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Lag time*</td>
<td>455.00 ± 52.99</td>
<td>424.40 ± 63.02</td>
<td>0.17</td>
<td>-16.35 to 77.55</td>
<td>446.90 ± 93.57</td>
<td>0.89</td>
<td>-58.39 to 51.19</td>
<td></td>
</tr>
<tr>
<td>Maximum absorption*</td>
<td>0.375 ± 0.050</td>
<td>0.390 ± 0.053</td>
<td>0.04</td>
<td>-0.32 to 0.01</td>
<td>0.378 ± 0.076</td>
<td>0.01</td>
<td>-0.03 to 0.01</td>
<td></td>
</tr>
<tr>
<td>Lysis time**</td>
<td>1500.33 (1261.25, 1822.92)</td>
<td>1570.83 (1386.92, 1868.00)</td>
<td>0.17</td>
<td>-1.38 (4)</td>
<td>1422.33 (1330.00, 1458.42)</td>
<td>0.29</td>
<td>-1.07 (7)</td>
<td></td>
</tr>
<tr>
<td>Lysis area*</td>
<td>658.20 ± 289.27</td>
<td>675.11 ± 215.61</td>
<td>0.64</td>
<td>-95.23 to 61.40</td>
<td>556.05 ± 157.71</td>
<td>0.02</td>
<td>-106.53 to 35.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Lag time*</td>
<td>436.11 ± 31.19</td>
<td>406.11 ± 45.50</td>
<td>0.04</td>
<td>-95.23 to 61.41</td>
<td>466.50 ± 76.64</td>
<td>0.02</td>
<td>9.28 to 78.32</td>
<td></td>
</tr>
<tr>
<td>Maximum absorption*</td>
<td>0.404 ± 0.068</td>
<td>0.413 ± 0.064</td>
<td>0.12</td>
<td>1.20 to 58.80</td>
<td>0.340 ± 0.082</td>
<td>0.374 ± 0.109</td>
<td>0.31</td>
<td>-0.03 to 0.08</td>
</tr>
<tr>
<td>Lysis time**</td>
<td>1628.33 (1284.83, 2100.50)</td>
<td>1632.33 (1305.25, 2488.25)</td>
<td>0.11</td>
<td>-1.60 (2)</td>
<td>1432.33 (1325.25, 1578.67)</td>
<td>0.20</td>
<td>-1.27 (4)</td>
<td></td>
</tr>
<tr>
<td>Lysis area**</td>
<td>732.77 (434.31, 1250.34)</td>
<td>772.05 (427.26, 1227.70)</td>
<td>0.09</td>
<td>-1.72 (1)</td>
<td>607.38 (496.64, 683.83)</td>
<td>0.33</td>
<td>-0.97 (3)</td>
<td></td>
</tr>
</tbody>
</table>

*paired t-test, **Wilcoxon signed rank test
5.4.4.2. Platelet function

There was no significant change in platelet activation during the normal day either in healthy volunteers or patients with type 2 diabetes (Table 26 and 27).

<table>
<thead>
<tr>
<th></th>
<th>Basal**</th>
<th>ADP 0.1 μm**</th>
<th>ADP 1 μm *</th>
<th>ADP 10 μm*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrinogen binding</strong></td>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>CI/ z (N)</td>
</tr>
<tr>
<td>Controls</td>
<td>2.38 (1.89, 2.86)</td>
<td>2.08 (1.85, 2.33)</td>
<td>0.24</td>
<td>-1.17 (7)</td>
</tr>
<tr>
<td>T2DM</td>
<td>4.55 (3.63, 7.00)</td>
<td>3.90 (3.01, 5.86)</td>
<td>0.51</td>
<td>-0.66 (6)</td>
</tr>
<tr>
<td><strong>P-selectin expression</strong></td>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>CI/ z (N)</td>
</tr>
<tr>
<td>Controls</td>
<td>1.70 (1.54, 4.03)</td>
<td>1.60 (1.53, 2.54)</td>
<td>0.21</td>
<td>-1.25 (6)</td>
</tr>
<tr>
<td>T2DM</td>
<td>4.20 (2.94, 6.71)</td>
<td>3.43 (2.90, 6.20)</td>
<td>0.96</td>
<td>-0.05 (5)</td>
</tr>
</tbody>
</table>

*paired t-test, **Wilcoxon signed rank test

Table 26. Platelet activation and response to ADP prior to and after the control environmental intervention.
Table 27. Platelet sensitivity to PGI₂ prior to and after the normal environmental intervention.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>T2DM</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>p</td>
<td>CI/ z (N)</td>
<td>Before</td>
</tr>
<tr>
<td>PGI2 1nM + ADP*</td>
<td>44.00 ± 11.38</td>
<td>40.33 ± 1.07</td>
<td>0.28</td>
<td>-3.57 to 10.91</td>
<td>40.03 ± 15.47</td>
</tr>
<tr>
<td>PGI2 10nM + ADP**</td>
<td>58.27 ± 16.65</td>
<td>57.55 ± 12.37</td>
<td>0.92</td>
<td>-15.07 to 16.50</td>
<td>50.51 ± 18.68</td>
</tr>
<tr>
<td>PGI2 100nM + ADP**</td>
<td>93.69 (83.17, 96.36)</td>
<td>94.50 (87.02, 96.86)</td>
<td>0.96</td>
<td>-0.05 (5)</td>
<td>93.61 (82.93, 95.20)</td>
</tr>
<tr>
<td>PGI2 100nM + ADP***</td>
<td>93.98 ± 8.97</td>
<td>42.35 ± 10.84</td>
<td>0.43</td>
<td>4.58 to 9.48</td>
<td>43.47 ± 14.91</td>
</tr>
<tr>
<td>PGI2 100nM + ADP***</td>
<td>60.35 ± 16.21</td>
<td>58.22 ± 14.62</td>
<td>0.78</td>
<td>-14.26 to 18.51</td>
<td>54.02 ± 16.46</td>
</tr>
<tr>
<td>PGI2 100nM + ADP***</td>
<td>93.90 (83.17, 96.36)</td>
<td>92.93 (85.83, 96.95)</td>
<td>0.96</td>
<td>-0.05 (5)</td>
<td>93.91 (85.80, 95.27)</td>
</tr>
</tbody>
</table>

*paired t-test, **Wilcoxon signed rank test

There was no significant change in platelet activity during the hypoxic experiment in healthy volunteers. In patients with type 2 diabetes there was a significant increase in basal platelet activation while platelet response to ADP and sensitivity to PGI2 was unchanged (Table 28 and 29). When analysed the data for the whole group (n=20), there was significant increase in basal fibrinogen binding after hypoxia compared to the basal baseline fibrinogen binding before hypoxia (2.28 ± 0.87 vs. 1.81 ± 0.52; p=0.04) while there was no change during the normal day.
<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Basal*</td>
<td>2.03±0.50</td>
</tr>
<tr>
<td>ADP 0.1 μm*</td>
<td>5.04±2.63</td>
</tr>
<tr>
<td>ADP 1 μm*</td>
<td>45.50±16.42</td>
</tr>
<tr>
<td>ADP 10 μm*</td>
<td>63.17±12.89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>P-selectin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Basal**</td>
<td>2.18 (1.65, 5.58)</td>
</tr>
<tr>
<td>ADP 0.1 μm*</td>
<td>6.61±3.97</td>
</tr>
<tr>
<td>ADP 1 μm*</td>
<td>48.43±19.61</td>
</tr>
<tr>
<td>ADP 10 μm*</td>
<td>67.58±18.04</td>
</tr>
</tbody>
</table>

*paired t-test, ** Wilcoxon signed rank test

Table 28. Platelet activation and response to ADP prior to and after the hypoxic environmental intervention.
<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td><strong>Fibrinogen binding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGI₂ 1nM + ADP*</td>
<td>42.40 ± 15.58</td>
<td>37.37 ± 17.85</td>
</tr>
<tr>
<td>PGI₂ 10nM + ADP**</td>
<td>56.72 ± 15.73</td>
<td>54.62 ± 14.08</td>
</tr>
<tr>
<td>PGI₂ 100nM + ADP***</td>
<td>95.98 (86.68, 97.09)</td>
<td>94.19 (91.06, 95.62)</td>
</tr>
</tbody>
</table>

|                      |                  |                |            |              |                  |                |            |              |
| **P-selectin expression** |                  |                |            |              |                  |                |            |              |
| PGI₂ 1nM + ADP*      | 41.71 ± 15.98  | 40.25 ± 18.35  | 0.54       | -3.66 to 6.57  | 33.88 ± 9.15  | 36.42 ± 7.97  | 0.55       | -11.97 to 6.89 |
| PGI₂ 10nM + ADP**     | 50.90 (49.31, 57.06) | 57.85 (39.63, 70.93) | 0.96       | -0.05 (4)       | 49.52 (33.42, 53.04) | 53.73 (40.88, 55.12) | 0.59       | -0.53 (4)       |
| PGI₂ 100nM + ADP***   | 91.99 (84.57, 94.79) | 92.82 (86.96, 95.84) | 0.66       | -0.96 (6)       | 86.53 (78.21, 91.43) | 89.15 (84.64, 92.57) | 0.59       | -0.53 (4)       |

*paired t-test, **Wilcoxon signed rank test

Table 29. Platelet sensitivity to PGI₂ prior to and after the hypoxic environmental intervention.
5.4.4.3. **Endothelial function**

There was a general increase in MP counts during the hypoxic experiment, while a general decrease during the control day, however these changes were not clinically significant. Reactive Hyperaemia Index remained unchanged (Table 30).

<table>
<thead>
<tr>
<th></th>
<th>Hypoxia Before</th>
<th>Hypoxia After</th>
<th>p</th>
<th>CI/±z(N) Before</th>
<th>Hypoxia After</th>
<th>T2DM Before</th>
<th>T2DM After</th>
<th>p</th>
<th>CI/±z(N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD42b/annexin V**</td>
<td>147.36 (96.57, 460.05)</td>
<td>184.34 (122.57, 448.11)</td>
<td>0.80</td>
<td>-0.26 (5)</td>
<td>372.81 (75.58, 869.62)</td>
<td>263.74 (106.84, 458.50)</td>
<td>0.14</td>
<td>-1.48 (6)</td>
<td></td>
</tr>
<tr>
<td>CD42b**</td>
<td>4004.59 (1994.87, 8655.10)</td>
<td>4655.41 (1555.78, 10662.96)</td>
<td>0.72</td>
<td>-0.36 (7)</td>
<td>3198.58 (1739.98, 7144.48)</td>
<td>3437.92 (2221.17, 7654.41)</td>
<td>0.80</td>
<td>-0.26 (4)</td>
<td></td>
</tr>
<tr>
<td>TotalCD42b+ MPs**</td>
<td>4292.98 (2151.07, 9388.84)</td>
<td>4916.24 (1646.06, 10833.03)</td>
<td>0.72</td>
<td>-0.36 (7)</td>
<td>372.81 (75.58, 869.62)</td>
<td>3664.46 (2535.02, 8246.59)</td>
<td>0.80</td>
<td>-0.26 (4)</td>
<td></td>
</tr>
<tr>
<td>CD45/annexin V**</td>
<td>27.64 (18.06, 57.75)</td>
<td>29.92 (19.06, 79.81)</td>
<td>0.95</td>
<td>-0.05 (5)</td>
<td>29.99 (12.60, 72.67)</td>
<td>36.86 (18.07, 63.31)</td>
<td>0.33</td>
<td>-0.97 (6)</td>
<td></td>
</tr>
<tr>
<td>CD45*</td>
<td>204±168</td>
<td>229±151</td>
<td>0.50</td>
<td>-105.09 to 55.26</td>
<td>200±145</td>
<td>312±517</td>
<td>0.36</td>
<td>-3.37 to 174.91</td>
<td></td>
</tr>
<tr>
<td>Total CD45+ MPs*</td>
<td>244±167</td>
<td>273±156</td>
<td>0.53</td>
<td>-128.48 to 70.46</td>
<td>242±150</td>
<td>370±508</td>
<td>0.37</td>
<td>-3.68 to 179.90</td>
<td></td>
</tr>
<tr>
<td>CD106/annexin V**</td>
<td>26.73 (14.14, 58.18)</td>
<td>36.03 (22.67, 65.41)</td>
<td>0.11</td>
<td>-1.58 (2)</td>
<td>75.73 (24.82, 92.47)</td>
<td>47.91 (25.54, 88.78)</td>
<td>0.51</td>
<td>-0.66 (6)</td>
<td></td>
</tr>
<tr>
<td>CD106*</td>
<td>170±105</td>
<td>262±240</td>
<td>0.24</td>
<td>-256.78 to 71.95</td>
<td>328±190</td>
<td>466±606</td>
<td>0.39</td>
<td>-4.84 to 208.71</td>
<td></td>
</tr>
<tr>
<td>Total CD106+ MPs*</td>
<td>203±97</td>
<td>331±313</td>
<td>0.25</td>
<td>-362.12 to 106.79</td>
<td>395±196</td>
<td>525±591</td>
<td>0.42</td>
<td>-4.78 to 218.31</td>
<td></td>
</tr>
<tr>
<td>CD144/annexin V**</td>
<td>24.95 (13.58, 63.49)</td>
<td>36.59 (16.04, 71.95)</td>
<td>0.65</td>
<td>-0.46 (5)</td>
<td>54.10 (20.70, 118.77)</td>
<td>32.87 (13.44, 101.34)</td>
<td>0.06</td>
<td>-2.09 (8)</td>
<td></td>
</tr>
<tr>
<td>CD144*</td>
<td>182.74 (40.49, 314.85)</td>
<td>275.16 (28.84, 325.08)</td>
<td>0.58</td>
<td>-0.56 (5)</td>
<td>348.74 (117.99, 515.52)</td>
<td>312.84 (89.30, 699.90)</td>
<td>0.45</td>
<td>-0.76 (3)</td>
<td></td>
</tr>
<tr>
<td>Total CD144+ MPs**</td>
<td>211.63 (69.91, 367.67)</td>
<td>307.32 (52.82, 381.58)</td>
<td>0.72</td>
<td>-0.36 (5)</td>
<td>467.65 (146.15, 574.59)</td>
<td>375.56 (110.27, 801.24)</td>
<td>0.45</td>
<td>-0.76 (7)</td>
<td></td>
</tr>
<tr>
<td>RHI**</td>
<td>1.73 (1.61, 2.44)</td>
<td>1.74 (1.65, 1.88)</td>
<td>0.59</td>
<td>-0.53 (5)</td>
<td>1.57 (1.43, 1.71)</td>
<td>1.59 (1.37, 1.83)</td>
<td>0.48</td>
<td>-0.70 (4)</td>
<td></td>
</tr>
</tbody>
</table>
### Table 30. Microparticle counts and Reactive Hyperaemia Index in healthy volunteers and patients with type 2 diabetes before and after the control day and the hypoxic experiment.

<table>
<thead>
<tr>
<th>Microparticle Counts</th>
<th>Normal day</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>T2DM</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td><strong>CD42b/annexin V</strong></td>
<td>168.08</td>
<td>140.10</td>
</tr>
<tr>
<td>(109.88, 297.11)</td>
<td>(89.36, 216.65)</td>
<td></td>
</tr>
<tr>
<td><strong>Before</strong></td>
<td>1624.99</td>
<td>1536.79</td>
</tr>
<tr>
<td>(705.81, 2190.47)</td>
<td>(1149.63, 2016.62)</td>
<td></td>
</tr>
<tr>
<td><strong>Total CD42b+ MPs</strong></td>
<td>1700.04</td>
<td>166.52</td>
</tr>
<tr>
<td>(959.32, 3518.37)</td>
<td>(124.97, 309.83)</td>
<td></td>
</tr>
<tr>
<td><strong>CD45/annexin V</strong></td>
<td>58 ± 23</td>
<td>57 ± 36</td>
</tr>
<tr>
<td>(26.07, 142.50)</td>
<td>(20.02, 149.66)</td>
<td></td>
</tr>
<tr>
<td><strong>Total CD45+ MPs</strong></td>
<td>132.94</td>
<td>136.20</td>
</tr>
<tr>
<td>(77.95, 222.72)</td>
<td>(1149.63, 2016.62)</td>
<td></td>
</tr>
<tr>
<td><strong>CD106/annexin V</strong></td>
<td>72.15</td>
<td>79.60</td>
</tr>
<tr>
<td>(26.07, 142.50)</td>
<td>(20.02, 149.66)</td>
<td></td>
</tr>
<tr>
<td><strong>Total CD106+ MPs</strong></td>
<td>189 ± 109</td>
<td>149 ± 67</td>
</tr>
<tr>
<td>(22.20, 187.08)</td>
<td>(1149.63, 2016.62)</td>
<td></td>
</tr>
<tr>
<td><strong>CD144/annexin V</strong></td>
<td>83.91</td>
<td>64.29</td>
</tr>
<tr>
<td>(22.20, 187.08)</td>
<td>(20.02, 149.66)</td>
<td></td>
</tr>
<tr>
<td><strong>Total CD144+ MPs</strong></td>
<td>234 ± 147</td>
<td>198 ± 147</td>
</tr>
<tr>
<td>(50.82, 212.86)</td>
<td>(1149.63, 2016.62)</td>
<td></td>
</tr>
<tr>
<td><strong>RHI</strong></td>
<td>1.68 (1.54, 1.83)</td>
<td>1.75 (1.54, 2.07)</td>
</tr>
</tbody>
</table>

*paired t-test, **Wilcoxon signed rank test*
5.4.5. Percentage changes in clotting parameters, platelet function and endothelial function during the normal and hypoxic experiments

5.4.5.1. Clotting indices

There was no significant difference in the degree of change in clotting parameters between healthy controls and patients with type 2 diabetes, either during the control or hypoxic experiment (Table 31).

<table>
<thead>
<tr>
<th></th>
<th>Normal day</th>
<th>Hypoxia</th>
<th>Total change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy controls</td>
<td>T2DM</td>
<td>p</td>
</tr>
<tr>
<td>Lag time</td>
<td>-6.86 ± 8.83</td>
<td>-9.16 ± 10.01</td>
<td>0.60</td>
</tr>
<tr>
<td>Maximum absorption</td>
<td>1.75 (-0.93, 5.49)</td>
<td>0.82 (-7.46, 3.00)</td>
<td>0.35</td>
</tr>
<tr>
<td>Lysis time</td>
<td>6.51 (-0.20, 18.37)</td>
<td>2.37 (-1.38, 7.53)</td>
<td>0.45</td>
</tr>
<tr>
<td>Lysis area</td>
<td>15.43 ± 16.65</td>
<td>5.82 ± 12.00</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*unpaired t-test, **Mann-Whitney U test

Table 31. Percentage change in clotting parameters during the normal day and the hypoxic environmental intervention.
5.4.5.2.  Platelet function

There was no significant difference in the degree of change in platelet activation (response to ADP and sensitivity to PGI2) between healthy controls and patients with type 2 diabetes during the control or the hypoxic experiment (Table 32 and 33).

<table>
<thead>
<tr>
<th></th>
<th>Normal day</th>
<th>Healthy controls</th>
<th>T2DM</th>
<th>p</th>
<th>CI/U</th>
<th>Total change</th>
<th>Healthy controls</th>
<th>T2DM</th>
<th>p</th>
<th>CI/U</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fibrinogen binding</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td></td>
<td>-17.82</td>
<td>6.12</td>
<td>0.48</td>
<td>40.00**</td>
<td>19.54 ± 45.94</td>
<td>50.74 ± 58.32</td>
<td>0.22</td>
<td>-82.87 to 20.46*</td>
<td>20.68 ± 89.73</td>
</tr>
<tr>
<td>ADP 0.1 μm</td>
<td>-4.47 ± 45.10</td>
<td>-9.15 ± 33.91</td>
<td>0.80</td>
<td>-3.31 to 42.38*</td>
<td>5.42</td>
<td>4.05 (-48.95, 79.17)</td>
<td>0.91</td>
<td>43.00**</td>
<td>9.80 ± 69.65</td>
<td>52.44 ± 108.23</td>
</tr>
<tr>
<td>ADP 1 μm</td>
<td>5.54 ± 14.06</td>
<td>-7.16 ± 15.88</td>
<td>0.08</td>
<td>-1.41 to 26.80*</td>
<td>1.29</td>
<td>6.83 (-29.71, 12.96)</td>
<td>0.72</td>
<td>40.00**</td>
<td>-3.29 (-22.53, 26.01)</td>
<td>15.40</td>
</tr>
<tr>
<td>ADP 10 μm</td>
<td>-0.78 ± 9.03</td>
<td>14.78 ± 4.67</td>
<td>0.79</td>
<td>-10.19 to 13.17*</td>
<td>8.51 ± 14.23</td>
<td>-1.02 ± 32.09*</td>
<td>0.43</td>
<td>-16.08 to 35.13*</td>
<td>9.29 ± 16.76</td>
<td>1.78 ± 39.12</td>
</tr>
</tbody>
</table>

|                      |            |                  |      |    |      |              |                  |      |    |      |
| **P-selectin expression** |          |                  |      |    |      |              |                  |      |    |      |
| Basal                |            | -11.78           | -12.12 | 0.85 | 47.00** | -4.75 (-24.00, 19.44) | 31.82 (24.24, 87.18) | 0.06 | 18.00** | 8.91 (-24.00, 30.00) | 43.71 (26.19, 55.45) | 0.16 | 27.00** |
| ADP 0.1 μm           | 1.97       | 5.63 (-37.87, 21.52) | 0.58 | 42.00** | 1.52 (-15.69, 14.29) | 26.32 (0.00, 36.78) | 0.24 | 30.00** | -6.55 (-31.78, 22.48) | -5.63 (54.85) | 0.45 | 35.00** |
| ADP 1 μm             | 10.72 ± 15.94 | 0.21 ± 14.41 | 0.14 | -3.78 to 24.80* | 0.23 (-4.96, 25.35) | -7.35 (-15.30, 28.88) | 0.72 | 40.00** | -8.19 (-24.24, 14.18) | -0.96 (-2.97, 0.65) | 0.40 | 34.00** |
| ADP 10 μm            | 2.33 ± 10.42 | 3.00 ± 12.70 | 0.90 | -11.61 to 10.28* | 7.39 ± 14.55 | 7.74 ± 24.24 | 0.97 | -20.47 to 19.76* | 5.05 ± 11.01 | 5.24 ± 19.64 | 0.98 | -16.30 to 15.92* |

*unpaired t-test, **Mann-Whitney U test

Table 32. Percentage change in platelet activation and response to ADP during the normal day and the hypoxic environmental intervention.
Table 33. Percentage change in platelet sensitivity to PGI₂ (% inhibition) during the normal day and the hypoxic environmental intervention.

<table>
<thead>
<tr>
<th></th>
<th>Normal day</th>
<th></th>
<th></th>
<th>Hypoxia</th>
<th></th>
<th></th>
<th>Net change</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy controls</td>
<td>T2DM</td>
<td>p</td>
<td>CI/U</td>
<td>Healthy controls</td>
<td>T2DM</td>
<td>p</td>
<td>CI/U</td>
</tr>
<tr>
<td>Fibrinogen binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGI₂ 1nM + ADP</td>
<td>-7.31 (-24.96,5.62)</td>
<td>8.54 (-6.61,16.59)</td>
<td>0.35</td>
<td>37.00**</td>
<td>-8.81 (-14.95,5.15)</td>
<td>16.07 (-22.35,25.37)</td>
<td>0.18</td>
<td>28.00**</td>
</tr>
<tr>
<td>PGI₂ 10nM + ADP</td>
<td>-4.01 ± 23.07</td>
<td>19.58 ± 57.78</td>
<td>0.25</td>
<td>-36.67 to 23.66*</td>
<td>-7.17 ± 30.93</td>
<td>15.60 ± 43.46</td>
<td>0.21</td>
<td>-39.30 to 34.55*</td>
</tr>
<tr>
<td>PGI₂ 100nM + ADP</td>
<td>0.23 (-2.35,7.61)</td>
<td>1.30 (-2.69,2.09)</td>
<td>0.63</td>
<td>43.00**</td>
<td>-1.82 (-5.58,6.40)</td>
<td>0.76 (-1.49,10.63)</td>
<td>0.36</td>
<td>33.00**</td>
</tr>
<tr>
<td>P-selectin expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGI₂ 1nM + ADP</td>
<td>-6.08 (-21.57,8.40)</td>
<td>9.59 (-2.82,23.94)</td>
<td>0.28</td>
<td>35.00**</td>
<td>2.59 ± 23.86</td>
<td>15.77 ± 39.56</td>
<td>0.40</td>
<td>-46.03 to 19.68*</td>
</tr>
<tr>
<td>PGI₂ 10nM + ADP</td>
<td>-0.87 ± 14.49</td>
<td>17.04 ± 50.33</td>
<td>0.32</td>
<td>-35.81 to 21.33*</td>
<td>1.35 ± 11.16</td>
<td>11.28 ± 28.13</td>
<td>0.34</td>
<td>-45.63 to 50.23*</td>
</tr>
<tr>
<td>PGI₂ 100nM + ADP</td>
<td>-0.31 (-3.41,8.93)</td>
<td>0.38 (-1.05,1.91)</td>
<td>0.63</td>
<td>43.00**</td>
<td>-0.05 (-3.60,6.32)</td>
<td>1.70 (-3.41,4.94)</td>
<td>0.84</td>
<td>42.00**</td>
</tr>
</tbody>
</table>

*unpaired t-test, **Mann-Whitney U test
HbA1c did not affect the net percentage change between the hypoxic and the normal day experiment in basal platelet function neither in the control (fibrinogen binding: \( p=0.22, z=0.43 \); \( P=\) selectin expression: \( p=0.09, z=0.67 \)) or the diabetes group (fibrinogen binding: \( p=0.56, z=-0.22 \); \( P=\) selectin expression: \( p=0.47, z=0.28 \)). Weight had no effect on the net percentage change between the hypoxic and the normal day experiment in basal platelet function neither in the control (fibrinogen binding: \( p=0.62, z=-0.18 \); \( P=\) selectin expression: \( p=0.52, z=0.23 \)) or the diabetes group (fibrinogen binding: \( p=0.33, z=-0.37 \); \( P=\) selectin expression: \( p=0.30, z=0.39 \)).

### 5.4.5.3. Endothelial function

There was no significant difference in the degree of change in endothelial parameters between healthy controls and patients with type 2 diabetes during the control or the hypoxic experiment. Results are summarized in Table 34.
### Table 34. Percentage change in MP numbers and Reactive Hyperaemia Index (RHI) during the normal day and the hypoxic environmental intervention. Statistical significance was calculated using paired t-test.

<table>
<thead>
<tr>
<th></th>
<th>Normal day</th>
<th></th>
<th></th>
<th>Hypoxia</th>
<th></th>
<th></th>
<th>Net change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy controls</td>
<td>T2DM</td>
<td>p</td>
<td>CI/U</td>
<td>Healthy controls</td>
<td>T2DM</td>
<td>p</td>
</tr>
<tr>
<td>CD42b/annexin V</td>
<td>6.00</td>
<td>-45.97</td>
<td>0.53</td>
<td>41**</td>
<td>1.03</td>
<td>-20.67</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>(6.46, 38.77)</td>
<td>(-57.86, 62.84)</td>
<td></td>
<td></td>
<td>(32.69, 140.66)</td>
<td>(-55.84, 133.81)</td>
<td></td>
</tr>
<tr>
<td>CD42b</td>
<td>-27.76</td>
<td>-46.18</td>
<td>0.00</td>
<td>46**</td>
<td>-13.15</td>
<td>10.53</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>(-44.89, 35.63)</td>
<td>(-63.48, 71.19)</td>
<td></td>
<td></td>
<td>(-38.68, 81.00)</td>
<td>(-33.54, 106.68)</td>
<td></td>
</tr>
<tr>
<td>Total CD42b</td>
<td>-12.23</td>
<td>-46.81</td>
<td>0.08</td>
<td>44**</td>
<td>14.30</td>
<td>7.27</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>(-44.94, 38.20)</td>
<td>(-64.13, 74.00)</td>
<td></td>
<td></td>
<td>(-38.08, 81.78)</td>
<td>(-32.28, 108.88)</td>
<td></td>
</tr>
<tr>
<td>CD45/annexin V</td>
<td>-6.48 ± 41.97</td>
<td>-4.00 ± 55.84</td>
<td>0.91</td>
<td>-49.08 to 44.26*</td>
<td>0.38</td>
<td>-16.26</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>(33.10, 62.80)</td>
<td>(-26.79, 59.32)</td>
<td></td>
<td></td>
<td>(-16.29, 49.01)</td>
<td>(-3.45, 89.81)</td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>17.91 ± 44.25</td>
<td>-1.01 ± 31.40</td>
<td>0.29</td>
<td>-17.42 to 55.24*</td>
<td>-9.93</td>
<td>8.75</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>(-14.25, 52.18)</td>
<td>(-15.50, 23.63)</td>
<td></td>
<td></td>
<td>(-16.29, 49.01)</td>
<td>(-3.45, 89.81)</td>
<td></td>
</tr>
<tr>
<td>Total CD45</td>
<td>-0.80 ± 26.88</td>
<td>-0.64 ± 41.63</td>
<td>0.99</td>
<td>-33.48 to 33.17*</td>
<td>-9.90</td>
<td>-8.80</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>(-14.25, 52.18)</td>
<td>(-15.50, 23.63)</td>
<td></td>
<td></td>
<td>(-16.29, 49.01)</td>
<td>(-3.45, 89.81)</td>
<td></td>
</tr>
<tr>
<td>CD106/annexin V</td>
<td>-9.54 ± 22.17</td>
<td>-10.02 ± 52.70</td>
<td>0.98</td>
<td>-39.88 to 40.85*</td>
<td>1.72</td>
<td>-7.74</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>(0.38, 94.13)</td>
<td>(-37.23, 20.78)</td>
<td></td>
<td></td>
<td>(0.38, 94.13)</td>
<td>(-37.23, 20.78)</td>
<td></td>
</tr>
<tr>
<td>CD106</td>
<td>-7.81 ± 37.39</td>
<td>-10.74 ± 24.96</td>
<td>0.80</td>
<td>-26.46 to 33.91*</td>
<td>2.19</td>
<td>3.46</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>(-14.77, 46.29)</td>
<td>(-17.92, 53.98)</td>
<td></td>
<td></td>
<td>(-14.77, 46.29)</td>
<td>(-17.92, 53.98)</td>
<td></td>
</tr>
<tr>
<td>Total CD106</td>
<td>-9.94 ± 30.09</td>
<td>-15.23 ± 34.70</td>
<td>0.68</td>
<td>-24.36 to 36.75*</td>
<td>2.66</td>
<td>6.33</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>(-11.99, 47.80)</td>
<td>(-20.98, 18.74)</td>
<td></td>
<td></td>
<td>(-11.99, 47.80)</td>
<td>(-20.98, 18.74)</td>
<td></td>
</tr>
<tr>
<td>CD144/annexin V</td>
<td>-13.41 ± 24.62</td>
<td>0.06 ± 48.14</td>
<td>0.44</td>
<td>-50.30 to 23.35*</td>
<td>7.53</td>
<td>-15.08</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>(-13.96, 58.33)</td>
<td>(-45.65, 7.86)</td>
<td></td>
<td></td>
<td>(-13.96, 58.33)</td>
<td>(-45.65, 7.86)</td>
<td></td>
</tr>
<tr>
<td>CD144</td>
<td>-2.09 ± 38.08</td>
<td>9.73 ± 32.62</td>
<td>0.45</td>
<td>-44.11 to 24.49*</td>
<td>1.09</td>
<td>7.64</td>
<td>0.00</td>
</tr>
<tr>
<td>Total CD144</td>
<td>-1.99 ± 27.47</td>
<td>-1.91 ± 32.54</td>
<td>0.66</td>
<td>-34.32 to 22.28*</td>
<td>-1.27</td>
<td>-3.73</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>(-17.42, 67.04)</td>
<td>(-24.49, 13.02)</td>
<td></td>
<td></td>
<td>(-17.42, 67.04)</td>
<td>(-24.49, 13.02)</td>
<td></td>
</tr>
<tr>
<td>RHI</td>
<td>14.91 ± 35.35</td>
<td>9.47 ± 28.19</td>
<td>0.72</td>
<td>-26.64 to 37.52*</td>
<td>-4.27 ± 31.52</td>
<td>6.23 ± 18.36</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*unpaired t-test, ***Mann-Whitney U test
5.5. Discussion

This is the first study to assess the physiological changes that might affect thrombus formation when simulating a short duration commercial air flight, comparing patients with type 2 diabetes and healthy individuals. There was increased maximum clot absorption in T2DM during hypoxia indicating denser clot structure. Basal fibrinogen binding and P-selectin expression on platelets were also increased in T2DM patients after hypoxia, indicating platelet hyperactivity. Endothelial function measured by microparticles and reactive hyperaemia index did not change during hypoxia or the control experiment. We have found no differences in the degree of change in clotting parameters, platelet activation and endothelial function as a response to hypoxia compared to a control day, between healthy subjects and T2DM patients.

As part of the WRIGHT project a hypobaric hypoxia study was performed (468) that did not support the hypothesis that a simulated flight would cause elevated risk for the development of VTE among healthy individuals who were at low risk of VTE.

In another hypobaric hypoxia study (469) markers of activated coagulation changed significantly during an eight hour exposure. Prothrombin fragments 1 and 2 reached a maximum at two hours into exposure. The study was criticized as the baseline values of these markers were elevated at the zero time point however no problems with venesection were reported. This might raise the question whether the changes in these markers are more significant in individuals where the coagulation is already impaired and so the basal activation is greater than normal.

The risk factors to develop VTE during air travel identified so far can be environment-related and individual. The possible environmental risk factors are prolonged travel duration, immobilisation, cramped sitting position, relative hypoxia, low air pressure and low humidity (470-478). The possible role of stress, air pollution and infection have also been considered (479). Individual predisposing factors for VTE are known to be coagulation defects (480),
thrombophilia and/or the usage of oral contraceptive pills. The latter two represent a 14- to 16-fold increase of risk of developing VTE (472). Other passenger-related risk factors include overweight, chronic diseases such as chronic heart disease, oestrogen therapy, pregnancy and puerperium, family history of DVT or previous history of DVT, recent surgery or trauma, advanced age (over 40 years) and chronic venous insufficiency that leads to stasis. Some studies suggested that long-haul flights on their own are not risk factors for VTE however the risk is significantly greater if additional risk factors are present (481).

Type 2 diabetes is a chronic condition with elevated cardiovascular risk arising from advanced atherosclerosis, and abnormalities in endothelial and platelet function along with abnormal clotting mechanism. The evidence so far is not clear if T2DM is associated with increased risk of VTE. Some authors confirmed it while others did not. Heit et al concluded that diabetes mellitus is not an independent risk factor to develop VTE (482) while diabetes mellitus is a reported risk factor for VTE and PE according to others (441, 483-485). A meta-analysis also found a 1.4-fold increased risk to develop VTE in patients with diabetes (486).

In this study we found increased basal fibrinogen binding and P-selectin expression after the hypoxic experiment in T2DM patients. The increase in fibrinogen binding was strong enough to have an impact on the results when looked at for the whole group suggesting and independent effect of diabetes. However there was no difference in the percentage change of platelet activation when comparing healthy controls to patients with type 2 diabetes, either during the control or the hypoxic experiment. These findings are in accord with data showing that Type 2 diabetes has altered platelet function and metabolism (487). However a general trend was observed that the total percentage change in basal platelet activation and in the response to ADP was enhanced in T2DM patients compared to healthy controls, though it did not reach statistically significance. There was no difference in platelet function between T2DM patients and healthy subjects at baseline. Toff et al found no difference in platelet function between an 8 hour experiment in hypobaric hypoxia when compared to a control day in healthy subjects (468). These suggest that platelet function is affected by aircraft
environmental conditions in patients with type 2 diabetes however our findings
did not suggest that any change would be more marked in diabetic patients, and
may not be clinically important.

We have shown increased clot maximum absorption in both healthy volunteers
and T2DM patients after hypoxia, however the difference between the percentage
change in healthy and diabetic subjects was not significant. This suggests that
some elements of the clotting mechanism are affected both in healthy subjects and
patients with type 2 diabetes, but this impairment is not greater in diabetes. This is
the converse to that reported by Toff et al were no difference in soluble
coagulation factors were found (468). A larger study will be needed to determine if
this is finding is clinically relevant.

In our study we found higher concentration of VCAM-1 positive MPs in the T2DM
group, while there was no difference in VE Cadherin positive EMPs between
healthy controls and patients with type 2 diabetes. Microparticle levels are
elevated in type 2 diabetes either from platelets, monocytes or endothelial origin
(488-492). Diabetes mellitus is reportedly associated with elevated VCAM-1
positive EMP counts (373) and elevated level of VE_Cadherin positive MPs is
associated with coronary heart disease in diabetes (451). Tissue-factor bearing
MPs are involved in the formation of tissue factor-platelet hybrids and these are
critical in thrombus formation (493). MPs were also suggested to be one of the
significant procoagulant entities in type 2 diabetes (169). A recent study
conducted at the University of Hull including healthy volunteers, demonstrated
that hypoxia caused by simulated high altitude (3000 m), increased endothelial
dysfunction as measured by raised levels of microparticles in the circulation (378).
In another study the same group found that in healthy young men, the
procoagulant TF- and VCAM-1 positive MPS show a circadian rhythm and their
concentration decreases in the morning between 9am and 11am (494). Our
subjects entered the environmental chamber between 9am and 9:20am, and
stayed in the chamber for two hours. These findings are consistent with our results
from this study showing that there was a general increase in MPs during the
hypoxic experiment compared to the control day, however there was no significant
difference in the magnitude of this change in patients with type 2 diabetes compared to healthy controls.

Overall our findings suggests that there are some elements of clotting mechanism affected both in healthy subjects and patients with type 2 diabetes after simulated flight which was overall not greater in patients with type 2 diabetes. However platelet activation seems to be effected by hypoxia to a degree in T2DM that it altered the platelet activation in the whole group. These findings need to be confirmed in a larger study.
6. CHAPTER Summary discussion

6.1. Summary

This thesis was designed to seek further knowledge concerning some environmental factors which may influence the health of patients with type 2 diabetes, a metabolic disease that is rapidly increasing in prevalence (1, 2). The effects of different dietary soy interventions were examined and compared to the changes in healthy volunteers as was the environmental intervention of short-term hypoxia simulating a commercial air flight.

Type 2 diabetes is a chronic metabolic condition associated with insulin resistance, severe vascular complications and increased mortality (22-24). It is important to be aware how the condition can be prevented and what precautions are needed for patients with this condition.

The cornerstone of management of type 2 diabetes is lifestyle modification, with an emphasis on diet and physical activity (272-275). Polyphenols, such as soy and cocoa, have long been known to carry potential health benefits (195, 298-304, 319-331). Soy is rich in isoflavones, while cocoa is rich in flavanols. Previous studies have suggested their positive effect in various metabolic conditions including type 2 diabetes, however the effect of these food products have not been compared in a double-blind placebo controlled study before. In our study we focused on glycemic control and lipids, while also exploring the effects on endothelial function by measuring soluble endothelial markers and investigating the effect on thyroid function. Our aim was to explore if soy isoflavones are effective on their own, whether soy protein without isoflavones carries health benefits, or if both soy and isoflavones need to be present together to confer metabolic advantage. Furthermore, we wished to establish if any soy or isoflavone benefit can be further enhanced with the addition of cocoa.

It is important to evaluate endothelial function in patients with type 2 diabetes as dysfunction is a feature of the condition and has a close link with atherosclerosis.
and other vascular complications of the disease (113-116, 118). Soy and cocoa have previously been shown to improve endothelial function but was not found in this current study. Thyroid function is also often affected in patients with diabetes and soy might worsen subclinical hypothyroidism. However, we have not seen changes in thyroid hormones in our patients in any of the groups.

Our findings were consistent with the results of others (411) in terms that soy protein or isoflavones alone were ineffective, while the combination improved glycaemic control as shown by the decrease in HbA1c. Cocoa made no additional impact on any of these parameters.

An unexpected finding was that while HbA1c improved, there was no difference in fasting glucose, fasting insulin or insulin resistance. To gain more insight into the possible underlying mechanism for this discrepancy, the second study in the thesis compared the acute glycaemic response following consumption of two different soy snack bars, one containing soy protein only and the other containing both soy protein and isoflavones, in a cross-over study involving patients with type 2 diabetes. We collected insulin, glucose and HOMA-IR area under the curves (AUC) data and calculated the early insulin response to glucose (AIRg) and the Oral Disposition Index (ODI). AUC values and the overall glucose absorption were no different between groups, but there was a delay in glucose absorption, as indicated by the lower AIRg, after consuming the soy bars with isoflavones. A reduced postprandial response to soy ingestion has previously been identified, but delayed gastric emptying, caused by the fibre content of soy products, has been speculated as being the underlying reason (302, 307). However, there was no difference in fibre between our bars. It means that the underlying mechanism by which soy with isoflavones modulates glycaemic control may lie with the postprandial glucose response. However, this a further study, looking at postprandial glycaemia before and after a longer period of soy dietary intervention, will be required to help prove this.

The third study in this thesis attempted to determine whether type 2 diabetes patients showed changes following a simulated commercial air flight which were
likely to increase their risk of developing venous thromboembolism (VTE) compared to healthy individuals. This has never been investigated in patients with type 2 diabetes before even though obesity, which is common in this group of patients, is one of the risk factors known to be associated with developing air travel related VTE.

Commercial air flights have been long known to increase the risk of developing venothrombous events (VTE) (470-478). Type 2 diabetes itself is linked to impaired endothelial function, platelet dysfunction and impairment of the clotting indices (209, 229, 239) – all of them are crucial in developing VTE. However, the data linking T2DM to increase the incidence of VTE remains controversial (203, 431, 441-445).

Type 2 diabetes and healthy controls participated in a simulated flight within an environmental chamber for 2 hours. Participants were also subjected to a ‘control’ day, when the environmental conditions were not modified. We assessed endothelial function using peripheral arterial tonometry and microparticle counting, platelet activation by measuring fibrinogen binding and P-selectin expression with and without adding ADP and PGI₂, and clotting indices using a turbidity assay: evaluating clotting time, maximum clot absorbance, lysis time and lysis area.

Endothelial function did not change in either of the groups during the hypoxic or the control experiment. Basal fibrinogen binding and P-selectin expression increased during the simulated flight in T2DM patients. The increase in basal fibrinogen binding was high enough to cause an overall increase when the data for both groups were combined, however we found no difference between the percentage change in platelet function between T2DM patients and healthy controls. Clot maximum absorption increased during hypoxia in both groups, however the percentage change did not differ between T2DM patients and healthy subjects. These findings suggested that a commercial air flight may expose T2DM patients to greater risk to develop VTE than healthy controls. These preliminary findings may need to be further explored in a larger study, perhaps including a
longer duration of a simulated air flight. It would also be of interest to investigate other patient groups, for example Polycystic Ovary syndrome (PCOS) patients, where patients are also often obese with the features of insulin resistance and are frequently taking the oral contraceptive pill (OCP) as part of the management of their condition.

In conclusion, based on the findings of this thesis, we suggest that soy with isoflavones might improve glycaemic control in type 2 diabetes by modulating the postprandial glucose response. We also established that while some clotting parameters changed detrimentally during a simulated aircraft flight, the magnitude of the change was reassuringly in type 2 diabetes as it was in healthy controls.

6.2. Limitations

The main limitations of the studies included in this thesis were the relatively low number of participants and the short period of interventions. However being pilot studies, the numbers and timescales were sufficient for the purpose of the studies.

6.3. Future work

Providing that these studies were pilot, future work should focus on expanding these studies to more participants and longer intervention periods. When evaluating the effect of dietary interventions in patients with type 2 diabetes, a next obvious action would be to design a study where oral glucose tolerance test or a meal test is incorporated to check for possible changes in postprandial glycaemia.

For future work it would be an exciting project to explore whether simulated flight conditions expose other patients to higher risk to develop deep vein thrombosis, eg. young women with polycystic ovary syndrome, who are active, frequently obese and taking oral contraceptive pill.
7. CHAPTER References


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8. CHAPTER APPENDIX I. Normality testing and distribution histograms.


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<td>LDL</td>
<td>0.06</td>
<td>vWF</td>
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<td>E-selectin</td>
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<td>Waist/hip ratio</td>
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<td>ICAM-1</td>
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<td>VCAM-1</td>
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<td>Insulin</td>
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<tr>
<td>Triglycerides</td>
<td>&lt;0.01</td>
<td>HOMA-IR</td>
<td>0.01</td>
<td>T3</td>
<td>&lt;0.01</td>
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<tr>
<td>HDL</td>
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<td>QUICKI</td>
<td>0.12</td>
<td>T4</td>
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Distribution histograms
8.2. Effects of isolated soy protein versus combined soy protein and isoflavones on postprandial glycaemia and insulin resistance in patients with type 2 diabetes.

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<thead>
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<th>Active</th>
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<td>0.27</td>
</tr>
<tr>
<td>AUC insulin</td>
<td>0.87</td>
<td>0.20</td>
</tr>
<tr>
<td>AUC HOMA-IR</td>
<td>0.28</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>Active</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Peak glucose value</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Peak insulin value</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Peak HOMA-IR value</td>
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<td>0.03</td>
</tr>
<tr>
<td>Time of glucose peak</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>Time of insulin peak</td>
<td>0.06</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Time of HOMA-IR peak</td>
<td>0.06</td>
<td>n/a - constant</td>
</tr>
</tbody>
</table>

**Placebo**

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)

![Graph 4](image4)

**Active**

![Graph 5](image5)

![Graph 6](image6)

![Graph 7](image7)

![Graph 8](image8)
<table>
<thead>
<tr>
<th></th>
<th>AIRg</th>
<th>ODI</th>
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<tr>
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<td>Active (p)</td>
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<tr>
<td>0-30</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>30-60</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>30-90</td>
<td>0.20</td>
<td>0.20</td>
</tr>
</tbody>
</table>

**Early Insulin Response to glucose (AIRg)**

**Placebo**

**Active**
Oral Disposition Index (ODI)

Placebo

Active
### 8.3. FlyBar study normality testing and distribution histograms

#### 8.3.1. Baseline characteristics, clotting indices, platelet and endothelial function

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<td>cIMT</td>
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Patient characteristics

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</thead>
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<td>Maximum absorption</td>
<td>0.72</td>
<td>Lysis time</td>
</tr>
<tr>
<td>Lysis area</td>
<td>0.06</td>
<td>Basal fibrinogen binding</td>
<td>0.01</td>
<td>Basal P-selectin expression</td>
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<td>P-selectin expression ADP</td>
<td>0.02</td>
<td>Fibrinogen binding ADP 1μm</td>
</tr>
<tr>
<td>0.1μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-selectin expression ADP</td>
<td>0.43</td>
<td>Fibrinogen binding ADP 10μm</td>
<td>0.21</td>
<td>P-selectin expression ADP 10μm</td>
</tr>
<tr>
<td>1μm</td>
<td></td>
<td></td>
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</table>

Clotting indices, basal platelet activation and response to ADP

<table>
<thead>
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<td>P-selectin expression PGI₂</td>
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<td>1nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-selectin expression PGI₂</td>
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<td>Fibrinogen binding PGI₂ 100nM</td>
<td>0.00</td>
<td>P-selectin expression PGI₂ 100nM</td>
</tr>
<tr>
<td>10nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Platelet sensitivity to PGI₂

<table>
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<tbody>
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<td>0.06</td>
<td>CD42b</td>
<td>0.07</td>
<td>Total CD42b</td>
</tr>
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<tr>
<td>RHI</td>
<td>0.12</td>
<td></td>
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<td></td>
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Microparticles and Reactive Hyperaemia Index (RHI)
8.3.2. Absolute changes in clotting parameters, platelet function and endothelial function during the normal and the hypoxic experiments

**Clotting parameters**

<table>
<thead>
<tr>
<th></th>
<th>Hypoxia</th>
<th>Normal day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time</td>
<td>p</td>
<td>p</td>
</tr>
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<td>Maximum absorption</td>
<td>0.90</td>
<td>0.09</td>
</tr>
<tr>
<td>Lysis time</td>
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<td>&lt;0.01</td>
</tr>
<tr>
<td>Lysis area</td>
<td>0.32</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Hypoxia

Normal day
Hypoxia

Normal day
Platelet function – normal day

<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen binding</th>
<th>P-selectin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>Basal</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ADP 0.1 μm</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>ADP 1 μm</td>
<td>0.70</td>
<td>0.80</td>
</tr>
<tr>
<td>ADP 10 μm</td>
<td>0.16</td>
<td>0.87</td>
</tr>
<tr>
<td>PGI₂ 1nM + ADP</td>
<td>0.62</td>
<td>0.68</td>
</tr>
<tr>
<td>PGI₂ 10nM + ADP</td>
<td>0.27</td>
<td>0.96</td>
</tr>
<tr>
<td>PGI₂ 100nM + ADP</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Fibrinogen binding

[Graph of Fibrinogen binding]

P-selectin expression

[Graph of P-selectin expression]
Fibrinogen binding

P-selectin expression
Fibrinogen binding

P-selectin expression
Platelet function – hypoxia

<table>
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<tr>
<th></th>
<th>Fibrinogen binding</th>
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<td>( p )</td>
<td>( p )</td>
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<td>0.23</td>
<td>&lt;0.01</td>
</tr>
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</tr>
<tr>
<td>ADP 1 μm</td>
<td>0.07</td>
<td>0.72</td>
</tr>
<tr>
<td>ADP 10 μm</td>
<td>0.24</td>
<td>0.63</td>
</tr>
<tr>
<td>PGI(_2) 1nM + ADP</td>
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<td>0.60</td>
</tr>
<tr>
<td>PGI(_2) 10nM + ADP</td>
<td>0.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PGI(_2) 100nM + ADP</td>
<td>&lt;0.01</td>
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</table>
Fibrinogen binding

P-selectin expression
Fibrinogen binding  P-selectin expression
### Endothelial function

<table>
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</tr>
</thead>
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<tr>
<td>Endothelial function</td>
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<td></td>
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<tr>
<td>CD42b/annexin V</td>
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<td>&lt;0.01</td>
</tr>
<tr>
<td>CD42b</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total CD42b+ MPs</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD45/annexin V</td>
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<td>0.82</td>
</tr>
<tr>
<td>CD45</td>
<td>0.32</td>
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<td>Total CD45+ MPs</td>
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<td>&lt;0.01</td>
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<td>&lt;0.01</td>
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<tr>
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Hypoxia

Normal day
Hypoxia

Normal day
Hypoxia

Normal day
8.3.3. Percentage changes

Clotting indices

<table>
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<th>Total change</th>
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<tr>
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<td>Lag time</td>
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<td>Maximum absorption</td>
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<td>&lt;0.01</td>
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<td>Lysis time</td>
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<td>&lt;0.01</td>
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<td>Lysis area</td>
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</table>

Normal day           

Hypoxia              

Total change         

![Histograms for Lag time, Maximum absorption, Lysis time, and Lysis area](image)
### Percentage changes in platelet function

<table>
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<th>Hypoxia</th>
<th>Total change</th>
</tr>
</thead>
<tbody>
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<td><strong>Fibrinogen binding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>&lt;0.01</td>
<td>0.28</td>
<td>0.10</td>
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<tr>
<td>ADP 0.1 μm</td>
<td>0.07</td>
<td>&lt;0.01</td>
<td>0.28</td>
</tr>
<tr>
<td>ADP 1 μm</td>
<td>0.08</td>
<td>&lt;0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>ADP 10 μm</td>
<td>0.09</td>
<td>0.37</td>
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<td><strong>P-selectin expression</strong></td>
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<td>&lt;0.01</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ADP 1 μm</td>
<td>0.07</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ADP 10 μm</td>
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<td>0.43</td>
<td>0.82</td>
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<table>
<thead>
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<th>Total change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PGI₂ 1nM + ADP</strong></td>
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<td></td>
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<td>Basal</td>
<td>&lt;0.01</td>
<td>0.03</td>
<td>&lt;0.01</td>
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<tr>
<td>ADP 10nM + ADP</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>PGI₂ 100nM + ADP</strong></td>
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</tr>
<tr>
<td>Basal</td>
<td>&lt;0.01</td>
<td>0.13</td>
<td>&lt;0.01</td>
</tr>
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<td>0.15</td>
<td>0.82</td>
</tr>
<tr>
<td>ADP 10nM + ADP</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.06</td>
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</table>
Response to ADP
Fibrinogen binding

Normal day

Hypoxia

Total change
Response to ADP

P-selectin expression

Normal day

Hypoxia

Total change
Sensitivity to PGI$_2$

Fibrinogen binding

Normal day  Hypoxia  Total change
P-selectin expression

Normal day

Hypoxia

Total change
### Percentage changes in endothelial function

<table>
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<td>CD42b/annexin V</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<td>CD42b</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
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</table>

National Research Ethics Service
NRES Committee Yorkshire & The Humber - Humber Bridge
Yorkshire and the Humber Research Ethics Office
First Floor
Millside
Mill Pond Lane
Leeds
LS6 4RA
Telephone: 0113 3050127

Professor Stephen Atkin
Head of Academic Endocrinology, Diabetes and Metabolism
University of Hull
Michael White Diabetes Centre, Brocklehurst Building 220-236 Anlaby Road Hull HU3 2RW

Dear Professor Atkin

Study title: A Pilot Study Investigating the Effects of the Combined Effects of Cocoa and Soy Polyphenols in a Soy Protein Matrix on Insulin Resistance and Cardiovascular Disease Risk in Type 2 Diabetes – A Randomised Placebo-Controlled Double-Blind Parallel Study.
REC reference 11NH/0219
Protocol number not applicable

Thank you for your letter of 06 July 2011, responding to the Committee’s request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Alternate Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start
of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present.

We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA.

In the meantime no study procedures should be initiated at non-NHS sites.

**Conditions of the favourable opinion**

The favourable opinion is subject to the following conditions being met prior to the start of the study.

*Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.*

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation’s role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"); guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advertisement</td>
<td>1</td>
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</tr>
<tr>
<td>Covering Letter</td>
<td></td>
<td>03 June 2011</td>
</tr>
<tr>
<td>GP/Consultant Information Sheets</td>
<td>2</td>
<td>05 July 2011</td>
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<tr>
<td>Letter of invitation to participant</td>
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<td>Other: CV - J Kanya (Student)</td>
<td></td>
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<tr>
<td>Other: CV - E Kilpatrick</td>
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</tr>
<tr>
<td>Other: 7 day food diary</td>
<td>1</td>
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</tr>
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</table>
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

• Notifying substantial amendments
• Adding new sites and investigators
• Notification of serious breaches of the protocol
• Progress and safety reports
• Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review 11NH/0219 Please quote this number on all correspondence

With the Committee’s best wishes for the success of this project

Yours sincerely

Dr David Horton
Chair

Email: nicola.mallender-ward@nhs.net

Enclosures: “After ethical review – guidance for researchers” [SL-AR2]

Copy to: Dr Judit Konya, Hull and East Yorkshire Hospitals NHS Trust
Mr James Illingworth, Hull and East Yorkshire Hospitals NHS Trust
Study protocol - The effects of soy protein with or without isoflavones on glycaemic control in type 2 diabetes. A parallel, randomized, double-blind, placebo-controlled pilot study.

Full Title: A Pilot Study Investigating the Effects of the Combined Effects of Cocoa and Soy Polyphenols in a Soy Protein Matrix on Insulin Resistance and Cardiovascular Disease Risk in Type 2 Diabetes – A Randomised Placebo-Controlled Double-Blind Parallel Study.

Short title: Effects of combining cocoa and soy in type 2 diabetes


EudraCT number: Not Applicable, as the study is based on food components. Correspondence in appendix (appendices 1 and 2) states the MHRA is not interested in the food components which are used in the study.

Chief/Principal Investigator:
Professor Stephen L Atkin
Head of Academic Diabetes, Endocrinology and Metabolism
Hull York Medical School
220-236 Anlaby Road
HULL, HU3 2RW
E-mail: stephen.atkin@hyms.ac.uk
Telephone: 01482 675365

Sponsor:
Hull and East Yorkshire Hospitals NHS Trust,
R & D department, Office 6, 2nd Floor Daisy Building,
Castle Hill Hospital, Castle Rd,
Cottingham, East Yorkshire HU16 5JQ.

Funder:
Diabetes Charitable Funds
C/o Professor Stephen L Atkin

Statistician:
Mr Alan Rigby
Department of Medicine
University of Hull
Department of Cardiology
Castle Hill Hospital, Castle Road
Cottingham, HU16 5JQ
Email: asr1960@hotmail.com

Biochemical analysis will be undertaken in the clinical laboratories at Hull Royal Infirmary.

Development and manufacture of all bars will be undertaken by Halo Foods in accordance with Good Manufacturing Practice.
Background information

Soy is a staple in the diet of the Japanese, and consumption of soy has been shown to have an inverse relationship with mortality from CVD. Cardiovascular benefits from consuming in particular the isoflavones, genistein and daidzein have been demonstrated in the management of diabetes. The antidiabetic action of soy phytoestrogens has been linked from in vitro investigations to it being an inhibitor of the α-glucosidase in the brush border of the intestine, alongside its action inhibiting tyrosine kinase. Diets containing soy have been shown to improve insulin resistance in both primates and postmenopausal women. In a study of the effect of soy phytoestrogen a 30g per day dose providing 132mg per day of isoflavones was found to reduce insulin resistance by 6.5%, with no significant effect on weight, blood pressure and the hypothalamic-pituitary-ovarian axis.

Epidemiological studies suggest dietary flavonoids decrease the risk of death from coronary heart disease, cancer, and stroke. In a cohort of elderly men, cocoa intake was inversely associated with blood pressure and 15-year cardiovascular and all-cause mortality. Flavonoid-rich foods include fruits and vegetables as well as tea, red wine, and chocolate. Short-term administration of dark chocolate was followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation. In healthy humans, consumption of flavanol-rich dark chocolate decreased daytime and night time BP, reduced insulin resistance, and improved NO-dependent vaso-relaxation. This has led to the hypothesis that chocolate containing high cocoa solids may help to reduce the risk of developing type 2 diabetes.

Type 2 diabetes is being acknowledged as a potential public health time bomb, incidence is predicted to double over the next 10 years in the UK, the linked to this increase are thought to be associated with the rise in obesity and increasing sedentary lifestyles. Increased insulin resistance (IR) has been shown to be an important feature of type 2 diabetes (especially in those presenting with obesity and in particular visceral or abdominal obesity). IR is implicated as a risk factor of cardiovascular disease and may lead to pancreatic dysfunction owing to an increase demand on the β-cell of the pancreas. This leads to the manifestation of increased blood glucose levels and type 2 diabetes. The main cause of morbidity and mortality in type 2 diabetes is cardiovascular disease as the condition is associated with impaired vascular functioning and increased levels of oxidation markers.

The benefits of soy protein, with isoflavones in diabetes, have been demonstrated, although this did not appear to be the case with isoflavones alone. The aim of this study is to determine if polyphenol rich cocoa powder adds to any beneficial effects that soy protein and soy isoflavones may have in subjects with stable type 2 diabetes. To date no study has aimed to look at the combined effect of soy protein with 2 sources of polyphenol on insulin resistance and cardiovascular risk. All the results will be compared with results originated from effect of placebo.

Both soy isoflavones and cocoa polyphenols have been shown to improve mood in certain groups of patients but these effects are still not well-characterised.

Aims and Objectives

The aim of this study is to perform a double-blind, parallel, randomized, placebo-controlled study using a snack bar of 15g (7.5g twice daily) of soy protein as a base with added isoflavones and/or cocoa polyphenols to assess their effects on insulin resistance and other markers of diabetes control and cardiovascular risk. The placebo bar will be identical with rice flour base without any protein, isoflavons or polyphenol.
This research is also being carried out as part of the work for a higher research MD degree.

**Primary Research Question**

Does a snack bar enriched with soy protein and/or cocoa (with or without soy isoflavones) improve parameters of glucose control, insulin resistance, oxidative stress, vascular reactivity and other cardiovascular risk parameters in individuals with type 2 diabetes?

**Study Design**

This is a randomized, parallel, double-blind, placebo-controlled trial involving 100 individuals with type 2 diabetes.

Two bars containing a base of 15g (7.5 g each) of 70% isolated soy protein powder (Solcon F) will be given daily for 8 weeks following a 2 week run in period except the placebo bar which doesn’t contain soy protein powder The hundred subjects will be randomly allocated into 1 of 5 treatment arms.

The four arms will be:
- **A**, placebo bar without soy protein, isoflavones or cocoa polyphenols
- **B**, soy protein alone (soy isoflavone and cocoa polyphenol free);
- **C**, soy protein with soy isoflavones (each bar containing 16mg, 2 bars per day =32mg)
- **D**, soy protein alone with cocoa (each bar containing 400mg, 2 bars per day= 800mg)
- **E**, soy protein with isoflavones (each bar containing 16mg isoflavones, 2 bars per day=32mg isoflavones) and cocoa (each bar containing 400mg cocoa polyphenols, 2 bars per day=800mg polyphenols)

All the bars used in the study will be matched for taste and macronutrient content. The dose of soy protein and isoflavones was chosen as it was shown to be effective in our previous studies (submitted for publication). Each box will contain the number of soy bars required for four weeks of treatment plus 5 bars marked as reserve. The boxes and bars will only be identified by the randomisation number (randomisation will be held by Essential Nutrition, Brough, East Yorkshire).

**Study Visit Schedule (See Appendix 3)**

**Visit 1: (Screening visit)**
Consent
Anthropometric measurement – Height, Weight, Waist circumference
Blood pressure
Baseline bloods:
Hba1c, hs-CRP, lipid profile, biochemical profile and full blood count
Interview with a dietitian to screen for an excessive intake of isoflavones and to request avoidance of high sources of polyphenol rich foods for the duration of the study.
Subjects will be given the option to have a BVI (Body Volume Index) assessment

**Visit 2A: (1 day prior to Visit 2B)**
Patients will attend the Diabetes Centre to have the 24hour blood pressure machine placement. Patients will be given urine containers and the 24 hour urine collection starts.
**Visit 2B: Randomisation (2 weeks after visit 1)**
Following a 10 hour fast
Anthropometric measurement – Height, Weight, Waist circumference
Randomisation
Bloods:
Labs: Plasma glucose, HbA1c, Lipids, hs-CRP
Two plasma (yellow tops) and 2 purple, 1 light blue and 1 green top for oxidative stress markers, insulin, glucagon (to be stored in -80 freezer)
24 hour blood pressure machine removal
Collection of the urine containers to assess dietary phytoestrogen intake and urinary isoprostanes
Endothelial dysfunction assessment using Endopat 2000
Dispensing of snack bars (2 per day for 4 weeks)

**Visit 3: Dispensing Visit (4 weeks from visit 2)**
Anthropometric measurement – Height, Weight, Waist circumference.
Blood pressure
Compliance assessment: counting the empty wrappers and unused bars brought back by the patient
Dispensing of snack bars (2 per day for 4 weeks)

**Visit 4A: 1 day prior to Visit 4B.**
Patients will attend the Diabetes Centre to have the 24 hour blood pressure machine placement. Patients will be given urine containers and the 24 hour urine collection starts.

**Visit 4B: Final visit (after 4 weeks from visit 3)**
Following a 10 hour fast, subjects will have:
Anthropometric measurement - Weight, Waist circumference,
Bloods:
Labs: Plasma glucose, HbA1c, Lipids, hs-CRP
Two plasma (yellow tops) and 2 purple, 1 light blue and 1 green top for oxidative stress markers, insulin, glucagon (to be stored in -80 freezer)
Remove the 24 hour blood pressure machine
Collection of the urine containers to assess dietary phytoestrogen intake and urinary isoprostanes
For subjects opting into the BVI arm a second BVI measurement will be made
Endothelial dysfunction assessment using Endopat 2000
Compliance assessment: counting the empty wrappers and unused bars brought back by the patient

For 1 week prior to visit 2 and visit 4 subjects will be asked to complete a 7 day food diary and complete hourly visual analogue scales for hunger for the last 3 days of this 7 day period. The food diary contains mood rating scale to assess the changes in mood.

For assays for insulin, glucagon, oxidative stress and urinary measures, samples will be processed and stored at -80oC until completion of the study so that they can be analysed as a single batch.
Criteria for discontinuation of individual subjects, parts of the study or the entire study

As it is a relatively short intervention with a well tolerated food stuff this is felt to be very unlikely.

*Individuals may be withdrawn:*
- If they lose capacity
- If they develop an allergy or adverse reaction to the soy or the ingredients in the bar
- If compliance with the bar is less than 85%
- If they require a change in medication during the study, excluding self treatment with over the counter treatments, e.g. cold remedies and painkillers.

*For the part or the whole study to be withdrawn:*
- If the European Food Safety Agency or Food Standard Agency said that soy or any other component of the snack bars was unsafe as a food ingredient.

## Plan for the Study

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Visit 1</th>
<th>Visit 2A</th>
<th>Visit 2B</th>
<th>Visit 3</th>
<th>Visit 4A</th>
<th>Visit 4B</th>
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</table>
Primary Endpoints

Soy with isoflavones and cocoa polyphenols have a greater impact on insulin resistance, lipid parameters in type 2 diabetes than seen with soy protein alone, soy protein with isoflavones and soy protein with cocoa polyphenols.

Secondary Endpoints

Soy with isoflavones reduces cardiovascular risk as measured by fasting lipids and blood pressure

Soy with isoflavones increases plasma isoflavones

Soy with isoflavones does not negatively affect endothelial function as assessed by the EndoPAT 2000

Polyphenol rich cocoa improves endothelial architecture as assessed by EndoPAT 2000

Soy with isoflavones and polyphenol rich cocoa reduces insulin resistance as measured by HOMA

Changes in weight, body volume and circumferences between the bars and compared to baseline

Changes in mood from baseline to the end of the trial and comparison between the different arms of the study.

Subject selection

Patients will be identified through diabetes clinics, then an invitation letter will be sent to their home address along with the patient information sheet.

Local GPs will be approached in order to send an invitation letter with the participant information sheet to their patients who might be suitable for the study.

Subjects will be recruited by an advert in local papers.

Number of centres involved:

Michael White Diabetes Centre, 220-236 Anlaby Road, Hull

The optional BVI measurement will take place:
Department of Sport, Health and Exercise Science
Room 108. Washburn Building
West Campus of University of Hull, Cottingham Road, Hull

Inclusion Criteria

- The diagnosis of type 2 diabetes will be based on the WHO guidelines. These are 2 fasting plasma glucose readings of >7.0mmoll-1 or 2 random plasma glucose readings >11mmoll-1 in the absence of symptoms or concurrent illness or medication which might lead to hyperglycaemia (e.g. thiazide diuretics). Or one reading meeting the diagnostic level with the presence of symptoms of polyuria, polydipsia, nocturia, fatigue or blurring of vision. The final diagnostic method of diagnosis type 2 diabetes is a positive oral glucose tolerance
test (OGTT) using a 75g glucose load. If doubt exists on the diagnosis of diabetes an OGTT will be performed.

- Patients will be on stable medication (treatment only metformin) for their diabetes, hypertension, lipids and gout (if appropriate) for 3 months prior to entry into the study.
- Age between 45-80 years at the start of the study.

**Exclusion Criteria**

- Patients with concurrent illness or any medication in the last 3 months
- Patients not wishing to allow disclosure to their GPs.
- Patients who are taking hormone replacement therapy
- Patients who are currently or have taken antibiotics in the last 3 months
- HbA1c at recruiting stage of >9%
- Patients with known food allergies
- Smokers
- Vegans and vegetarians
- Patients not willing to consume snack bar
- Pre-menopausal women

**Subject recruitment**

Recruitment process: Males and post menopausal women with type 2 diabetes will be identified and contacted, available information will be reviewed to assess suitability including:

Patients will be identified through diabetes clinics, then an invitation letter will be sent to their home address along with the patient information sheet. They will be asked to ring the Diabetes Research Team if they are interested in the study.

Letters will be sent to local GPs in order to ask them to send an invitation letter with the patient information sheet to their patients who might be suitable for the study. In this invitation letter patients will be asked to contact the Diabetes Research Team if they express interest for the study.

Subjects also will be recruited by an advert in local papers. In the advertisement they will be asked to call the Diabetes Research Team, in case they want to know more information about the study. Contact details will be recorded and an invitation letter will be sent with the patient information sheet.

Following the opportunity to review the participant information sheet, individuals will be able to discuss the protocol with a trained member of the research team, who subject to their being comfortable that the individual understands the study will take informed consent. This will be counter signed by the research team member. As this study is aimed at individuals following a westernised diet and not having a habitually high isoflavone intake, only Western Europeans will be recruited, this along with cost implications mean that it will not be possible to include non-English speakers. Due to the short nature of the study it is unlikely that individuals will lose capacity, if this is the case, a serious adverse event would be reported,
and the subject discontinued. All data would be removed from analysis. As subjects will need to complete food diaries and questionnaires it will not be possible to include those with dementia and severe mental illness.

Patients will have enough time (at least one week) between they first read the patient information sheet and the time when they decide (and probably sign the consent form) about participation.

**Randomisation**

The randomisation will be performed by Essential Nutrition Ltd, UK. A computer generated randomisation list will be used to provide balanced blocks of patient numbers for each of the four treatment groups. A 1:1:1:1 treatment allocation will be used. The block size will not be revealed. A copy of the randomisation code will be held in a sealed envelope held in the study master file in case of clinical emergency.

**Blinding and other measures taken to avoid bias**

The study will be double-blind, with the ‘combined active’ being a dose of 15g soy protein per day with 32mg of isoflavones and 800mg of cocoa polyphenols, the placebo will contain rice flour without soy base, additional isoflavones or cocoa polyphenols. Comparator arms of 15g soy protein alone, 15g soy protein with 32mg of isoflavones and 15g of soy protein with 800mg of cocoa polyphenols will also be included, so potential synergistic effects of combined polyphenols can be explored. Blinding will be done at manufacture and the code will be placed in a sealed envelope only to be opened in emergency.

**Subject compliance**

Empty wrappers and uneaten bars will be returned and counted by the study team (at visit 3 and Visit 4B). If compliance is less than 85% then the subject will be withdrawn. Subjects will be reviewed by the study team 4 weeks after their withdrawal to monitor any adverse events.

**Withdrawal of Subjects**

If a subject is withdrawn, follow-up with the study team will be offered within 4 weeks to review any adverse events. Where possible they will be replaced in the study.

**Treatment of subjects**

The treatment will be with a snack bar to be taken twice daily. This will contain 7.5g of soy protein per bar with or without added isoflavones 16 per bar and with or without 400mg per bar of cocoa polyphenols. The placebo bar will not contain any soy protein, added isoflavones or cocoa polyphenols, only rice flour. Other ingredients include fruit concentrate flavouring, oats, sugar and palm oil.

**General information**

A snack bar based on soy protein isolates to be consumed twice daily.
Use of treatment within the trial

The snack bar is to be taken orally twice daily as between meal snacks for a period of 8 weeks. This duration is known to be long enough to assess an effect on the primary and secondary endpoints.

Patients will be taking the bars themselves and retaining empty packaging for compliance checks.

Each bar contains 7.5g soy protein, and depending on the study arm may also contain 16mg of soy isoflavones and/ or 400mg of cocoa polyphenols in 1.6g of cocoa powder. The placebo bar will contain none of these ingredients.

A dispensing / compliance log will be kept with the case report form.

All the bars will be produced and supplied by Halo Foods Ltd.

The snack bars will be made in accordance with Good Manufacturing Practice. Shelf life will be adequate for the duration of the trial. All ingredients will be fully traceable to source.

The bar will not necessarily be available for commercial use following the trial; a registered dietitian will be available for the participants to discuss how to incorporate soy into their diet following the study.

It is important that the participants are on stable medication prior to entry onto the study. The only treatments that need to be avoided are antibiotics for at least 3 months.

No changes in medications are permitted during the trial except general over the counter treatments, e.g. painkillers.

Efficacy Assessments

Efficacy will be measured by HOMA, lipids and weight for the primary outcome. Secondary outcome measures will be insulin, glucose, Hba1c, EndoPAT 2000, blood pressure, oxidative stress and inflammatory markers.

All samples will be stored and batched for analysis at the end of the study. Samples will be stored for two years after the completion of the study and will then be destroyed. Permission will be request to use the samples for additional analysis on cardiovascular risk indices during this period of time. The samples will be used for ethically approved future studies if those are intended to commence before the end of this particular study.

Safety Assessments

Adverse events will be reported in accordance with HEYHT R & D department adverse event reporting procedures. Serious adverse events will be notified to HEYHT R & D dept. within 24hrs of investigators becoming aware of the event using the SAE/SUSAR initial and follow-up report forms provided by R & D. All adverse events (serious and non-serious) will be recorded in patients data collection forms (CRFs) using R & D's adverse event report form. All adverse events will be recorded in patients' medical records.

All subjects following an adverse event will be followed by the research team for 4 weeks following the reported event.
Data collection

Data will be collated in patient notes and a case report form in line with trust guidelines and good clinical practice.

This study consists of 6 visits, at 3 blood samples will be taken, at 3 anthropometric data will be collected and at 2 assessment of endothelial function will be undertaken. For individuals who have opted to have a BVI scan, these will be undertaken at 2 visits. Also there will be two 24hour blood pressure measurement and 24 hour urine collection twice during the study.

Data will be collected by the study team. This will include research nurses, dietitians and medical practitioners. Data will be anonymised, with only be identified by the patient identification number.

Sample Size

The sample size is based on the effect of soy phytoestrogen in postmenopausal women with diabetes and effects on HDL seen with a similar dose of cocoa polyphenols provided by chocolate and was performed using N-Query software. Powered specifically for HOMA and HDL the minimum difference worth detecting/observed difference was 1.0, estimated within group SD was 0.9; therefore, for 80% power and a significance level of 5%, a sample size of 16 per group was calculated. If a 20% drop out rate is to be adjusted for then 20 per group would be needed, thus 100 patients in total would need to be recruited for the entire study.

Statistical Analysis

The analysis will be performed at the end of the study by Mr Alan Rigby, Medical Statistician, University of Hull.

The findings of the trial will be presented in line with CONSORT guidelines. Continuous normally distributed data are expressed as mean±SD. Within each treatment group, changes in oxidative stress markers, fasting and postprandial plasma glucose, insulin and other parameters from baseline will be analyzed by 1-factor analysis of variance. The assumptions of ANOVA will be tested by residual inspection. Where significant difference between groups reported, post-hoc analysis will be performed by Tukey’s honestly significant difference (HSD) test.

Monitoring

Arrangements for monitoring/auditing conduct of the research: The study will be monitored in accordance with HEYHT R & D department’s standard operating procedures to ensure compliance with UK GCP regulations. All trial related documents will be made available upon request for monitoring by R & D monitors.

Ethical considerations

The main consideration with this study is that care needs to be undertaken to exclude individuals who may have food allergies and intolerances to the ingredients in the bars. Care will also be taken to check that the individuals who are participating in the study do not gain weight from consuming the snack bars. From experience with previous soy studies, this has not been the case. However if an individual did gain weight then they would be offered repeated counselling with the study dietitian.
Ethics and R&D approval

The study will be performed subject to a Local Research Ethics Committee favourable opinion, Site Specific Assessment (SSA) approval and HEY Trust R & D approval.

Research Governance

This study will be conducted in line with the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines; and the Research Governance Framework for Health and Social Care.

Data handling and record keeping

Professor Atkin will act as the data custodian and is responsible for the storage, handling and quality of the study data

Utilising the Trust’s IT Services Department facility which has a backup procedure approved by auditors for disaster recovery. Servers are backed up to tape media each night. The tapes run on a 4 week cycle. Files stay on the server unless deleted by accident or deliberately. Anything deleted more than 4 weeks previously is therefore lost. Additional ‘archive’ backups are taken for archived data, so data should not be lost from this type of system e.g. FileVision which stores Medical Records. Tapes are stored in a fireproof safe.

Data will be collected in the case notes and in the case report form to allow for cross referencing to check validity.

Study documents (paper and electronic) will be retained in a secure (kept locked when not in use) location during and after the trial has finished. All essential documents including source documents will be retained for a minimum period of 5 years after study completion (last patient, last visit). A label stating the date after which the documents can be destroyed will be placed on the inside front cover of the casenotes of trial participants.

Data will be collected and retained in accordance with the Data Protection Act 1998.

Access to Source Data

Monitoring, audits, REC and Food Standards Agency reviews will be permitted. Direct access to source data and other study-specific documents will be provided.

Finance

The research project is funded by the Diabetes Endowment Fund.

In relation to the study visits, all travel and parking fees will be reimbursed for the patients.

Indemnity

This is an NHS-sponsored research study. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS indemnity covers NHS staff and medical academic staff with honorary contracts only when the trial has been approved by the Trust R & D department. NHS indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm.
Reporting and dissemination

This study will be reported at national and international conferences along with a number of papers in peer reviewed academic journals. A review of the study will also be made available to participants. Reporting of the study findings will be in accordance with the CONSORT statement (Consolidated Standards of Reporting Trials http://www.consort-statement.org/).
Signature page

Chief/ Principal Investigator
Professor Stephen Atkin
Signed ______________________________  Date _______________________________

Academic Co-Supervisor
Professor Eric Kilpatrick
Signed ______________________________  Date _______________________________

Sponsor, on behalf of Hull And East Yorkshire Hospitals NHS Trust
Name Mr James Illingworth
Signed ______________________________  Date _____________________________
Appendix 1

Correspondence with MHRA Regarding Soy in previous clinical trials

-----Original Message-----
From: Clinical Trial Helpline [mailto:ctdhelpline@mhra.gsi.gov.uk]
Sent: 12 February 2009 14:37
To: Stephen Atkin
Subject: RE: scope- protocol review - SOPHY protocol 2.2.doc (58 KB)

Dear Stephen

Thank you for your email dated 05 February 2009

I can confirm that your proposal is not a Clinical Trial of an
Investigational Medicinal Product (IMP) as defined by the EU Directive
2001/20/EC. You therefore are not required to submit a Clinical Trial
Authorisation (CTA) to the MHRA.

Please note that this e-mail is a normal form of communication for this
advice by the MHRA and no letter will be sent out for this.

Kind regards

Clinical Trial Helpline
MHRA
Dear Professor Atkin,

Thank you for your email dated 28th April 2008.

I can confirm that your proposal is not a Clinical Trial of an Investigational Medicinal Product (IMP) as defined by the EU Directive 2001/20/EC. You therefore are not required to submit a Clinical Trial Authorisation (CTA) to the MHRA.

Please note that this e-mail is a normal form of communication for this advice by the MHRA and no letter will be sent out for this.

Kind regards

Clinical Trial Helpline
MHRA

From: Stephen Atkin [mailto:Stephen.Atkin@hyms.ac.uk]
Sent: 28 April 2008 22:40
To: Clinical Trial Helpline
Cc: Mellor, Duane
Subject: Scope- protocol review for chocolate study

Dear Sir

from the information on medicinal products in clinical trials, the use of a commercially available chocolate in a clinical trial would not seem to be under your remit. I would be grateful if you could confirm that this is indeed the case

Best wishes

Stephen Atkin

Professor SL Atkin
Head, Academic Endocrinology, Diabetes and Metabolism,
Hull York Medical School,
Michael White Diabetes Centre,
220-236, Anlaby Road,
Hull,
HU3 2RW,
Tel. 01482 675365
Fax 01482 675370
Appendix 3

List of references


Participant Information Sheet - The effects of soy protein with or without isoflavones on glycaemic control in type 2 diabetes. A parallel, randomized, double-blind, placebo-controlled pilot study.

Participant Information Sheet (Version 4.17.10.2011.)

Full title: A Pilot Study Investigating the Effects of the Combined Effects of Cocoa and Soy Polyphenols in a Soy Protein Matrix on Insulin Resistance and Cardiovascular Disease Risk in Type 2 Diabetes – A Randomised Placebo-Controlled Double-Blind Parallel Study.

Short title: Effects of combining cocoa and soy in type 2 diabetes


Introduction
We would like to invite you to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it would involve for you. Please take the time to read the following information carefully and discuss it with friends, relatives and your GP if you wish.

If there is anything that is not clear, or if you would like more information, please ask us. Take time to decide whether or not you wish to take part. The research is being carried out by doctors and dietitians who have specialised in diabetes. This study is designed to look at potential food based therapies for diabetes. Thank you for taking the time to read this information sheet.

What is the purpose of the study?
Diabetes is an increasingly common condition which affects millions of people in the United Kingdom. A cornerstone of the treatment is lifestyle which includes looking at the way how people eat. It is known that food containing compounds called polyphenols can reduce the risk of heart disease and other problems in people with diabetes. Two foods which are rich sources of polyphenols are cocoa (epicatechins) and soy (isoflavones). Studies in the past have shown the benefits of these foods in the diets of people with diabetes. What is not known is whether there is any extra benefit of combining soy protein and isoflavones with cocoa. It has also been shown that soy isoflavones and cocoa polyphenols can improve the mood in certain groups of patients.

The aim of the study is to have more knowledge about the effects of soy and/or cocoa in patients with type 2 diabetes. We also would like to determine whether there is any additional benefit when extra cocoa and soy are added together to the diet.

We plan to enrol 100 patients to the study. You will be asked to eat two soy bars per day for 8 weeks. The bars will contain:
- soy protein alone, or
- soy protein with additional isoflavones, or
- soy protein with cocoa, or
- soy protein with isoflavones and cocoa, or
- Inert bars without soy protein, extra isoflavones or cocoa. The base of this bar will be rice flour.
The study is randomised which means a computer based allocation schema will be used to decide which bars you will need to eat. You will have the same 20% chance to be participant of any of the above groups. The study is placebo-controlled which means we will use placebo bars without soy protein, additional isoflavones and cocoa. The study is double-blind which means nor you or the research team will know which bars you will take (it is possible because the foil wrapper won’t allow us to see what is in the package).

**Why have I been chosen?**
You have type 2 diabetes and you are on stable medication.

**Do I have to take part?**
No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the study and you would like to participate will you be asked to sign a consent form (attached to this information sheet) and will be given a copy of the patient information sheet and the consent form to keep. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will happen to me if I take part?**
If you decide to take part, you would be expected to attend the Michael White Diabetes Centre, Brocklehurst Building, 220-236 Anlaby Road, Kingston Upon Hull for 6 visits over a period of 2-3 months.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Visit 1</th>
<th>Visit 2A</th>
<th>Visit 2B</th>
<th>Visit 3</th>
<th>Visit 4A</th>
<th>Visit 4B</th>
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<tbody>
<tr>
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<tr>
<td>Height, weight and waist measurement</td>
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<td>X</td>
<td>X</td>
<td></td>
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<td>X</td>
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<tr>
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<td></td>
<td>X</td>
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<tr>
<td>Blood pressure Remove 24h blood pressure machine</td>
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<td>X</td>
<td>X</td>
<td></td>
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<td>X</td>
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<tr>
<td>EndoPAT 2000</td>
<td>X</td>
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<tr>
<td>Fasting blood test</td>
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<tr>
<td>Dispensing of containers for 24 hour urine collection</td>
<td>X</td>
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<td>X</td>
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<tr>
<td>Collection of filled in urine containers</td>
<td>X</td>
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<td>X</td>
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<tr>
<td>Dispensing of snack bars</td>
<td>X</td>
<td>X</td>
<td></td>
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</tr>
<tr>
<td>Optional Body Volume Index (BVI) measurement</td>
<td>X</td>
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<td></td>
<td>X</td>
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<tr>
<td>Collection of empty wrappers and uneaten bars</td>
<td>X</td>
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<td>X</td>
</tr>
</tbody>
</table>
At the first visit you will discuss the study with a study doctor who will explain each aspect of the study to you. Then if you are still interested in, you will be asked to sign a consent form, which is attached to this patient information sheet. Then we will take some blood and measure your blood pressure, weight, height and waist circumference. A dietitian will explain to you about eating a normal diet and the foods we would like you avoid during the study. If you are vegetarian you may include too much soy in your diet and for this reason you will not be able to take part in the study. As smoking affects heart disease risk, if you smoke unfortunately you will not be able to enter the study. If later in the study at any point you wish to discuss your diet and lifestyle with a dietitian, an additional appointment can be made.

Two weeks later you need to attend the Diabetes Centre to have a 24 hours blood pressure machine placement. This machine automatically will measure your blood pressure during the next 24 hours once in every hour. You will also be given urine sample containers in order to collect urine during this 24 hours.

The next day you will need to come back to remove the 24 hours blood pressure measurement machine and you will need to bring back the filled in urine containers. We will take bloods and it is important to come fasting to this visit. You will be provided hot drink and biscuits after taking the blood. Then a weight, height and waist circumference measurement will be done. You will have a blood vessel health test (Endopat 2000); this will take between 20-30 minutes. It involves placing probes on the index finger of each hand and a blood pressure cuff on one arm. It is slightly uncomfortable as the test needs the blood pressure cuff to be inflated for 3-5 minutes. You will be given enough snack bars to have 2 a day for 4 weeks. You will be asked to have 1 bar mid morning and 1 bar mid afternoon.

The next visit is due after four weeks. You will need to bring back all uneaten bars and the empty wrappers. We will measure your height, weight, abdominal circumference and we will also measure your blood pressure. You will be given enough snack bars to have 2 a day for 4 weeks. You will be asked to have 1 bar mid morning and 1 bar mid afternoon.

After four weeks again you will need to come back to have the 24 hour blood pressure measurement machine placement the second time. We will give you urine containers in order to collect your urine in the next 24 hours.

The next day will be the final visit when the 24 hour blood pressure machine will be removed and you will need to bring back the filled in urine collection containers. It is important to come back after fasting as we will take some blood. You will be provided hot drink and biscuits after taking the blood. You need to bring back all the uneaten bars and the empty wrappers. We will measure your height, weight and abdominal circumference. Then you will have the second blood vessel test (Endopat 2000).

The maximum amount of blood we will take is 25ml (about 50 drops of blood) during each of the three occasions. During the study you will be asked to complete a one week food diary and a three day hunger questionnaire before the fasting blood tests. The food diary will include mood rating scales to assess the effect of the consumption of the bars on mood.

All information will be confidential and treated in accordance with the Data Protection Act 1998.
**Body Volume Index (BVI) measurement**

At the first visit you will be asked if you would agree to have your body volume measured (BVI – Body Volume Index). This is optional, if you chose not to have this done you can still participate in the study. This is an entirely new method to measure body shape using a 3D scan without radiation. It is thought that this is a more accurate measure than Body Mass Index measurement as it takes into account body shape as well as height and weight. Body volume involves the participant having a scan (a photograph which does not include the face so is anonymous) in underwear that shows what the body shape is.

If you wish to have your body volume measured, this will be done at the beginning and the end of the study in the Department of Sport, Health and Exercise Science, Room 108. Washburn Building, West Campus of University of Hull, Cottingham Road, Hull.

**Expenses and payments**

You will not be paid for your participation in this study however we will reimburse all your travel and parking expenses for your research visits to the centre. Please keep the receipts whenever you can obtain them.

At the time of the two fasting visits we will provide you hot drink and biscuits after taking the blood samples.

**What are the possible benefits of taking part?**

You may become more aware of the importance of food in your diabetes.

The basic snack bar contains the ingredients shown in previous studies to improve control of diabetes and risk of heart disease. The addition of cocoa to this may or may not add further benefits. The research team are specialists in diabetes and will review your diabetes at each of your visits during the study.

It is hoped that the results of the study will help us to treat diabetes better in the future. It is also hoped that it will help to develop tasty foods that can assist in this aim.

**Are there any risks to taking part in the study?**

There are only very small risks of taking part in this study. These include the risk of weight gain as you will be having two snack bars daily. To help with this a research dietitian will be available to discuss diet with you before you start the study and while you are taking part.

It is inconvenient to have the blood and blood vessel health checks, there is also a risk of discomfort and bruising from these tests. These will be undertaken by trained professionals who are experienced in performing these procedures.

The Endopat 2000 test may be uncomfortable because the blood pressure cuff will be inflated for 3-5 minutes.

The bars may cause allergy or abdominal discomfort. You will be given 24/7 contact details for the research staff so you can contact them if you experience unpleasant symptoms.

**Will my taking part in the study be kept confidential?**

Yes. Unless you tell anyone that you are taking part, only your GP will know. The blood samples and test results that are collected will be anonymous when the results are studied.
What will happen to the results of the study?
The results will be published in appropriate medical journals. However, individual people will not be identified and complete anonymity will be maintained in line with Trust policy and the Data Protection Act 1998.

What if something goes wrong?
We do not anticipate any problems with the study. However, in the unlikely event that this occurs you will be covered under the NHS compensation scheme, and details on our complaint procedure can be obtained on our trust website (http://www.hey.nhs.uk).

Ethical considerations
This study has been given a favourable opinion by the Humber Bridge Research Ethics Committee, which has not objected to the study taking place.

Problems or concerns can be discussed with Professor Atkin or Dr Judit Konya or any members of the clinical trial team, please telephone 01482 675314, 01482 675387 or 01482 675372 from 9am to 5pm weekdays or the Hull Royal Infirmary switchboard on 01482 328541 out of hours, and ask for Dr Judit Konya.

Thank you for your kind collaboration!

This information sheet and a copy of the consent form that you have signed are for you to keep for future reference.
CONSENT FORM

Full title: A Pilot Study Investigating the Effects of the Combined Effects of Cocoa and Soy Polyphenols in a Soy Protein Matrix on Insulin Resistance and Cardiovascular Disease Risk in Type 2 Diabetes – A Randomised Placebo-Controlled Double-Blind Parallel Study.

Short title: Effects of combining cocoa and soy in type 2 diabetes

Name of Researchers: Prof S.L. Atkin
Prof ES Kilpatrick
Dr Judit Konya

Please write your Initials all the boxes to show that you have read, understood and where needed had the meaning of the points explained to you by a member of the research team.

1. I confirm that I have read and understand the Participant Information Sheet Version 4. 17. 10. 2011. for the above study and have had the opportunity to ask questions and I am prepared to take part in the above study.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. For any remaining blood samples after the study I agree to have those blood samples stored to be used solely for future cardiovascular research, and to be destroyed if used within a period of two years after study completion.

4. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the research team or the representatives of the Hull East Yorkshire Hospitals NHS Trust for this study. I give permission for these individuals to have access to my records.

5. I give permission for the research team to inform my general practitioner about my participation in this study.

6. I wish to have Body Volume Index measurement twice during the study.

Yes    No

Name of the Patient      Signature      Date

………………………    ……………………..    ……………………

Name of Researcher      Signature      Date

………………………    ……………………..    ……………………

One for patient, one for researcher, one to be kept with source documents.
Ethics approval – Effects of isolated soy protein versus combined soy protein and isoflavones on postprandial glycaemia and insulin resistance in patients with type 2 diabetes.

**Study title:** Modelling endothelial function with hypoxia and low humidity (simulated flight environment) in healthy volunteers and patients with type 2 diabetes: effect of soy and cocoa on the endothelial changes.

**EC reference:** 12/YH/0016

**Amendment number:** 3.5

**Amendment date:** 08 October 2013

**IRAS project ID:** 97429

**Overview of amendment**

1. Change of Principal Investigator
2. To incorporate a new sub study

The above amendment was reviewed by the Sub-Committee in correspondence.

**Ethical opinion**

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

**Approved documents**

The documents reviewed and approved at the meeting were:
The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hra-training/

Yours sincerely

Dr Lynn Cawkwell
Chair

E-mail: nrescommittee.yorkandhumber-humberbridge@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Mr James Illingworth, Hull and east Yorkshire NHS Trust
NRES Committee Yorkshire & The Humber - Humber Bridge

Attendance at Sub-Committee of the REC meeting on 30 October 2013

<table>
<thead>
<tr>
<th>Name</th>
<th>Profession</th>
<th>Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Stephen Beer</td>
<td>Consultant Physician</td>
<td>Expert</td>
</tr>
<tr>
<td>Dr Lynn Cawkwell</td>
<td>Senior Lecturer in Cancer Genetics</td>
<td>Expert</td>
</tr>
</tbody>
</table>
Study protocol - Effects of isolated soy protein versus combined soy protein and isoflavones on postprandial glycaemia and insulin resistance in patients with type 2 diabetes.

Full title: Modelling endothelial function with hypoxia and low humidity (simulated flight environment) in healthy volunteers and patients with type 2 diabetes: effect of soy and cocoa on the endothelial changes

Short title: FlyBar


Principal Investigator:
Dr Thozhukat Sathyapalan
Reader and honorary consultant in Diabetes, Endocrinology and Metabolism
Hull York Medical School
220-236 Anlaby Road
HULL, HU3 2RW
E-mail: thozhukat.sathyapalan@hyms.ac.uk
Telephone: 01482 675365

Sponsor:
Hull and East Yorkshire Hospitals NHS Trust,
R & D department, Office 6, 2nd Floor Daisy Building,
Castle Hill Hospital, Castle Rd,
Cottingham, East Yorkshire HU16 5JQ.

Funder: Diabetes Charitable Funds

Statistician:
Mr Alan Rigby
Department of Medicine
University of Hull
Department of Cardiology
Castle Hill Hospital, Castle Road
Cottingham, HU16 5JQ
Email: asr1960@hotmail.com

Biochemical analysis will be undertaken in the clinical laboratories at Hull Royal Infirmary.
Flow cytometry tests will be undertaken in the Hull York Medical School at the premises of the University of Hull.
The environmental chamber is located in the Department of Sport, Health and Exercise Science, University of Hull.
List of abbreviations and definitions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>ADMA</td>
<td>Asymmetric Dimethylarginine</td>
</tr>
<tr>
<td>CAA</td>
<td>Civil Aviation Authority</td>
</tr>
<tr>
<td>cIMT</td>
<td>Carotid Intima Media Thickness</td>
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<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>EMPs</td>
<td>Endothelial Microparticles</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Agency</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
</tr>
<tr>
<td>Hs-CRP</td>
<td>High-sensitivity C-Reactive Protein</td>
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<td>Microparticle</td>
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<td>National Air Traffic Services</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
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</tr>
<tr>
<td>PO₂</td>
<td>Partial Pressure of Oxygen</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
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<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
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</table>
1. Background information

This proposal focuses on how changes in the environmental conditions experienced during flight may affect the pathophysiology of patients with type 2 diabetes and whether it is affected by dietary intervention.

1.1 Flight. In recent years the number of passengers travelling by aircraft has rapidly increased. In 2010 the National Air Traffic Services (NATS) in the United Kingdom handled 2.1 million flights, involving more than 200 million passengers (15). Every year, over one billion people travel by air and that figure is predicted to double in the next two decades according to the Civil Aviation Authority (CAA) (16).

The special environmental conditions on an aircraft have several effects on passengers. Aircraft cabins are pressurised to maintain good oxygenation and the cabin altitude is around 8000 ft (2438 m). This results in reduced barometric pressure with a concomitant decrease in the partial pressure of oxygen (PO$_2$). The percentage of oxygen in the air on board an aircraft is the same as at sea level, but because of the lower atmospheric pressure (equal to 8000 ft), the PO$_2$ is decreased. Consequently, the driving pressure of the gas exchange in the lungs is also lower, causing hypoxia. While the barometric pressure is 760 mmHg at sea level, with a corresponding partial arterial O$_2$ pressure (PaO$_2$) of 98 mmHg, the barometric pressure at 8000 ft will be 565 mmHg with a PaO$_2$ of about 55 mmHg. Due to the shape of the oxy-haemoglobin dissociation curve, this results in a fall of oxygen saturation to 90% (16, 17). The humidity in an aircraft is very low (10–20%) compared to the humidity at sea level in an average building (40–50%) (16). Despite these environmental changes there is no special cautions regarding a flight for patients with well-controlled type 2 diabetes (17).

An environment with similar conditions as a flight can be simulated in an environmental chamber, by adjusting the chamber settings to replicate the oxygen and humidity level of a cabin during a typical commercial flight.

1.2 Type 2 diabetes cardiovascular risk, endothelial function and microparticles.

Patients with type 2 diabetes (T2DM) are at increased morbidity and mortality from cardiovascular events. It is well recognised that there are a number of cardiovascular risks that can predispose to these events including hyperlipidaemia and hypertension that are actively treated with proven benefit (1). Endothelial function is thought to be a key determinant in cardiovascular risk in T2DM, but it is difficult to quantify in clinical practice and difficult to modulate in the research setting in order to investigate therapeutic interventions. A number of methodological approaches have been developed to assess EC dysfunction including Endothelium-dependent vasodilatation, or circulating soluble markers (MPs) reflects the endothelial function (14). Other functional measures of endothelial function include the use of the EndoPAT 2000. The EndoPAT 2000 is a Food and Drug Agency (FDA) cleared, reactive hyperaemia-based, operator independent device for diagnosing endothelial dysfunction.

Endothelial microparticles (EMPs) are vesicles shed by the endothelial cells and consist of cell membrane, cytoplasmic and nuclear elements. EMPs express surface markers reflective to their cell of origin (2). Under normal physiological conditions microparticles (MPs) are constantly shed into the circulation of healthy individuals (3). However, the level of EMPs is elevated in the circulation after damage, activation or apoptosis of the endothelial cell (EC)s. This phenomenon is also observed in disease states that are associated with vascular dysfunction such as acute coronary syndromes and diabetes (4). Furthermore it has been demonstrated that number of EMPs in circulation in vivo correlate with other indices of EC dysfunction, with the complications of diabetes type 2 (5), and as a result of high-fat meals (6). These observations raise the possibility that plasma EMP levels
may be measurable markers of EC dysfunction (7-12).

A recent study conducted at the University of Hull, demonstrated that hypoxia caused by simulated high altitude (3000 m), increased endothelial dysfunction as measured by raised levels of microparticles in the circulation (13). In studies where multiple indices of endothelial function has been monitored it has emerged that dietary intervention can help modulate this potentially damaging pathophysiological changes. In the context of the work proposed in the present application, it has been shown that consumption of soy and cocoa products improves endothelial function (18-21). In the present application we wish to pull together this important observations and will use hypoxia and low humidity (modelling a simulated flight experience) to investigate endothelial function. This will be measured using the EndoPAT 2000, EMPs and other serum markers such as von Willebrand Factor (vWF), Plasminogen Activator Inhibitor-1 (PAI-1), Asymmetric Dimethylarginin (ADMA), Hypoxia Inducible Factor (HIF) (22, 23). Urinary isoprostanes will also be measured (24). We will then investigate whether the use of soy isoflavones and cocoa polyphenols can attenuate any endothelial dysfunction in response to a simulated flight.

As an addition, a carotid artery intima media thickness (cIMT) measurement will be taken during the screening visit as multiple studies have shown that cIMT is a surrogate marker of cardiovascular disease (29) and relates to endothelial dysfunction (30). We’d like to determine whether there is any relationship between cIMT and the degree of change in endothelial function during the simulated flight.

In order to be able to evaluate and fully interpret the hypothesized changes in the level of the investigated markers (31), a control day will be offered for the participants as a flight sub-study when they’ll be invited for a “control flight” to sit in the environmental chamber. Environmental conditions will not be manipulated.

Soy food products have shown to improve endothelial dysfunction, lipid profile and hyperglycaemia in type 2 diabetes. In some studies, there was an improvement in the lipid profile (32) and decrease in HbA1c in patients with type 2 diabetes through improvement in insulin resistance and fasting glucose or post prandial hyperglycaemia (33, 34). In our recent preliminary study we found a significantly decreased HbA1c levels in patients with type 2 diabetes after chronic dietary intervention with soy products. However, there were no changes in fasting glucose or insulin resistance. (in process of publication). This suggests that a significant improvement in the postprandial hyperglycaemia may be involved in the effect of the soy products. Literature is very limited to assess the postprandial metabolic effects of acute soy ingestion (35). It is also not clear yet whether this effect is caused by the soy protein or the soy isoflavones.

2. Aim

The aims of the study are:

i. To determine the effect of a simulated flight (hypoxia and low humidity in an environmental chamber) on the endothelial function in healthy volunteers and patients with type 2 diabetes

ii. To determine whether these changes can be modified with the consumption of soy or cocoa food products.

3. Hypothesis

The simulated flight (hypoxia and low humidity in the environmental chamber) causes significant endothelial dysfunction (measured by endothelial-dependent vasodilatation and increased level of EMPs detectable in the circulation) in type 2 diabetic patients and healthy
controls. This effect can be modified with consumption of food products (soy isoflavones, cocoa polyphenols).

4. Primary endpoints

- To determine if the simulated flight causes a measurable degree of endothelial dysfunction, indicated by elevated levels of MPs in the circulation and impaired endothelial-dependent vasodilatation.
- To determine if the degree of endothelial dysfunction differs between patients with type 2 diabetes and healthy participants.
- To determine if any endothelial dysfunction caused by the simulated flight can be attenuated with previous acute and chronic soy or cocoa food product consumption.

5. Secondary endpoints

- The intervention with the environmental chamber does not cause significant impairment in the glucose and lipid homeostasis measured by blood glucose, insulin, glucagon and lipid levels.
- Eating soy or cocoa food product for a longer period (3 weeks) further attenuates any impact on the endothelial dysfunction caused by the simulated flight, compared to eating these food products only once shortly before the simulated flight.
- Consumption of the soy protein+soy isoflavones+cocoa polyphenol bars has greater preventive effect on endothelial dysfunction than the placebo or soy protein+soy isoflavones bars and the soy protein+ soy isoflavones bars have greater preventive effect than the placebo bar (soy protein only).

6. Food products to be used in the study

Three different types of bars will be used during the study:

- soy protein alone (placebo group – washed soy, isoflavones have been removed)
- soy protein with soy isoflavones (each bar containing 16mg, 2 bars per day =32mg)
- soy protein with soy isoflavones and cocoa (each bar containing 16 mg soy isoflavone and 400mg cocoa polyphenol, 2 bars per day= 32 mg isoflavone and 800mg polyphenol)

The dose of soy protein and isoflavones was chosen as it was shown to be effective in our previous studies (submitted for publication).

The participants will be randomised to one of the three parallel arms as detailed above.

7. Study Design

This is a randomised placebo-controlled feasibility study involving 18 healthy volunteers and 18 patients with type 2 diabetes. The participants will be randomised into 3 arms (placebo, soy isoflavones, soy isoflavones + cocoa polyphenols).
7.1 Randomisation
The randomisation will be performed by Essential Nutrition Ltd, UK. The randomisation will be done before the second simulated flight (Visit 3). A computer generated randomisation list will be used to provide balanced blocks of patient numbers for each of the three treatment groups. A 1:1:1 treatment allocation will be used. A copy of the randomisation code will be held in a sealed envelope in the trial master file.

7.2 Visit schedule

Visit 1 (Week 0) Screening visit

Inclusion/exclusion criteria
Medical history (for patients with diabetes, detailed diabetes history for the previous 12 months; concomitant medications)
Full physical examination
Anthropometric measurements: weight, height, waist circumference
Blood pressure, pulse
cIMT measurement
Completion of the Activity Questionnaire for Adults and Adolescents (AQeAA)
Baseline bloods: biochemical profile, full blood count, glucose, hs-CRP, HbA1c, lipid profile
Discussion with the dietician about the diet and get advice how to avoid high sources of isoflavones and polyphenols.

Visit 2 (Week 2) Fasting

1. Pre-simulated flight bloods (glucose, biochemistry, insulin, glucagon, lipids, hs-CRP, blood for flow-cytometry; endothelial stress markers)
   EndoPAT

2. The participants will be subjected to a simulated flight in the environmental chamber. The length of time they will spend in the environmental chamber is 2 hours.

3. At the end of the simulated flight:
   Bloods (glucose, biochemistry, insulin, glucagon, lipids, hs-CRP, blood for flow-cytometry; endothelial stress markers)
   EndoPAT

Patients will be asked to complete a mood rating scale and a memory task before entering the chamber, whilst being inside the chamber, and after leaving the chamber.

Visit 3 (Week 3) Fasting

Visit 3 procedures will be the same as those during Visit 2, but the participants will eat one bar (depending on the randomisation) after the initial blood sampling and EndoPAT, but before entering the environmental chamber.

Three weeks supply of the bars will be dispensed to the participants (placebo, soy+isoflavones or soy+isoflavones+cocoa, depending on the randomisation results). The participants will be asked to ingest two bars daily between visit 3 and visit 4.
Visit 4 (Week 6) Fasting

Visit 4 procedures will be the same as those during Visit 2. Patients will be asked to bring back empty wrappers and uneaten bars for Visit 4 to assess compliance.

A phone call one week after the simulated flight will be made to check the participants’ health (record any adverse events, changes in medication) and to record the blood glucose results (4, 6 and 8 hours after the intervention) for the patients with type 2 diabetes.

<table>
<thead>
<tr>
<th>Visit 1 (Week 0) Screening</th>
<th>Visit 2 (Week 2)</th>
<th>Visit 3 (Week 3)</th>
<th>Visit 4 (Week 6)</th>
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</thead>
<tbody>
<tr>
<td>Consent</td>
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<tr>
<td>Medical history</td>
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<tr>
<td>Inclusion/Exclusion</td>
<td>X</td>
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<tr>
<td>Full physical examination</td>
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<td>X</td>
<td>X</td>
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<tr>
<td>BP, pulse</td>
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<tr>
<td>Concomittant medication</td>
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<tr>
<td>Anthropometric measurements (height, weight, circumference)</td>
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<td>AQuAA</td>
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<tr>
<td>Blood test</td>
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<td>X **</td>
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<tr>
<td>Interview with a dietician</td>
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<tr>
<td>Environmental chamber – simulated flight</td>
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<td>X</td>
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<tr>
<td>EndoPAT</td>
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<tr>
<td>Dispensing of the bars</td>
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<tr>
<td>Collect and count empty wrappers</td>
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<tr>
<td>Adverse event recording</td>
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<tr>
<td>Mood rating scale, memory task</td>
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<tr>
<td>cIMT measurement</td>
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</tbody>
</table>

*: Screening bloods: biochemical profile, full blood count, glucose, hs-CRP, HbA1c, lipid profile

**: Bloods for the chamber visits (MPs: CD54, 62E, 105, 106, 144; Hsp32, PBMC; other endothelial stress markers such as vWF, PAI-1, ADMA, hypoxia inducible factor)

Patients will need to follow a diet throughout the trial to avoid high sources of isoflavones and polyphenols. Participants will also be asked to complete a 7 day food diary before the visits in the chamber (3 times all together during the trial).
7.2.1. Flight Sub-study

All participants will be offered to take part in a sub-study. The sub-study includes an extra fasting visit to the environmental chamber without altering the environmental conditions. Blood samples will be taken and EndoPAT will be performed as per Visit 2 in the main study.

7.2.2. Soy sub-study

Six patients with type 2 diabetes will be invited for an additional soy sub-study that will involve two separate visits to the Diabetes Research Centre, Broxclehurst Building, Hull Royal Infirmary. After securing a peripheral venous access through a cannula, patients will be asked to consume a soy bar contains soy protein without isoflavones during the first, and soy protein + isoflavones bar during the second visit. Thirty minutes later an oral glucose tolerance test will be performed by asking the participants to drink the standard 75g glucose solution. Venous blood samples will be collected prior to consuming the snack bar, then immediately prior to the glucose load, and 30, 60, 120, 180 and 240 minutes after. Blood samples will be analysed for glucose, insulin, lipids, endothelial and oxidative stress markers. Approximately 80 ml of blood will be collected during each visit. Lunch will be provided after each visit. The timeframe between the two visits is one week.

7.3 Inclusion Criteria

Diabetes group:

- Diagnosis of type 2 diabetes based on the WHO guidelines
- Patients on stable medication (for their diabetes metformin being the only treatment if any), for 3 months prior to entry into the study
- Age between 45-75 years at the start of the study; women need to be postmenopausal and not undergoing hormone replacement therapy (HRT)
- No episodes of hypoglycaemia or hyperglycaemia that required medical intervention/treatment for at least one year
- The patient has not been hospitalised for any reason connected with diabetes in the last year

Healthy volunteers group:

- No current disease which would have effect on the safety of the subjects or interfere with the study results
- No current medication that would interfere with the study results
- Age between 45-75 years at the start of the study; women need to be postmenopausal and not undergoing HRT

7.4 Exclusion criteria

For both groups:

- Patients with concurrent illness or any medication in the last 3 months that would interfere with the study based on the investigator’s judgement
- Acute coronary syndrome in the last 3 months or any major clinical event in the previous 3 months that would effect the study based on the investigator’s judgement
- Patients not wishing to allow disclosure to their general practitioner (GP)s.
- HbA1c >9%
7.5 Use of food products within the trial

To assess the acute effect of the bars: Participants will eat one bar 1 hour before entering the environmental chamber during visit 3, but after the EndoPAT and initial blood tests.

To assess the chronic effect of the bars: After visit 3 the snack bar is to be taken orally twice daily as between-meal snacks for a period of 3 weeks. Patients will be taking the bars themselves and retaining empty packaging and uneaten bars for compliance checks. A dispensing / compliance log will be kept with the case report form.

All the bars will be produced and supplied by Halo Foods Ltd. in accordance with Good Manufacturing Practice. Shelf life will be adequate for the duration of the trial. All ingredients will be fully traceable to source.

7.6 Subject recruitment

Patients with type 2 diabetes will be identified from clinic database and the diabetes research database. After identifying potentially suitable patients, an invitation letter from the Diabetes Research Team will be sent along with a participant information sheet to them in which they'll be asked to contact the research centre in case they are interested in taking part. Healthy volunteers will be recruited by contacting subjects who participated in previous trials in the Centre (and they have already given verbal consent regarding to be contacted in such cases) and by advertisement in local papers and also on notice boards on premises of the University of Hull and Diabetes Research Centre.

When they contact the research team an appointment will be booked to meet with a study doctor to discuss the study. They can then go home and discuss the opportunity with members of the family or with the GP. There will be different participant information sheets and consent forms for the healthy volunteers and patients with type 2 diabetes.

If they decide to take part the participants will sign the relevant informed consent form (for healthy participants or patients with type 2 diabetes) in the research centre before or at the time of the screening visit. Informed consent will be taken by a study doctor. No vulnerable subjects or non-English speakers will be recruited.

Participants (healthy volunteers and patients with type 2 diabetes) will be given £35 Amazon Vouchers per completed visit in the environmental chamber including the visit that is part of the sub-study. Reasonable parking and travel expenses will be reimbursed to each participant (patients with type 2 diabetes and healthy volunteers).
7.7 Withdrawal of a subject from the study

Individuals may be withdrawn from the study:
- If they lose capacity
- If they wish to pull out of the study
- If they need to use any medication that would interfere with the test results
- If they can’t tolerate being in the chamber (developing psychiatric, hypoxia or hypoglycaemia related symptoms – these will be recorded as adverse events)

Withdrawn patients will be replaced using the proposed methods of recruitment unless there is a tendency in the occurrence of adverse events. Withdrawn subjects will be followed-up by a phone call to them one week after the withdrawal.

7.8 The simulated flight

The environmental chamber is situated in the Department of Sport, Health and Exercise Science (Design and Manufacture of Environmental Test Chambers, A3897 - SSR60-20H).

The intervention in the environmental chamber involves each participant sitting in the chamber for 2 hours. The humidity level will be set at 15% and the oxygen concentration will be 15% equivalent to that of an altitude of 2500 m. During the sub-study visit, the environmental conditions will not be modified.

There will be a 2 week run-in period when the patients establish the diet.

Centres involved in the study

The consent will be taken and the screening visit will be done in the Diabetes Research Centre (Brocklehurst Building, Hull Royal Infirmary, 220-236 Anlaby Road, Hull, HU3 2RW).

The environmental chamber is situated in the Department of Sport, Health and Exercise Science, University of Hull, HU6 7RX.

Efficacy measurements

For the primary outcome: Change in endothelial function (EndoPAT 2000, circulating MPs, endothelial markers).

For the secondary outcomes: blood glucose, insulin, glucagon and lipid measurements.

Safety measurements

The collection and reporting of data on adverse events and serious adverse events will be in accordance with ICH GCP and the Research Governance Framework 2005.

Definitions

**AE (Adverse event):** An adverse event is any untoward medical occurrence in a subject to
whom a research treatment or procedure has been administered, including occurrences which are not necessarily caused by or related to that treatment or procedure.

**AR (Adverse Reaction):** An adverse reaction is any untoward and unintended response in a subject which is caused by or related to a research treatment or procedure.

**SAE (Serious Adverse Event):** An adverse event becomes serious if it:
- results in death
- is life-threatening
- requires hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability or incapacity
- is a congenital anomaly or birth defect
- is otherwise considered medically significant by the investigator

The term “life-threatening” refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

Hospitalizations planned prior to enrolment in the trial (elective surgery) or for social reasons should not normally be considered as SAEs unless the hospitalization has to be prolonged.

**Reporting adverse events**

The AE reporting period for this trial begins on at visit 1, and ends 30 days after the patients final research clinic appointment.

Each trial subject will be questioned about adverse events at each visit. The investigator will record all directly observed AEs and all AEs spontaneously reported by the trial subject.

A pre-existing condition (i.e. a disorder present before the AE reporting period started and noted on the pre-treatment medical history/physical examination form/medical notes), is not to be reported as an AE unless the condition worsens or episodes increase in frequency during the AE-reporting period.

All adverse events (serious and non-serious) will be recorded in patients data collection forms (CRFs) using R&D’s adverse event report form. All adverse events will be recorded in patients’ medical records.

All AEs will be followed-up until the event has resolved or a decision has been taken for no further follow-up.

If a clinically significant abnormal laboratory value occurs, this abnormality will be recorded as an adverse event/reaction.

**Reporting serious adverse events**

If a trial subject experiences a serious adverse event which in the opinion of the chief investigator is both

- **Related** - that is, it resulted from administration of any of the research treatments or procedures; and
- **Unexpected** - that is, the type of event is not listed in the protocol as an expected occurrence.

Then it will be reported to the Research Ethics Committee that gave a favourable opinion of the study and the sponsor (Hull and East Yorkshire Hospitals NHS Trust R&D department) **within 15 days** of the chief investigator becoming aware of the event using the NRES safety report form available from:

[http://www.nres.npsa.nhs.uk/applications/after-ethical-review/safetyreports/safety-reports-for-all-other-research/#safetynonCTIMPrepotingSAEs](http://www.nres.npsa.nhs.uk/applications/after-ethical-review/safetyreports/safety-reports-for-all-other-research/#safetynonCTIMPrepotingSAEs)

**Urgent safety measures**

The chief/principal investigator may take appropriate urgent safety measures in order to protect research participants against any immediate hazard to their health or safety. These safety measures should be taken immediately and may be taken without prior authorization from the REC or Trust.

However, the chief/principal investigator must alert the sponsor (HEYHT R&D) as soon as possible of the urgent measures by contacting the R&D Office telephone number 461883 or 461903 (Mon - Fri 8am - 6pm) or the Trust Switchboard 875875 (out-of-office hours) and...
asking for either the R&D Director or the R&D Manager. The chief/principal investigator or sponsor should phone the main REC to discuss the issue as soon as possible. If notified by the principal investigator, the relevant local REC should also be informed. The main REC and Trust should be notified within 3 days after the urgent measures have been taken by submitting a Notification of Amendment form and covering letter setting out the reasons for the urgent safety measures and the plan for further action.

Annual Progress Reports
An annual progress report will be submitted to the main REC which gave the favourable opinion 12 months after the date on which the favourable opinion was given and thereafter until the end of the study according to the NRES website below: http://www.nres.npsa.nhs.uk/applications/after-ethical-review/annual-progress-reports/

Data collection

Data will be collected in patient case notes and a case report form (CRF) designed specifically for the study in line with the sponsor’s guidelines and Good Clinical Practice.

Data will be collected by the study team. This will include research nurses and medical practitioners. Data will be anonymised and will only be identified by the patient identification number.

End of trial

End of the trial is the last participant’s last visit day. The trial will be terminated early in case of tendency in the adverse events or if a new data becomes available that diving is not safe for patients with type 2 diabetes. An end of study declaration form (using the NRES form) will be submitted to the main REC and Trust R&D within 90 days from completion of the trial and within 15 days if the trial is discontinued prematurely. A summary of the trial report/publication will be submitted to the main REC and Trust R&D within 1 year of the end of trial.

Power and sample size

The primary endpoint is circulating EMPs. The study has been powered on the between group comparison (diabetes versus healthy volunteers). Eighteen patients per group will allow us to detect a 0.90 of a standard deviation difference between the two groups (80% power, 5% significance, two-tailed). This equates to a large effect size. The sample size allows for 10% to follow-up (non-differential between groups). The study lacks sufficient power for within-group comparisons (after cereal bar intervention) which will be presented as a descriptive account.

Statistical methods

The trial will be reported according to CONSORT guidelines (25). Continuous data will be summarised using the median and 25th/75th centiles; categorical data by percentages. Missing data will be handled using methods outlined elsewhere (26). The between group comparisons will be made by the independent t-test (Mann-Whitney U test for non-normal data). 95% confidence intervals will be reported. No correction for multiple comparisons will be made (27). Paired differences will be computed for within-group comparisons but not compared statistically. P-values for secondary outcomes will be nominal. Depending on data quality subgroup comparisons by gender may be made. The Stata statistical computer package will be used to analyse the data (28).
We'll perform the initial analysis on data obtained from the first visit in the chamber for 12 healthy individuals and 12 patients with type 2 diabetes. For these volunteers the visit window will likely be longer than 1 week between Visit 2 (first simulated flight) and Visit 3 (second simulated flight). Patient suitability and any change in medication/medical history will be checked and eligibility will be reconfirmed before the second simulated flight to ensure patient safety along with consistency and reliability of the study results.

Monitoring

The study may be monitored in accordance with HEY R & D department’s standard operating procedures to ensure compliance with ICH GCP and the Research Governance Framework 2005. All trial related documents will be made available upon request for monitoring by R&D monitors.

Ethics and R&D approval

The study will be performed subject to Research Ethics Committee favourable opinion, Site Specific Assessment (SSA) approval and HEY Trust R&D approval.

Research Governance

This study will be conducted in accordance with the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines and the Research Governance Framework for Health and Social Care.

Data handling and record keeping

Data will be collected in the case notes and in the case report form to allow for cross referencing to check validity. The data will be transferred to password protected NHS computers in adherence to Trust Information Governance Policy. Professor Atkin will act as the data custodian and is responsible for the storage, handling and quality of the study data.

IT Services Department has a backup procedure approved by auditors for disaster recovery. Servers are backed up to tape media each night. The tapes run on a 4 week cycle. Files stay on the server unless deleted by accident or deliberately. Anything deleted more than 4 weeks previously is therefore lost. Additional ‘archive’ backups are taken for archived data, so data should not be lost from this type of system e.g. FileVision which stores Medical Records. Tapes are stored in a fireproof safe.

Study documents (paper and electronic) will be retained in a secure (kept locked when not in use) location during and after the trial has finished. All essential documents including source documents will be retained for 5 years after study completion (last patient, last visit). A label stating the date after which the documents can be destroyed will be placed on the inside front cover of the casenotes of trial participants.

Data will be collected and retained in accordance with the Data Protection Act 1998.

Access to Source Data

Monitoring, audits, REC review will be permitted.

Finance

All participants (patients with type 2 diabetes and healthy volunteers) will be given
£35.00 after each completed visit, including the flight and soy sub-study visits, excluding screening visit. Reasonable parking and travel expenses will be reimbursed to each participant after each visit, including screening.

**Indemnity**

This is an NHS-sponsored research study. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS indemnity covers NHS staff and medical academic staff with honorary contracts only when the trial has been approved by the Trust R&D department. NHS indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Where the Principal investigator is employed by the University of Hull, the University has an insurance policy that includes cover for no-fault compensation in respect of accidental injury to a research subject.
Principal Investigator

Dr Thozhukat Sathyapalan

Signed ______________________________  Date ________________________

Sponsor, on behalf of Hull And East Yorkshire Hospitals NHS Trust

Name Mr James Illingworth

Signed ______________________________  Date ________________________
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5. Hidenobu Koga, MD, Seigo Sugiyama, MD, PhD, Kiyotaka Kugiyama, MD, PhD; Keisuke Watanabe, MD, Hironobu Fukushima, MD, Tomoko Tanaka, MD, Tomohiro Sakamoto, MD, PhD, Michihiro Yoshimura, MD, PhD, Hideaki Jinnouchi, MD, PhD; Hisao Ogawa, MD, PhD. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. Journal of the American College of Cardiology. 2005; 45(10):1622-30.

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Patient Information sheet - Effects of isolated soy protein versus combined soy protein and isoflavones on postprandial glycaemia and insulin resistance in patients with type 2 diabetes.

Additional Participant Information Sheet (Version 1. 07 October 2013.) – Soy Sub-Study

Full title: Modelling endothelial function with simulated flight environment in healthy volunteers and patients with type 2 diabetes


Introduction

We would like to invite you to participate in an additional part of the FlyBar study.

What is the purpose of the sub-study?

The main purpose of this sub-study is to gain deeper knowledge about the effect of soy for patients with type 2 diabetes. We already know that consumption of foods that contain soy is beneficial for patients with type 2 diabetes as it improves blood sugar control. This can happen through two mechanisms – either the fasting blood sugar levels become lower, or after consuming a meal, the blood sugar does not increase as much as it would without soy. Yet it is not clear which mechanism is more important and also we don’t know whether eating just one soy bar before a meal would cause an acute favourable effect. Soy contains molecules called isoflavones that might be responsible for the favourable effects. However it is not fully understood if it is the whole soy with the isoflavones or the soy protein without the isoflavones that would carry the nutritional benefits.

Why have I been chosen?

You have been chosen because you have type 2 diabetes and agreed to participate in the FlyBar study.

Do I have to take part?

No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the sub-study and you would like to participate, will you be asked to sign a consent form (attached to this information sheet) and will be given a copy of the patient information sheet and the consent form to keep. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

As part of the soy sub-study, you’ll be asked to attend the Diabetes Research Centre in the Brocklehurst Building on two separate occasions. It is important to come to these visits fasting. These visits will take for approximately 5 hours, including lunch.

We will measure your blood pressure and ask about your recent health and well-being. Then
we’ll put a cannula into one of your arms to make blood sampling more comfortable for you. Then we will take a blood sample and ask you to consume a snack bar that contains either soy protein only or soy with isoflavones. 30 minutes later we’ll take another blood sample and ask you to drink a sugary drink with 75 g glucose. You might have had this test done before the diagnosis of your diabetes. 30, 60, 120 and 240 minutes later we’ll again take blood samples and then we’ll remove the cannula and provide lunch.

The total amount of blood we will take is approximately 80ml (about 150 drops of blood). Blood samples either will be processed immediately in the clinical laboratories at Hull Royal Infirmary or will be stored at -80oC in the Diabetes Department, Hull Royal Infirmary. Stored samples will be processed at the end of the study. If you give consent to this, remaining samples will be used in future Ethics Committee approved cardiovascular research.

The only difference between the two visits will be the type of soy bar you'll be required to eat. Taste and consistency will be similar.

What are the possible benefits of taking part?

You may become more aware of the importance of diet and different food components in your diabetes management.

Are there any risks to taking part in the study?

It might be uncomfortable to have the cannula inserted and there is also a risk of bruising and a small risk of local infection. Cannulation will be performed by adhering to the Trust’s approved protocol. Cannula will be inserted after careful disinfection of the skin. We will carefully inspect the cannula every 30 minutes and will remove it in case of discomfort or redness of the skin. All study procedures will be undertaken by trained professionals who are experienced in performing these procedures.

Expenses and payments

We’ll pay £35 after each completed soy sub-study visit. In addition, we will reimburse all your travel and parking expenses for your research visits. Please keep the receipts whenever you can obtain them.

Will my taking part in the study be kept confidential?

Yes. Unless you tell anyone that you are taking part, only your GP will know. The blood samples and test results that are collected will be anonymous when the results are studied. All information will be confidential and treated in accordance with the Data Protection Act 1998.

What will happen to the results of the study?

The results will be published in appropriate medical journals. However, individual people will not be identified and complete anonymity will be maintained in line with Trust policy and the Data Protection Act 1998.

What if something goes wrong?

We do not anticipate any problems with the study. However, in the unlikely event that this
occurs you will be covered under the NHS compensation scheme, and details on our complaint procedure can be obtained on our trust website (http://www.hey.nhs.uk).

**Ethical considerations**

This study has been given a favourable opinion by the Humber Bridge Research Ethics Committee, which has not objected to the study taking place.

Problems or concerns can be discussed with Dr Thozhukat Sathyapalan or Dr Judit Konya or any members of the clinical trial team, please telephone 01482 675314, 01482 675387 or 01482 675372 from 9am to 5pm weekdays or the Hull Royal Infirmary switchboard on 01482 328541 out of hours, and ask for Diabetes Research Team.

Thank you for your kind collaboration!

This information sheet and a copy of the consent form that you have signed are for you to keep for future reference.
CONSENT FORM  Version 1. 07. October 2013.  FlyBar Soy Sub-Study

Full title: Modelling endothelial function with simulated flight environment in healthy volunteers and patients with type 2 diabetes

Short title: FlyBar Soy Sub-Study
Name of Researchers: Dr Thozhukat Sathyapalan
Dr Judit Konya
Prof Stephen L Atkin

Please write your Initials all the boxes to show that you have read, understood and where needed had the meaning of the points explained to you by a member of the research team.

1. I confirm that I have read and understand the Participant Information Sheet Version 1. 07. October 2013. for the FlyBar Soy Sub-Study and have had the opportunity to ask questions, and I am prepared to take part in the above study.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

7. For any remaining blood samples after the study I agree to have those blood samples stored to be used solely for future cardiovascular research.

8. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the research team or the representatives of the Hull East Yorkshire Hospitals NHS Trust for this study. I give permission for these individuals to have access to my records.

9. I give permission for the research team to inform my general practitioner about my participation in this study.

Name of the Patient  Signature  Date and time
………………………………  …………………………………  …………………………………

Name of Researcher  Signature  Date and time
………………………………  …………………………………  …………………………………

One for patient, one for researcher, one to be kept with source documents.
Ethics approval – The effect of simulated flight environment with hypoxia and low humidity on endothelial function in healthy volunteers and patients with type 2 diabetes.

NRES Committee Yorkshire & The Humber - Humber Bridge
HRA NRES Centre North West
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Tel: 0161 625 7816
Fax: 0161 625 7299

25 July 2013

Professor Stephen Atkin
Head of Diabetes and Endocrinology
Department University of Hull
Michael White Diabetes Centre, Brocklehurst Building
220-236 Anlaby Road
Hull
HU3 2RW

Dear Professor Atkin

Study title: Modelling endothelial function with hypoxia and low humidity (simulated flight environment) in healthy volunteers and patients with type 2 diabetes: effect of soy and cocoa on endothelial changes.

REC reference: 12/YH/0016
Amendment number: Substantial amendment 2.
Amendment date: 08 July 2013.
IRAS project ID: 97429

Overview of amendment
The amendment proposed to add an optional sub-study that contains an extra fasting visit to the environmental chamber.

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion
The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents
The documents reviewed and approved at the meeting were:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participant Information Sheet: and informed consent form</td>
<td>1.</td>
<td>08 July 2013</td>
</tr>
<tr>
<td>Protocol</td>
<td>4.</td>
<td>08 July 2013</td>
</tr>
<tr>
<td>Notice of Substantial Amendment (non-CTIMPs)</td>
<td></td>
<td>08 July 2013</td>
</tr>
<tr>
<td>Covering Letter</td>
<td></td>
<td>08 July 2013</td>
</tr>
</tbody>
</table>
Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hra-training/

Yours sincerely

Dr Lynn Cawkwell
Chair

E-mail: nrescommittee.yorkandhumber-humberbridge@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Mr James Illingworth, Hull & East Yorkshires Hospitals NHS Trust
NRES Committee Yorkshire & The Humber - Humber Bridge

Attendance at Sub-Committee of the REC meeting held by correspondence

<table>
<thead>
<tr>
<th>Name</th>
<th>Profession</th>
<th>Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Stephen Beer</td>
<td>Consultant Physician</td>
<td>Expert</td>
</tr>
<tr>
<td>Mrs Kate Bollington</td>
<td>Customer Services Manager</td>
<td>Expert</td>
</tr>
</tbody>
</table>
Study protocol – The effect of simulated flight environment with hypoxia and low humidity on endothelial function in healthy volunteers and patients with type 2 diabetes.

Full title: Modelling endothelial function with hypoxia and low humidity (simulated flight environment) in healthy volunteers and patients with type 2 diabetes: effect of soy and cocoa on the endothelial changes

Short title: FlyBar


Principal Investigator:
Professor Stephen L Atkin
Head of Academic Diabetes, Endocrinology and Metabolism
Hull York Medical School
220-236 Anlaby Road
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E-mail: stephen.atkin@hyms.ac.uk
Telephone: 01482 675365

Sponsor:
Hull and East Yorkshire Hospitals NHS Trust,
R & D department, Office 6, 2nd Floor Daisy Building,
Castle Hill Hospital, Castle Rd,
Cottingham, East Yorkshire HU16 5JQ.

Funder: Diabetes Charitable Funds
C/o Professor Stephen L Atkin

Statistician:
Mr Alan Rigby
Department of Medicine
University of Hull
Department of Cardiology
Castle Hill Hospital, Castle Road
Cottingham, HU16 5JQ
Email: asr1960@hotmail.com

Biochemical analysis will be undertaken in the clinical laboratories at Hull Royal Infirmary.
Flow cytometry tests will be undertaken in the Hull York Medical School at the premises of the University of Hull.
The environmental chamber is located in the Department of Sport, Health and Exercise Science, University of Hull.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA</td>
<td>Asymmetric Dimethylarginine</td>
</tr>
<tr>
<td>CAA</td>
<td>Civil Aviation Authority</td>
</tr>
<tr>
<td>cIMT</td>
<td>Carotid Intima Media Thickness</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>EMPs</td>
<td>Endothelial Microparticles</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Agency</td>
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<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
</tr>
<tr>
<td>Hs-CRP</td>
<td>High-sensitivity C-Reactive Protein</td>
</tr>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>NATS</td>
<td>National Air Traffic Services</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial Arterial Oxygen Pressure</td>
</tr>
<tr>
<td>PO₂</td>
<td>Partial Pressure of Oxygen</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
</tbody>
</table>
1. Background information

This proposal focuses on the how changes in the environmental conditions experienced during flight may effects the pathophysiology of patients with type 2 diabetes and whether is affected by dietary intervention.

1.1 Flight. In recent years the number of passengers travelling by aircraft has rapidly increased. In 2010 the National Air Traffic Services (NATS) in the United Kingdom handled 2.1 million flights, involving more than 200 million passengers (15). Every year, over one billion people travel by air and that figure is predicted to double in the next two decades according to the Civil Aviation Authority (CAA) (16).

The special environmental conditions on an aircraft have several effects on passengers. Aircraft cabins are pressurised to maintain good oxygenation and the cabin altitude is around 8000 ft (2438 m). This results in reduced barometric pressure with a concomitant decrease in the partial pressure of oxygen (PO$_2$). The percentage of oxygen in the air on board an aircraft is the same as at sea level, but because of the lower atmospheric pressure (equal to 8000 ft), the PO$_2$ is decreased. Consequently, the driving pressure of the gas exchange in the lungs is also lower, causing hypoxia. While the barometric pressure is 760 mmHg at sea level, with a corresponding partial arterial O$_2$ pressure (PaO$_2$) of 98 mmHg, the barometric pressure at 8000 ft will be 565 mmHg with a PaO$_2$ of about 55 mmHg. Due to the shape of the oxy-haemoglobin dissociation curve, this results in a fall of oxygen saturation to 90% (16, 17). The humidity in an aircraft is very low (10-20%) compared to the humidity at sea level in an average building (40-50%) (16). Despite these environmental changes there is no special cautions regarding a flight for patients with well-controlled type 2 diabetes (17).

An environment with similar conditions as a flight can be simulated in an environmental chamber, by adjusting the chamber settings to replicate the oxygen and humidity level of a cabin during a typical commercial flight.

1.2 Type 2 diabetes cardiovascular risk, endothelial function and microparticles.

Patients with type 2 diabetes (T2DM) are at increased morbidity and mortality from cardiovascular events. It is well recognised that there are a number of cardiovascular risks that can predispose to these events including hyperlipidaemia and hypertension that are actively treated with proven benefit (1). Endothelial function is thought to be a key determinant in cardiovascular risk in T2DM, but it is difficult to quantify in clinical practice and difficult to modulate in the research setting in order to investigate therapeutic interventions. A number of methodological approaches have been developed to assess EC dysfunction including Endothelium-dependent vasodilatation, or circulating soluble markers (MPs) reflects the endothelial function (14). Other functional measures of endothelial function include the use of the EndoPAT 2000. The EndoPAT 2000 is a Food and Drug Agency (FDA) cleared, reactive hyperaemia-based, operator independent device for diagnosing endothelial dysfunction.

Endothelial microparticles (EMPs) are vesicles shed by the endothelial cells and consist of cell membrane, cytoplasmic and nuclear elements. EMPs express surface markers reflective to their cell of origin (2). Under normal physiological conditions microparticles (MPs) are constantly shed into the circulation of healthy individuals (3). However, the level of EMPs is elevated in the circulation after damage, activation or apoptosis of the endothelial cell (EC)s. This phenomenon is also observed in disease states that are assoaicated with vascular dysfunction such as acute coronary syndromes and diabetes (4). Furthermore it has been demonstrated that number of EMPs in circulation in vivo correlate with other indices of EC dysfunction, with the complications of diabetes type 2 (5), and as a result of high-fat meals (6). These observations raise the possibility that plasma EMP levels
may be measurable markers of EC dysfunction (7-12).

A recent study conducted at the University of Hull, demonstrated that hypoxia caused by simulated high altitude (3000 m), increased endothelial dysfunction as measured by raised levels of microparticles in the circulation (13). In studies where multiple indices of endothelial function has been monitored it has emerged that dietary intervention can help modulate this potentially damaging pathophysiological changes. In the context of the work proposed in the present application, it has been shown that consumption of soy and cocoa products improves endothelial function (28-31). In the present application we wish to pull together this important observations and will use hypoxia and low humidity (modelling a simulated flight experience) to investigate endothelial function. This will be measured using the EndoPAT 2000, EMPs and other serum markers such as von Willebrand Factor (vWF), Plasminogen Activator Inhibitor-1 (PAI-1), Asymmetric Dimethylarginin (ADMA), Hypoxia Inducible Factor (HIF) (22, 23). Urinary isoprostanes will also be measured (24). We will then investigate whether the use of soy isoflavones and cocoa polyphenols can attenuate any endothelial dysfunction in response to a simulated flight.

As an addition, a carotid artery intima media thickness (cIMT) measurement will be taken during the screening visit as multiple studies have shown that cIMT is a surrogate marker of cardiovascular disease (29) and relates to endothelial dysfunction (30). We'd like to determine whether there is any relationship between cIMT and the degree of change in endothelial function during the simulated flight.

In order to be able to evaluate and fully interpret the hypothesized changes in the level of the investigated markers (31), a control day will be offered for the participants as a sub-study when they'll be invited for a “control flight” to sit in the environmental chamber. Environmental conditions will not be manipulated.

2. Aim

The aims of the study are:

i. To determine the effect of a simulated flight (hypoxia and low humidity in an environmental chamber) on the endothelial function in healthy volunteers and patients with type 2 diabetes.

ii. To determine whether these changes can be modified with the consumption of soy or cocoa food products.

3. Hypothesis

The simulated flight (hypoxia and low humidity in the environmental chamber) causes significant endothelial dysfunction (measured by endothelial-dependent vasodilatation and increased level of EMPs detectable in the circulation) in type 2 diabetic patients and healthy controls. This effect can be modified with consumption of food products (soy isoflavones, cocoa polyphenols).

4. Primary endpoints

- To determine if the simulated flight causes a measurable degree of endothelial dysfunction, indicated by elevated levels of MPs in the circulation and impaired endothelial-dependent vasodilatation.

- To determine if the degree of endothelial dysfunction differs between patients with type 2 diabetes and healthy participants.
• To determine if any endothelial dysfunction caused by the simulated flight can be attenuated with previous acute and chronic soy or cocoa food product consumption.

5. Secondary endpoints

• The intervention with the environmental chamber does not cause significant impairment in the glucose and lipid homeostasis measured by blood glucose, insulin, glucagon and lipid levels.

• Eating soy or cocoa food product for a longer period (3 weeks) further attenuates any impact on the endothelial dysfunction caused by the simulated flight, compared to eating these food products only once shortly before the simulated flight.

• Consumption of the soy protein+soy isoflavones+cocoa polyphenol bars has greater preventive effect on endothelial dysfunction than the placebo or soy protein+soy isoflavones bars and the soy protein+ soy isoflavones bars have greater preventive effect than the placebo bar (soy protein only).

6. Food products to be used in the study

Three different types of bars will be used during the study:

- soy protein alone (placebo group – washed soy, isoflavones have been removed)
- soy protein with soy isoflavones (each bar containing 16mg, 2 bars per day =32mg)
- soy protein with soy isoflavones and cocoa (each bar containing 16 mg soy isoflavone and 400mg cocoa polyphenol, 2 bars per day= 32 mg isoflavone and 800mg polyphenol)

The dose of soy protein and isoflavones was chosen as it was shown to be effective in our previous studies (submitted for publication).

The participants will be randomised to one of the three parallel arms as detailed above.

7. Study Design

This is a randomised placebo-controlled feasibility study involving 18 healthy volunteers and 18 patients with type 2 diabetes. The participants will be randomised into 3 arms (placebo, soy isoflavones, soy isoflavones + cocoa polyphenols).

7.1 Randomisation

The randomisation will be performed by Essential Nutrition Ltd, UK. The randomisation will be done before the second simulated flight (Visit 3). A computer generated randomisation list will be used to provide balanced blocks of patient numbers for each of the three treatment groups. A 1:1:1 treatment allocation will be used. A copy of the randomisation code will be held in a sealed envelope in the trial master file.

7.2 Visit schedule

Visit 1 (Week 0) Screening visit
Inclusion/exclusion criteria
Medical history (for patients with diabetes, detailed diabetes history for the previous 12 months; concomitant medications)
Full physical examination
Anthropometric measurements: weight, height, waist circumference
Blood pressure, pulse
cIMT measurement
Completion of the Activity Questionnaire for Adults and Adolescents (AQuAA)
Baseline bloods: biochemical profile, full blood count, glucose, hs-CRP, HbA1c, lipid profile
Discussion with the dietician about the diet and get advice how to avoid high sources of isoflavones and polyphenols.

Visit 2 (Week 2) Fasting

4. Pre-simulated flight bloods (glucose, biochemistry, insulin, glucagon, lipids, hs-CRP, blood for flow-cytometry; endothelial stress markers)
   EndoPAT

5. The participants will be subjected to a simulated flight in the environmental chamber. The length of time they will spend in the environmental chamber is 2 hours.

6. At the end of the simulated flight:
   Bloods (glucose, biochemistry, insulin, glucagon, lipids, hs-CRP, blood for flow-cytometry; endothelial stress markers)
   EndoPAT

Diabetic patients will also check their blood glucose 60 and 30 minutes before then 4, 6 and 8 hours after the simulated flight.

Patients will be asked to complete a mood rating scale and a memory task before entering the chamber, whilst being inside the chamber, and after leaving the chamber.

Visit 3 (Week 3) Fasting

Visit 3 procedures will be the same as those during Visit 2, but the participants will eat one bar (depending on the randomisation) after the initial blood sampling and EndoPAT, but before entering the environmental chamber.

Three weeks supply of the bars will be dispensed to the participants (placebo, soy+isoflavones or soy+isoflavones+cocoa, depending on the randomisation results). The participants will be asked to ingest two bars daily between visit 3 and visit 4.

Visit 4 (Week 6) Fasting

Visit 4 procedures will be the same as those during Visit 2.
Patients will be asked to bring back empty wrappers and uneaten bars for Visit 4 to assess compliance.
A phone call one week after the simulated flight will be made to check the participants’ health (record any adverse events, changes in medication) and to record the blood glucose results (4, 6 and 8 hours after the intervention) for the patients with type 2 diabetes.

<table>
<thead>
<tr>
<th></th>
<th>Visit 1 (Week 0) Screening</th>
<th>Visit 2 (Week 2)</th>
<th>Visit 3 (Week 3)</th>
<th>Visit 4 (Week 6)</th>
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<tr>
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<tr>
<td>Collect and count empty wrappers</td>
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<td>cIMT measurement</td>
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*: Screening bloods: biochemical profile, full blood count, glucose, hs-CRP, HbA1c, lipid profile

**: Bloods for the chamber visits (MPs: CD54, 62E, 105, 106, 144; Hsp32, PBMC; other endothelial stress markers such as vWF, PAI-1, ADMA, hypoxia inducible factor)

Patients will need to follow a diet throughout the trial to avoid high sources of isoflavones and polyphenols. Participants will also be asked to complete a 7 day food diary before the visits in the chamber (3 times all together during the trial).

7.2.1. Sub-study

All participants will be offered to take part in a sub-study. The sub-study includes an extra fasting visit to the environmental chamber without altering the environmental conditions. Blood samples will be taken and EndoPAT will be performed as per Visit 2 in the main study.
7.3 Inclusion Criteria

Diabetes group:

- Diagnosis of type 2 diabetes based on the WHO guidelines
- Patients on stable medication (for their diabetes metformin being the only treatment if any), for 3 months prior to entry into the study
- Age between 45-75 years at the start of the study; women need to be postmenopausal and not undergoing hormone replacement therapy (HRT)
- No episodes of hypoglycaemia or hyperglycaemia that required medical intervention/treatment for at least one year
- The patient has not been hospitalised for any reason connected with diabetes in the last year

Healthy volunteers group:

- No current disease which would have effect on the safety of the subjects or interfere with the study results
- No current medication that would interfere with the study results
- Age between 45-75 years at the start of the study; women need to be postmenopausal and not undergoing HRT

7.4 Exclusion criteria

For both groups:

- Patients with concurrent illness or any medication in the last 3 months that would interfere with the study based on the investigator's judgement
- Acute coronary syndrome in the last 3 months or any major clinical event in the previous 3 months that would effect the study based on the investigator's judgement
- Patients not wishing to allow disclosure to their general practitioner (GP)s.
- HbA1c >9%
- Smokers
- Pre-menopausal women or post-menopausal women on HRT
- Claustrophobia, panic attacks in the anamnesis
- Individuals with known food allergies
- Vegans and vegetarians
- Individuals not willing to consume snack bar
- Subjects must not have been subjected to a commercial flight within 4 weeks of the study

7.5 Use of food products within the trial

To assess the acute effect of the bars:
Participants will eat one bar 1 hour before entering the environmental chamber during visit 3, but after the EndoPAT and initial blood tests.

To assess the chronic effect of the bars:
After visit 3 the snack bar is to be taken orally twice daily as between-meal snacks for a period of 3 weeks.
Patients will be taking the bars themselves and retaining empty packaging and uneaten bars for compliance checks.
A dispensing / compliance log will be kept with the case report form.
All the bars will be produced and supplied by Halo Foods Ltd. in accordance with Good Manufacturing Practice. Shelf life will be adequate for the duration of the trial. All ingredients will be fully traceable to source.

7.6 Subject recruitment

Patients with type 2 diabetes will be identified from clinic database and the diabetes research database. After identifying potentially suitable patients, an invitation letter from the Diabetes Research Team will be sent along with a participant information sheet to them in which they'll be asked to contact the research centre in case they are interested in taking part. Healthy volunteers will be recruited by contacting subjects who participated in previous trials in the Centre (and they have already given verbal consent regarding to be contacted in such cases) and by advertisement in local papers and also on notice boards on premises of the University of Hull and Diabetes Research Centre.

When they contact the research team an appointment will be booked to meet with a study doctor to discuss the study. They can then go home and discuss the opportunity with members of the family or with the GP. There will be different participant information sheets and consent forms for the healthy volunteers and patients with type 2 diabetes.

If they decide to take part the participants will sign the relevant informed consent form (for healthy participants or patients with type 2 diabetes) in the research centre before or at the time of the screening visit. Informed consent will be taken by a study doctor. No vulnerable subjects or non-English speakers will be recruited.

Participants (healthy volunteers and patients with type 2 diabetes) will be given £35 Amazon Vouchers per completed visit in the environmental chamber including the visit that is part of the sub-study. Reasonable parking and travel expenses will be reimbursed to each participant (patients with type 2 diabetes and healthy volunteers).

7.7 Withdrawal of a subject from the study

Individuals may be withdrawn from the study:
- If they lose capacity
- If they wish to pull out of the study
- If they need to use any medication that would interfere with the test results
- If they can't tolerate being in the chamber (developing psychiatric, hypoxia or hypoglycaemia related symptoms – these will be recorded as adverse events)

Withdrawn patients will be replaced using the proposed methods of recruitment unless there is a tendency in the occurrence of adverse events. Withdrawn subjects will be followed-up by a phone call to them one week after the withdrawal.

7.8 The simulated flight

The environmental chamber is situated in the Department of Sport, Health and Exercise Science (Design and Manufacture of Environmental Test Chambers, A3897 - SSR60-20H).
The intervention in the environmental chamber involves each participant sitting in the chamber for 2 hours. The humidity level will be set at 15% and the oxygen concentration will be 15% equivalent to that of an altitude of 2500 m. During the sub-study visit, the environmental conditions will not be modified.

There will be a 2 week run-in period when the patients establish the diet.

Centres involved in the study

The consent will be taken and the screening visit will be done in the Diabetes Research Centre (Brocklehurst Building, Hull Royal Infirmary, 220-236 Anlaby Road, Hull, HU3 2RW).

The environmental chamber is situated in the Department of Sport, Health and Exercise Science, University of Hull, HU6 7RX.

Efficacy measurements

For the primary outcome: Change in endothelial function (EndoPAT 2000, circulating MPs, endothelial markers).

For the secondary outcomes: blood glucose, insulin, glucagon and lipid measurements.

Safety measurements

The collection and reporting of data on adverse events and serious adverse events will be in accordance with ICH GCP and the Research Governance Framework 2005.

Definitions

AE (Adverse event): An adverse event is any untoward medical occurrence in a subject to whom a research treatment or procedure has been administered, including occurrences which are not necessarily caused by or related to that treatment or procedure.

AR (Adverse Reaction): An adverse reaction is any untoward and unintended response in a subject which is caused by or related to a research treatment or procedure.

SAE (Serious Adverse Event): An adverse event becomes serious if it:
- results in death
- is life-threatening
- requires hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability or incapacity
- is a congenital anomaly or birth defect
- is otherwise considered medically significant by the investigator

The term “life-threatening” refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

Hospitalizations planned prior to enrolment in the trial (elective surgery) or for social reasons should not normally be considered as SAEs unless the hospitalization has to be prolonged.

Reporting adverse events

The AE reporting period for this trial begins on at visit 1, and ends 30 days after the patients final research clinic appointment.

Each trial subject will be questioned about adverse events at each visit. The investigator will record all directly observed AEs and all AEs spontaneously reported by the trial subject.
A pre-existing condition (i.e. a disorder present before the AE reporting period started and noted on the pre-treatment medical history/physical examination form/medical notes), is not to be reported as an AE unless the condition worsens or episodes increase in frequency during the AE-reporting period.

All adverse events (serious and non-serious) will be recorded in patients data collection forms (CRFs) using R&D’s adverse event report form. All adverse events will be recorded in patients’ medical records. All AEs will be followed-up until the event has resolved or a decision has been taken for no further follow-up.

If a clinically significant abnormal laboratory value occurs, this abnormality will be recorded as an adverse event/reaction.

Reporting serious adverse events
If a trial subject experiences a serious adverse event which in the opinion of the chief investigator is both

- **Related** - that is, it resulted from administration of any of the research treatments or procedures; and
- **Unexpected** - that is, the type of event is not listed in the protocol as an expected occurrence.

Then it will be reported to the Research Ethics Committee that gave a favourable opinion of the study and the sponsor (Hull and East Yorkshire Hospitals NHS Trust R&D department) **within 15 days** of the chief investigator becoming aware of the event using the NRES safety report form available from:

[http://www.nres.npsa.nhs.uk/applications/after-ethical-review/safetyreports/safety-reports-for-all-other-research/#safetynonCTIMPrepotingSAEs](http://www.nres.npsa.nhs.uk/applications/after-ethical-review/safetyreports/safety-reports-for-all-other-research/#safetynonCTIMPrepotingSAEs)

Urgent safety measures
The chief/principal investigator may take appropriate urgent safety measures in order to protect research participants against any immediate hazard to their health or safety. These safety measures should be taken immediately and may be taken without prior authorization from the REC or Trust.

However, the chief/principal investigator must alert the sponsor (HEYHT R&D) as soon as possible of the urgent measures by contacting the R&D Office telephone number 461883 or 461903 (Mon - Fri 8am - 6pm) or the Trust Switchboard 875875 (out-of-office hours) and asking for either the R&D Director or the R&D Manager.

The chief/principal investigator or sponsor should phone the main REC to discuss the issue as soon as possible. If notified by the principal investigator, the relevant local REC should also be informed.

The main REC and Trust should be notified within 3 days after the urgent measures have been taken by submitting a Notification of Amendment form and covering letter setting out the reasons for the urgent safety measures and the plan for further action.

Annual Progress Reports
An annual progress report will be submitted to the main REC which gave the favourable opinion 12 months after the date on which the favourable opinion was given and thereafter until the end of the study according to the NRES website below:


Data collection
Data will be collected in patient case notes and a case report form (CRF) designed specifically for the study in line with the sponsor’s guidelines and Good Clinical Practice.

Data will be collected by the study team. This will include research nurses and medical practitioners. Data will be anonymised and will only be identified by the patient identification number.
**End of trial**

End of the trial is the last participant's last visit day. The trial will be terminated early in case of tendency in the adverse events or if a new data becomes available that diving is not safe for patients with type 2 diabetes. An end of study declaration form (using the NRES form) will be submitted to the main REC and Trust R&D within 90 days from completion of the trial and within 15 days if the trial is discontinued prematurely. A summary of the trial report/publication will be submitted to the main REC and Trust R&D within 1 year of the end of trial.

**Power and sample size**

The primary endpoint is circulating EMPs. The study has been powered on the between group comparison (diabetes versus healthy volunteers). Eighteen patients per group will allow us to detect a 0.90 of a standard deviation difference between the two groups (80% power, 5% significance, two-tailed). This equates to a large effect size. The sample size allows for 10% to follow-up (non-differential between groups). The study lacks sufficient power for within-group comparisons (after cereal bar intervention) which will be presented as a descriptive account.

**Statistical methods**

The trial will be reported according to CONSORT guidelines (25). Continuous data will be summarised using the median and 25th/75th centiles; categorical data by percentages. Missing data will be handled using methods outlined elsewhere (26). The between group comparisons will be made by the independent t-test (Mann-Whitney U test for non-normal data). 95% confidence intervals will be reported. No correction for multiple comparisons will be made (27). Paired differences will be computed for within-group comparisons but not compared statistically. P-values for secondary outcomes will be nominal. Depending on data quality subgroup comparisons by gender may be made. The Stata statistical computer package will be used to analyse the data (28).

We'll perform the initial analysis on data obtained from the first visit in the chamber for 12 healthy individuals and 12 patients with type 2 diabetes. For these volunteers the visit window will likely be longer than 1 week between Visit 2 (first simulated flight) and Visit 3 (second simulated flight). Patient suitability and any change in medication/medical history will be checked and eligibility will be reconfirmed before the second simulated flight to ensure patient safety along with consistency and reliability of the study results.

**Monitoring**

The study may be monitored in accordance with HEY R &D department's standard operating procedures to ensure compliance with ICH GCP and the Research Governance Framework 2005. All trial related documents will be made available upon request for monitoring by R&D monitors.

**Ethics and R&D approval**

The study will be performed subject to Research Ethics Committee favourable opinion, Site Specific Assessment (SSA) approval and HEY Trust R&D approval.

**Research Governance**
This study will be conducted in accordance with the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines and the Research Governance Framework for Health and Social Care.

**Data handling and record keeping**

Data will be collected in the case notes and in the case report form to allow for cross referencing to check validity. The data will be transferred to password protected NHS computers in adherence to Trust Information Governance Policy. Professor Atkin will act as the data custodian and is responsible for the storage, handling and quality of the study data.

IT Services Department has a backup procedure approved by auditors for disaster recovery. Servers are backed up to tape media each night. The tapes run on a 4 week cycle. Files stay on the server unless deleted by accident or deliberately. Anything deleted more than 4 weeks previously is therefore lost. Additional ‘archive’ backups are taken for archived data, so data should not be lost from this type of system e.g. FileVision which stores Medical Records. Tapes are stored in a fireproof safe.

Study documents (paper and electronic) will be retained in a secure (kept locked when not in use) location during and after the trial has finished. All essential documents including source documents will be retained for 5 years after study completion (last patient, last visit). A label stating the date after which the documents can be destroyed will be placed on the inside front cover of the casenotes of trial participants.

Data will be collected and retained in accordance with the Data Protection Act 1998.

**Access to Source Data**

Monitoring, audits, REC review will be permitted.

**Finance**

All participants (patients with type 2 diabetes and healthy volunteers) will be given £35.00 after each completed visit in the environmental chamber, including the sub-study visit. Reasonable parking and travel expenses will be reimbursed to each participant.

**Indemnity**

This is an NHS-sponsored research study. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS indemnity covers NHS staff and medical academic staff with honorary contracts only when the trial has been approved by the Trust R&D department. NHS indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Where the Principal investigator is employed by the University of Hull, the University has an insurance policy that includes cover for no-fault compensation in respect of accidental injury to a research subject.


40. Hidenobu Koga, MD, Seigo Sugiyama, MD, PhD, Kiyotaka Kugiyama, MD, PhD; Keisuke Watanabe, MD, Hironobu Fukushima, MD, Tomoko Tanaka, MD, Tomohiro Sakamoto, MD, PhD, Michihiro Yoshimura, MD, PhD, Hideaki Jinnouchi, MD, PhD; Hisao Ogawa, MD, PhD. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. Journal of the American College of Cardiology. 2005; 45(10):1622-30.

41. Alexandre C. Ferreira, MD; Arley A. Peter, MD; Armando J. Mendez, PhD; Joaquín J. Jimenez, MD; Lucia M. Mauro; Julio A. Chirinos, MD; Reyan Ghany, MD; Salim Virani, MD; Santiago Garcia, MD; Lawrence L. Horstman; Joshua Purow, MD; Wenche Jy, PhD; Yeon S. Ahn, MD; Eduardo de Marchena, MD: Postprandial Hypertriglyceridemia Increases Circulating Levels of Endothelial Cell Microparticles. Circulation. 2004; 110: 3599-3603.


Introduction

We would like to invite you to participate in an additional part of the FlyBar study.

What is the purpose of the sub-study?

The main purpose of this sub-study is to prove that the molecular changes that are happening during a simulated flight are due to the different environmental conditions and not only due to the fasting or sedentary state. The aim of the sub-study is to show this.

Why have I been chosen?

You have been chosen because you agreed to participate in the FlyBar study.

Do I have to take part?

No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the sub-study and you would like to participate, will you be asked to sign a consent form (attached to this information sheet) and will be given a copy of the patient information sheet and the consent form to keep. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

As part of the sub-study you’ll be asked to attend the environmental chamber fasting on one more occasion. The visit procedures are the same as during the other environmental chamber visit as part of the main FlyBar study. The only difference is that the environmental conditions won’t be manipulated, it will feel like sitting in any other indoor environment, for example a seminar room in an ordinary building.

The environmental chamber is located in the Department of Sport, Health and Exercise Science (Washburn Building, University of Hull, Cottingham Road HU6 7RX). It is important to come to this visit fasting.

Before entering the chamber:
We will measure your blood pressure and perform an EndoPAT test, then take blood samples before entering the chamber.
At the end of the simulated flight:
We will take bloods again and repeat the EndoPAT test.
When we finish you'll be provided lunch and then you can leave.

We will ask you to complete a mood rating questionnaire and a memory task before entering the chamber, while being in the chamber and after leaving the chamber.

The approximate amount of blood we will take is 50ml (about 100 drops of blood). Blood samples either will be processed immediately in the clinical laboratories at Hull Royal Infirmary or in the University of Hull, or will be stored at -80°C in the Diabetes Department, Hull Royal Infirmary. Stored samples will be processed at the end of the study. If you give consent to this, remaining samples will be used in future Ethics committee approved cardiovascular research.

What are the possible benefits of taking part?

You may become more aware of the importance of different environmental conditions.

Are there any risks to taking part in the study?

It is inconvenient to have the bloods taken and blood vessel health checked, there is also a risk of discomfort and bruising from these tests. These will be undertaken by trained professionals who are experienced in performing these procedures.

The EndoPAT 2000 test may be uncomfortable because the blood pressure cuff will be inflated for 5 minutes.

Expenses and payments

We can pay you £35 after each completed visit in the environmental chamber. In addition, we will reimburse all your travel and parking expenses for your research visits. Please keep the receipts whenever you can obtain them.

Will my taking part in the study be kept confidential?

Yes. Unless you tell anyone that you are taking part, only your GP will know. The blood samples and test results that are collected will be anonymous when the results are studied. All information will be confidential and treated in accordance with the Data Protection Act 1998.

What will happen to the results of the study?

The results will be published in appropriate medical journals. However, individual people will not be identified and complete anonymity will be maintained in line with Trust policy and the Data Protection Act 1998.

What if something goes wrong?

We do not anticipate any problems with the study. However, in the unlikely event that this occurs you will be covered under the NHS compensation scheme, and details on our complaint procedure can be obtained on our trust website (http://www.hey.nhs.uk).

Ethical considerations

This study has been given a favourable opinion by the Humber Bridge Research Ethics Committee, which has not objected to the study taking place.
Problems or concerns can be discussed with Professor Atkin or Dr Judit Konya or any members of the clinical trial team, please telephone 01482 675314, 01482 675387 or 01482 675372 from 9am to 5pm weekdays or the Hull Royal Infirmary switchboard on 01482 328541 out of hours, and ask for Diabetes Research Team.

Thank you for your kind collaboration!

This information sheet and a copy of the consent form that you have signed are for you to keep for future reference.
CONSENT FORM  Version 1. 08. July. 2013.  FlyBar Sub-Study

Full title: Modelling endothelial function with simulated flight environment in healthy volunteers and patients with type 2 diabetes

Short title:  FlyBar Sub-Study
Name of Researchers:  Prof S.L. Atkin
Dr Judit Konya

Please write your initials all the boxes to show that you have read, understood and where needed had the meaning of the points explained to you by a member of the research team.

1. I confirm that I have read and understand the Participant Information Sheet Version 1. 08. July 2013. for the FlyBar sub-study and have had the opportunity to questions, and I am prepared to take part in the above study.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

10. For any remaining blood samples after the study I agree to have those blood samples stored to be used solely for future cardiovascular research.

11. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the research team or the representatives of the Hull East Yorkshire Hospitals NHS Trust for this study. I give permission for the individuals to have access to my records.

12. I give permission for the research team to inform my general practitioner about participation in this study.

Name of the Patient  Signature  Date and time
…………………………………….  …………………………….  …………………………….

Name of Researcher  Signature  Date and time
…………………………………….  …………………………….  …………………………….

One for patient, one for researcher, one to be kept with source documents.
Participant Information Sheet (Version 3. 26. 02. 2013.) Healthy participants group

Full title: Modelling endothelial function with simulated flight environment in healthy volunteers and patients with type 2 diabetes

Short title: FlyBar

Introduction

We would like to invite you to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it would involve for you. Please take the time to read the following information carefully and discuss it with friends, relatives and your GP if you wish.

If there is anything that is not clear, or if you would like more information, please ask us. Take time to decide whether or not you wish to take part. The research is being carried out by doctors and dietitians who have specialised in diabetes. This study is designed to look at potential food based therapies for diabetes. Thank you for taking the time to read this information sheet.

What is the purpose of the study?

The present study has two main purposes. First, we would like to investigate the effect of food compounds in healthy volunteers and patients with type 2 diabetes. Second, we would like to explore a new research method to model how blood vessels may alter their function by changes in their diameter.

Diabetes is an increasingly common condition which affects millions of people in the United Kingdom. Patients with type 2 diabetes are at increased risk to develop severe heart disease and vascular complications and these can result in death. A cornerstone of the treatment is lifestyle and diet. It is known that food containing compounds called polyphenols can reduce the risk of heart disease and other problems in people with diabetes. Two foods which are rich sources of polyphenols are cocoa (epicatechins) and soy (isoflavones).

In the blood there are certain compounds called microparticles. These microparticles can be found in the blood even under normal circumstances but the concentration correlates with how well blood vessels’ inner cell layer (endothelium) works. It was recently shown that the concentration of the microparticles is higher in diseases with increased cardiovascular risk such as type 2 diabetes as these are linked with changes in the endothelium.

The number of travellers is increasing quickly and there are more people than ever travelling by airplane. During a commercial flight the pressure of the air is lower than the pressure on the surface and that results in lower concentration of oxygen in the air we are breathing while flying. Also the humidity is very low inside an aircraft. There is no data regarding that flying would involve risks for healthy individuals. A flight with an aircraft can be simulated in an environmental chamber. It has recently been shown that low oxygen concentration increases the level of microparticles in the blood and this suggests that a commercial flight causes changes in the endothelial cells in a transient way and improved rapidly. There is no suggestion that this will cause any long-term damage or health risk for the research patients.

The aims of this study are to show if we can use a simulated flight as a method to look at
how the endothelial cells react and to see if soy can alter any changes and whether this is different between patients with type 2 diabetes mellitus and healthy volunteers. If the study is successful we hope that in the future we can use the environmental chamber to investigate the effects of other protective factors.

We plan to enrol 36 participants to the study (18 patients with type 2 diabetes and 18 healthy volunteers). The duration of the study is 6 weeks.

The proposed research is part of an educational project towards a PhD higher qualification.

**Why have I been chosen?**

You are 45-75 years old, healthy, and you expressed an interest to participate in the study.

**Do I have to take part?**

No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the study and you would like to participate, will you be asked to sign a consent form (attached to this information sheet) and will be given a copy of the patient information sheet and the consent form to keep. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will happen to me if I take part?**

During the 6 weeks you will need to attend 4 visits. Please find the schedule below.

<table>
<thead>
<tr>
<th>Visit 1 (Week 0) Screening</th>
<th>Visit 2 (Week 2)</th>
<th>Visit 3 (Week 3)</th>
<th>Visit 4 (Week 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical history, eligibility assessment, recording of medication</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical examination</td>
<td>x</td>
<td></td>
<td></td>
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<tr>
<td>Blood pressure, pulse</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Height, weight, waist circumference measurement</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Physical questionnaire</td>
<td>X</td>
<td></td>
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</tr>
<tr>
<td>Blood test</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Interview with a dietician</td>
<td>x</td>
<td></td>
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<tr>
<td>Environmental chamber – simulated flight</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>EndoPAT</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Mood rating scale, memory task</td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
<td>Ultrasound scan of the neck</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of the visit</td>
<td>85 minutes</td>
<td>4 hours</td>
<td>4 hours</td>
</tr>
</tbody>
</table>

At the first visit in the Diabetes Research Centre (220-236 Anlaby Road, Hull Royal Infirmary,
Hull, HU3 2RW) you will discuss the study with a study doctor who will explain the study to you. Then if you are still interested in, you will be asked to sign a consent form, which is attached to this patient information sheet. Then the study doctor will assess your eligibility for the trial through taking a medical history and your list of medication. If you are eligible, the doctor will do a physical examination. We will measure your blood pressure, pulse, weight, height and waist circumference. We will take some blood. A dietitian will explain to you about eating a normal diet and the foods we would like you to avoid during the study. During the screening visit we'll perform an ultrasound investigation of your neck. The procedure is non-invasive, we are going to take pictures of your neck using an ultrasound machine. This means we'll place an ultrasound probe on your neck while you are in a comfortable lying position. The investigation itself won't take longer than 10 minutes.

If you are vegan or vegetarian you may include too much soy in your diet and for this reason you will not be able to take part in the study. As smoking affects heart disease risk, if you smoke unfortunately you will not be able to enter the study. If later in the study at any point you wish to discuss your diet and lifestyle with a dietitian, an additional appointment can be made.

The next three visits (Visit 2, visit 3 and visit 4) will include a simulated flight experience in the environmental chamber. The environmental chamber is located in the Department of Sport, Health and Exercise Science (Washburn Building, University of Hull, Cottingham Road HU6 7RX). It is important to come to these visits fasting.

**Before entering the chamber:**
We will measure your blood pressure, pulse, height, weight and waist circumference. We will take some blood and do a procedure we call EndoPAT. Then you’ll enter the chamber. We plan to do the simulated flights with four-six participants at the same time.

**At the end of the simulated flight:**
We will take bloods again and repeat the EndoPAT test. When we finish you’ll be provided lunch and then you can leave.

We will ask you to complete a mood rating questionnaire and a memory task before entering the chamber, while being in the chamber and after leaving the chamber.

These procedures before and after the simulated flight will be the same during the three visits which involve the environmental chamber.

In addition, you’ll be asked to eat one food bar before entering the chamber during visit 3. Before going home after this Visit 3, we’ll give 3 weeks supply of food bars to you and from that time you’ll need to take two bars a day for three weeks when the last visit (Visit 4) is due. We will also ask you to complete a food diary for a week before each visit in the environmental chamber, so all together three occasions during the study.

The maximum amount of blood we will take is 50ml (about 100 drops of blood) during each of the four occasions. Blood samples either will be processed immediately in the clinical laboratories at Hull Royal Infirmary or in the University of Hull, or will be stored at -80°C in the Diabetes Department, Hull Royal Infirmary. Stored samples will be processed at the end of the study. If you give consent to this, remaining samples will be used in future Ethics committee approved cardiovascular research.

Three different types of bars will be used during the study:
- soy protein alone
- soy protein with soy isoflavones (each bar containing 16mg, 2 bars per day =32mg)
- soy protein with isoflavones and cocoa (each bar containing 400mg, 2 bars per day= 800mg)

The dose of soy protein and isoflavones was chosen as it was shown to be effective in our previous studies (submitted for publication).

The study is randomised which means a computer based allocation schema will be used to decide which bars you will need to eat. You will have the same 33.3% chance to be participant of any of the above groups. The study is placebo-controlled which means we will use placebo bars without soy isoflavones or cocoa polyphenols (the placebo bar will only contain soy protein). The study is double-blinded that means nor you or the research team will know which bars you will take (it is possible because the foil wrapper won’t allow us to see what is in the package).

We are planning to undertake an early analysis of the study results after the first 24 volunteers’ first simulated flight visit. If you are one of the first 24 participants, the time difference between Visit 2 (first visit in the simulated flight environment) and Visit 3 (second visit in the simulated flight environment) will likely be longer than 1 week. A decision will be made at this point whether to continue the study with the soy bars. In either case we are going to inform you as soon as possible and we’ll assess you again if needed due to the longer time period between the second and third visit.

**EndoPAT**

The EndoPAT 2000 test is designed to assess the condition of the inner layer of the blood vessels – this is a predictor of the health of the circulation. During this test a blood pressure cuff will be inflated for 5 minutes. This might cause some discomfort.

**Diet**

You will need to follow dietary recommendations for throughout the study. A dietician will explain this to you at your first visit. Information about the necessary diet is attached to this Participant Information Sheet.

**What will happen during the simulated flight?**

The flight is simulated in an environmental chamber located in the Department of Sport, Health and Exercise Science (Washburn Building, University of Hull, Cottingham Road HU6 7RX). The environmental chamber is used to research purposes to simulate different environments manipulating the temperature, humidity and/or oxygen concentration inside the chamber. You’ll sit down on comfortable chairs for 2 hours during each simulated flight visit. The conditions will be the following: 15% oxygen and 15% humidity, like the conditions in a commercial airplane.

You will be able to move and talk while being in the chamber and a study doctor as well as the operational staff of the chamber will always be there to ensure your safety and that you are feeling comfortable.
When coming for a session it is useful to bring something to read, as it helps to pass the time. It is also useful to bring a light cardigan or jacket to avoid being cold.

What are the possible benefits of taking part?

You may become more aware of the importance of food in your everyday life.

The basic snack bar contains the ingredients shown in previous studies to improve control of diabetes and risk of heart disease. The addition of cocoa to this may or may not add further benefits.

Are there any risks to taking part in the study?

One of the minimal risks are weight gain as you will be having two snack bars daily for three weeks. To help with this a research dietitian will be available to discuss diet with you before you start the study and while you are taking part.

It is inconvenient to have the blood and blood vessel health checks, there is also a risk of discomfort and bruising from these tests. These will be undertaken by trained professionals who are experienced in performing these procedures.

The EndoPAT 2000 test may be uncomfortable because the blood pressure cuff will be inflated for 5 minutes.

The bars may cause allergy or abdominal discomfort. You will be given 24/7 contact details for the research staff so you can contact them if you experience unpleasant symptoms.

The simulated flight itself doesn’t involve any additional risk.

Expenses and payments

We can pay you £35 after each completed visit in the environmental chamber in the form of an Amazon voucher to compensate you for your time. In addition, we will reimburse all your travel and parking expenses for your research visits. Please keep the receipts whenever you can obtain them.

At the end of the three visits including the simulated dive we will provide you lunch after
finishing the visits.

**Will my taking part in the study be kept confidential?**

Yes. Unless you tell anyone that you are taking part, only your GP will know. The blood samples and test results that are collected will be anonymous when the results are studied. All information will be confidential and treated in accordance with the Data Protection Act 1998.

**What will happen to the results of the study?**

The results will be published in appropriate medical journals. However, individual people will not be identified and complete anonymity will be maintained in line with Trust policy and the Data Protection Act 1998.

**What if something goes wrong?**

We do not anticipate any problems with the study. However, in the unlikely event that this occurs you will be covered under the NHS compensation scheme, and details on our complaint procedure can be obtained on our trust website ([http://www.hey.nhs.uk](http://www.hey.nhs.uk)).

**Ethical considerations**

This study has been given a favourable opinion by the Humber Bridge Research Ethics Committee, which has not objected to the study taking place.

Problems or concerns can be discussed with Professor Atkin or Dr Judit Konya or any members of the clinical trial team, please telephone 01482 675314, 01482 675387 or 01482 675372 from 9am to 5pm weekdays or the Hull Royal Infirmary switchboard on 01482 328541 out of hours, and ask for Diabetes Research Team.

Thank you for your kind collaboration!

This information sheet and a copy of the consent form that you have signed are for you to keep for future reference.
CONSENT FORM  

Healthy participants group

Full title: Modelling endothelial function with simulated flight environment in healthy volunteers and patients with type 2 diabetes

Short title: FlyBar

Name of Researchers: Prof S.L. Atkin
Dr Judit Konya

Please write your Initials all the boxes to show that you have read, understood and where needed had the meaning of the points explained to you by a member of the research team.

1. I confirm that I have read and understand the Participant Information Sheet Version 3. 26. 02. 2013. for the above study and have had the opportunity to ask questions, and I am prepared to take part in the above study.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

13. For any remaining blood samples after the study I agree to have those blood samples stored to be used solely for future cardiovascular research.

14. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the research team or the representatives of the Hull and East Yorkshire Hospitals NHS Trust for this study. I give permission for the individuals to have access to my records.

15. I give permission for the research team to inform my general practitioner about my participation in this study.

Name of the Patient  Signature  Date and time  
…………………………………………………………………………………………

Name of Researcher  Signature  Date and time  
…………………………………………………………………………………………

One for patient, one for researcher, one to be kept with source documents.
Screening for excessive intake of isoflavones

Isoflavones are nutrients that are mainly found in soybeans and soy products. As this study is looking at the effect of soy on insulin resistance and cardiovascular disease in type 2 diabetes, we need to ensure that you are not already having a high intake of isoflavones. It is also important that you avoid foods high in isoflavones throughout the study.

The study dietitian will ask you a number of questions about the types of food that you eat, please be honest with your answers as this may affect the results of the study. If your isoflavones intake is high unfortunately you will be unable to take part in the study.

Please avoid the following foods:

- All products containing soya as a main ingredient
- Soya Mince, burgers, sausages
- TVP (textured vegetable protein) or any foods containing this.
- Tofu
- Soya milk/ drinks
- Soya yoghurts
- Soya desserts
- Soya cheese
- Soya ice cream
- Soya beans/ nuts
- Soya spreads (e.g. Pure, Suma)
- Soya and linseed breads (Burgen, Vogel)
- Soy sauce
- Soya Sprouts
- Soya Snacks (crisps)
- Soya based ready meals

This list is not exhaustive so if you come across other foods which contain soya please avoid.
Low polyphenol dietary advice sheet

Polyphenols are found naturally in many foods. Because we are testing the effect of polyphenols on insulin resistance and cardiovascular disease in type 2 diabetes, it is important you do not have foods high in polyphenols. Below is a list of foods which we would like you to avoid or reduce your intake of:

Please avoid:
- Chocolate
- Any foods containing chocolate or chocolate flavoured foods
- Berries
- Red wine
- Phytosterol products (margarines, yoghurt drink and cheese advertised to reduce cholesterol.) (Flora Proactive/ Bencol/ Danone)
- Grapefruit
- Licorice
- Vitamin and mineral supplements
- Onion and garlic (try to avoid completely small amounts in ready meals is acceptable if there is no alternative)

Please restrict your intake of the following to:
- Fruit juice 150mls 1 glass per day.
- Vegetables and Fruit please do not exceed 5 portions per day.
- Coffee and Tea please limit to a maximum of 3 cups per day.
- Please avoid Herbal tea or green tea
- Alcohol: please keep within recommended limits 2-3 units per day for females, 3-4 units per day for males.

Please try and keep you intake of the above food and drink consistent.

Portion Guide
- 3 heaped tablespoons of vegetables, beans or pulses
- 1 apple, banana, pear, orange or other similar sized fruit
- 2 plums, satsuma’s, kiwi fruit or other similar sized fruit
- One to two handfuls of grapes
- A portion of dried fruit is around 30g
Introduction

We would like to invite you to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it would involve for you. Please take the time to read the following information carefully and discuss it with friends, relatives and your GP if you wish.

If there is anything that is not clear, or if you would like more information, please ask us. Take time to decide whether or not you wish to take part. The research is being carried out by doctors and dietitians who have specialised in diabetes. This study is designed to look at potential food based therapies for diabetes. Thank you for taking the time to read this information sheet.

What is the purpose of the study?

The present study has two main purposes. First, we would like to investigate the effect of food compounds in healthy volunteers and patients with type 2 diabetes. Second, we would like to explore a new research method to model how blood vessels may alter their function by changes in their diameter.

Diabetes is an increasingly common condition which affects millions of people in the United Kingdom. Patients with type 2 diabetes are at increased risk to develop severe heart disease and vascular complications and these can result in death. A cornerstone of the treatment is lifestyle and diet. It is known that food containing compounds called polyphenols can reduce the risk of heart disease and other problems in people with diabetes. Two foods which are rich sources of polyphenols are cocoa (epicatechins) and soy (isoflavones).

In the blood there are certain compounds called microparticles. These microparticles can be found in the blood even under normal circumstances but the concentration correlates with how well blood vessels’ inner cell layer (endothelium) works. It was recently shown that the concentration of the microparticles is higher in diseases with increased cardiovascular risk such as type 2 diabetes as these are linked with changes in the endothelium.

The number of travellers is increasing quickly and there are more people than ever travelling by airplane. During a commercial flight the pressure of the air is lower than the pressure on the surface and that results in lower concentration of oxygen in the air we are breathing while flying. Also the humidity is very low inside an aircraft. There is no data regarding that flying would involve risks for patients with well-controlled type 2 diabetes. A flight with an aircraft can be simulated in an environmental chamber. It has recently been shown that low oxygen concentration increases the level of microparticles in the blood and this suggests that a commercial flight causes changes in the endothelial cells in a transient way and improved rapidly. There is no suggestion that this will cause any long-term damage or health risk for the research patients.

The aims of this study are to show if we can use a simulated flight as a method to look at
how the endothelial cells react and to see if soy can alter any changes and whether this is different between patients with type 2 diabetes mellitus and healthy volunteers. If the study is successful we hope that in the future we can use the environmental chamber to investigate the effects of other protective factors.

We plan to enrol 36 participants to the study (18 patients with type 2 diabetes and 18 healthy volunteers). The duration of the study is 6 weeks.

The proposed research is part of an educational project towards a PhD higher qualification.

**Why have I been chosen?**
You have type 2 diabetes and you are on a stable dose of metformin or not taking any other medication for the diabetes.

**Do I have to take part?**
No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the study and you would like to participate, will you be asked to sign a consent form (attached to this information sheet) and will be given a copy of the patient information sheet and the consent form to keep. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will happen to me if I take part?**
During the 6 weeks you will need to attend 4 visits. Please find the schedule below.

<table>
<thead>
<tr>
<th>Visit 1 (Week 0) Screening</th>
<th>Visit 2 (Week 2)</th>
<th>Visit 3 (Week 3)</th>
<th>Visit 4 (Week 6)</th>
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<tbody>
<tr>
<td>Medical history, eligibility assessment, recording of medication</td>
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<td>Physical examination</td>
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<td>Height, weight, waist circumference measurement</td>
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<td>Physical activity questionnaire</td>
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<td>Blood test</td>
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<td>Interview with a dietician</td>
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<tr>
<td>Environmental chamber – simulated flight</td>
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<td>X</td>
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<tr>
<td>EndoPAT</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Mood rating scale, memory task</td>
<td>X</td>
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<tr>
<td>Ultrasound scan of the neck</td>
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</tr>
<tr>
<td>Length of the visit</td>
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<td>4 hours</td>
<td>4 hours</td>
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</tbody>
</table>

At the first visit in the Diabetes Research Centre (220-236 Anlaby Road, Hull Royal Infirmary, Hull, HU3 2RW) you will discuss the study with a study doctor who will explain the study to you. Then if you are still interested in, you will be asked to sign a consent form, which is
attached to this patient information sheet. Then the study doctor will assess your eligibility for the trial through taking a medical history and your list of medication. If you are eligible, the doctor will do a physical examination. We will measure your blood pressure, pulse, weight, height and waist circumference. We will take some blood. A dietitian will explain to you about eating a normal diet and the foods we would like you to avoid during the study.

During the screening visit we'll perform an ultrasound investigation of your neck. The procedure is non-invasive, we are going to take pictures of your neck using an ultrasound machine. This means we'll place an ultrasound probe on your neck while you are in a comfortable lying position. The investigation itself won't take longer than 10 minutes.

If you are on more than one medication for your diabetes unfortunately you cannot take part in this research. If you are vegan or vegetarian you may include too much soy in your diet and for this reason you will not be able to take part in the study. As smoking affects heart disease risk, if you smoke unfortunately you will not be able to enter the study. If later in the study at any point you wish to discuss your diet and lifestyle with a dietitian, an additional appointment can be made.

The next three visits (Visit 2, visit 3 and visit 4) will include a simulated flight experience in the environmental chamber. The environmental chamber is located in the Department of Sport, Health and Exercise Science (Washburn Building, University of Hull, Cottingham Road HU6 7RX). It is important to come to these visits fasting.

Before entering the chamber:
We will measure your blood pressure, pulse, height, weight and waist circumference. We will take some blood and do a procedure we call EndoPAT. Then you'll enter the chamber. We plan to do the simulated flights with four-six participants at the same time.

At the end of the simulated flight:
We will take bloods again and repeat the EndoPAT test. When we finish you'll be provided lunch and then you can leave.

We will ask you to complete a mood rating questionnaire and a memory task before entering the chamber, while being in the chamber and after leaving the chamber.

These procedures before and after the simulated flight will be the same during the three visits which involve the environmental chamber.

In addition, you'll be asked to eat one food bar before entering the chamber during visit 3. Before going home after this Visit 3, we'll give 3 weeks supply of food bars to you and from that time you'll need to take two bars a day for three weeks when the last visit (Visit 4) is due. We will also ask you to complete a food diary for a week before each visit in the environmental chamber, so all together three occasions during the study.

The maximum amount of blood we will take is 50ml (about 100 drops of blood) during each of the four occasions. Blood samples either will be processed immediately in the clinical laboratories at Hull Royal Infirmary or in the University of Hull, or will be stored at -80oC in the Diabetes Department, Hull Royal Infirmary. Stored samples will be processed at the end of the study. If you give consent to this, remaining samples will be used in future Ethics committee approved cardiovascular research.

Three different types of bars will be used during the study:
- soy protein alone
- soy protein with soy isoflavones (each bar containing 16mg, 2 bars per day =32mg)
- soy protein with isoflavones and cocoa (each bar containing 400mg, 2 bars per day= 800mg)
The dose of soy protein and isoflavones was chosen as it was shown to be effective in our previous studies (submitted for publication).

The study is randomised which means a computer based allocation schema will be used to decide which bars you will need to eat. You will have the same 33.3% chance to be participant of any of the above groups. The study is placebo-controlled which means we will use placebo bars without soy isoflavones or cocoa polyphenols (the placebo bar will only contain soy protein). The study is double-blinded that means nor you or the research team will know which bars you will take (it is possible because the foil wrapper won’t allow us to see what is in the package).

We are planning to undertake an early analysis of the study results after the first 24 volunteers’ first simulated flight visit. If you are one of the first 24 participants, the time difference between Visit 2 (first visit in the simulated flight environment) and Visit 3 (second visit in the simulated flight environment) will likely be longer than 1 week. A decision will be made at this point whether to continue the study with the soy bars. In either case we are going to inform you as soon as possible and we’ll assess you again if needed due to the longer time period between the second and third visit.

**EndoPAT**

The EndoPAT 2000 test is designed to assess the condition of the inner layer of the blood vessels – this is a predictor of the health of the circulation. During this test a blood pressure cuff will be inflated for 5 minutes. This might cause some discomfort.

**Diet**

You will need to follow dietary recommendations for throughout the study. A dietician will explain this to you at you first visit. Information about the necessary diet is attached to this Participant Information Sheet.

**What will happen during the simulated flight?**

The flight is simulated in an environmental chamber located in the Department of Sport, Health and Exercise Science (Washburn Building, University of Hull, Cottingham Road HU6 7RX). The environmental chamber is used to research purposes to simulate different environments manipulating the temperature, humidity and/or oxygen concentration inside the chamber. You’ll sit down on comfortable chairs for 2 hours during each simulated flight visit. The conditions will be the following: 15% oxygen and 15% humidity, like the conditions in a commercial airplane.
You will be able to move and talk while being in the chamber and a study doctor as well as the operational staff of the chamber will always be there to ensure your safety and that you are feeling comfortable.
When coming for a session it is useful to bring something to read, as it helps to pass the time. It is also useful to bring a light cardigan or jacket to avoid being cold.

**What are the possible benefits of taking part?**

You may become more aware of the importance of food in your diabetes.

The basic snack bar contains the ingredients shown in previous studies to improve control of diabetes and risk of heart disease. The addition of cocoa to this may or may not add further benefits. The research team are specialists in diabetes and will review your diabetes at each of your visits during the study.

**Are there any risks to taking part in the study?**

One of the minimal risks are weight gain as you will be having two snack bars daily for three weeks. To help with this a research dietitian will be available to discuss diet with you before you start the study and while you are taking part.

It is inconvenient to have the blood and blood vessel health checks, there is also a risk of discomfort and bruising from these tests. These will be undertaken by trained professionals who are experienced in performing these procedures.

The EndoPAT 2000 test may be uncomfortable because the blood pressure cuff will be inflated for 5 minutes.

The bars may cause allergy or abdominal discomfort. You will be given 24/7 contact details for the research staff so you can contact them if you experience unpleasant symptoms.

The simulated flight itself doesn’t involve any additional risk.

**Expenses and payments**

We can pay you £35 after each completed visit in the environmental chamber in the form of an Amazon voucher to compensate you for your time. In addition, we will reimburse all your travel and parking expenses for your research visits. Please keep the receipts whenever you can obtain them.
At the end of the three visits including the simulated dive we will provide you lunch after finishing the visits.

**Will my taking part in the study be kept confidential?**

Yes. Unless you tell anyone that you are taking part, only your GP will know. The blood samples and test results that are collected will be anonymous when the results are studied. All information will be confidential and treated in accordance with the Data Protection Act 1998.

**What will happen to the results of the study?**

The results will be published in appropriate medical journals. However, individual people will not be identified and complete anonymity will be maintained in line with Trust policy and the Data Protection Act 1998.

**What if something goes wrong?**

We do not anticipate any problems with the study. However, in the unlikely event that this occurs you will be covered under the NHS compensation scheme, and details on our complaint procedure can be obtained on our trust website (http://www.hey.nhs.uk).

**Ethical considerations**

This study has been given a favourable opinion by the Humber Bridge Research Ethics Committee, which has not objected to the study taking place.

Problems or concerns can be discussed with Professor Atkin or Dr Judit Konya or any members of the clinical trial team, please telephone 01482 675314, 01482 675387 or 01482 675372 from 9am to 5pm weekdays or the Hull Royal Infirmary switchboard on 01482 328541 out of hours, and ask for Diabetes Research Team.

Thank you for your kind collaboration!

This information sheet and a copy of the consent form that you have signed are for you to keep for future reference.
Patient identification number:


Full title: Modelling endothelial function with simulated flight environment in healthy volunteers and patients with type 2 diabetes

Short title: FlyBar
Name of Researchers: Prof S.L. Atkin
Dr Judit Konya

Please write your Initials all the boxes to show that you have read, understood and where needed had the meaning of the points explained to you by a member of the research team.

1. I confirm that I have read and understand the Participant Information Sheet Version 3. 26. 03. 2013. for the above study and have had the opportunity to ask questions and I am prepared to take part in the above study.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

16. For any remaining blood samples after the study I agree to have those blood samples stored to be used solely for future cardiovascular research.

17. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the research team or the representatives of the Hull and East Yorkshire Hospitals NHS Trust for this study. I give permission for these individuals to have access to my records.

18. I give permission for the research team to inform my general practitioner about my participation in this study.

Name of the Patient  Signature  Date and time

……………………  ………………………  ………………………

Name of Researcher  Signature  Date and time

……………………  ………………………  ………………………

One for patient, one for researcher, one to be kept with source documents.
Screening for excessive intake of isoflavones

Isoflavones are nutrients that are mainly found in soybeans and soy products. As this study is looking at the effect of soy on insulin resistance and cardiovascular disease in type 2 diabetes, we need to ensure that you are not already having a high intake of isoflavones. It is also important that you avoid foods high in isoflavones throughout the study.

The study dietitian will ask you a number of questions about the types of food that you eat, please be honest with your answers as this may affect the results of the study. If you isoflavones intake is high unfortunately you will be unable to take part in the study.

Please avoid the following foods:
• All products containing soya as a main ingredient
• Soya Mince, burgers, sausages
• TVP (textured vegetable protein) or any foods containing this.
• Tofu
• Soya milk/ drinks
• Soya yoghurts
• Soya desserts
• Soya cheese
• Soya ice cream
• Soya beans/ nuts
• Soya spreads (e.g. Pure, Suma)
• Soya and linseed breads (Burgen, Vogel)
• Soy sauce
• Soya Sprouts
• Soya Snacks (crisps)
• Soya based ready meals

This list is not exhaustive so if you come across over foods which contain soya please avoid.
Low polyphenol dietary advice sheet

Polyphenols are found naturally in many foods. Because we are testing the effect of polyphenols on insulin resistance and cardiovascular disease in type 2 diabetes, it is important you do not have foods high in polyphenols. Below is a list of foods which we would like you to avoid or reduce your intake of:

Please avoid:
- Chocolate
- Any foods containing chocolate or chocolate flavoured foods
- Berries
- Red wine
- Phytosterol products (margarines, yoghurt drink and cheese advertised to reduce cholesterol.) (Flora Proactive/ Bencol/ Danone)
- Grapefruit
- Licorice
- Vitamin and mineral supplements
- Onion and garlic (try to avoid completely small amounts in ready meals is acceptable if there is no alternative)

Please restrict your intake of the following to:
- Fruit juice 150mls 1 glass per day.
- Vegetables and Fruit please do not exceed 5 portions per day.
- Coffee and Tea please limit to a maximum of 3 cups per day.
- Please avoid Herbal tea or green tea
- Alcohol: please keep within recommended limits 2-3 units per day for females, 3-4 units per day for males.

Please try and keep you intake of the above food and drink consistent.

Portion Guide
- 3 heaped tablespoons of vegetables, beans or pulses
- 1 apple, banana, pear, orange or other similar sized fruit
- 2 plums, satsuma’s, kiwi fruit or other similar sized fruit
- One to two handfuls of grapes
- A portion of dried fruit is around 30g
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<tr>
<th><strong>Production Data Sheet</strong></th>
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<td><strong>DIMENSIONS (INT):-</strong></td>
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<td><strong>COOLING RANGE :-</strong></td>
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<tr>
<td><strong>TEST LOAD WEIGHT:-</strong></td>
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</table>
Design and Manufacture of Environmental Test Chambers

**Schematic Drawings**

A3897 - SSR60-20H

The University Of Hull
Cottingham Road,
North Hum,
HU6 7RX,
Design and Manufacture of Environmental Test Chambers

**Mechanical Drawings**

**And Parts List**
10/03/10
* APPROX POSITION OF AIR RECEIVER

3/02/10
Suspended ceiling tiles etc. to be removed in area above chamber

* * *
* STANDARD 15mm water supply req'd in this area for supply to humidifying boilers

8" Min. Concrete Base req'd approx 2mx1.5m to fill alcove.
Alcove to be cleared of existing air conditioning units to a height of 1.8M

POWER SUPPLY INTO CABINET
APPROX REQ'D POSITION OF CUSTOMER ISOLATOR FOR POWER SUPPLY - 415V - 3 PHASE N & E - 100A (supply to be taken to cabinet by customer)

CONTROL PANEL
300 x 400 x 180mm

ISOLATOR SWITCH
80A

18.5Kw COMPRESSOR
HPC ASK32
Compressor for Nitrogen Gen in this area

Housed refrigeration plant (Bitzer LH114/4PCS-10.2y) in this area

Hypoxic ring main with diffusers to surface drain

Pressur relief port A3897M-18

Lighting units seen in drawing A3897M-44

Refrigeration pipework & oxygen rich vent (warning sign may be req'd) to exit room in this area

SEE A3897M-22

4/01/10
Hypoxic ring main with 6 diffusers (see separate Parts List)

1000 APPROX - TO SUIT PANEL LAYOUT

H2 O To Drain
2558 TO U/SIDE OF TILES
APPROX 150mm SQ.

Area along wall for pipe work and looms (capping req'd)

SIDE VIEW THRO' ROOM/CHAMBER

2 off Air Handling units ECO STE 337BLED

Humidity Boiler tanks

Humidity Boiler / Supply tank

HYPOXIC CABINET AND CONTROL PANEL

VIEWING WINDOW 380mm X 380mm

VIEWING WINDOW 960mm X 960mm

DRINKS HATCH A3897M-25

300mm X 300mm

ENTRY PORT 1 OFF A125mm CABLE LC

PLAN VIEW OF ROOM/CHAMBER

INTERNAL SIZE OF ROOM
4600mm WIDE
4600mm DEEP
2400mm HIGH

DOOR DETAILS
LEFT HAND HUNG
FACE MOUNTING AND SEALING
(4 SIDED GASKET)

2000mm HIGH OPENING

TEMPERATURE RANGE
-20°C TO +50°C

HUMIDITY RANGE
15% TO 80% RH

STANDARD ASSEMBLY
CAMLOCKED WALL PANELS
WHITE INTERNAL LINER AND FLOOR
WHITE EXTERNAL FINISH

POWER SUPPLY - 415V - 3 PHASE N & E - 100A

4/01/10
2600
4800
6960
7200

2000 c/o 6200

Design Environmental Ltd.
4800

2724 TO U/SIDE OF SLAB

790
565
1115

275
1040
60
240
565
60
195
1755
19
2030
2215

3306
15

schunk

Design Environmental Ltd.
Hypoxic Nitrogen generator

MATERIAL - VARIOUS, NOTED.

DESCRIPTION -

A

DATE - 01/10

ISSUE - A

CUSTOMER - Uni of Hull

DRAWING No. A3897NITRO

DESIGNER - RJL

APPROVED -

Tolerance +/- 1.0mm Unless Otherwise Stated

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Design Environmental Limited and is the property of

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<td>003-8503</td>
<td>Chamber light sealing patch</td>
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<td>19</td>
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<td>RUBBER EXTRUSION 'U' (BLACK) OUTSIDE OF WINDOW</td>
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<td><strong>S</strong></td>
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<td>Ø32mm STAINLESS STEEL TUBE (EXPRESS METALS)</td>
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<td>18SWG STAINLESS STEEL BRIGHT ANNEALED</td>
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Design and Manufacture of Environmental Test Chambers

Fridge Drawings
And Parts List
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</table>
Design and Manufacture of Environmental Test Chambers

**Electrical Drawings**

**And Parts List**
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MINI 8 CONTROLLER

FAULT PLUG/SKT1

EVENT1 PLUG/SKT2

UNI OF HULL

MINI 8 CONTROLLER

DRAWING TITLE SHEET

DRAWING No. A3897E-2

CURRENT DRAWING

FINISH - MATERIAL -

DESCRIPTION - Issued To Production.

DATE - SIGNATURE - DY

ISSUE - APPROVED -

TYPE - SSR60-20H

DRAWN. DY DATE. SHEET

CUSTOMER

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Ebbw Vale,
Gwent, NP3 5SD
Tel: 01495 305 555
Fax: 01495 303 595

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Ebbw Vale,
Gwent, NP3 5SD
Tel: 01495 305 555
Fax: 01495 303 595

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MINI 8 CONTROLLER

DRAWING TITLE SHEET

DRAWING No. A3897E-2

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FINISH - MATERIAL -

DESCRIPTION - Issued To Production.

DATE - SIGNATURE - DY

ISSUE - APPROVED -

TYPE - SSR60-20H

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Gwent, NP3 5SD
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Fax: 01495 303 595

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CONNECT ALL INPUT N/C TERMINALS TO WIRE NUMBER 128 EXCEPT FS CTRL AND DEHUM

Design Environmental Limited
32 Rassau Industrial Estate,
Ebbw Vale,
Gwent. NP3 5SD
Tel : 01495 305 555
Fax : 01495 303 595

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CUSTOMER

LOGIC CONTROLLER

ISSUE - A
APPROVED -

ISSUE -

DRAWING No. A3897E-3
DRAWN. DY DATE. 01/10 SHEET

UNI OF HULL
CHAMBER HEATERS

**Type:** SSR60-20H

**Description:**
- Chamber Heaters

**Issue:** A

**Customer:** Uni of Hull

**Drawing No.:** A3897E-4

**Drawing Title:** Chamber Heaters

**Date:** 01/07

**Signatures:**
- D.Y.

---

**Design Environmental Limited**

32 Rassau Industrial Estate,
Ebbw Vale,
Gwent. NP3 5SD

Tel: 01495 305 555
Fax: 01495 303 395

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NOTE: ALL INCOMING WIRES TO ISOLATOR MUST NOT BE WIRED TO DIN RAIL TERMINALS

MOUNT ON PLANT
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Current wiring regulations for destination country must be used.

Fan, Lights & EM Stop Relay

10 OFF POWER SOCKETS 230VOLT MOUNTED INSIDE ROOM

RCBO 1 20A 30mA
N1 660 661
N2 0 9

TYPE - SSR60-20H
FINISH - MATERIAL -
DESCRIPTION - Issued To Production.
DATE - 10/06 SIGNATURE - DY
ISSUE - A APPROVED -

CUSTOMER
Uni of Hull

DRAWING No. A3897E-10

Design Environmental Limited
32 Rassau Industrial Estate,
Ebbw Vale,
Gwent. NP3 5SD
Tel : 01495 305 555
Fax : 01495 303 595
9 WAY D PLUG FITTED TO CONVERTER B&B485USB9F-2W

25 WAY D PLUG FITTED TO CHAMBER

3M SCREENED LEAD (D.E.L. MANUFACTURED)

YELLOW
GREEN
GROUND

5 WAY D PLUG FITTED TO CONVERTER B&B485USB9F-2W

1 WAY D PLUG FITTED TO CHAMBER

3M SCREENED LEAD (D.E.L. MANUFACTURED)
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