Oral thiamine (Vitamin B1) supplementation in subjects with type 2 diabetes mellitus: a randomised, double-blind, placebo-controlled crossover trial assessing biophysical markers of endothelial function, oxidant stress, insulin sensitivity and vascular inflammation.

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This thesis is submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Medicine of the University of Portsmouth

In collaboration with:

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Abstract

Background and Aims
Type 2 diabetes is increasing in prevalence and is associated with a threefold increased risk of cardiovascular mortality despite management of the traditional risk factors. Novel risk factors have been hypothesised that contribute to the pathogenesis of both type 2 diabetes and atherosclerosis and include oxidative stress, inflammation and endothelial dysfunction. Thiamine has been shown to be an important cofactor in the attenuation of these novel risk factors and people with type 2 diabetes have been shown to be thiamine deficient. This study tested the hypothesis that thiamine supplementation may improve endothelial function, oxidative stress, vascular inflammation and insulin resistance in subjects with type 2 diabetes who have a high cardiovascular risk profile.

Methods
Subjects with type 2 diabetes underwent a randomised, double blind, placebo-controlled crossover pilot study receiving 300mg daily of oral thiamine hydrochloride or placebo for eight weeks with a two week washout period. Measurements were taken for endothelial function (change in the reflective index post salbutamol using digital photoplethysmography, plasma cyclic GMP, plasma sVCAM-1, urinary albumin:creatinine ratio), insulin resistance (HOMA-IR), oxidative stress (glutathione ratio, CuPRAC-BCS) and inflammation (hsCRP) at the beginning and end of treatment with both thiamine and placebo.

Results
34 patients (20 male) completed the study. Mean age 61 ± 9.4 years, HbA1c 7.46 ± 0.88 %, blood pressure 137/77 ± 18/9 mmHg, total cholesterol 4.01 ± 1.11 mmol/l, HDL cholesterol 1.00 ± 0.30 mmol/l, triglycerides 1.87 ± 1.39
mmol/l. The majority of the patients were on two or more glucose lowering therapies with 88% on metformin. Most of the patients were on other cardiovascular disease modifying medications (statins or antihypertensive agents). Treatment with thiamine demonstrated a significant increase in thiamine diphosphate levels (310 ± 82 nmol/l post thiamine vs. 178 ± 32 nmol/l post placebo, p=<0.001) but no significant difference in markers of endothelial function, insulin resistance, oxidative stress or inflammation or other metabolic markers.

**Conclusion**

In this cohort of patients treatment with 300mg per day of oral thiamine for eight weeks in well-controlled type 2 diabetes at high cardiovascular risk, demonstrated no significant improvement in endothelial function, insulin resistance, oxidative stress or inflammation.
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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award.

The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Abbreviations

ACE  Angiotensin Converting Enzyme
AEAC  Ascorbic Acid Equivalent Antioxidant Capacity
AGE  Advanced Glycation Endproducts
ANOVA  Analysis of Variation
BCS  Bathocuproinedisulphonic Acid
BMI  Body Mass Index
CABG  Coronary Artery Bypass Graft
cGMP  Cyclic Guanosine Monophosphate
CRP  C-Reactive Protein
CuPRAC-BCS  Copper(II)reduction assay with bathocuproinedisulfonic acid disodium salt
CV  Coefficient of Variance
DTNB  5,5'-dithiobis-(2-nitrobenzoic acid
DVP  Digital Volume Pulse
ECG  Electrocardiogram
EDTA  Ethylenediaminetetraacetic acid
eNOS  Endothelial Nitric Oxide Synthase
ET-1  Endothelin-1
FMD  Flow Mediated Dilatation
GSH  Glutathione
GSSG  Glutathione disulphide
GTN  Glyceryl Trinitrate
HbA1c  Glycated Haemoglobin
HDL  High Density Lipoprotein
HOMA  Homeostasis Model Assessment
HOMA-IR  Homeostasis Model Assessment – Insulin Resistance
HPLC  High-performance liquid chromatography
hsCRP  Highly sensitive C-Reactive Protein
hTHTR  Human thiamine transporter
ICAM-1  Intercellular Adhesion Molecule 1
IFG  Impaired Fasting Glucose
IGT  Impaired Glucose Tolerance
LDL  Low-density Lipoprotein
M2VP  1-methyl-2-vinyl-pyridinium trifluoromethanesulphonate
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHRA</td>
<td>Medicines and Healthcare Products Regulatory Agency</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>NFKB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Care Excellence</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>OHA</td>
<td>Oral Hypoglycaemic Agent</td>
</tr>
<tr>
<td>PWA</td>
<td>Pulse Wave Analysis</td>
</tr>
<tr>
<td>QAH</td>
<td>Queen Alexandra Hospital</td>
</tr>
<tr>
<td>RI</td>
<td>Reflective Index</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SSA</td>
<td>Sulphosalicylic acid</td>
</tr>
<tr>
<td>SST</td>
<td>Serum separator tube</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>Soluble vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>TAC</td>
<td>Total Antioxidant Capacity</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>TDP</td>
<td>Thiamine Diphosphate</td>
</tr>
<tr>
<td>TMP</td>
<td>Thiamine Monophosphate</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
</tbody>
</table>
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Poster Presentation
Is there an association between thiamine status and endothelial dysfunction in individuals with Type 2 diabetes?

Diabetes UK Annual Professional Conference. March 2013
Poster Presentation
Thiamine supplementation in patients with Type 2 diabetes: effect on endothelial function and vascular inflammation.
Introduction

Diabetes Mellitus

Definition

Diabetes Mellitus is a metabolic disorder where defective insulin secretion, insulin action or both leads to chronic hyperglycaemia. Patients may be asymptomatic at diagnosis or may present with the characteristic symptoms of polyuria, excess thirst, weight loss and blurring of the vision. The World Health Organisation defined the diagnostic criteria in 1998 (1) (table 1.1) and in 2011 2.9 million people in the UK were known to have diabetes. This is estimated to increase to 5 million people by 2025 (2). Long-term hyperglycaemia causes damage and dysfunction to various organs and an associated increase in morbidity and mortality, the major cause of which is cardiovascular disease.

Table 1.1: Diagnostic criteria for diabetes mellitus (3).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Glucose concentration plasma (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes Mellitus:</td>
<td></td>
</tr>
<tr>
<td>Fasting or 2-h post glucose load</td>
<td>≥7.0</td>
</tr>
<tr>
<td>or both</td>
<td>≥11.1</td>
</tr>
<tr>
<td>Impaired Glucose Tolerance (IGT):</td>
<td></td>
</tr>
<tr>
<td>Fasting concentration (if measured) and 2-h post glucose load</td>
<td>≤7.0</td>
</tr>
<tr>
<td></td>
<td>≥7.8 - 11.0</td>
</tr>
<tr>
<td>Impaired Fasting Glycaemia (IFG):</td>
<td></td>
</tr>
<tr>
<td>Fasting 2-h (if measured)</td>
<td>6.1 - 6.9</td>
</tr>
<tr>
<td></td>
<td>&lt;7.8</td>
</tr>
</tbody>
</table>
Pathophysiology

Diabetes is sub-classified into several aetiological types, the most common of which are type 1 and type 2 diabetes. Type 1 diabetes occurs as a result of cellular-mediated autoimmune destruction of the pancreatic beta cells. It accounts for 5-10% of those individuals with diabetes and affected individuals are dependent on insulin for survival. Type 2 diabetes is more prevalent accounting for 90-95% of those with diabetes worldwide. It is associated with insulin resistance and relative insulin deficiency but the specific pathophysiological change that causes type 2 diabetes is not yet known. Type 2 diabetes is also associated with the metabolic syndrome, a disordered state where individuals have a cluster of risk factors for cardiovascular disease.

Vascular disease

Vasculopathy associated with diabetes is commonly subdivided into macrovascular or microvascular disease. Macrovascular disease is characterised by atherosclerosis, a process whereby lipid-rich plaques form on the internal wall of blood vessels, the endothelium. Microvascular disease is a disorder only seen in diabetes and includes retinopathy, nephropathy and neuropathy. The pathophysiology of microvascular disease is less well understood than that of macrovascular disease but they share many pathophysiologicals and risk factors, and in view of this it can be argued that they can be treated as one disease entity (4).

Traditional risk factors for macrovascular disease include hypertension, dyslipidaemia, smoking and glycaemic control and individual risk predictions can be calculated by the measurement of these factors. One such tool to do this is the Framingham Cardiovascular Disease Risk Score Profile, one of several well-recognised risk scores developed as a result of long-term observational studies (5). Current treatment strategies target these factors where aggressive treatment has been shown to reduce morbidity and mortality. Within the UK the National Institute for Health and Care Excellence (NICE) publishes evidence-based guidance on the management of diabetes (6).
However, despite efforts to manage the traditional risk factors, people with type 2 diabetes, compared with people without diabetes, have a threefold increased risk of cardiovascular mortality (7). It is this increased morbidity that has lead to the thinking that there are other underlying processes that contribute to cardiovascular risk.

**Novel risk factors**

The pathogenesis of vascular complications in diabetes is a much-debated subject and despite extensive research no unifying mechanism has been discovered. Several hypotheses exist: capillary hypertension, insulin resistance syndrome, endothelial dysfunction, increased vascular inflammation and oxidative stress with advanced glycation end product (AGE) formation (8). While these are all important processes it would appear that one is not exclusive of the other and it is more likely that all the above processes are integrally related. Figure 1.1 shows the relationship between these processes and how they act synergistically to promote the development of diabetes and vascular disease. Through studying these mechanisms both in isolation and in relation to each other, further understanding of this complex process can be developed.
Figure 1.1: Diagram showing the relationship between the metabolic syndrome and its components to oxidative stress, inflammation and endothelial dysfunction. All these processes contribute to atherosclerosis.

**Oxidative stress**

Reactive Oxygen Species (ROS) are highly reactive atoms or molecules that are able to react with biological factors causing damage through oxidation. Within any organ system there is a balance between pro-oxidant factors and those that scavenge them, namely antioxidants. It is the tipping of this balance one way or another that determines the degree of oxidative damage done and this can be as a consequence of raised free radical production, insufficient antioxidant potential or both (9).

Hyperglycaemia is known to produce oxidative stress through free radical generation either by autoxidation leading to the production of reduced oxygen species or by glycoxidation resulting in advanced glycosylation end products (AGEs). Also in the hyperglycaemic state increased activity in the polyol pathway leads to depletion in NADPH stores and an associated increase in the cytosolic NADH-to-NAD⁺ ratio, so called hyperglycaemic
pseudohypoxia. Pseudohypoxia can lead to free radical formation via increased prostanoid metabolism and reduced NADPH levels affect other metabolic pathways, importantly the glutathione redox cycle leading to decreased levels of reduced glutathione, an antioxidant. It has also been shown that hyperglycaemia may suppress natural antioxidant defences such as superoxide dismutase or glutathione peroxidase (10).

Thus there are several processes by which hyperglycaemia leads to free radical production. These free radicals damage lipids, proteins and DNA through cross-linking and fragmentation, thus causing oxidative stress.

**Endothelial dysfunction**

Serving as a barrier between the circulating blood products and underlying tissue the endothelium is recognized to have many complex functions. These functions vary across the blood vessels according to location and vessel structure and include vasodilatation versus vasoconstriction, anti-thrombotic and anti-inflammatory effects, transport and delivery of nutrients and hormones as well as metabolic waste product disposal (11). The disruption of any of the functions of the endothelium can be termed endothelial dysfunction, thus it is a complex term and not a discreet entity (12). However an early sign of endothelial dysfunction is reduced nitric oxide (NO) bioavailability (13,14).

NO is a highly reactive compound with a very short half-life and its production requires the activation of endothelial NO synthase (eNOS), also termed nitric oxide synthase 3 (NOS3), and the conversion of L-arginine to L-citrulline. Once formed it is converted to more stable products such as nitrites and nitrates and NO bioavailability depends on the balance between its production and conversion. Decreased NO bioavailability can occur due to decreased or disordered eNOS production or accelerated NO degradation by ROS (11). Several molecular mechanisms that regulate NO are disrupted through metabolic derangements that occur in diabetes including hyperglycaemia, insulin resistance, hyperinsulinaemia and free fatty acid production (15).
There is a strong link between these metabolic derangements, endothelial dysfunction and oxidative stress. I have discussed the link between hyperglycaemia and oxidative stress and in turn ROS and superoxide generation negatively effects eNOS formation and function. Superoxide generation can also contribute to endothelial dysfunction through over expression of adhesion molecules, increased production and release of Endothelin-1 (ET-1) (the naturally occurring functional antagonist to NO in regulation of vascular homeostasis) and increased activation and expression of pro-inflammatory proteins (16-18). Free fatty acids may impair endothelial function in similar ways to hyperglycaemia, i.e. through ROS interference with NO production and pro-inflammatory signalling (19).

Insulin, in addition to its effects and glucose and lipid metabolism, contributes to vascular tone through a number of pathways, including endothelial cell production of NO and ET-1. The signalling pathway that leads to phosphorylation and activation of eNOS and NO production involves activation of the insulin tyrosine kinase receptor, phosphoinositide 3-kinase and protein kinase B. Similarly glucose uptake in skeletal muscle and adipose tissue involves the recruitment of GLUT4 glucose transporters to the cell membrane via the tyrosine kinase, phosphoinositide 3-kinase and protein kinase B pathway. It has been shown that activation of IKKβ and proinflammatory signalling inhibition of these pathways by free fatty acids leads to insulin-resistance as well as endothelial dysfunction, demonstrating a close association between the two (20). Insulin resistance can lead to hyperinsulinaemia as a compensatory mechanism to maintain euglycaemia. Hyperglycaemia, independently to insulin resistance, has been shown to enhance ET-1 secretion and adhesion molecule expression (21). Therefore there are many ways in which the metabolic derangements associated with diabetes can contribute to endothelial dysfunction.

**Vascular inflammation**

Chronic low-grade inflammation has been demonstrated in obese individuals and is thought to be due to the pro-inflammatory effect of leptin (22). Also reducing pro-inflammatory cytokines has been shown to reduce insulin resistance suggesting an inflammatory cause of type 2 diabetes (23).
Inflammatory cells, leucocytes and macrophages, are found within atherosclerotic plaques demonstrating the close relationship between inflammation and macrovascular disease (24). In all these situations there is a close link between inflammation and endothelial dysfunction and it may be that inflammation is central in the pathogenesis of type 2 diabetes and vascular disease.

**Insulin resistance**

Insulin resistance can be defined as a reduced sensitivity to the metabolic actions of insulin. In clinical practice it is seen in those individuals with type 2 diabetes who require large doses of insulin to achieve normoglycaemia. There are strong associations between insulin resistance and the metabolic syndrome and it is linked to the pathogenesis of type 2 diabetes and coronary heart disease (25, 26).

**Thiamine**

Thiamine is a member of the B-vitamin family and was the first water-soluble vitamin to be discovered in 1912 and isolated in 1926. Humans cannot synthesize thiamine and so a regular intake of thiamine, from exogenous sources, yeasts and plants, is necessary to maintain body stores and the recommended daily intake for adults in the UK is between 1 and 1.4 mg/day (27).

Thiamine is absorbed from the diet in the proximal part of the small intestine into the enterocyte via either simple diffusion or trans-phosphorylation to thiamine monophosphate (TMP) but the majority occurs through active transport (28). This active transport involves a carrier-mediated process that is believed to involve the human thiamine transporters hTHTR-1 and hTHTR-2. Thiamine is subsequently phosphorylated to thiamine diphosphate, the most abundant compound in the body, and an essential coenzyme for the transketolase enzyme and the dehydrogenase complexes for pyruvate, alpha-ketoglutarate and branched-chain keto acids. All of these enzymes are essential in carbohydrate metabolism.
Thiamine is excreted through the kidneys, where hTHTR-1 and hTHTR-2 are involved in the re-uptake of thiamine in the proximal tubules. These are adaptively upregulated in thiamine deficiency via transcriptional regulatory mechanisms, and thus the kidneys are responsible for thiamine homeostasis (29). The thiamine transporters are also necessary for the uptake and regulation of thiamine in the pancreas where it essential for its normal endocrine function (30).

Thiamine status can be assessed directly by measuring thiamine levels in blood or urinary excretion before and after loading (31). Alternatively a functional measure of thiamine status, erythrocyte transketolase activity can be measured, but this is influenced by many other factors other than thiamine deficiency, is relatively unstable on sampling and there is a lack of agreement over the upper limit of the reference range (32). Microbiological assays measure red cell thiamine concentration but are time consuming and the sensitivity of the assays is not always sufficient for analyses of human body fluids (33). The majority of the total thiamine content of whole blood is found in erythrocytes as TDP and has been shown to be a good indicator of body stores as it depletes at a rate similar to those of major organs. High performance liquid chromatography (HPLC) measurement of blood TDP levels has been determined to be a simple and precise way of assessing thiamine status (34).

**Thiamine deficiency**

Deficiency can occur as a result of inadequate intake, increased requirements (fever, pregnancy, breast feeding), excessive renal loss, consumption of anti-thiamine factors (tea, coffee, raw shellfish), or a combination of these factors. Severe deficiency results in beri-beri, which is termed, wet, dry, or cerebral (commonly known as Wernicke's Encephalopathy) depending on the system affected. In the western world Wernicke's Encephalopathy is most commonly associated with chronic alcoholism as a result of both a nutritional and absorptive deficiency.

Treatment of at risk patients with high-dose parenteral thiamine is a widely accepted practice to prevent the mortality associated with Wernicke’s
encephalopathy, or the development of Korsakoff’s psychosis, a chronic form of the disease characterised by severe short-term memory loss.

Studies into Wernicke’s encephalopathy have shown the neuro-degeneration that occurs is strongly associated with increased eNOS and inducible nitric oxide synthase (iNOS/NOS2) production, inter-cellular adhesion molecule 1 (ICAM-1) levels and the production of reactive oxygen species. It is not currently clear whether eNOS production induces neuronal damage through peroxynitrite formation or has a neuroprotective role in response to local inflammation (35). There has also been shown to be a breakdown in the blood-brain barrier as a result of endothelial dysfunction, and decreased cerebral energy due to impaired glucose metabolism, which occurs as a result of decreased activity of α-ketoglutarate dehydrogenase. Finally increased pro-inflammatory cytokines have been demonstrated in the thiamine deficient brain (36). All of these pathological processes seen in the thiamine deficient brain are strongly associated with the pathophysiology of insulin resistance and macrovascular disease as previously discussed.

**Thiamine deficiency and diabetes mellitus**

**Evidence**

Several studies have demonstrated thiamine deficiency in individuals with both type 1 and type 2 diabetes. Studies have shown altered erythrocyte transketolase activity indicating a risk of thiamine deficiency in both type 1 and type 2 diabetes, but the proportion of affected individuals varies from 17% to 79% across the studies (37-39). In addition, when comparing people with diabetes with the normal population, there was found to be no significant difference between erythrocyte transketolase activity levels in one study (40) but levels indicating a significantly higher risk of thiamine deficiency in another study (37).

Measurement of thiamine levels and its esters in blood, serum and plasma also reveals differing results across the studies. Three studies have shown reduced plasma thiamine concentrations in patients with diabetes,(38, 40,
however red cell thiamine levels were low in 15% of patients with diabetes in one study (42) but normal in another (40).

The reasons for these differing results are not well understood. While some may be down to sampling and assay problems as described previously, not all the studies comment on the alcohol intake in the individuals or other potential causes of thiamine deficiency. Only the Jermendy (2006) and Thornalley (2007) papers (37, 40) specifically sited excess alcohol intake as an exclusion criteria, but direct comparison of these two papers reveal opposing results in erythrocyte transketolase activity as described above.

**Mechanisms**

The reason for this deficiency is not well understood but several mechanisms may be responsible. Insulin deficiency is associated with a reduction in the rate of thiamine transport across the intestine and insulin deficient rats have been shown to have a net reduction in the transport of free thiamine and TMP but with a corresponding increase in TDP levels (43). Conversely thiamine deficiency leads to a marked impairment in insulin synthesis and secretion (30) thereby insulin deficiency may exacerbate thiamine deficiency and vice-versa.

Thornalley (2007) showed that in the thiamine deficient state there is increased renal clearance and fractional excretion of thiamine and it has been hypothesised that this is secondary to decreased re-uptake of thiamine in renal proximal tubules, possibly an early marker of renal proximal tubule dysfunction in diabetes (40). However a further study has shown increased plasma thiamine levels with progressive renal impairment and proteinuria suggesting decreased renal clearance of thiamine (44). The reason for these differing results remains unclear and may be due to changes in the number and/or function of thiamine transporter proteins or due to increased or reduced filtration. Altered functioning of thiamine transporter proteins could, in theory, affect the hTHTR-1 and 2 proteins in both the gut and pancreas, with a reduction in active thiamine absorption and reduced pancreatic endocrine function, thus exacerbating both thiamine deficiency and hyperglycaemia.
Replacement

Thiamine supplementation (>4mg per day) has been shown to normalise red cell thiamine levels in patients with diabetes, whereas increasing dietary thiamine intake above the recommended dietary intake of 1 to 1.4mg/day have not – suggesting a need for higher than normal thiamine intake in patients with diabetes compared with normal individuals (42). Oral thiamine replacement is widely available in the UK as thiamine hydrochloride, a water-soluble compound. Benfotiamine is a lipid-soluble allithiamine derivative that has better intestinal absorption and improved bioavailability but it is not currently available for prescription in the UK (45). Treatment with benfotiamine for 7 days has shown an improvement in erythrocyte transketolase activity, thus improving thiamine status (37).

Thiamine and hyperglycaemia

Thiamine diphosphate is essential for carbohydrate metabolism. In the thiamine deficient state glucose undergoes metabolism via alternate pathways that can result in vascular damage. These pathways are summarised in figure 2.
Figure 1.2: Diagram detailing pathways of glucose metabolism. Thiamine diphosphate is an essential cofactor for the enzymes depicted in *italics*. Inhibition of these enzymes results in increased superoxide production (O$_2^-$) and reduced flux through the pentose phosphate pathway with subsequent increased flux through the alternative pathways of glucose metabolism (46).

Several studies have been undertaken looking at the effect of thiamine and benfotiamine administration on these biochemical pathways. Animal studies have shown that high dose thiamine reduces activity through the hexosamine pathway (48). Thiamine supplementation can prevent hyperglycaemia-driven reductions in cell replication and proliferation as well as decreasing AGE formation and reducing lactate levels (49). *In vitro* studies with benfotiamine and thiamine have shown a reduction in protein kinase C activation in the glomeruli and decreased glomerular AGE levels (50). Benfotiamine has been shown to prevent increased markers of hexosamine pathway activity, intracellular AGE formation, intracellular protein kinase C activity and NF-κB activation seen with *in vitro* hyperglycaemic damage (47). Oral benfotiamine in combination with the anti-oxidant α-lipoic acid treatment normalises production of angiopoietin-2, a marker of increased intracellular methylglyoxals in endothelial cells which contribute to AGE formation, and N-acetylglucose modified protein, a marker of hexosamine pathway activity (51). Both thiamine and benfotiamine have been shown to reduce AGE formation in experimental diabetes (52). Treatment with both thiamine and benfotiamine has been shown to reduce activation of the polyol pathway of glucose metabolism and to increase transketolase expression in the presence of hyperglycaemia (53).

Collectively this data suggests that administration of thiamine or a derivative can influence carbohydrate metabolism by reducing metabolism through the alternate pathways of metabolism and improving metabolism via the pentose-phosphate pathway. This has been demonstrated in diabetic animal models where treatment with thiamine reduced fasting glucose and HbA1c levels (54) and also in humans with type 2 diabetes, where a short duration of treatment with thiamine (150mg per day for 1 month) showed a significant improvement in fasting glucose levels (55).

**Thiamine and oxidative stress**

The antioxidant properties of thiamine have been known since the 1950's. Rye breads are an important source of B vitamins and have a higher antioxidant activity than white wheat breads (56). Lukienko et al (2000)
demonstrated that thiamine inhibited lipid peroxidation *in vitro* (57) and thiamine deficiency is associated with reduced anti-oxidant capacity and increased oxidative stress in hepatocytes (58). Furthermore increased oxidative stress has been demonstrated in thiamine deficient cardiac myocytes with increased ROS production (59).

Schmid et al (2008) demonstrated the antioxidant properties of benfotiamine in kidney cell lines, although similar results with thiamine were not seen in these studies (60). *In vivo* animal studies have shown benfotiamine can reduce oxidative stress when induced either through chemical means (sodium arsenite, nicotine or uric-acid) or diabetes (61-63). Both thiamine and benfotiamine have been shown to reduce oxidative stress in a hyperglycaemic environment in animal models (50). The anti-oxidative capacity of benfotiamine has also been demonstrated in patients with diabetes and those undergoing dialysis (64, 65).

**Thiamine and endothelial function**

Improved endothelial function in a thiamine-rich environment has been demonstrated by a reversal of hyperglycaemia-induced reduction in endothelial cell migration and proliferation. In addition increased von Willibrand (vWF) factor levels, a marker of endothelial cell damage, are reduced when wounded endothelial cells are treated with thiamine (66). Animal models have shown benfotiamine activates endothelial nitric oxide synthase to enhance the generation and bioavailability of NO, subsequently improving the integrity of vascular endothelium and preventing induced experimental vascular endothelial dysfunction (61, 63). In individuals with type 2 diabetes there has been demonstrated a positive correlation between plasma thiamine levels and brachial artery flow-mediated dilatation (67) and a negative correlation with soluble vascular cellular adhesion molecule (sVCAM-1), a protein released from the endothelium (40). Further studies in individuals with diabetes have shown that endothelial dysfunction may be prevented by both oral benfotiamine and intravenous thiamine (64, 68).

Microalbuminuria, an early indicator of diabetic nephropathy, is associated with an increased cardiovascular risk in individuals with and without
diabetes (69). *In vitro* studies with benfotiamine and thiamine have shown the ability to prevent microalbuminuria and proteinuria in diabetic rats. *In vivo* studies have shown that high dose oral supplementation with thiamine compared with placebo significantly reduces urinary albumin excretion in individuals (70,71). However reduction in microalbuminuria, with benfotiamine, was not evident in patients already receiving angiotensin converting enzyme inhibitors or angiotensin II receptor blockers (72).

**Thiamine and inflammation**

Benfotiamine has been shown to have an anti-inflammatory effect and this may be through regulation of the arachidonic acid pathway in macrophages (73). Stirban et al (2006) demonstrated a reduction in C-reactive protein (CRP), a marker of inflammation, in individuals with diabetes following oral benfotiamine administration (64). However these results have not been replicated in other studies with either oral thiamine or benfotiamine (55).

**Summary**

Diabetes mellitus is endemic worldwide with increasing associated morbidity and mortality. Glucose metabolism is dependent upon thiamine as a cofactor and in the hyperglycaemic environment alternative pathways of metabolism (that are not thiamine dependent) are activated. This leads to increased formation of harmful by-products that contribute to the pathophysiology of diabetic complications. In addition thiamine has a direct action on the endocrine function of the pancreas and, therefore, deficiency may contribute to hyperglycaemia through mechanisms other than impaired glucose metabolism.

Thiamine and its derivatives have been shown to improve endothelial function and reduce oxidative stress in experimental and clinical situations. Given that these processes are closely linked with vascular inflammation it could be hypothesised that thiamine has anti-inflammatory properties. There is some evidence that this is seen in induced skin inflammation in mice but vascular inflammation has not been extensively studied (74).
Hypothesis and Aims

It has been demonstrated that thiamine has a beneficial effect upon several features of the metabolic syndrome such as microalbuminuria, a surrogate marker of vascular risk, and glycaemic control, and it could be hypothesised that thiamine supplementation may have beneficial effects upon integrally linked pathophysiological processes such as insulin resistance and hypertension. However there is a lack of long-term data examining whether thiamine therapy may have beneficial effects in individuals with diabetes through reducing cardiovascular risk and microvascular complications.

Therefore this study aims to test the hypothesis that

- Thiamine improves markers of oxidative stress, endothelial function and insulin resistance in subjects with type 2 diabetes.
- Thiamine improves markers of inflammation in these same subjects
- Thiamine improves traditional risk factors for cardiovascular disease such as LDL-cholesterol and blood pressure.

The secondary hypothesis states that insulin resistance, oxidative stress, endothelial function and vascular inflammation are interdependent and this study aims to test this by examining the relationship between endothelial function, markers of oxidative stress, insulin sensitivity and vascular inflammation at baseline and any thiamine induced changes.
Methods

Trial design
A randomised, double blind, placebo-controlled crossover pilot study of subjects with type 2 diabetes was undertaken to assess the effect of high dose thiamine on endothelial function, oxidant stress, insulin sensitivity and inflammation, and investigate any relationship between them.

Participants
Suitable participants were identified through the patient database of the Department of Diabetes at Queen Alexandra Hospital (QAH). Correspondence was sent to those patients identified, including a patient information sheet, giving them the option to participate via means of a reply slip. Prospective participants were screened for suitability either via a telephone or face-to-face interview.

Patients eligible for this study were individuals between the ages of 18 and 75 with known type 2 diabetes mellitus, an HbA1c less than 10%, and a more than 30% chance of cardiovascular disease over the next ten years but without established cardiovascular disease. Cardiovascular disease was defined as ischaemic heart disease, cerebrovascular disease or peripheral vascular disease. Cardiovascular risk was calculated using the Framingham 10-year cardiovascular disease risk score, a sex-specific multivariable risk factor algorithm that assesses age, total and high-density lipoprotein cholesterol, systolic blood pressure, treatment for hypertension, smoking, and diabetes status (5) and established disease was determined using clinical history, examination and electrocardiogram (ECG) recording. The other exclusion criteria were: allergy/intolerance to thiamine supplementation, insulin treatment, diuretic treatment, current multivitamin/thiamine therapy, abnormal thyroid function, chronic excess alcohol consumption (>21 units per week in females, >28 units per week in males; Department of Health Guidelines) or impaired liver function. Informed consent was obtained from all participants. Approval for the study was obtained from the Southampton and South West Hampshire
Regional Ethics Committee B (Ref: 09/H0504/137) and the Medicines and Health Regulatory Authority (MHRA).

**Interventions**

This was an 18-week randomised, double blind, placebo-controlled crossover-study and was conducted at the Diabetes Centre, QAH. Each patient attended a total of four times having fasted for a minimum of 12 hours prior to the visit. Patients who passed the screening criteria underwent an initial assessment that included a clinical history and a routine physical examination. Body weight was measured on an electronic column scale (Seca; model 778) without shoes and wearing light clothing and a 12 lead ECG was performed. The Waist: Hip ratio was calculated by measuring the waist midway between the bottom of the ribs and the top of the pelvis, and the hips at the widest point.

Following baseline assessment all patients were randomly assigned to one of two possible treatment sequences. Each patient received high-dose 300mg thiamine (75), a dose used in previous studies (70), or placebo for 8 weeks, followed by a minimum 2-week washout period. They then received a further 8 weeks of placebo or 300mg thiamine, whichever they had not received the first time. The patient and study investigators were blind to the randomisation schedule. Patients were evaluated for outcome measures at the beginning and end of each 8-week treatment period.
Assessment of suitability, “Patient information leaflet” supplied, Consent obtained

Visit 1
Interview and physical examination, 12 lead ECG. Urine sampling, blood sampling, and photoplethysmography

Randomisation

300mg thiamine daily for 8 weeks

Visit 2
Urine sampling, blood sampling, and photoplethysmography

2 week washout

Visit 3
Urine sampling, blood sampling, and photoplethysmography

Placebo equivalent daily for 8 weeks

Visit 2
Urine sampling, blood sampling, and photoplethysmography

2 week washout

Visit 3
Urine sampling, blood sampling, and photoplethysmography

Placebo equivalent daily for 8 weeks

Visit 4
Urine sampling, blood sampling, and photoplethysmography

300mg thiamine daily for 8 weeks

Visit 4
Urine sampling, blood sampling, and photoplethysmography
Outcomes

Primary outcomes
The primary outcomes were to detect changes in markers of:

- Endothelial dysfunction
- Insulin sensitivity
- Oxidant stress
- Vascular inflammation
- Glycaemic control
- Lipid parameters

Secondary outcomes
The secondary outcome was to examine any relationship between endothelial function, insulin sensitivity, oxidant stress and vascular inflammation.

Metabolic parameters
The metabolic parameters measured were systolic and diastolic blood pressure, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, fructosamine and HbA1c.

At each visit blood pressure was measured using an automated sphygmomanometer (Welch Allyn; 52000 series) in stress-free surroundings with the subject in the seated position for at least 5 minutes (British Hypertension Society Guidance (76)).

Fasting venous blood investigations were obtained to measure the participants’ lipid profile (Total cholesterol, HDL Chol, LDL Chol, Triglycerides) and glycaemic control (Fructosamine and HbA1c). Analysis of these parameters except Fructosamine was undertaken at the Department of Blood Sciences, QAH. Fructosamine was analysed at the Department of Biochemistry, Royal United Hospital, Bath.
Thiamine diphosphate (TDP) levels in red blood cells were measured at the Chemical Pathology Department, West Park Hospital, Epsom. The normal range of TDP is 66-200nmol/l.

Additional measurements were obtained through blood sampling and photoplethysmography and the methodologies described below. With the exception of hsCRP, blood analysis was undertaken at the School of Pharmacy and Biomedical Sciences, University of Portsmouth. The coefficient of variation of all measured parameters was <10%.

**Endothelial function**

Endothelial function was measured using several techniques.

**Venous photoplethysmography**

**Background**

One of the most widely used clinical endpoints for the assessment of endothelial function is endothelial-dependent vasodilatation whereby pharmacological stimulation of endothelial release of NO can be compared with the vascular response to endothelium-independent vasodilators (77). Initial vasomotor studies examined the coronary arteries by measuring variations in coronary artery diameter, enabling direct assessment of endothelial function in one of the key atherosclerotic sites (78). However these techniques are invasive and impaired endothelial responses, as seen in the coronary arteries, can be determined in the peripheral circulation.

Several techniques have been developed for measuring endothelial function in the peripheral circulation which include brachial flow-mediated dilatation (FMD), forearm perfusion studies, laser doppler flowmetry of the skin and pulse wave analysis. Pulse wave analysis (PWA) is a non-invasive technique that measures changes in the digital volume pulse (DVP) using finger photoplethysmography (pulse contour analysis) (79). Whilst the amplitude of the pulsatile component of the DVP can be influenced by factors that influence local perfusion (respiration, the autonomic nervous system), the contour of the pulse remains relatively constant, suggesting
that the contour is primarily influenced by characteristics of the systemic circulation (80).

Contour analysis of the DVP has been extensively described and compared with individuals from the Framingham cohort (81). Four classes of the DVP have been described which change with increasing age or the degree of atherosclerosis and these are shown in figure 2.2. Class I is seen in young healthy individuals and class IV seen in the elderly or those with atherosclerotic disease (81).

Class I: A distinct notch is seen on the downward slope of the pulse wave
Class II: No notch develops but the line of descent becomes horizontal
Class III: No notch is present but a well-defined change in the angle of descent is observed
Class IV: No evidence of a notch is seen or no change in angle of descent occurs.

Figure 2.2: The four classes of the digital volume pulse (81).

Further techniques have been developed which look at the reflection index (RI) of the DVP. The reflection index is a calculated ratio of the amplitudes of the transmitted pressure wave from the left ventricle and the reflected component from the aorta to the heart (figure 2.3). By measuring the change in this reading in response to an endothelium dependent vasodilator (i.e. salbutamol) endothelial function can be assessed (82). Endothelium independent vasodilatation can be assessed using glyceryl trinitrite (GTN) and this technique has been validated in individuals with type 2 diabetes (83).
Figure 2.3: The reflection index showing the transmitted (b) and the reflected (a) pressure waves.

**Technique**

The pulse trace probe was attached to subjects resting in the reclining position (Micro Medical Pulse Trace, Rochester, Kent, UK). Three baseline measurements of RI were obtained over a 10-minute period. Subjects were then given a GTN tablet 500µg sublingually and repeated measurements of RI were made after 3 and 5 minutes. The GTN tablet was removed and a washout period of 20 minutes commenced. A further reading was then taken to ensure the RI had returned to baseline. Participants were then given 400mcg inhaled salbutamol via a spacer using a standardised inhaler technique. RI was measure at 10, 12 and 15 minutes post inhalation. The mean of the readings at baseline, post GTN and post salbutamol were calculated and the change in RI calculated for salbutamol (endothelium-dependent) (ΔRISalb) and GTN (endothelium-independent) (ΔRIGTN).

**Albumin: Creatinine ratio**

**Background**

Microalbuminuria is defined as the presence of between 30-300mg/l of protein in the urine. It occurs when the renal glomerulus leaks small amounts of protein into the urine. It is commonly reported as the albumin: creatinine ratio and can be calculated from a spot urine collection, preferably of the first morning void (84). In addition to being an
independent predictor of cardiovascular disease it is well established as being a marker of endothelial dysfunction (85-87).

**Technique**
Participants provided a fresh early morning urine sample at each visit. This was analysed within the Blood Sciences Department at Queen Alexandra Hospital, Portsmouth. Urine albumin was measured by radioimmunoassay; urine creatinine concentration was measured by an endpoint Jaffe reaction.

**Soluble Vascular Cellular Adhesion Molecule 1 (sVCAM-1)**

**Background**
Vascular cellular adhesion molecule 1 (VCAM-1) is one of a class of proteins called adhesion molecules. Adhesion molecules are expressed on the surface of a cell and mediate adhesion of the cell to other cells or the extracellular matrix. VCAM1 is transcriptionally induced on endothelial cells and facilitates the adhesion of lymphocytes to activated endothelium. It undergoes proteolytic cleavage to produce a soluble form sVCAM-1 (88). sVCAM-1 is a circulating protein that can be measured directly in the blood. It has been identified as a strong independent predictor of future cardiovascular events in subjects with and without diabetes (89-92).

**Technique**
3ml of venous blood was collected from the patient in an EDTA bottle and centrifuged at 1000 x g for 15 mins. The supernatant plasma was extracted and stored in a plain tube at -85°C. sVCAM-1 was measured with the use of a Quantikine kit purchased through R&D Systems and employs the quantitative sandwich enzyme immunoassay technique. This uses 96 well microplates coated with mouse monoclonal antibody against human sVCAM-1. The plasma underwent a 20-fold dilution with calibrator diluent RD5P (buffered protein solution). All reagents were prepared according to kit instructions. 100µl sVCAM conjugate (monoclonal antibody against sVCAM-1 conjugated to horseradish peroxidase) and 100 µl of sample was added to each well and incubated for 90 minutes at room temperature. The plate was then aspirated and washed 4 times with a solution of buffered surfactant. 100µl of substrate solution (50:50 mix stabilised hydrogen
peroxide and chromogen) was added to each well, protected from light and incubated for 20 minutes at room temperature. The reaction was stopped with 5 µl of 2 N sulfuric acid. The optical density was measured at 450 nm with wavelength correction set at 540 nm. All samples were analysed in duplicate and the mean value calculated. Sample range from healthy volunteers 341 - 897 ng/ml.

**Cyclic guanosine monophosphate (cGMP)**

**Background**
The vasorelaxant effects of endothelium-induced nitric oxide are mediated via soluble guanylate cyclase leading to the formation of cyclic guanosine monophosphate (cGMP) (93). Thus measurement of cGMP levels can be interpreted as an indicator of nitric oxide production (94).

**Technique**
Venous blood EDTA samples were collected from the patient and prepared as for the sVCAM-1 assay. cGMP was measured with the use of a Quantikine kit purchased through R&D Systems. This uses 96 well microplates coated with goat anti-rabbit polyclonal antibody. All reagents were prepared according to kit instructions. The plasma underwent a 20-fold dilution with calibrator diluent RD5-5 (buffered protein solution). 100µl sample, 50µl cGMP conjugate (cGMP conjugated to horseradish peroxidase) and 50µl primary antibody solution (rabbit polyclonal antibody to cGMP) was added to the wells and then incubated at room temperature on a horizontal microplate shaker set at 500 ± 50 rpm for three hours. The plate was then aspirated and washed 4 times with a solution of buffered surfactant. 200µl of substrate solution (50:50 mix stabilised hydrogen peroxide and chromogen) was added to each well, protected from light and incubated for 30 minutes at room temperature. The reaction was stopped with 50 µl of 2 N sulfuric acid. The optical density was measured at 450 nm with wavelength correction set at 540 nm. All samples were analysed in
duplicate and the mean value calculated. Sample range from healthy volunteers 75-219 pmol/l.

Insulin Resistance

Several methods exist for measuring insulin resistance of which the gold standard test is the hyperinsulinaemic euglycaemic glucose clamp, a technique developed in 1979 (95). However this is a time consuming, expensive and technically difficult means of undertaking the assessment. Several more simplistic methods have been developed and one widely accepted model is the homeostatic model assessment (HOMA) developed in 1985 (96).

Homeostasis model assessment

Background

The HOMA model uses mathematical predictions to yield an estimate of insulin sensitivity and β-cell function from fasting plasma insulin and glucose concentrations. After its initial development in 1985 (HOMA1) it was updated in 1996 (HOMA2) where allowances for variations in hepatic and peripheral glucose resistance, increased insulin secretion with hyperglycaemia and the effects of proinsulin were taken into account (97). In 2004 the HOMA2 calculator was released as an online tool for researchers and is available to be downloaded from www.dtu.ox.ac.uk/homacalculator. It is well validated in assessing insulin resistance in those individuals who are on oral hypoglycaemic agents having been used in the UKPDS studies (98).

Figure 2.4: The HOMA2 calculator.
Technique

Three paired fasting insulin and glucose samples at five-minute intervals were collected from the participants. Glucose samples were analysed at the Department of Blood Sciences, QAH and insulin samples were frozen immediately and analysed at the Department of Chemical Pathology, Southampton General Hospital. Mean glucose and insulin concentrations were calculated and the results inputted into the HOMA calculator to generate HOMA-IR (figure 2.4). The use of three samples allows for a more accurate assessment than a single sample (97).

Oxidative stress markers

Glutathione ratio

Background

Glutathione (GSH) is the principal thiol intracellular antioxidant and most of the cellular GSH is found within the cytosol. GSH is readily oxidised to glutathione disulphide (GSSG) by free radicals and reactive oxygen species. The GSH:GSSG ratio is often used an indicator of cellular redox status (99).

Studies have shown a negative correlation between reduced erythrocyte glutathione concentration and diabetic complications and between the duration of diabetes and the levels of reduced glutathione. This suggests antioxidant defences are depleted by chronic oxidative stress of type 2 diabetes (100). Thornalley (1996) postulated that an inherent low level of reduced glutathione in an individual might predispose them to diabetic complications, a phenomenon that is noted clinically (101). In diabetic patients a significant increase in plasma free radical concentrations can contribute to a reduction in plasma GSH concentration and declining blood GSH:GSSG ratio. This can in turn affect insulin action. Using euglycaemic clamp studies, Paolisso (1992) found that supplementation of glutathione lead to an improvement of whole body glucose disposal (102).
**Technique**

2 ml of venous blood was collected from patients in an EDTA (Ethylenediamine tetraacetic acid) bottle. This was mixed with 1 ml 0.5mM EDTA/10% (w/v) SSA (Sulphosalicylic acid) in a plain bottle and then centrifuged at 1000 x g for 15 minutes. The supernatant was then stored at –85°C. Glutathione ratio was assessed using the GSSG reductase/5,5'-dithio-bis(2-nitrobenzoic acid) re-circulating method following derivatisation of GSSG with 2-vinylpyridine (103).

GSH was assessed photometrically in a microplate reader at 37°C. The final well comprised of 70µl DTNB (0.857 mM); 10µl β-NADPH (5 mM); 10µl sample diluted 1:20 with phosphate-buffered saline (PBS) (120mM, pH 7.4); 10µl glutathione reductase (25U/ml). Reagents were dissolved in PBS +6.3mM EDTA. The recirculating assay was initiated after 10 minute incubation at 37°C by the addition of GSH reductase and the initial rate determined from the absorbance increase measured at 405nm every 10 seconds over 1 minute.

For the selective measurement of GSSG (=2GSH), thiols were first derivatised (1 hour) with 1-methyl 2-vinylpyridine (M2VP) in the presence of triethanolamine (TEA) (10µl M2VP and 6 µl TEA added to 100µl of sample).

All samples were measured in duplicate and the mean value calculated. Analysis of results was undertaken to express GSH:GSSG as a ratio.

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**CuPrac-BCS**

**Background**

Oxidative stress can be measured by determining the total antioxidant capacity (TAC) and this can be achieved by simple *in vitro* methods. One method for determining TAC is based on cupric ion reduction by antioxidants in the presence of bathocuproinedisulphonic acid disodium salt (BCS), a chelating agent. This method has been validated as a suitable method for TAC assessment in human plasma (104).
Technique

2ml of venous blood was collected from the patients in a heparinised bottle and centrifuged at 1000 x g for 15 mins. The supernatant plasma was extracted and stored in a plain tube at -85°C. 15µl of sample was added to 585µl 0.25mM BCS dissolved in 10mM PBS (pH 7.4) in a 2ml microcentrifuge and mixed. 200µl was added to a 96-well plate in duplicate and the spectrophotometric absorbance at 490 nm was measured. Subsequently 50µl Copper (II) sulphate solution (0.5mM) was added and incubated at room temp. The reaction was stopped after 3 minutes with 50µl EDTA (10mM) and the spectrophotometric absorbance at 490 nm was measured again. The absorbance change due to reducing activity was calculated = (second read-first read for sample) – (second read-first read for blank). A standard curve was obtained by performing the process described above with standard dilutions of ascorbate (10mM solution). Linear regression was used to convert absorbance change into ascorbate equivalent antioxidant concentration (AEAC) for samples. All samples were analysed in duplicate and the mean value calculated.

Inflammation

Highly sensitive C-reactive protein (hsCRP)

Background

Atherosclerosis has been shown to be a chronic inflammatory process and inflammatory markers such as highly sensitive C-reactive protein (hsCRP) have been shown to be a strong independent predictor of future cardiovascular disease in both healthy individuals and those with established cardiovascular disease (105).

Technique

4 ml of venous blood was collected from the participants into a serum separation tube. Clotted blood was centrifuged at 1000 g for 10 minutes and the serum extracted and stored at -20°C. Serum was analysed at the Department of Chemical Pathology/Metabolic Medicine, Guys and St Thomas’ Hospital, London.
**Sample size**
Using 34 participants, this study was estimated to have a power of 80% to detect a two standard deviations change from the normal mean in sVCAM-1 concentration, from previous trial data (70).

**Randomisation**
A six-block randomisation method was used and randomisation was undertaken by the Clinical Trials Pharmacy Service, QAH at the time of dispensing of the study medication.

**Blinding**
Study participants and investigators were blinded to the interventions. A placebo tablet matching thiamine 100mg (Tyvera) was manufactured through Pharmacy Manufacturing, St Thomas’ Hospital, London. The Clinical Trials Pharmacy Service, QAH, packaged the thiamine and placebo tablets in identical, non-identifiable packaging.

**Statistical methods**
Data was analysed for normality using Komlogorov-Smirnov goodness of fit test. Baseline characteristics between the two groups were measured using mean and standard deviation (SD) analysis or median and inter-quartile ranges (IQR) for non-parametric data. Visit one and visit three blinded data was compared using repeated measures analysis of variance (RM-ANOVA) or Wilcoxon Signed Rank (WSR) test to ensure that adequate washout had occurred during the two week break. The data was then unblinded and RM-ANOVA or Friedman test was used to assess response to treatment or placebo. Post-hoc testing was undertaken using Bonferroni correction. Spearman’s correlation analysis was to be used to determine any association between measured markers pre-thiamine and after treatment, if seen.

Analysis was undertaken using SPSS statistics 20 (IBM). Data is presented as mean +/- standard deviation for parametric data and median with IQR for non-parametric data.
**Results**

**Participant flow**

In total 49 patients expressed an interest in the study. Eleven were excluded as they did not meet the required study criteria (already on insulin n=7; previous CABG n=1; on thiamine n=1; on other research intervention n=2). Following allocation two patients were withdrawn. One patient's initial bloods tests demonstrated they were outside the inclusion criteria (HbA1c >10%) and another patient was excluded, as we were unable to undertake photoplethysmography. Two patients discontinued the intervention, one because of side effects related to the medication (abdominal pain after ingestion) and one developed a chronic pain syndrome and felt unable to continue. 34 subjects successfully completed the study (figure 3.1).

**Recruitment**

Recruitment commenced in June 2010 and was completed by March 2011. Patient follow-up continued until June 2011. The trial ended when 34 participants were recruited and completed the study.

**Patient characteristics**

20 subjects were male and 14 female, all were Caucasian. Their age was 61 ± 9.4 years (mean ± standard deviation) with duration of diabetes from diagnosis of 9.6 ± 5.3 years. Their mean BMI was 32.3 ± 5.4 kg/m² and waist hip ratio was 0.97 ± 0.07 overall (0.93 ± 0.09 for the women, 1.00 ± 0.04 for the men) indicating the majority of the patients were obese (BMI 30-35 kg/m²) and had a more central distribution of body fat (associated with a higher risk of cardiovascular disease). Their calculated risk of developing cardiovascular disease over the next 10 years was 34.5 ± 5.6 % (Framingham equation). Full baseline demographic parameters are given in table 3.1.

The majority of patients were on two or more glucose lowering therapies for the control of their diabetes with two being on dietary modification alone (see figure 3.2). A high proportion of patients were on cardiovascular
disease prevention medication including 30/34 metformin, 26/34 a statin, 18/34 an ACE inhibitor, 19/34 aspirin and 14/34 another antihypertensive medication (see figure 3.3). None of these agents were adjusted/stopped during the study.

Figure 3.1: Patient flow diagram
Table 3.1: Baseline demographic parameters.

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<th>Age (years)</th>
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<th>Duration of DM from diagnosis (years)</th>
<th>Weight (kg)</th>
<th>Height (m)</th>
<th>BMI (kg/m^2)</th>
<th>Waist: Hip</th>
<th>Cardio vascular Risk (%)</th>
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<tr>
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<td>52</td>
<td>M</td>
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<td>82.0</td>
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<td>3</td>
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<td>41.7</td>
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<td>1.75</td>
<td>26.1</td>
<td>0.88</td>
<td>36.5</td>
</tr>
</tbody>
</table>
Figure 3.2: Percentage of subjects taking none, one or a combination of glucose lowering therapies with 30/34 (88%) on metformin and 21/34 (62%) on a sulphonylurea.

Figure 3.3: Percentage of subjects on vasoactive medication (HTN = hypertension medication).
Outcomes

Baseline metabolic markers
Pre trial measurements showed HbA1c measured 7.46 ± 0.88%, which showed the majority of subjects were above the NICE target of 6.5%. Lipid parameters were close to NICE guidance with a total cholesterol concentration measuring 4.01 ± 1.11 mmol/l, HDL cholesterol concentration measuring 1.00 ± 0.30 mmol/l and calculated LDL cholesterol concentration measuring 2.05 ± 0.73 mmol/l. Triglycerides measured 1.87 ± 1.39 mmol/l. Blood pressure control was good at 137/77 ± 18/9 mmHg. Full baseline metabolic parameters are given in table 3.2.

Comparison of visit 1 and visit 3
Analysis of measured parameters between visit 1 and visit 3 was undertaken to assess for any change that may be present due to a lack of washout from those that were randomised to receive thiamine first. The results are shown in table 3.3 and RM-ANOVA or Wilcoxon Signed Rank testing showed there was no significant difference across baselines except with respect to blood pressure where both systolic and diastolic blood pressure was significantly reduced between the first visit and third visit.
Table 3.2: Baseline metabolic parameters

<table>
<thead>
<tr>
<th>Trial No</th>
<th>Systolic BP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>Total cholesterol (mmol/l)</th>
<th>HDL cholesterol (mmol/l)</th>
<th>LDL cholesterol (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T001</td>
<td>124</td>
<td>81</td>
<td>3.15</td>
<td>0.85</td>
<td>1.64</td>
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<tr>
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<td>0.64</td>
<td>1.53</td>
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<tr>
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<td>1.01</td>
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<td>0.95</td>
<td>1.05</td>
<td>1.52</td>
<td>6.1</td>
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</table>
Table 3.3: Comparison of results between visit 1 and visit 3 (blinded data)

<table>
<thead>
<tr>
<th></th>
<th>Visit 1</th>
<th>Visit 3</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>TDP (nmol/l)</td>
<td>186 ± 24</td>
<td>193 ± 44</td>
<td>0.29</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>135.6 ± 18.1</td>
<td>127.6 ± 18.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76.8 ± 8.9</td>
<td>71.4 ± 8.9</td>
<td>0.003</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.01 ± 1.11</td>
<td>3.97 ± 1.05</td>
<td>0.70</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.10 ± 0.38</td>
<td>1.09 ± 0.43</td>
<td>0.60</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.01 ± 0.67</td>
<td>2.09 ± 0.80</td>
<td>0.30</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.88 ± 1.39</td>
<td>1.87 ± 1.01</td>
<td>0.85</td>
</tr>
<tr>
<td>Fructosamine (µmol/l)</td>
<td>276 ± 38</td>
<td>283 ± 44</td>
<td>0.11</td>
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<tr>
<td>HbA1c (%)</td>
<td>7.5 ± 0.9</td>
<td>7.6 ± 0.8</td>
<td>0.16</td>
</tr>
<tr>
<td>Insulin Resistance (HOMA-IR)</td>
<td>1.5 ± 0.8</td>
<td>1.6 ± 0.8</td>
<td>0.57</td>
</tr>
<tr>
<td>RI GTN</td>
<td>12.9 ± 7.1</td>
<td>13.6 ± 6.6</td>
<td>0.60</td>
</tr>
<tr>
<td>RI Salb</td>
<td>7.4 ± 9.8</td>
<td>6.6 ± 8.4</td>
<td>0.66</td>
</tr>
<tr>
<td>ACR</td>
<td>0.70 IQR 1.90</td>
<td>0.65 IQR 1.52</td>
<td>0.24§</td>
</tr>
<tr>
<td>sVCAM-1 (ng/ml)</td>
<td>799 ± 228</td>
<td>792 ± 239</td>
<td>0.66</td>
</tr>
<tr>
<td>cGMP (pmol/mL)</td>
<td>407 ± 185</td>
<td>405 ± 226</td>
<td>0.95</td>
</tr>
<tr>
<td>CuPRAC (mM Asc (AEAC))</td>
<td>0.40 ± 0.10</td>
<td>0.41 ± 0.10</td>
<td>0.78</td>
</tr>
<tr>
<td>GSH:GSSG</td>
<td>63 IQR 73</td>
<td>66 IQR 65</td>
<td>0.82§</td>
</tr>
<tr>
<td>Oxidised glutathione (GSSG) (µM)</td>
<td>7.1 IQR 7.5</td>
<td>8.3 IQR 6.1</td>
<td>0.49§</td>
</tr>
<tr>
<td>Total Glutathione (µM)</td>
<td>483 ± 156</td>
<td>509 ± 200</td>
<td>0.42</td>
</tr>
<tr>
<td>Reduced Glutathione (µM)</td>
<td>464 ± 195</td>
<td>494 ± 170</td>
<td>0.37</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>1.2 IQR 2.5</td>
<td>1.1 IQR 2.2</td>
<td>0.70§</td>
</tr>
</tbody>
</table>

§ Wilcoxon Signed Rank test
Primary outcome measure

TDP concentration was measured to assess treatment compliance by the trial subjects. There was a significant increase across the treatment arm from $183 \pm 28 \text{ nmol/l}$ pre-thiamine to $310 \pm 82 \text{ nmol/l}$ post-thiamine, $p<0.001$ (RM-ANOVA). In the placebo arm there was a decrease in TDP concentration across the 8-weeks although this did not reach statistical significance ($196 \pm 41 \text{ nmol/l}$ vs. $178 \pm 32 \text{ nmol/l}$, $p = 0.07$)

Effect of thiamine on primary outcome measures

Pre and post thiamine and placebo measurements are seen in table 3.4. RM-ANOVA or Friedman analysis show there to be no significant differences in markers of endothelial function, insulin resistance, oxidative stress or inflammation in either the treatment or placebo arm.

Table 3.4: Effect of thiamine vs. placebo on markers of endothelial function, insulin resistance, oxidative stress and inflammation.

<table>
<thead>
<tr>
<th></th>
<th>Pre-thiamine</th>
<th>Post-thiamine</th>
<th>Pre-placebo</th>
<th>Post-placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDP (nmol/l)</td>
<td>$183 \pm 28$</td>
<td>$310 \pm 82$</td>
<td>$196 \pm 41$</td>
<td>$178 \pm 32$</td>
</tr>
<tr>
<td>RI GTN</td>
<td>$13.4 \pm 7.3$</td>
<td>$13.0 \pm 7.0$</td>
<td>$13.1 \pm 6.3$</td>
<td>$13.1 \pm 8.3$</td>
</tr>
<tr>
<td>RI Salb</td>
<td>$6.7 \pm 6.9$</td>
<td>$5.3 \pm 9.2$</td>
<td>$7.3 \pm 10.9$</td>
<td>$6.8 \pm 6.9$</td>
</tr>
<tr>
<td>ACR</td>
<td>$0.60 \text{ IQR 1.55}$</td>
<td>$0.80 \text{ IQR 2.35}$</td>
<td>$0.70 \text{ IQR 2.00}$</td>
<td>$0.70 \text{ IQR 2.34}$</td>
</tr>
<tr>
<td>sVCAM-1 (ng/ml)</td>
<td>$808 \pm 234$</td>
<td>$792 \pm 257$</td>
<td>$783 \pm 233$</td>
<td>$785 \pm 231$</td>
</tr>
<tr>
<td>cGMP (pmol/ml)</td>
<td>$432 \pm 213$</td>
<td>$431 \pm 183$</td>
<td>$380 \pm 196$</td>
<td>$380 \pm 188$</td>
</tr>
<tr>
<td>Insulin Resistance (HOMA-IR)</td>
<td>$1.5 \pm 0.8$</td>
<td>$1.7 \pm 1.0$</td>
<td>$1.6 \pm 0.9$</td>
<td>$1.7 \pm 0.8$</td>
</tr>
<tr>
<td>GSH:GSSG</td>
<td>$73 \text{ IQR 162}$</td>
<td>$49 \text{ IQR 93}$</td>
<td>$53 \text{ IQR 65}$</td>
<td>$65 \text{ IQR 67}$</td>
</tr>
<tr>
<td>Oxidised glutathione (GSSG) (µM)</td>
<td>$7.1 \text{ IQR 5.9}$</td>
<td>$9.6 \text{ IQR 10.9}$</td>
<td>$8.9 \text{ IQR 7.0}$</td>
<td>$10.1 \text{ IQR 22.7}$</td>
</tr>
<tr>
<td>Total Glutathione (µM)</td>
<td>$523 \pm 151$</td>
<td>$492 \pm 171$</td>
<td>$469 \pm 201$</td>
<td>$472 \pm 162$</td>
</tr>
<tr>
<td>Reduced Glutathione (µM)</td>
<td>$507 \pm 190$</td>
<td>$451 \pm 170$</td>
<td>$452 \pm 173$</td>
<td>$454 \pm 174$</td>
</tr>
<tr>
<td>CuPRAC (mM Asc (AEAC))</td>
<td>$0.40 \pm 0.09$</td>
<td>$0.41 \pm 0.09$</td>
<td>$0.41 \pm 0.11$</td>
<td>$0.41 \pm 0.09$</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>$1.1 \text{ IQR 3.2}$</td>
<td>$1.3 \text{ IQR 3.2}$</td>
<td>$1.1 \text{ IQR 2.1}$</td>
<td>$1.3 \text{ IQR 3.1}$</td>
</tr>
</tbody>
</table>
On analysis of the metabolic markers RM-ANOVA with Bonferroni correction showed a significant decrease in systolic blood pressure with thiamine treatment from $134.9 \pm 18.7$ mmHg to $121.7 \pm 12.6$ mmHg, $p = 0.001$. There was no significant change seen in the placebo arm ($127.8 \pm 17.7$ vs. $124.7 \pm 17.8$ mmHg, $p = 1.00$). No further statistically significant changes in metabolic markers were seen (table 3.5).

Table 3.5: Effect of thiamine vs. placebo on blood pressure and metabolic markers

<table>
<thead>
<tr>
<th></th>
<th>Pre-thiamine</th>
<th>Post-thiamine</th>
<th>Pre-placebo</th>
<th>Post-placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDP (nmol/l)</td>
<td>183 ± 28</td>
<td>310 ± 82</td>
<td>196 ± 41</td>
<td>178 ± 32</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>134.9 ± 18.7</td>
<td>121.7 ± 12.6*</td>
<td>127.8 ± 17.7</td>
<td>124.7 ± 17.8</td>
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<td>Diastolic BP (mmHg)</td>
<td>74.7 ± 8.9</td>
<td>72.6 ± 9.5</td>
<td>73.0 ± 8.9</td>
<td>71.2 ± 9.9</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
<td>3.92 ± 1.09</td>
<td>3.94 ± 0.94</td>
<td>4.06 ± 1.07</td>
<td>4.08 ± 1.14</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.10 ± 0.43</td>
<td>1.09 ± 0.41</td>
<td>1.08 ± 0.37</td>
<td>1.12 ± 0.42</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.05 ± 0.79</td>
<td>2.01 ± 0.65</td>
<td>2.12 ± 0.74</td>
<td>2.04 ± 0.77</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.82 ± 1.04</td>
<td>1.95 ± 1.14</td>
<td>1.93 ± 1.36</td>
<td>2.10 ± 1.25</td>
</tr>
<tr>
<td>Fructosamine (µmol/l)</td>
<td>281 ± 42</td>
<td>295 ± 49</td>
<td>280 ± 40</td>
<td>286 ± 42</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.55 ± 0.90</td>
<td>7.59 ± 0.94</td>
<td>7.51 ± 0.82</td>
<td>7.65 ± 0.92</td>
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</tbody>
</table>

* - $p = 0.001$ compared with Pre-thiamine value.

In view of the change in systolic blood pressure further analysis was undertaken to assess the effect of thiamine on cardiovascular risk. On recalculating the Framingham risk score pre and post thiamine administration there was a significant reduction in the risk of cardiovascular disease over 10 years from $32.2 \pm 12.4\%$ to $27.3 \pm 8.8\%$ ($p = <0.05$).
Secondary outcomes

The results from visit 1 were examined to look for any associations between markers of oxidative stress, endothelial dysfunction, vascular inflammation and insulin resistance using Spearman’s rank order correlation. The results show a moderate correlation between hsCRP and HOMA-IR (ρ = 0.438, p = 0.01) (fig 3.4). There was a moderate correlation between hsCRP and CuPRAC (ρ = 0.359, p = 0.04) (fig 3.5) and a moderate correlation between cGMP and CuPRAC (ρ = 0.346, p = 0.05) (fig 3.6). Further analysis was undertaken to see if these associations persisted after thiamine administration but this was not seen. No other significant associations between the measured markers were detected (table 3.6). To further check whether these associations were independent multiple regression analysis was undertaken using BMI as a constant factor. Subsequently no associations were seen. Further analysis was undertaken to see if these associations persisted after thiamine administration but this was not seen.

Figure 3.4: Correlation between hsCRP and HOMA-IR

----- 95% confidence interval
Figure 3.5: Correlation between hsCRP and CuPRAC
----- 95% confidence interval

Figure 3.6: Correlation between cGMP and CuPRAC
----- 95% confidence interval
### Table 3.6: Table of Correlations

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<tr>
<td>sVCAM vs. GSH:GSSG</td>
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</tr>
<tr>
<td>sVCAM vs. hsCRP</td>
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<tr>
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<td>ACR vs. HOMA-IR</td>
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<td>CuPRAC vs. GSH:GSSG</td>
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<td>0.99</td>
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<td>CuPRAC vs. hsCRP</td>
<td>0.359</td>
<td>0.04*</td>
</tr>
<tr>
<td>GSH:GSSG vs. hsCRP</td>
<td>0.295</td>
<td>0.10</td>
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</tbody>
</table>
Harms

Only one patient in the study reported any side effects related to the study medication. They experienced abdominal pain after taking the medication, which resolved on stopping, and as a result felt unable to continue in the study. Unblinding revealed that the patient was taking the thiamine when they experienced the symptoms. Abdominal pain is not a recognised side effect of thiamine, which is known to be a well-tolerated medication at the prescribed dose.
Discussion
This study was undertaken to see whether thiamine supplementation in patients with type 2 diabetes receiving standard treatment from primary care providers resulted in an improvement in oxidative stress, vascular inflammation, endothelial dysfunction, and insulin resistance.

The key findings of this study were

1. A significant increase in serum thiamine diphosphate levels across the treatment arm. This increase was not seen in the placebo arm thus suggesting good treatment compliance, absorption of thiamine and correct dose administration.

2. A statistically significant reduction in systolic blood pressure across the treatment arm in contrast to the placebo arm.

3. The subjects showed no evidence of oxidative stress, endothelial dysfunction, vascular inflammation and insulin resistance at baseline and treatment with thiamine showed no significant change in these biophysical markers.

4. A significant association between inflammation and insulin resistance was demonstrated at baseline, as was an association between inflammation and oxidative stress.

5. A correlation between cGMP and CuPRAC at baseline was seen.

To review the strength and significance of these findings we need to compare this study's findings with previously published clinical trials as well as review the limitations of this study.

Comparison with relevant findings from other published studies.
To date, only a few studies have been published exploring the effect of oral thiamine administration upon individuals with type 2 diabetes. In 2009 Rabbani et al published the results of a pilot study looking at the effect of administration of 300mg oral thiamine daily for three months, or placebo to individuals with type 2 diabetes and persistent microalbuminuria. This
study showed no significant effect of thiamine supplementation upon glycaemic control, dyslipidaemia, blood pressure or markers of vascular dysfunction (sVCAM1); however they did demonstrate a reduction in microalbuminuria (70). Riaz et al (2011) also showed a reduction in microalbuminuria after three months of 300mg daily oral thiamine therapy (71). In 2010 Gonzalez-Ortiz et al published their comparison study of 150mg of thiamine daily vs. placebo for 1 month in drug naïve patients with type 2 diabetes. This study showed a small but significant reduction in fasting glucose levels after one month of treatment (55).

Other work has examined the effect of the lipophilic thiamine derivative benfotiamine and its effect on those with diabetes. In those with type 1 diabetes four weeks of oral benfotiamine combined with α-lipoic acid showed no effect on glycaemic control (HbA1c, fructosamine or fasting glucose) but did show a reduction in AGE formation and hexosamine pathway activity (51). A long term, 24-month study of benfotiamine on individuals with type 1 diabetes also showed no effect of administration on glycaemic control (HbA1c). Also there was no effect seen on lipid parameters (Cholesterol, HDL), VCAM-1 or hsCRP despite an increase in TDP levels in the treatment arm (106). Stirban et al (2006) have studied benfotiamine administration in those with type 2 diabetes. Short term, three day administration of benfotiamine showed a reduction in micro and macro-vascular dysfunction (induced by a high AGE content meal). In addition benfotiamine prevented an increase in serum markers of endothelial dysfunction (including VCAM-1), inflammation (hsCRP) and oxidative stress (thiobarbituric acid reacting substances) (64). However a longer-term study by the same group showed that six weeks of treatment with benfotiamine did not reveal the same positive results as seen in their previous study (107).

Direct comparison with these studies cannot be undertaken due to differences with the studies’ inclusion and exclusion criteria but some observations can be made. In the studies showing a reduction in microalbuminuria 24-hour urinary albumin excretion was measured rather than ACR, measurements known to correlate with each other (108) and both
showed a higher level of microalbuminuria at baseline than that of this patient cohort (43.7mg/24 h and 56.9µg/ml). This study did not specifically look to analyse fasting blood glucose values but the patients did not demonstrate a change in glycaemic control with respect to either HbA1c or fructosamine reduction. Similarly a change in HbA1c was not seen in the Gonzalez-Ortiz study despite the reduction in fasting glucose. No other significant changes in lipid parameters, blood pressure or inflammatory markers (hsCRP) were seen in any of these studies. The Rabbani study did show a negative linear regression between plasma thiamine levels and sVCAM, which was not replicated in this study (70).

In the 6-week crossover trial of benfotiamine sub-group analysis was undertaken dividing the cohort into those with highest and lowest FMD at baseline. They found that in those with a lesser degree of FMD showed some benefit to treatment with benfotiamine – consistent with their earlier study (107). Thus it can be hypothesised that benfotiamine and possibly thiamine is more beneficial when given to those with earlier, reversible vascular dysfunction rather than those with advanced vascular damage. The study cohort had a higher mean duration of diabetes than the Stirban group and so may have asymptomatic, irreversible vascular damage, with increased arterial stiffness but normal arterial diameter (109, 110).

Limitations of this study
There are many factors that can be considered to confound our findings, and these can be broadly categorised into factors related to the patient cohort, the study methodology and the chosen measured parameters.

Patient Cohort
It can be seen from the baseline measurements that the patient cohort, despite being of high cardiovascular risk, all showed good control of metabolic parameters known to affect atherosclerosis. The baseline measurements of blood pressure, lipid profile and glycaemic control were close to the NICE guidance targets for people with type 2 diabetes (6). Also the majority of patients were on metformin (88%), a statin (76%) and/or an
ACE-inhibitor (53%) with three-quarters of the cohort taking two or more of these medications. These medications are known to modulate endothelial function, prevent oxidative stress, and reduce vascular inflammation (111-113). If this is achieved to a degree that already normalises the underlying processes then it could be argued that we are unlikely to see any further change with thiamine administration.

On designing this study it was assumed that the study cohort would have a degree of thiamine deficiency as has been reported in people with type 2 diabetes. However this study population showed normal thiamine diphosphate levels at baseline. Erythrocyte transketolase activity, a functional measure of thiamine status, was not measured in our cohort. Thiamine deficiency in the diabetic population has been associated with increased renal excretion of thiamine (114). This study cohort showed they had a normal albumin: creatinine ratio and therefore low levels of microalbuminuria (a marker of increased renal filtration) thereby it could be postulated this was the reasoning behind the lack of thiamine deficiency in our study cohort.

Methodology

It is important to review the study design and there are several parts of the study methodology that may have contributed to the study limitations. This study was powered to detect a two standard deviations change from the mean in sVCAM-1 concentration from previous trial data (70). However the study numbers were still small and our patient cohort had better metabolic control than the group on which our sample size estimation was based.

Thiamine hydrochloride tablets have a distinct smell and taste, which is yeasty in nature and although we were able to use a matched placebo in appearance we were unable to match these other characteristics. Patients commented on noticing the characteristic smell and taste of the thiamine, with some finding it unpleasant but not to a degree that they were unable to continue with the study. Patients were not asked to comment on whether they thought they were taking the placebo or treatment medication and
therefore no comment can be made on whether this difference in the medications contributed to the success, or not, of the blinding process. The change in thiamine diphosphate levels show that the participants had good compliance with the thiamine medication and that it was well absorbed across the gastrointestinal barrier. Thiamine hydrochloride has a bioavailability between 3.7% and 5.3%(115, 116). It is absorbed both actively and passively across the gastro-intestinal tract with peak absorption after approximately 4 hours and is best given in three divided doses (117). Whilst the patient cohort was asked to do this, several patients reported taking the three doses of thiamine at once in order to improve compliance. Despite this variance in the way the medication was taken all patient showed improved serum values. Importantly there was no difference in the thiamine concentration before each treatment arm across the two groups (table 3.3) showing that there was good washout of thiamine within the two-week timeframe allowed.

Our cohort was given thiamine or placebo for an eight-week period and whilst this improved their thiamine diphosphate levels it may not have been a long enough time for the measured markers to change, however Arora et al (2006) showed an improvement in endothelium-dependent vasodilatation after a single 100mg intravenous dose of thiamine (68). Other studies, as described previously, showed changes in microalbuminuria after 3 months of therapy and improved glycaemic control after only 1 month of therapy. (55, 70, 71). Stirban et al (2006) showed an improvement in endothelial function after only three days of treatment with high dose benfotiamine (64). Experimental studies where thiamine and/or benfotiamine have shown improvements in endothelial function and oxidative stress, the duration of treatment has been significantly longer (24-26 weeks) than in clinical studies (54, 118).

Comparison of baselines showed an elevated systolic blood pressure at visit one compared to the other visit attendances. Anxiety is well recognised to cause an increase in blood pressure due to stimulation of the sympathetic nervous system (119). At visit 1 the patient cohort were attending for the first set of trial measurements and it is possible that the difference in the
blood pressures seen at baselines is related to anxiety. If this increase in blood pressure is related to anxiety rather than true hypertension then it can be questioned whether this would be associated with underlying pathophysiological changes. In addition as all patients were randomised equally to the two treatment arms following the initial visit any bias caused by this change in blood pressure will be distributed across the two treatment arms.

The change in blood pressure seen in the treatment arm compared with the placebo arm is statistically significant with a 12mmHg reduction seen. This level of BP reduction is clinically significant and it is widely accepted that blood pressure reduction is key in reducing the risk of cardiovascular disease as seen in the UKPDS and other studies (120, 121). The associated reduction in 10-year cardiovascular risk that is seen with this reduction in blood pressure is not surprising given the significant weighting systolic blood pressure has on the Framingham risk algorithm. In those on antihypertensive agents a systolic pressure over 140mmHg influences over 20% of the Framingham risk score and has more influence than either total or HDL cholesterol (5)

Upon further evaluation it can be seen that the pre-thiamine value is significantly higher, as shown in table 3.5, than the other systolic blood pressure measurements. The reasoning for this is unclear. Due to randomisation it is unlikely that the anxiety factor that was seen between the baselines would have contributed to this anomaly.

In light of this finding subgroup analysis of ten individuals who were not on hypertensive treatment was undertaken. RM-ANOVA with Bonferroni correction showed no significant change in systolic blood pressure across the treatment arm (128.3 ± 20.1 mmHg vs. 120.6 ± 20.7 mmHg pre and post-placebo (p = 0.17) and 131.6 ± 20.2 mmHg vs. 119.1 ± 11.6 mmHg pre and post thiamine (p = 0.24)).

No other studies have shown thiamine supplementation, or any derivatives, to affect blood pressure and therefore the change observed is a novel finding.
of this study. Further evaluation would be necessary to follow-up this finding with a larger study.

Chosen measured parameters
On designing this study it was assumed that because the patients have type 2 diabetes and were at high cardiovascular risk based upon Framingham analysis they would have elevated markers of oxidative stress, vascular inflammation etc at baseline. By undertaking a crossover study the study cohort acts as his or her own controls and therefore we have not directly compared the study population with a matched, non-diabetic population.

Endothelial function
There are many ways in which endothelial function can be assessed and there is no consensus as to which methods are used in its evaluation. In the peripheral vasculature vasomotor changes can be assessed and in this study we employed pulse wave analysis via digital photoplethysmography with the administration of GTN as an endothelium-independent stimulus and salbutamol as an endothelium-dependent stimulus. Flow-mediated dilatation is another established technique for measuring endothelial function in the peripheral vasculature and direct comparison of these techniques has shown that FMD is superior to PWA in terms of reproducibility and is the non-invasive technique of choice (122). However FMD is a more complex technique requiring experienced technical training and is operator-dependent, compared with PWA, which is a more simple technique.

The cohort baseline values showed reduced measures of reflection index after salbutamol, an established test of NO dependent endothelial vasodilatory function, compared with the normal healthy population (7.4 ± 9.8 vs. 11.8 ± 1.8) (83). Similar levels to this study were seen in a previous study examining PWA in type 2 diabetes where our study cohort was well-matched in terms of age, BMI and glycaemic control, although our cohort had a longer duration of diabetes (9.6 ±5.3 vs.5.9 ± 2.1 yrs) (123).
Microalbuminuria has been shown to be closely associated with endothelial dysfunction although the relationship between these two is complex and the underlying pathophysiology is not well understood (85). The cohort had low levels of microalbuminuria at baseline and this could be a reflection of low levels of endothelial dysfunction. The concomitant use of statins and ACE-inhibitors are likely to have played a significant role in reducing this (109, 123).

The cGMP results showed elevated levels compared with values from the sample ranges provided by the assay manufacturers. Previous studies have shown lower levels of cGMP in people with type 2 diabetes compared with the normal population (661.12 ±179.24 vs. 965.25± 102.12 fmol/ml) (94). The higher levels observed in the study cohort may show up-regulation of cGMP as an adaptive response to combat endothelial dysfunction.

**Oxidative stress**

The baseline total glutathione results are lower than seen in other studies where this has been measured in individuals with diabetes (2360 ± 823 vs. 649 ± 324 vs. 483 ± 156 µM) (123, 125). Also the reduced:oxidised glutathione ratio was higher in this patient population than seen in a similar study (12.5 ± 12.7 vs. 63 ± 73) (123). CuPRAC is a relatively new assay and so there is no comparable data available where this has been studied in diabetes populations, however it correlates with other methods of measuring TAC such as the ferric reducing ability of plasma (FRAP) (126). Comparison with other studies measuring TAC as ascorbic acid equivalents (AEAC) reveals significantly lower levels of oxidative stress within this cohort despite a comparable metabolic profile (123, 124, 127).

These markers show low levels of oxidative stress at baseline in this population, once again likely to be due to good metabolic control and high use of cardiovascular disease-modifying medications.
Secondary outcome measures showed a significant correlation between the CuPRAC and cGMP assays at visit 1. This supports the evidence showing a close linkage between oxidative stress and endothelial function (128).

**Inflammation**

Both diabetes and obesity are associated with a low-grade chronic inflammatory state and hsCRP is well known to be a robust marker of inflammation with good predictive value of future vascular events (129).

In this study the mean baseline levels of hsCRP were very low (1.1mg/l) and in the range seen in a normal healthy population (<1.5mg/l) (130). This demonstrates low levels of inflammation in this cohort and there was no change in hsCRP with either treatment thiamine or placebo. A review undertaken by the American Heart Association on the application of markers of inflammation to clinical health practice formulated a stratification system whereby the relative risk of cardiovascular disease was stratified into low, average and high risk corresponding to approximate tertiles of values <1.0, 1.0-3.0 and >3.0mg/L respectively (131). Good metabolic control, statins and metformin have all been shown to reduce hsCRP (132) and it is possible that due to the high use of these medications in this cohort, plus their good metabolic control this precluded any further change that may have been seen with thiamine.

Despite the low levels of inflammation a positive correlation between hsCRP and markers of both oxidative stress and insulin resistance was seen. The association between inflammation and insulin resistance is well recognised and was extensively investigated by Festa et al (2000) (133). The correlation seen with CuPRAC is surprising given that it is a positive correlation. Inflammation has been shown to increase oxidative stress and thus it would be expected that as hsCRP rises the CuPRAC level decreases. However, it could be possible that antioxidant status is up-regulated due to increased inflammation and therefore the positive correlations seen represent this adaptive response. This may represent an early phase in the
relationship before the underlying inflammatory process overwhels the adaptive response.

**Insulin Resistance**

The hyperinsulinaemic euglycaemic glucose clamp was originally developed in 1979 by DeFronzo et al and is widely accepted as the reference standard for the direct determination of metabolic insulin sensitivity in humans (95). However it is time consuming, expensive and labour intensive and therefore is not felt to be appropriate for large clinical studies (96). In addition to this there has been shown to be considerable intersubject variability, especially in those with type 2 diabetes where it is as high as 46 percent (134).

The HOMA-IR model is one of several mathematical models for measuring insulin resistance and has a reasonable linear correlation with glucose clamp models of insulin sensitivity estimation (135). It is useful for evaluation of basal insulin resistance (determined by hepatic insulin resistance) in those with mild to moderate diabetes; however it may not be appropriate in those with severely impaired or absent beta-cell function. It does not, however, measure stimulated insulin resistance, which is more reflective of skeletal muscle glucose handling. In order to improve the intrasubject variation, the use of three samples taken at 5-min intervals is better than a single sample, as was done in this study (97).

The study cohort showed low levels of insulin resistance at baseline with no change seen with either thiamine or placebo. It is likely that the high use of metformin contributed with this low level of insulin resistance and reduced HOMA-IR in this patient population (136).

**Glycaemic Control**

In clinical practice glycated haemoglobin (HbA1c) is the standard method for assessing long-term glycaemic control. However it could be argued that four or eight weeks of treatment, as in this study, is not sufficient time to allow a significant change in glycated haemoglobin (137). We measured fructosamine in addition to HbA1c as this has been shown to be useful in
assessing shorter-term (three to six week) changes in glycaemic control (138).

Implications of this work

This study does not provide any supportive evidence for the routine use of thiamine supplementation in people with well-controlled diabetes. Dietary and lifestyle changes are the mainstay of the initial management of people with type 2 diabetes and in light of the evidence provided by the Gonzalez-Ortiz study (55), thiamine supplementation may be a useful part of the dietary changes that could contribute to a delay in patients requiring oral hypoglycaemic therapy. This is further supported by the findings in a recent study where thiamine supplementation in newly diagnosed, drug naïve individuals showed a significant reduction in fasting glucose (139).

Furthermore it may be possible that thiamine supplementation is beneficial in those individuals who have poorly controlled diabetes despite the use of oral hypoglycaemic medications or insulin. This hypothesis is supported by the fact that the majority of the experimental studies showing improved endothelial function have been undertaken in a hyperglycaemic vs. normoglycaemic environment.

This study is consistent with the premise that when individuals with type 2 diabetes with high cardiovascular risk are treated appropriately with glucose lowering, blood pressure and lipid modifying therapy then their underlying levels of endothelial function, oxidative stress and vascular inflammation may be similar to levels seen in the normal healthy population.

There are many possibilities for future research as a result of this study. Studies could be undertaken to assess whether thiamine supplementation can delay the onset or advancement of diabetes in drug naïve individuals. Also studies assessing the effect on those participants with poor glycaemic control with or without underlying endothelial dysfunction would be another avenue in which research could be undertaken. There is an increasing international prevalence of diabetes and a significant focus on
strategies that can identify pre-diabetes and prevent or delay the onset to type 2 diabetes. This is a further area in which thiamine may have a role given the importance of thiamine diphosphate in the glucose metabolism pathway. Given that thiamine is a cheap, well-tolerated medication this would make it a very attractive treatment option.
References


100. Jain SK, McVie R. Effect of glycemic control, race (white versus black), and duration of diabetes on reduced glutathione content in erythrocytes of diabetic patients. Metabolism. 1994;43:306-309.


FORM UPR16
Research Ethics Review Checklist

Please complete and return the form to Research Section, Quality Management Division, Academic Registry, University House, with your thesis, prior to examination.

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<td>Student Name: Georgina Page</td>
<td></td>
</tr>
<tr>
<td>Department: PBMS</td>
<td>First Supervisor: Prof MH Cummings</td>
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<td>Start Date: or progression date for Prof Doc students</td>
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If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University’s Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study.

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at http://www.ukr io.org/what-we-do/code-of-practice/finished-researchers/

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame? **YES**

b) Have all contributions to knowledge been acknowledged? **YES**

c) Have you complied with all agreements relating to intellectual property, publication and authorship? **YES**

d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration? **YES**

e) Does your research comply with all legal, ethical, and contractual requirements? **YES**

*Delete as appropriate*

UPR 16 (2011) – August 2011
**Student Statement:**

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s).

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If you have not submitted your work for ethical review, and/or you have answered No to one or more of questions a) to j), please explain why this is so.

| Signed: | (Student) |
| Date: | |
National Research Ethics Service

SOUTHAMPTON & SOUTH WEST HAMPSHIRE
RESEARCH ETHICS COMMITTEE (B)

11 January 2010

Dr. M.H. Cummings
Consultant Physician & Endocrinologist, Honorary Reader
Academic Department of Diabetes & Endocrinology
Level C, Queen Alexandra Hospital, Cosham, Portsmouth
PO6 3LY

Dear Dr. Cummings,

Study Title: Oral thiamine (Vitamin B1) supplementation in subjects with type 2 diabetes mellitus: a randomised, double-blind, placebo-controlled crossover trial assessing biophysical markers of endothelial function, oxidant stress, insulin sensitivity and vascular inflammation

REC reference number: 09/H0504/137
Protocol number: 1
EudraCT number: 2009-017537-21

Thank you for your letter of 04 January 2010, responding to the Committee’s request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the 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

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

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

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

Management permission or approval must be obtained from each host organisation prior to

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the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) 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 the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

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

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

Participant Information Sheet:
On page 2 under "What will happen if I decided to take part?", third to the last line, the word "it" between steadily and into should be removed so the sentence should say "It holds the medicine mist long enough for it to be inhaled slowly and steadily into the lungs." Please ensure the revised copy of the information sheet is submitted to the Committee for information.

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:

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<td>Listing of Serious Adverse Events/Reactions</td>
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<td>Appendix X - Listing of Adverse Events/Reactions</td>
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Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

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

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

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.

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
- 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.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@bree.npa.nhs.uk.

09/ii0504/137 Please quote this number on all correspondence

Yours sincerely

Professor R King
Alternate Vice-Chair

Email: scsrw.SWHRECS@nhs.net

Enclosures: “After ethical review – guidance for researchers” SL-AR1 for CTIMPs.

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Mr M Cummings  
QUEEN ALEXANDRA HOSPITAL  
DEPARTMENT OF DIABETES  
SOUTHWICK HILL ROAD, COSHAM  
PORTSMOUTH  
P06 8LY  
UNITED KINGDOM  
02/03/2010  

Dear Mr M Cummings  

THE MEDICINES FOR HUMAN USE (CLINICAL TRIALS) REGULATIONS 2004 SI 2004/1031  

Our Reference: 131422027/001-0001  
Brand Number: 2009/017397-21  
Protocol Number: PHT/2009/06  
Product: Thiamine  

NOTICE OF ACCEPTANCE OF AMENDED REQUEST  

I am writing to inform you that the Licensing Authority accepts your amended request for a clinical trial authorisation (CTA), received on 01/03/2010.  

The authorisation is effective from the date of this letter although your trial may be suspended or terminated at any time by the Licensing Authority in accordance with regulation 31. You must notify the Licensing Authority within 30 days of the trial starting.  

Finally, you are reminded that a favourable opinion from the Ethics Committee is also required before this trial can proceed; changes made as part of your amended request may need to be notified to the Ethics Committee.  

Yours sincerely,  

Clinical Trials Unit  
MHRA