The effects of garlic upon endothelial function, vascular inflammation, oxidative stress and insulin resistance in patients with type 2 diabetes at high cardiovascular risk. A double blind randomised placebo controlled trial.

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

Submitted May 2011

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.

Signature:

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ACKNOWLEDGEMENTS

I would like to thank:

Professor Michael Cummings – First Supervisor

Dr David Laight - Supervisor

Ms Sharon Allard- Research Nurse, QAH. - for help in dealing with subjects

Dr Anthony Wierzbicki & Dr Joseph Cheung - for their laboratories help with processing the HsCRP samples

Professor Ian Cree – for use of the cancer laboratory, QAH

Dr Partha Kar – for his support and guidance

Dr Georgina Page – for help in consolidating results

Dr Nagatoshi Ide, Wakunaga Pharmaceuticals Ltd – for guidance in use of AGE

Wakunaga Pharmaceuticals Ltd – for supply of AGE capsules

Ms Heather Cuell, Pharmacist – for dispensing AGE and randomisation

Reuben Ogollah – for statistical advice

The GP practices who allowed me to recruit subjects from their diabetes patients' database.

The subjects who volunteered to take part in the study.

My family- for putting up with the years of study

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ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

ACEI: Angiotensin converting enzyme inhibitor

ACR: Albumin Creatinine ratio

ACSO: S-Allyl-L-cysteine Sulphoxides

AEAC: Ascorbate Equivalent Antioxidant Concentration

AGE: Aged Garlic Extract

AGEPs: Advanced Glycation End Products

AMP: Adenosine monophosphate

AMS: Allyl methyl Sulphide

AMSO: Allyl methyl Sulphone

ANOVA: Analysis of variance

BMI: Body mass index

CO: Crossover study

CHD: Coronary Heart disease

CV: Coefficient of variance

DAS: Diallyl suphides

DATS: Diallyl trisulfide

DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid)

DVP: Digital Volume Pulse

DVW: Digital Volume waveform

ECG: Electrocardiogram

EDTA: Ethylenediamine tetraacetic acid

ELAM-1: Endothelial leucocyte adhesion molecule-1

eNOS: Endothelial nitric oxide synthase

FFA: Free fatty acids

FMD: Flow mediated dilatation

FRAP: Ferric reducing ability of plasma

GIP: Gastrointestinal Peptide

GLP: Glucagon-like peptide

GLUT-1: Glucose transporter protein type 1

GSH/GSSG: Glutathione ratio

GSH: Glutathione

GSSG: Oxidized glutathione

GTN: Glyceryltrinitrate

HbA1c: Glycosylated haemoglobin

HDL: High density lipoprotein

HMG-CoA: 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A

HOMA-B: Homeostasis Model Assessment- Beta cells

HOMA-IR: Homeostasis Model Assessment- Insulin Resistance

HPLC: High Performance Liquid Chromatography

HsCRP: Highly sensitive C-reactive protein

ICAM: Intercellular adhesion molecule

IL-6: Interleukin 6

iNOS: Inducible nitric oxide synthase

INR: International Normalised Ratio

JNK: c-Jun N-Terminal Kinase

KS test: Kolmogorov-Smirnov test

LDL: Low density lipoprotein

LHP: Lipid hydroperoxides

MAPK: Mitogen activated Protein Kinase

MCP-1: Monocyte chemotactic protein-1

MDA: malonaldehyde

NADPH: Nicotinamide adenine dinucleotide

NF-kappa B: Nuclear factor kappa beta

NO: Nitric oxide

NOS: Nitric Oxide Synthase

PC: Placebo controlled study

PWV: Pulse wave velocity

RAGE: Receptor for Advanced glycation end products

RI: Reflective index

RIA: Radioimmunoassay

ROS: Reactive Oxygen Species

SAC: S-Allyl Cysteine

SAMC: S- Allyl Methyl Cysteine

SAPK: Stress-activated protein kinase

SD: Standard Variation

sICAM-1: soluble intercellular adhesion molecule type 1

SSA: Sulpho-salicylic acid

TAC: Total Anti-oxidant capaciity

TAOS: Total antioxidant status

TBARS: ThioBarbituric Acid Reactive Substances

TEA: Triethonalamine

TNF-α: Tumour necrosis factor alpha

UKPDS: United Kingdom Prospective Diabetes Study

VCAM-1: Vascular cell adhesion molecule-1

WHO: World Health Organisation

WHO: World Health Organisation

Dissemination

Diabetes UK Annual Professional Conference, Liverpool, March 2010

- The effects of Garlic upon endothelial function and vascular inflammation in patients with type 2 diabetes at high cardiovascular risk

- Is there an association between markers of inflammation and endothelial function in individuals with type 2 diabetes?

Medical Research Society, Royal College of Physicians, Feb 2010

- The effects of Garlic upon oxidative stress in patients with type 2 diabetes at high cardiovascular risk

University of Portsmouth Postgraduate Research Review, July 2009

- The effects of Garlic upon endothelial function, vascular inflammation, oxidative stress and insulin resistance in patients with type 2 diabetes at high cardiovascular risk.

Association British Clinical Diabetologists Meeting, Harrogate, Nov 2009

- The effects of Garlic upon modifiable cardiovascular risk factors and insulin resistance in patients with type 2 diabetes at high cardiovascular risk

Abstract

Background and aims

Endothelial dysfunction, vascular inflammation and oxidative stress have been integrally linked to the pathogenesis of both type 2 diabetes and cardiovascular disease. Aged Garlic Extract (AGE), a potent antioxidant, has been shown in previous studies to attenuate these novel risk factors in a non-diabetic population.

Aims

This study tested the hypothesis that AGE may improve endothelial function , oxidative stress, vascular inflammation and insulin resistance in high risk cardiovascular subjects with type 2 diabetes (defined as >30% Cardiovascular risk over 10 yrs).

Methods

A double blind, placebo controlled cross-over study was performed in 26 type 2 diabetic patients who received 1200mg of AGE or placebo daily for 4 weeks with a 4 week washout period. Plasma HsCRP was measured as a marker of inflammation. TAOS,GSH/GSSG and LHP were measured as markers of oxidative stress/anti-oxidant defence. Insulin resistance was measured using the HOMA-IR method. Endothelial function was measured using change in the reflective index (RI) post salbutamol using digital photoplethysmography and urinary albumin/creatinine ratio was measured as a biochemical surrogate. Measurements were taken at baseline and after intervention with AGE or placebo.

Results

Of the 26 patients studied (Male 17, Female 9), mean age was 61 ± 8 yrs, HbA1c 7.2 ±1.1%, BP 130/75 ±15.9/9.8 mmHg , total cholesterol 4.2 ±0.81mmol/l, triglyceride 2.11 ± 1.51mmol/l, HDL-cholesterol 1.04 ± 0.29mmol/l. The majority of patients were being treated with metformin (59%), aspirin (50%) and statin (96%) therapy. 36% were treated with an ACEI. There were no changes in these therapies throughout the study.

Treatment with AGE had no significant effect upon the above metabolic parameters including insulin resistance. Systolic blood pressure pre AGE 130± 15mmHg vs post AGE 130± 14mmHg. Total cholesterol pre AGE 4.2±0.9mmol/l vs post AGE 4.2±0.8mmol/l. Triglycerides pre AGE 1.4 IQ range 0.7 mmol/l vs post AGE 1.4 IQ range 0.8 mmol/l. HDL cholesterol pre AGE 1.0 ± 0.3 mmol/l vs post AGE 1.0 ± 0.3mmol/l. In addition, no statistically significant difference was found in plasma HsCRP (pre AGE: median 2.0mg/l, IQ range 0.8-2.7 vs post AGE: median 1.83mg/l, IQ range 1.1-3.2, p =0.89) or urinary albumin/creatinine ratio (pre AGE: 0.55, IQ range 0.4-1.65 vs post AGE: 0.6, IQ range 0.47-1.5, p = 0.43) endothelial function (change in RI pre AGE: 6.5%, IQ range 2.75-11 vs post AGE: 6.5%, IQ range 2.75-13, p = 0.95) with AGE or placebo.

Conclusion

In this group of type 2 diabetic patients at high cardiovascular risk, 4 weeks treatment with AGE did not significantly improve endothelial function, vascular inflammation, oxidative stress or insulin resistance.

Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus describes a collection of metabolic derangements characterised by chronic hyperglycaemia. The hyperglycaemic state may derive from defects in insulin secretion, end organ resistance or a combination of both. Persistent hyperglycaemia and insulin deficiency can cause acute complications with significant mortality or more commonly, chronic complications in both the macro and microvasculature which shorten life expectancy and full health.

1.1.1 Definition

The World Health Organisation (WHO) criteria for diagnosis of diabetes mellitus is a fasting glucose greater than or equal to 7.0mmol/l, a random plasma glucose of greater than or equal to 11.1mmol/l with symptoms or 2 abnormal blood tests, or a plasma glucose of greater than or equal to 11.1mmol/l 2 hours after a 75g oral glucose tolerance test. [1]

Table 1: WHO classification of Diabetes

Type of Diabetes	Main pathological Defect	Percentage of DM cases
Type 1	Autoimmune, Islet cell destruction (insulin deficiency)	5-25%
Type 2	Peripheral insulin resistance	75-95%
Type 3	Insulin deficiency ±resistance (genetic, endocrine causes etc)	<1%
Type 4	Insulin resistance in pregnancy (gestational diabetes)	Approx 3.5% pregnancies[2]

The WHO (World Health Organisation) has classified diabetes into several types (table), the majority of which fall into type 1 and type 2 diabetes. Type 1 is characterized by an immune mediated destruction of pancreatic beta cells which results in an absolute deficiency of insulin secretion. Diagnosis can be aided by measuring circulating antibodies and a reduction in plasma C- peptide levels as a marker of beta cell dysfunction.

The more common Type 2 diabetes represents over 90% of cases and develops as a consequence of end organ resistance to insulin and an inadequate compensatory insulin secretory response.

1.1.2 Epidemiology

It is estimated that 2.4 million people had diabetes in England in 2008 [3], giving a prevalence of 4.67%. A recent study showed an average annual increase in prevalence of diabetes of 4.9% or a 10 year increase of 54%. Most of this rise is due to type 2 diabetes (97%). The cause of this increase has been attributed to increasing obesity and an ageing population [4]. Type 2 diabetes is more common amongst certain ethnic groups and in under privileged areas [5].

The rapid rise in the prevalence of diabetes is not simply an English phenomenon. Worldwide some 246 million people were estimated to have diabetes in 2004, which is predicted to rise to 333 million by 2025 [6].

1.1.3 Mortality & Morbidity in Diabetes

Diabetes continues to inflict a huge cost on individuals and health care systems.

In England people with diabetes aged between 20 and 79 years are approximately twice as likely to die as people without diabetes (Diabetes in England) and it is estimated that there were 26,300 excess deaths from diabetes in 2005. This equates to an approximate reduction of life expectancy of 17 years in a 45 year old male with type 2 diabetes [7, 8].

Cardiovascular and cerebrovascular disease accounts for approximately 80% [3] of mortality in those with diabetes. Mortality rates from heart disease in adults with diabetes are between two to four times higher than in adults without the condition [9, 10] and those with diabetes have a risk of a stroke two to four times higher than those without diabetes. [11].

For those that live with diabetes, chronic complications are a burden. It is estimated within 20 years of diagnosis, nearly all people with Type 1 diabetes and approximately 60% of individuals with Type 2 diabetes will have a degree of diabetic retinopathy [12]. Diabetes is

also the single largest cause of blindness among people of work age in the United Kingdom [13].

About 30% of people with Type 2 diabetes develop overt renal disease [14]. In 2006/07 there were 5015 lower limb amputations in people with diabetes in England [15].

In 2006/7 the total NHS expenditure on the diabetes care programme was £1,043 million and this does not include expenditure in general practice [3].

1.1.4 Pathophysiology

Type 1 diabetes

Type 1 diabetes is caused by a destruction of pancreatic beta cells which is thought to be autoimmune in origin. This is supported histologically by the presence of an inflammatory infiltrate in the Islets of Langerhans in the pancreas and the presence of circulating islet cell antibodies in those with newly diagnosed type 1 diabetes, although these findings are not universal.

The cause of type 1 diabetes remains uncertain. Population clusters suggest a genetic predisposition but there is speculation that this requires an environmental trigger. Several triggers have been implicated such as gut viruses [16] and Vitamin D deficiency [17].

Type 2 diabetes

Type 2 diabetes is thought to originate from progressive beta cell failure on the background of insulin resistance, giving a relative insulin deficiency and subsequent hyperglycaemia.

End organ resistance is characterized by increased hepatic glucose production and impaired glucose handling by skeletal muscle and adipose tissue. This gives a compensatory hyperinsulinaemia. Initially this is adequate to prevent hyperglycaemia, however, as beta cell

function declines the ability to maintain this insulin hypersecretion is lost and hyperglycaemia and type 2 diabetes ensue. (See figure 1)

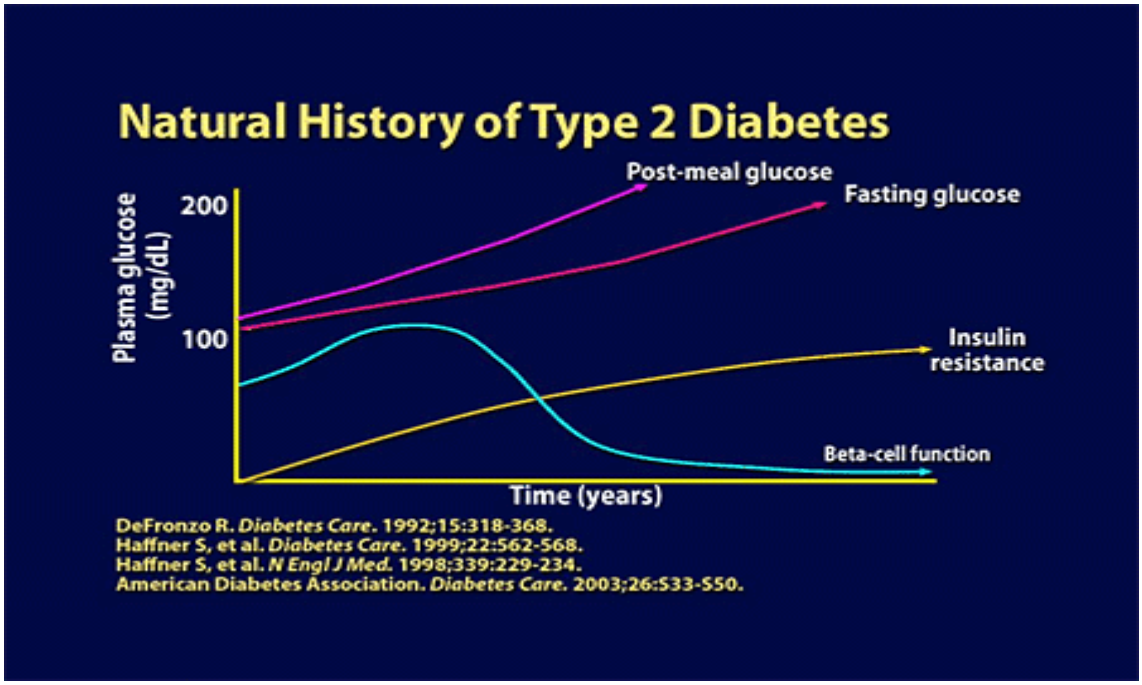


Figure 1: Progression of metabolic decompensation over time with type 2 diabetes. PPG = Post-prandial plasma glucose levels, FPG = Fasting Plasma glucose levels

Normal Physiology of Glucose transport and insulin secretion

Glucose Transporters

There are a variety of facilitative glucose transporters (GLUTs) on the cellular membrane which vary in their affinity and their specificity for sugars and tissue distribution. Initially 5 GLUTs were identified and are the best described, GLUT 1-4 being glucose transporters and GLUT-5 a fructose transporter. GLUTs 6 -14 have since been identified [411]. GLUT distribution in mammalian cells is widespread including liver skeletal muscle, vascular endothelial cells and the blood brain barrier.

GLUT-1 is widely found in human tissues but is particularly abundant in blood brain barrier endothelial cells, fibroblasts and erythrocytes. GLUT-1 is thought to be responsible for the high volume transport of glucose across the blood brain barrier and is generally insensitive to insulin. However, GLUT-1 in adipose tissue has been shown to be insulin sensitive and to translocate to the plasma membrane in the presence of insulin in a manner similar to GLUT-4 in skeletal muscle suggesting a regulatory role [409]. This effect has also been found to be enhanced by the insulin sensitiser metformin which may be a window on its mode of action [410].

GLUT-2 is a low affinity high turnover glucose transporter found in liver cells, small intestine and kidney and may regulate glucose transport in response to nutritional status [408]. It is also the main glucose transporter in pancreatic beta cells. One of the characteristics of the GLUT 2 transporter is that its glucose transport is dependent on the prevailing glucose concentration rather than the expression of the transporter thus it is not saturated by physiological concentrations of glucose [411].

Glucose metabolism and transport in endothelial cells

Glucose is metabolised by endothelial cells mainly by glycolytic processes at normal physiological glucose concentrations. This gives the endothelial cell some degree of protection against low glucose levels [412]. However 98% of all glucose transported across the endothelial cell is not metabolised [413].

There are 2 types of membrane associated carrier proteins responsible for glucose transport in eukaryotic cells; the sodium dependent transporters (SGLT) and the facilitated transporters known as GLUTs. Glucose transport into and across endothelial cells is principally brought about by facilitated glucose transport via GLUT. This has been mainly investigated in the

blood brain barrier in isolated animal cells and the process was found to be sodium independent and related to the concentration gradient across the membrane suggesting the GLUT facilitated transport is the predominant process [414]. Glucose transport out of the endothelial cell also seems to facilitated by GLUT with a distribution ratio of 1:4 between the luminal and abluminal membranes in bovine brain endothelium [415].

GLUT 1 is the most abundant glucose transporter present in endothelial cells and has been extensively studied in the blood brain barrier. GLUT 1 has also been found in human retinal endothelial cells [416], Human placental endothelium [417], Human testis microvessels [418] and Rat aorta endothelium [419].

GLUT 3 has been found on human placental microvessels [420], GLUT 4 on rat brain endothelium [421] and GLUT 5 on human brain endothelial cells, though this did not seem to have an effective glucose transport role [422].

GLUT 2 role in glucose sensing

When extracellular glucose concentrations rise the pancreatic beta cell transports glucose into the cell via the high turnover GLUT 2 transporter where it is then phosphorylated by glucokinase to glucose-6 phosphate. Oxidative and glycolytic metabolism of glucose raises the ATP/ADP ratio which causes depolarisation of the beta cell by the K_{ATP} channel, allows an influx of calcium and thus secretion of insulin. [411]. Cyclic AMP also plays an important role in potentiating this glucose dependent insulin secretion and acts in response to gut hormones (GLP-1, GIP and glucagon) [407].

There is also a central glucose sensing mechanism in the brain which operates in a similar but inverse fashion to the pancreatic beta cell and its output is the release of counter-regulatory hormones to prevent hypoglycaemia. Thus this sensing system protects the glucose dependent

brain tissue from hypoglycaemia. The GLUT transporter involved in this process it not yet known, though GLUT 2 could be involved [433].

GLUT-3 is widespread in neuronal tissue and is likely to be the main transporter for nerves and brain [434].

GLUT-4 is the main insulin sensitive glucose transporter expressed in skeletal muscle, cardiac muscle and adipose tissue [408]. In adipocytes and skeletal muscle cells it is located in perinuclear vesicles which are translocated to the plasma membrane in the presence of insulin [435].

GLUT-5 has been found in intestinal tissues, kidney, skeletal muscle and adipocytes and its distribution is not affected by insulin. GLUT 6 & 8 are glucose transporters but are structurally different to GLUT 1-5 and do not respond to insulin [436]. GLUT 11 is only expressed in human cardiac and skeletal muscle. GLUT 6, 8 and 11 do not seem to be present in vascular endothelium [436].

Insulin Receptors

Insulin receptors are present in virtually all human tissues though the concentration varies between 40 on erythrocytes to 200,000 on hepatocytes and adipose tissue [437].

The insulin receptor compromises 2 α subunits which are extramembraneous and act as the insulin-binding domains. There are also 2 intramembraneous β units. After binding with insulin tyrosine kinase is activated stimulating autophosphorylation in several regions of the β subunit [439]. These changes in the subunit activate the various intracellular insulin receptor substrate proteins which in turn trigger cytosolic post receptor cascades such as PI3K, Akt and MAP kinase which are responsible for the various metabolic actions of insulin within cell types [438].

In skeletal muscle and adipose tissue the main actions of insulin are glucose transport and storage. In these tissues insulin achieves this by stimulating the translocation of the glucose transporter GLUT-4 from perinuclear storage vesicles to the cell membrane [438]. Insulin also slows the rate of internalisation of membrane GLUT-4 receptors therefore increasing the ability to import glucose in the presence of insulin [440]. Furthermore, insulin may aid docking of the GLUT-4 vesicles to the cellular membrane [440]. A similar process to increase cellular glucose transport takes place during exercise but it is not insulin dependent [436].

Interestingly insulin's role in overall body glucose handling is seemingly more important in some tissues than others and may go beyond glucose handling. Although skeletal muscle is responsible for 75% of glucose disposal, knockout mice bred with no insulin receptor in skeletal muscle have normal glucose tolerance [441]. However, mice with knockout of adipose insulin receptors have impaired glucose tolerance which originates from the muscle and liver [442], suggesting that metabolically, adipose tissue glucose handling may be more important. Neural brain specific receptor knockout mice show increased food intake, adiposity and insulin resistance [443].

Normal insulin secretion is a complex process which is influenced by a number of factors. Prevailing blood glucose concentration however is the most important factor influencing insulin release. Insulin is produced in a dose response curve that is sigmoidal in shape, giving large amounts of insulin secretion that correspond to usual post-prandial glucose concentrations. In this way, the pancreatic beta cell is able to maintain post-prandial blood glucose concentration within a tight range whatever the nutritional challenge.

Once activated, insulin is released in a biphasic fashion with an initial rapid peak followed by a second, slowly rising peak. The clinical relevance of the first phase peak is unclear but the second phase response is directly related to the prevailing blood glucose concentration [405].

Furthermore, exposure of the beta cell to chronically raised blood glucose concentrations primes its response to subsequent blood glucose concentrations, giving a greater insulin secretion for each given level of blood glucose. This increase in beta cell insulin sensitivity seems to be brought about by the over expression of hexokinase in relation to glucokinase. Hexokinase is a more potent enzyme giving a greater insulin secretion response.

Insulin is then produced as the proinsulin molecule which is broken down intracellularly to give two chains of active insulin and C peptide which are secreted by exocytosis [403]. The latter can be used as a marker of insulin release. Insulin is secreted into the portal circulation and travels through to the liver where it reduces hepatogluconeogenesis and then enters the peripheral circulation to facilitate glucose disposal, particularly in adipose and skeletal muscle tissue.

Insulin secretion in type 2 Diabetes

Beta cell dysfunction is a key pathological process in the development of type 2 diabetes. In the prediabetes stage insulin secretion is actually enhanced as a compensation for reduced insulin sensitivity. However, in certain predisposed individuals progressive beta cell failure leads eventually to loss of glucose homeostasis.

Beta cells in diabetes show loss of the first phase insulin secretion response, reduced insulin content [18] and reduced insulin mRNA expression [19, 20]. The insulin secretion response to the gut hormones is also attenuated as shown in the incretin effect. Insulin secretion is also deranged, with a higher proportion of the less efficacious proinsulin released [21].

The most important factor in beta cell dysfunction is a gradual loss of beta cell mass. This is supported by studies that have consistently found reduced beta cell mass in the pancreas of individuals with type 2 diabetes (up to 40%) in comparison to non-diabetic control subjects,

even in lean individuals [22,23,24]. Furthermore, *in vivo* studies have shown dynamic parameters of beta cell function to worsen over time and can be used to predict those individuals that progress from a prediabetes state to diabetes [25, 26]. In the Belfast Diet study [27], in individuals whose type 2 diabetes was controlled with diet alone and whose insulin sensitivity remained stable, markers of beta cell function closely predicted failure of diabetes treatment (diabetes progression). This also mirrors the data produced in the landmark study UKPDS which followed patients over 20 years and showed that irrespective of treatment type, plasma glucose concentrations deteriorated with time and this was closely followed by the beta cell function as measured by HOMA-B.

Insulin Resistance and Diabetes

The pathological processes that underpin insulin resistance are unclear, but a major role seems to be played by a delay in insulin delivery to the tissues responsible for glucose disposal [28]. Skeletal muscle accounts for up to 80% of insulin-mediated glucose disposal in euglycaemic-hyperinsulinaemic clamp studies in humans [29] and is the metabolic tissue with the capacity to vary rates of glucose disposal. Insulin stimulated suppression of gluconeogenesis in the liver is a rapid response to physiological (relatively low) levels of insulin [29] and furthermore its rate is similar between healthy and insulin resistant individuals [30], suggesting it does not play an extensive role in insulin resistance.

Insulin delivery to the skeletal muscle is dependent upon muscle blood flow, the available microvascular exchange surface and trans-endothelial transport of insulin in the muscle capillary beds into the myocyte. Once at the myocyte, insulin stimulates storage of glucose as glycogen, thus regulating blood glucose.

Vasoactive actions of insulin

Insulin has vasoactive actions in the metabolic capillary beds as well as metabolic actions and by doing so may facilitate its own delivery to the myocytes. At the level of the resistance vessels insulin can cause vasodilation so increasing overall limb blood flow [31] and can dilate terminal arterioles allowing for capillary bed recruitment, thus increasing the microvascular exchange surface. There have been studies that have showed a strong correlation between total limb blood flow and whole body glucose uptake [32] although these have not always been replicated, particularly in insulin resistant individuals [33]. At the level of the endothelium, insulin can act on the endothelial cell via its insulin receptor and stimulate several intracellular signalling pathways. There are 2 main pathways of post receptor signalling, PI3K and MAPK. (Figure 2)

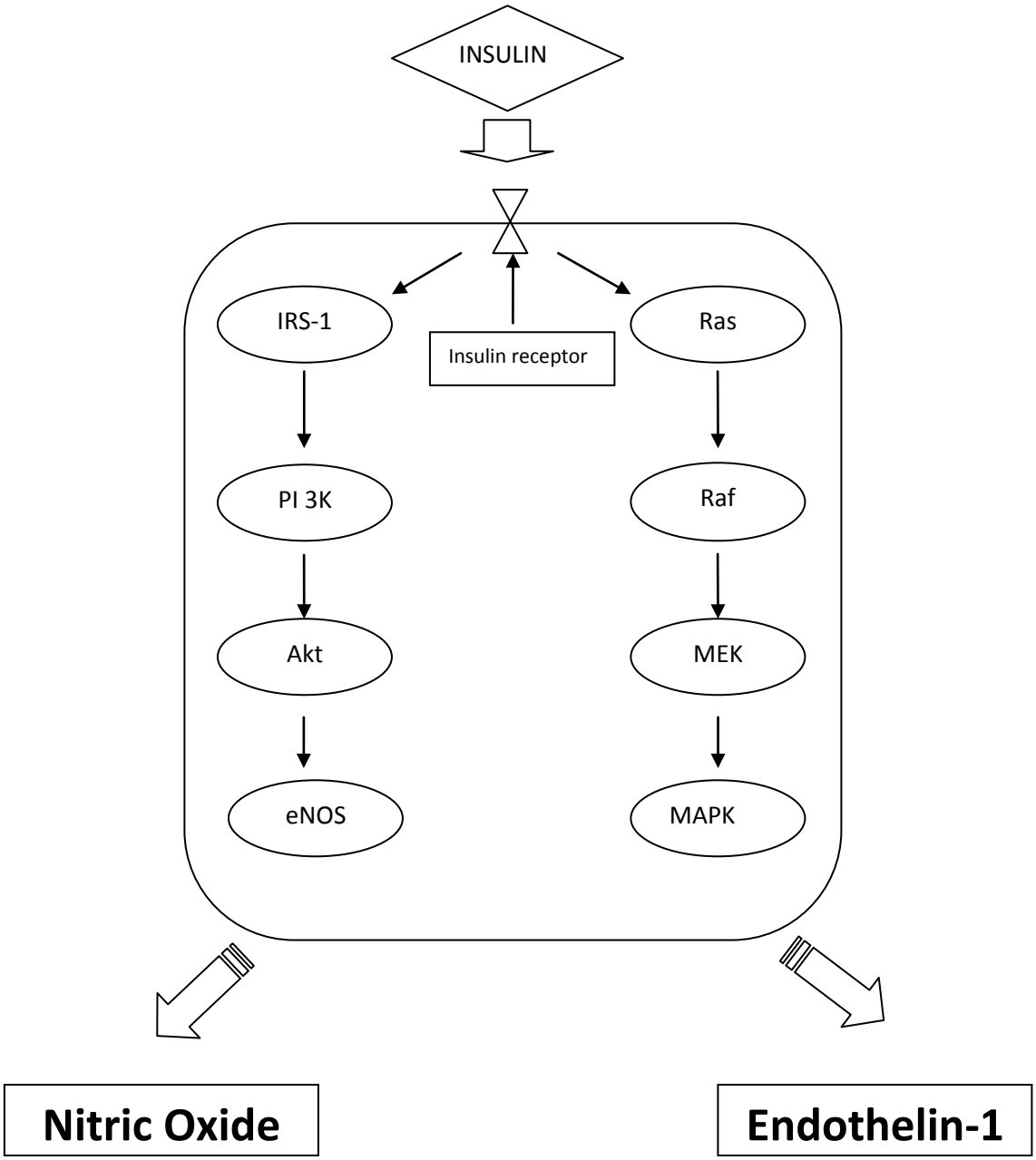


Figure 2. Insulin signalling pathways in the endothelial cell.

The PI 3K pathway is responsible for the metabolic actions of insulin in the skeletal muscle and adipose tissue and also has been shown to stimulate vasodilation via nitric oxide (NO) production in human umbilical vein endothelial cells [404,34]. In contrast, the MAPK pathway induces vasoconstriction via the potent vasoconstrictor endothelin -1. The MAPK

pathway also stimulates intracellular cytokine production and expression of cellular adhesion molecules, both of which are detrimental to endothelial function. In healthy states the balance of these pathways favours vasodilation in response to insulin but in states of insulin resistance, insulin triggers the MAPK pathway and thus vasoconstriction [35] which may be why the insulin-mediated vasodilation phenomenon is absent or blunted in insulin resistant individuals.

Many of the biochemical processes that typify diabetes push the balance of the insulin signalling in the endothelium towards the MAPK pathway. These include vascular inflammation, oxidative stress, hyperinsulinaemia, hyperglycaemia, and increased free fatty acids (FFA) [35]. This results in endothelial dysfunction which can lead to reduced glucose handling by the skeletal muscle and favours pro-atherogenic factors which, in larger vessels, may lead to early atherosclerosis.

Reducing the potential activators of the MAPK pathway may therefore serve to tackle insulin resistance.

1.2 Traditional risk factors for cardiovascular disease

As cardiovascular and cerebrovascular disease are responsible for 80% of diabetes related mortality [36], adequate treatment of the risk factors for vascular disease is given equal priority to blood glucose control in patients with diabetes.

There are long established risk factors for cardio and cerebrovascular disease which can be categorised into reversible and non reversible.

Table 2: Reversible and non reversible risk factors for vascular disease

Reversible	Non Reversible
LDL Cholesterol	Age
Blood pressure	Gender
Glycaemic control	Family History
Smoking	

Traditional risk factors such blood pressure, high serum LDL cholesterol and glycaemic control have been aggressively treated in numerous studies in those with diabetes [37,38] and have shown mortality and morbidity benefits. The land mark study which established the reversible link between glycaemic control and vascular (particularly micro) complications was the UKPDS [37] which ran for 20 years (1977-1997).

Interventions addressing these reversible risk factors have produced reductions in the incidence of myocardial infarction and stroke in diabetes [5]. However, despite this cardiovascular complications of diabetes continue to cause significant morbidity and mortality [10].

1.3 Novel Risk factors

The additional risk of coronary heart disease in people with diabetes cannot be explained in terms of the traditional risk factors mentioned above. [39, 40, 41].

This suggests there are underlying processes prominent in diabetic complications, which as yet, remain untreated by ameliorating traditional risk factors.

Many of the traditional risk factors share similar underlying biochemical processes such as oxidative stress, vascular inflammation and endothelial dysfunction which could explain their contribution to the complications of diabetes. Further consideration shows these processes to be present even before the development of diabetes [42, 43, 44, 45] and they also seem to have a fundamental role in the pathogenesis of diabetic complications. (See figure 3)

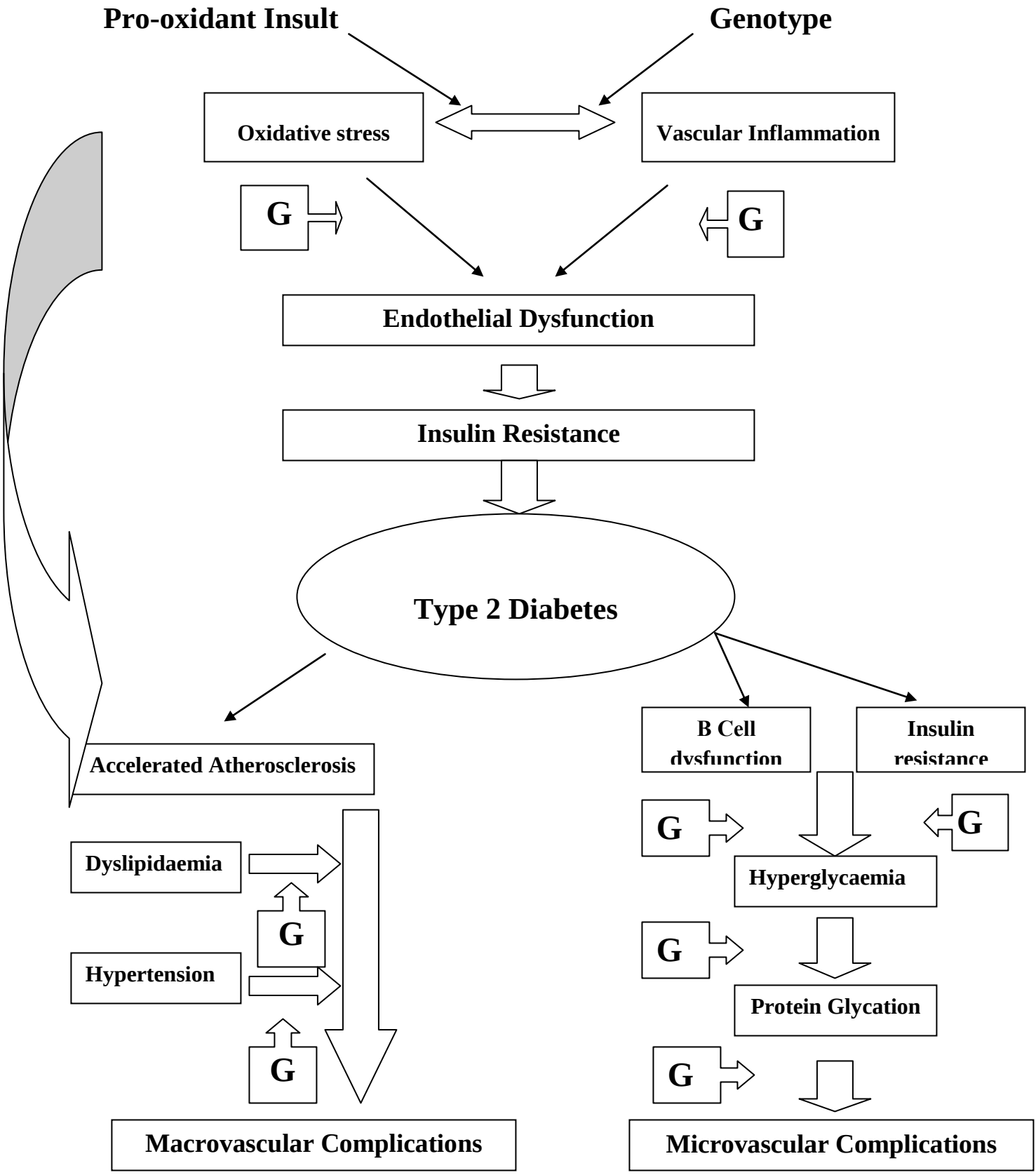


Figure 3. Diagram to show the common soil hypothesis of the inter-relationship between oxidative stress, vascular inflammation and endothelial dysfunction in type 2 diabetes. The points at which garlic may be effective are marked with a **G**

1.3.1 Role of Oxidative Stress in Diabetes

Free radicals are naturally created during biochemical processes, but usually these are metabolised harmlessly. Free radicals are molecules containing one or more unpaired electrons and as such have a high degree of reactivity. Free radicals derived from oxygen represent one of the most important radical species and are termed reactive oxygen species (ROS). Oxidative Stress results from an imbalance in the generation of ROS and the organism's ability to metabolise them. Thus oxidative stress can occur as a result of increased oxygen radical production or reduced antioxidant defence, or both.

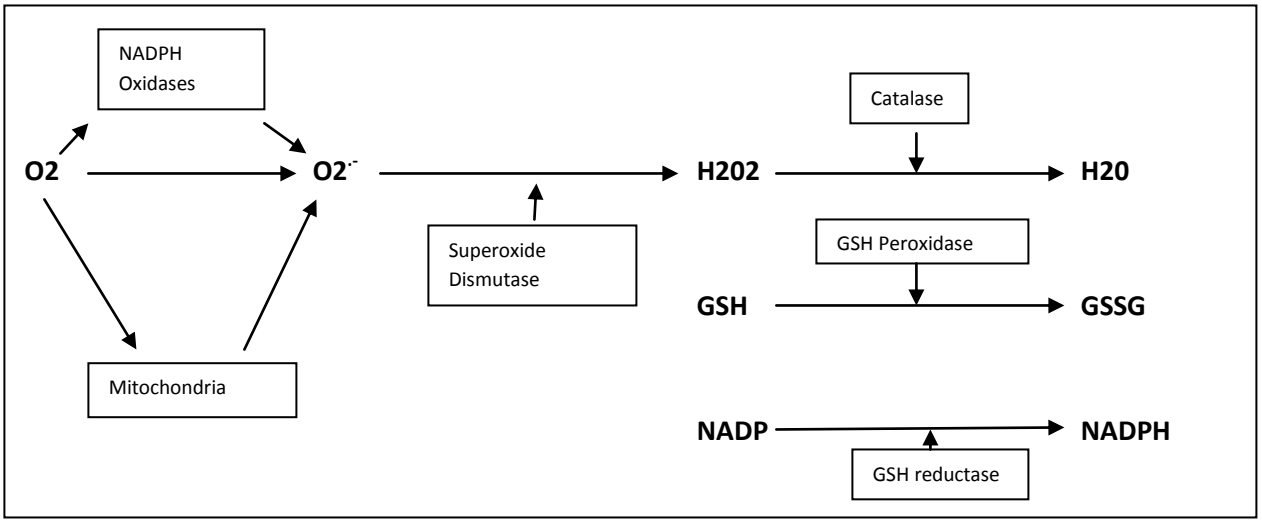


Figure 4: Pathways of reactive oxygen species production and clearance.

Many studies have found markers of oxidative stress are increased in type 2 diabetes. An increase in superoxide levels has been found in hypertensive diabetes patients [46] and direct markers of oxidative stress have also been found to be raised in diabetes [47, 48]. *In vivo* studies suggest endothelial cells cultured in hyperglycaemic media show increased superoxide production and an upregulation of antioxidant genes such as those coding for glutathione peroxidase 1, peroxiredoxin 6 and thioredoxin 2 [49, 50]. Furthermore clinical studies have shown that fluctuations in blood glucose in diabetes act as a strong predictor of oxidative stress levels [51, 52, 53].

During oxidative stress, the resultant excess of free radicals leads to damage of cell components, particularly lipids, proteins and DNA. This can manifest itself in the complications of diabetes as described below. There is also stimulation of numerous intracellular biochemical pathways, many of which perpetuate oxidative stress, therefore initiating a downward spiral.

1.3.2 Potential sources of ROS production in Diabetes

Oxidative Phosphorylation

Between 0.4 and 4% of mitochondrial oxidative phosphorylation in normal cells produces ROS [54] but this figure is increased in diabetes. It has been suggested from animal and *in vitro* studies that hyperglycaemia and a deficiency of uncoupling proteins both act on the mitochondria to increase intracellular ROS production [55, 56].

However, hyperglyceamia induces ROS production even in cells lacking mitochondria [57]. These cells are thought to be subject to glucose autoxidation, a reaction of glucose with molecular oxygen in the presence of transition metals to give hydrogen peroxide, superoxide ions and hydroxyl radicals, one of the most reactive ROS [58]. Chelators of transition metals have been used in diabetic models to prevent superoxide production in these cells [59].

NADPH oxidase

NADPH oxidase is a membrane-associated enzyme present in many cells and in normal cells produces small numbers of ROS but its activity is increased in disease states and diabetes. Superoxide production by NADPH has been shown to be increased in aortic endothelial cells exposed to a high glucose environment [60] and in the renal cortex of diabetic rats [61].

Nitric Oxide Synthase

Another potential source of ROS is Nitric Oxide Synthase (NOS). Nitric oxide is an important modulator of vascular function and its production from L-arginine is catalysed by endothelium derived NOS (eNOS). eNOS is inducible and its production is upregulated in diabetes in human [62] and animal models [63,64]. In the pro-oxidant state of diabetes, the increased levels of NO react with ROS to give peroxynitrite, a highly reactive oxidant [65]. Furthermore, the hyperglycaemic state favours peroxynitrite to react with NOS, which

uncouples the enzyme encouraging it to produce superoxide anions rather than NO [66]. In this way ROS not only quench NO, which has antioxidant properties, but also prevent NOS replenishing NO levels. This lack of NO has been suggested to directly affect endothelial function [67].

Advanced Glycation End Products

Chronic exposure of proteins, DNA and lipids to high glucose causes the spontaneous formation of a glycated Amadori product. These products can undergo further reactions to form advanced glycation end products (AGEP), which are almost universally found in diabetic tissues. This process is accelerated by ROS [68]. AGEPs have been implicated in the formation of diabetic complications, both directly and indirectly [69]. AGEPs can also generate ROS through the Malliard reaction and through their interaction with RAGE receptors on the endothelial cell membrane [70], which increases ROS production by NADPH oxidase [71].

Increased Polyol Pathway

At normal glucose concentrations the activity of aldose reductase in the Polyol Pathway accounts for only a small percentage of glucose metabolism.

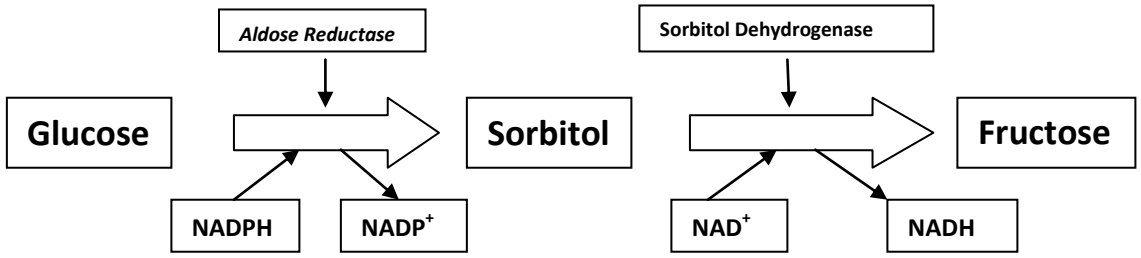


Figure 5: Polyol pathway of glucose metabolism

However, in the hyperglycaemic state much more glucose is put through the pathway leading to increased intracellular sorbitol and reduced NADPH concentrations. It has been postulated that increased intracellular sorbitol may increase oxidative stress and the polyol pathway itself consumes NADPH therefore reducing the cells ability to produce glutathione and so reducing cellular anti oxidant defence [72].

1.3.3 Reduced antioxidant defence in diabetes

Oxidative stress is potentiated in diabetes by the inhibition of antioxidant enzymes and depletion of antioxidants themselves.

Vitamins E and C are major antioxidants in the plasma and the cytosol of many cells and they act synergistically. The amounts of these vitamins in the plasma has been shown to be reduced in diabetes [73,74] and are inversely proportional to the presence of diabetic complications [75] and the duration of type 2 diabetes [73].

Glutathione (GSH) is the principal thiol intracellular antioxidant and as such the relative amount of intracellular reduced and oxidised GSH is a measure of the cells redox status. Decreased intracellular concentrations of reduced GSH have been found in numerous cell types in diabetes [73, 75, 76] and has been found to be inversely proportional to severity of diabetic complications [77] and the duration of diabetes [73] and has shown a strong negative correlation with plasma HbA1c levels [78].

The principal antioxidant enzymes involved in antioxidant defence are Glutathione peroxidase, Catalase and Superoxide Dismutase [79, 58]. The activity of these enzymes has been shown to be reduced in diabetes [80, 81] and supplementations of their actions have been therapeutic targets with variable success [82, 83, 84, 85].

1.3.4 Oxidative stress and complications of diabetes

There is a large body of evidence suggesting a link between oxidative stress and both micro and macrovascular diabetic complications. Plasma concentrations of antioxidants are reduced in those with diabetic complications [47, 86, 75]. The risk of diabetic complications is not distributed equally among diabetic patients with similar hyperglycaemic exposure. Some individuals seem to have innate protection against developing complications. This could be explained by differences in the genetically determined cellular redox status of individuals [87]. Similarly, the redox status of particular organs make them more susceptible to hyperglycaemic damage, including the beta cell which has a physiologically low level of oxidant defence [88]. Increased oxidative stress also causes increased vascular inflammation and consequent endothelial dysfunction suggesting oxidative status could play a pivotal role in the pathogenesis of diabetic complications and even diabetes per se. [89, 58, 90].

Much of the evidence for a role of oxidative stress in microvascular complications is *in vitro* or indirect. Serum AGEP concentrations are increased in diabetic patients with retinopathy and the concentrations are correlated positively with the severity of the retinopathy [91, 92]. Use of AGEP inhibitors in diabetic rats reduced pericyte apoptosis and abnormal endothelial cell proliferation, both early changes in the development of diabetic retinopathy [93].

AGEP formation has been implicated in both neuropathy and nephropathy and the use of AGEP inhibitors has prevented early changes in both these complications [94, 95, 96, 93]. Antioxidants such as N acetyl cysteine [97], α-lipoic acid [98] and L propyl carnithine [99] have all had beneficial effects in animal models.

Macrovascular complications, principally atherosclerosis, start even before the onset of diabetes and there is strong evidence that that oxidative stress plays a role in this process,

often via its effect upon endothelial function. Some clinically used medications have been shown to have intracellular antioxidant properties such as TZDs (thiazolidinediones) [100], Statins [101], ACEI [102] and Beta Blockers [103] and there is evidence that all of these improve cardiovascular outcome in diabetes [104, 105, 38, 106].

Epidemiological studies have suggested that individuals with a high intake of antioxidant vitamins have a lower than average risk of cardiovascular disease [393, 394, 395]. However, large studies using antioxidant supplementation have been disappointing in improving cardiovascular outcomes [107, 108, 109, 38, 110, 111, 112]. This may be due to single antioxidants being used and, given that naturally occurring antioxidants often work synergistically, it may be that using a cocktail of antioxidants could give a more positive finding.

1.3.5 Role of vascular inflammation in diabetes

The aetiology of diabetes has been suggested to be linked to a state of low-grade systemic inflammation [113,114, 115, 116]. This has been supported by numerous studies showing an increase in inflammatory markers in those with diabetes [117,118, 119] and epidemiological data suggests chronically raised inflammatory markers can predict those who will go on to develop diabetes [113]. High dose salicylates and anti-inflammatories have also been used to successfully lower glucose concentrations [120, 121].

The source of this inflammatory state in diabetes is probably multi-factorial. Oxidative stress can trigger intracellular signalling pathways, which stimulate production of a proinflammatory milieu including NF-κB [122, 123], JNK/SAPK [124] and Protein Kinase C [125, 126, 127]. These stimulate inflammation by gene transcription [128], growth factor [129] and cytokine production and may induce insulin resistance by disrupting intracellular insulin signalling

pathways [130, 131]. Furthermore, hyperglycaemia itself is pro inflammatory [127] and central obesity, which often coexists with diabetes, is thought to induce inflammation by reducing adiponectin (an insulin sensitizer) [132, 133] and production of inflammatory cytokines [134, 113].

Markers of inflammation such as high sensitivity CRP (HsCRP), interleukin 6 (IL-6) [135] and tumour necrosis factor α (TNF-α) have all been positively correlated with insulin resistance in people without diabetes [135, 136] and have been found to be high in patients with diabetes [137, 113] and have been associated with diabetes complications [138]. This has been supported by animal studies suggesting IL-6 modifies glucose stimulated insulin release from pancreatic β cells in rats and reduces insulin-stimulated hepatocyte glycogen production *in vitro* [139]. Suggested consequences of this chronic inflammatory process include the effect of cytokines on the liver where they increase the synthesis of acute phase proteins [140, 141]. Acute phase proteins have been shown to be raised in diabetes [142, 143, 144] and, by means of their actions in the liver, give the typical atherogenic lipid profile of type 2 diabetes [145]. Furthermore, they have been directly implicated in the pathogenesis of atherosclerotic plaques [117, 146,147,148]. Insulin is also noted to reduce acute phase protein synthesis and the presence of insulin resistance reduces this effect [149].

1.3.6 Vascular inflammation and complications of diabetes

Atherosclerosis, as with diabetes, has been shown to be an inflammation driven process [150]. Elevated HsCRP, TNF α, IL-6, ICAM-1 and fibrinogen have been found to be independent predictors of cardiovascular risk [148, 151, 152], Indeed, HsCRP has been shown to be a more potent predictor of cardiovascular risk than LDL cholesterol [153]. Many of these inflammatory markers have also been linked to insulin resistance suggesting a common basis [154, 155, 156]. The endothelium responds to both hyperglycaemia and hyperlipidaemia with

an inflammatory response and this pushes the normal endothelial equilibrium in a pro-atherogenic direction [157, 158].

Several well established pharmaceutical agents have been found to have anti-inflammatory properties. Large clinical trials with agents such as statins (cholesterol lowering agent) and angiotensin converting enzyme inhibitors (ACEIs – anti-hypertensives) and TZDs (hypoglycaemic agents), have shown reductions in levels of circulating proinflammatory markers [159, 160, 161], reductions in insulin resistance [162, 163] and reduced LDL levels [164], and may even delay the development of diabetes in high risk individuals [165].

1.3.7 Role of endothelial dysfunction in diabetes

Definition

The endothelium is a single cell layer lining the blood vessels, which acts as the first line of vascular defence. The endothelium maintains vessel function by regulation of tone, inflammation, growth and homeostasis. It is a metabolically active organ whose surface area would cover the surface of a football pitch and whose weight would be similar to that of the liver [166]. The endothelium modulates vascular permeability and maintains the balance of coagulant and fibrinolytic mediators. It also has an anti-inflammatory role within the vessel wall including control of leucocyte adhesion and migration. It also plays a vital role in the actions of other cells in the vasculature such as smooth muscle, platelets, leucocytes and large artery macrophages.

Endothelial dysfunction is the inability of the endothelium to maintain vascular function and is multifaceted.

Endothelial cells exposed to chronic high glucose concentrations exhibit increased cell apoptosis [167], increased extracellular matrix production and increased pro-coagulant protein

production [168]. Endothelial function can also be impaired by obesity, hypertension, increased triglyceride, reduced HDL, hyperinsulinaemia and low-grade inflammation, many of which are present in type 2 diabetes [169]. However, endothelial dysfunction is also present in individuals before the diagnosis of diabetes, [170] leading to the speculation that endothelial dysfunction may play an important role in the insulin resistance that typifies type 2 diabetes.

1.3.8 Endothelial dysfunction as a source of insulin resistance

Insulin resistance is the pathological process that underpins type 2 diabetes and many associated conditions such as obesity, hypertension and hyperlipidaemia. In an insulin-sensitive individual insulin acts as a vasodilator in metabolically active tissues such as adipose tissue, liver and skeletal muscle. After a meal, physiological levels of insulin recruit capillary beds and insulin mediated vasodilation increases blood volume in skeletal muscle and thus increases glucose and insulin delivery to the appropriate tissues maximizing glucose disposal.

It has been suggested that endothelial function has different effects in different vascular beds [79]. Large vessel endothelial dysfunction is a precursor of the accelerated atherosclerosis seen in those with diabetes [171, 172]. However, endothelial dysfunction in metabolically active capillary beds, such as skeletal muscle, may play an important role in insulin action and glucose handling [173, 174] (see vasoactive actions of insulin).

The endothelium is a target for insulin action and is vital for insulin delivery to the tissues. Insulin is a vasodilator and its presence increases blood flow in skeletal muscle capillary beds, even when overall limb blood flow is not affected [175]. In insulin resistant skeletal muscle, capillarisation has been proved to be reduced [176]. Both of those pathophysiologies would contribute to reduced glucose disposal and increase insulin resistance. Mice that are homozygous null for the eNOS gene are also found to be insulin resistant [177]. The

endothelium also acts as a barrier to insulin reaching the interstitium [174] and the transport of this molecule across the endothelium is delayed in insulin resistant individuals [173]. This seems to be particularly important in fluctuations of blood glucose suggesting that a delay in insulin delivery to the glucose handling tissues may be important in controlling hyperglycaemia.

1.3.9 Endothelial dysfunction and complications of diabetes

In diabetic retinopathy there is hyperpermeability and thickening of the basement membrane and eventually microthrombosis of the retinal microvasculature [178]. Here there is evidence of increased cell apoptosis [179], increased growth factors [180], and increased endothelin-1 secretion [181] all suggesting underlying endothelial dysfunction.

There is also evidence of microvascular endothelial dysfunction in diabetic nephropathy, again with an increase of apoptosis [97], increased growth factors [182] and impaired extracellular matrix turnover [183]. These give the hallmark pathological changes of diabetic nephropathy of basement membrane thickening and mesangial cell expansion.

Similar changes have also been reported in the microvasculature surrounding nerves involved in diabetic neuropathy [184] with a suggestion of endoneurial hypoperfusion. However, the link between endothelial dysfunction and diabetic neuropathy is less clear.

Atherosclerosis is a disease of the large vessels although endothelial dysfunction is thought to be a pivotal trigger in its pathogenesis [185, 186]. However, generalised endothelial dysfunction may play a part at distant sites. The small vessels represent a much larger surface area and synthetic capacity than the large vessels and changes at this level could influence pathology elsewhere [187].

Loss of endothelial lipoprotein lipase action in the small vessels increases the exposure of large vessels to pro-atherogenic lipid particles [188] and these small LDL particles are also toxic to endothelial cells [189] propagating endothelial dysfunction. Changes in the extracellular matrix in the large vessels as a result of endothelial dysfunction increase retention of these particles, [190] encouraging atherosclerosis.

It has also been suggested that endothelial dysfunction within adipose tissue prevents adequate insulin delivery and thus increases lipolysis and subsequent release of free fatty acids (FFA). FFA's have been shown to impair endothelial dependant vasodilation [191], increase insulin resistance [192] and effect changes in the extracellular matrix that promote LDL retention [193].

It has also been suggested that loss of endothelium dependant vasodilation of the small vessels may contribute to the systemic hypertension [187] that forms part of the common pathway to the pathogenesis of atherosclerosis in type 2 diabetes.

1.4 The Common Soil Hypothesis of diabetes; The relationship between insulin resistance, endothelial function, oxidative stress and vascular inflammation

The precise pathophysiology of the insulin resistance and beta cell dysfunction that underlies type 2 diabetes is uncertain. Furthermore, the precise pathophysiology of the vascular disease that makes up the majority of diabetes mortality is also unclear and is incompletely addressed by treatment of traditional risk factors. It is clear that diabetes and other pro-atherogenic states, such as hypertension and obesity, share an upscaling of oxidative stress, endothelial dysfunction and inflammation. Increasingly it is being suggested that these processes are inter-related and synergistic [51, 156, 194,195,196,197, 198]. (See Figure 3)

In individuals with a genotype which impairs their ability to cope with such biochemical stresses, a pro-oxidant and pro-inflammatory insult (usually obesity) increases the cells exposure to oxidative stress and an increased inflammatory load. The result of this insult is permanent damage to DNA, proteins and lipids and triggering of further signalling pathways which perpetuate the oxidative stress and inflammation and create a cycle which propagates the initial biochemical insult [195,199].

In certain, more susceptible tissues, this oxidative stress and inflammatory load, results in dysfunction and loss of homeostasis [200,201,202]. These tissues include the pancreas and the vascular endothelium.

The pancreatic beta cells are low in indigenous oxidant defence enzymes such as SOD, glutathione peroxidase and catalase, as are endothelial cells. In the face of oxidative stress this predisposes to beta cell failure [128, 203, 204,205], a preliminary pathological stage of diabetes.

In the endothelium, oxidative stress and excess inflammation result in endothelial dysfunction. In the metabolic capillary beds such as skeletal muscle, this can affect delivery of insulin to metabolic tissues and thus blunt its glucose handling effects and subsequently create insulin resistance [206, 207, 208]. Moreover, oxidative stress and inflammation can interrupt insulin dependent intracellular signalling pathways, directly affecting the ability of insulin to handle glucose in skeletal muscle [208].Together these processes eventually lead to persistent hyperglycaemia which in itself perpetuates oxidative stress and inflammation [201] and so the cycle continues.

In the larger vessels, adequate endothelial function is necessary to protect against atherosclerosis and therefore the synergistic interaction of oxidative stress and inflammation

create endothelial dysfunction early in the course of diabetes and subsequently predispose an individual to early vascular complications.

If these processes are inter-related and fundamental to the pathophysiology and vascular behaviour in diabetes then they would make attractive targets for potential intervention. One potential therapeutic strategy would be the use of garlic given that there is evidence suggesting garlic may have potent anti-inflammatory and anti-oxidant properties.

1.5 Garlic

1.5.1 Medicinal history of garlic

Garlic has been used as a medicinal product throughout antiquity and is currently one of the most common alternative medicines taken by those with diabetes [209]. Use of garlic for infections and "treating growths" was documented by the ancient Egyptians in the period 1550-200BC [210]. The Chinese also used garlic for respiratory and digestive complaints in a similar era [211]. Later the ancient Indians and Ancient Greeks both recommended garlic for heart disease and arthritis and Hippocrates himself, the father of medicine, used garlic for pulmonary complaints and as a purgative. The Romans used garlic for "cleansing the arteries" [212]. It is intriguing that cultures that rarely came in to contact, shared similar views on garlic as a medicinal product. Garlic has been found to have anticancer, antimicrobial, antifungal and cardiovascular protective effects.

More recently, medicinal properties of garlic have been exposed to scientific scrutiny with mixed results [213, 214, 215, 216]. However, there is a growing body of evidence, *in vitro* and *in vivo*, suggesting garlic has antioxidant and anti-inflammatory properties [217, 218, 219, 220, 221].

1.5.2 Active ingredients and metabolism of garlic

Chemistry/Preparations

Garlic is a member of the allium family along with onions and shallots. The main ingredients within garlic that are thought to be responsible for its antioxidant effects are the sulphur compounds, though steroid saponins have also been implicated [222].

Garlic in its natural state contains very few of the active sulphur compounds that are found in its prepared state. Instead, it contains storage compounds termed S allyl L cysteine sulphoxides, mainly in the form of alliin. Upon cutting or maceration, enzymes (alliinase) within garlic tissue break these compounds down to volatile organosulphur products which give garlic the familiar smell and taste. The principal initial product of alliin is allicin.

The first products to be produced upon activation of alliinase are sulphenic acids and thiosulphates. These are the precursors to many other compounds, the precise nature and balance of which are determined by the environmental conditions. Block et al [223] characterised the origin of these compounds as

1. Chemicals derived from cutting or maceration of garlic at room temperature
2. Decomposition products derived from thiosulphates at room temperature. These change with time.
3. Oil components – chemicals derived from vigorous preparation techniques such as steam distillation

Up to 50 of these products have so far been identified [224]. It is these products that have been implicated as the bioactive components of garlic.

Metabolism of garlic

Despite a multitude of studies of studies examining the *in vivo* actions of garlic, little is known about the metabolism of garlic or the component organosulphur compounds.

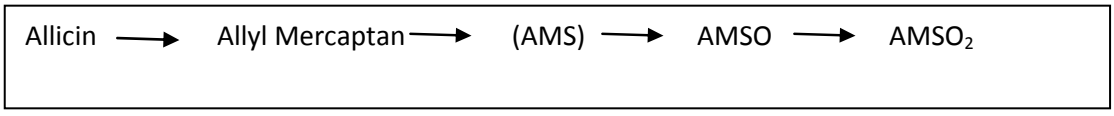
Alliin itself is not metabolised upon ingestion [225], presumably due to the inactivation of alliinase in the acidic environment of the stomach [226]. Alliinase activity is also reduced by cooking [227].

Allicin when added to blood is quickly converted to allyl mercaptan, which is not present in blood or urine in subjects that have ingested garlic [228], suggesting further metabolism takes place. This also suggests that allicin plays no meaningful role in any medicinal actions of garlic. [222,229].

The main volatile metabolite detected after ingestion of garlic is allyl methyl sulphide (AMS) [230], detected on the exhaled breath of subjects. The amount of AMS detected has also been found to relate to the amount of raw garlic ingested [228].

Davenport et al [231] and Germain [232] found oxidised forms of AMS in the urine; allyl methyl sulphoxides (AMSO) and allyl methyl sulphone ($AMSO_2$) suggesting the pathway of metabolism may be as suggested in figure 6.

Figure 6: Proposed metabolism of Allicin [Adapted from 229].



A comprehensive understanding of the metabolism of garlic in humans remains elusive. Many of these studies were performed on rodents and as such should be extrapolated to human

subjects with caution. Those conducted in human subjects concentrated on breath studies which will only detect volatile metabolites. Analysis of blood metabolites may be a more informative and complete measure of bioavailability.

Different preparations of garlic

There are numerous preparations of garlic available and due to the nature of their preparation the constituent compounds can be numerous and vary widely (figure 7). This makes interpretation of studies using garlic compounds challenging since they may be comparing different constituent compounds.

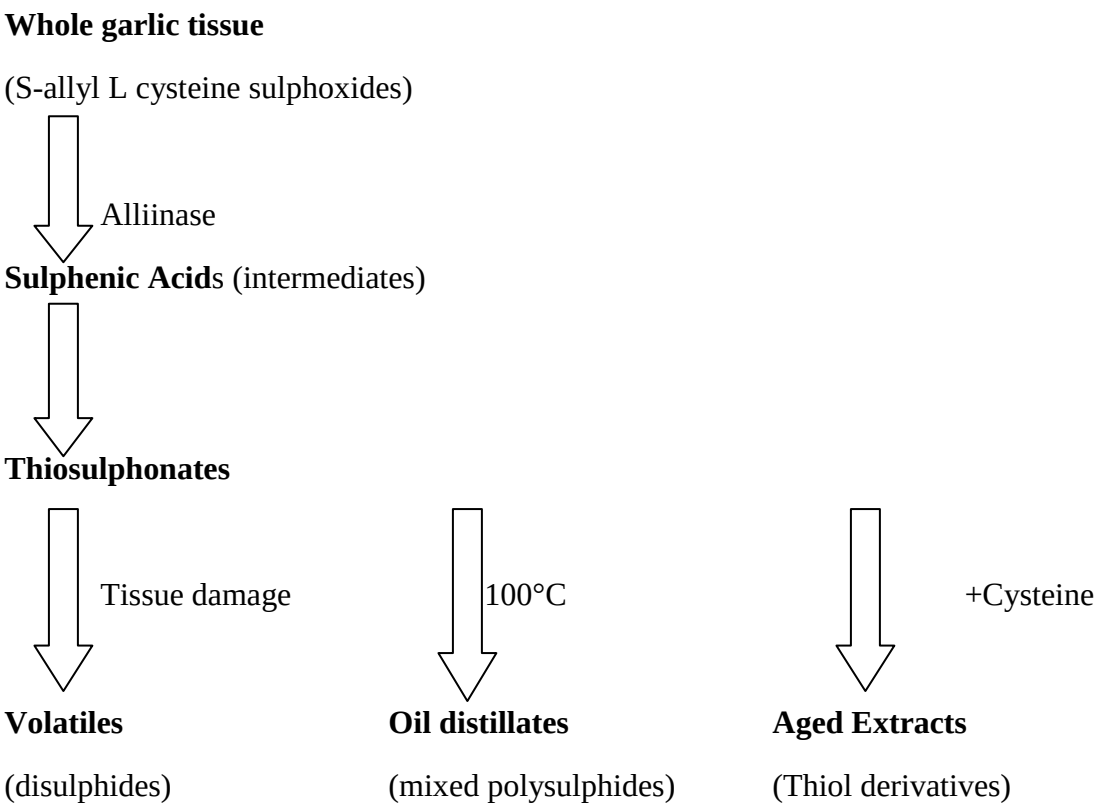


Figure 7. Processing of garlic gives different end products (adapted from [222] and [224])

Table 3: Major constituents of widely used garlic preparations

Garlic Preparation	Major constituents
Garlic Essential oil	Oil soluble sulphur compounds; diallyl suphides (DAS), diallyl disulphide (DADS), diallyl trisulfide (DATS), 1,2-epithiopropane, vinyldithiins.
Garlic Macerate oil	Oil soluble sulphur compounds; diallyl suphides (DAS), diallyl disulphide (DADS), diallyl trisulfide (DATS), 1,2-epithiopropane, vinyldithiins
Garlic Powder	g-glutamyl-S-allyl-L-cysteines, S-allyl-L-cysteine sulphoxides, small amounts DAS, DADS
Garlic Extracts	S-Allyl Cysteine (SAC), S-Allyl mercaptocysteine (SAMC), small amounts DAS, DADS

Many products are processed with a view to maximising the bioavailability of the active ingredients and reducing the harsh taste and smell. However, the true bioactive compounds within garlic remain unclear and controversial.

Garlic oil contains only oil soluble sulphur compounds but not water soluble compounds or allicin.

Garlic powder is produced from cloves of garlic which are pulverised into powder. Garlic powder contains alliin and small amounts of oil soluble sulphur compounds, very similar to that of raw garlic.

Distilled garlic is prepared by steam distilling whole garlic cloves ground in water. This gives small amounts of essential oils which are then mixed with soya oil. The main active ingredients are oil-soluble compounds such as diallyl sulphide (DAS) and diallyl disulfide (DADS)

Aged Garlic Extract (AGE) is prepared by storing sliced raw garlic in 15-20% ethanol solution for 20 months at room temperature. Almost all of the allicin is degraded over this period. The extraction process is thought to encourage the development of stable, bioavailable products such as S allylcysteine (SAC), S allyl mercaptocysteine (SAMC), allixin and selenium [233] all of which have been shown to exhibit anti oxidant properties in their own right [234] and have been suggested to be more potent than fresh garlic [235,236]. There are also small amounts of oil soluble compounds (DAS, DADS) and saponins [233].

The largest constituent of AGE is SAC followed by SAMC. Studies on the metabolism of these substances suggest they have shown that SAC is present in blood after ingestion and its concentration is related to the amount ingested [237].

1.5.3 Garlic as a therapeutic agent

Any research study that aims to study the effects of garlic needs to recognise the uncertainties surrounding the bioactive constituents of garlic-containing preparations. Therefore garlic preparations studied should satisfy the following criteria:

1. Well standardised constituents
2. Evidence of *in vitro* antioxidant efficacy
3. Evidence of antioxidant effect *in vivo* in similar population to our own
4. Evidence of a feasible mechanism of action, with identified bioactive, bioavailable constituents
5. Evidence of safety

AGE represents the preparation with the largest body of evidence satisfying the above criteria [222, 233, 235, 237, 246, 247]. The evidence was largely restricted to cell lines and in-vitro studies or small *in vivo* studies and often performed by the manufacturers' own employees. However, this criticism can equally be levelled at all other preparations of garlic.

AGE is prepared by storing sliced raw garlic in 15-20% ethanol solution for 20 months at room temperature. The extraction process maximises the content of stable water soluble organosulphur compounds, largest amongst which are S allyl cysteine (SAC) and S allyl mercaptocysteine (SAMC).This process, according to the manufacturers, reduces the harsh taste and odour of garlic and gives a standardised preparation (205g/l dry weight of the active ingredients) The extract is produced by Wakunaga Pharmaceuticals, California, USA and marketed as Kyolic.

Its constituents have been identified [233] and the pharmacokinetics and bioavailability investigated [237]. AGE and its constituents have been shown to have antioxidant effects *in*

vitro [238, 239, 240, 241] and *in vivo* [242, 243]. It has also shown anti-inflammatory effects *in vitro* [244] and its safety has been investigated [245, 246]. There is also some evidence that its antioxidant capabilities are greater than other garlic preparations but this is based on unpublished data from the manufacturer [247].

1.5.4 Garlic and oxidative stress

There is *in vitro* evidence that garlic reduces oxidative stress, both in relevant cell lines [248,249,250] and in diabetic animal models [251, 252].

Lee et al [251] used db/db (+/+) C57BL/KsL mice, a genetically modified diabetic model that mimics the insulin resistance, hyperinsulinaemia and pancreatic β cell failure seen in human diabetes. This study investigated the effects of diet supplementation of aged black garlic on lipid peroxidation (TBARS) and the effect on antioxidant enzyme activity in the liver and found that Aged black garlic reduced hepatic TBARS and increased antioxidant enzyme activity. However, this study used very young mice (3 weeks) and these models do not reach the peak of hyperglycaemia until 3-4 months [253]. Moreover, measuring antioxidant activity in the liver is likely to only be relevant to fasting hyperglycaemia. This study also employed high doses of garlic extract (5% of diet) and for a relatively long period of time (7 weeks). Kanth et al [252] investigated the effect of garlic on oxidative stress markers (glutathione and antioxidant enzymes) in hepatic and intestinal tissues in a streptozocin induced diabetes model in Wistar rats and found garlic extract to be protective against oxidative stress. This study used large doses (500mg/kg), investigated tissue specific antioxidant concentrations rather than systemic concentrations and the streptozocin diabetic rat model is more synonymous with type 1 than type 2 diabetes in humans.

Garlic has been shown to have beneficial effects upon markers of oxidative stress in human subjects [200, 254, 255, 256] but these studies have been small (10-50 subjects), of varying duration (2weeks -6 months) and have used numerous preparations and doses of garlic and different measurements of oxidative stress. They have also all used very different cohorts of subjects, which may derive their oxidative stress from different mechanisms. Furthermore, none of these studies included subjects with diabetes.

AGE has been shown to be an effective antioxidant both *in vitro* and in clinical studies [237, 239, 241]. Thus, garlic may have the potential to be an effective anti-oxidant agent in diabetes.

AGE has been shown to have free radical scavenging properties and also to increase antioxidant defence [239] *in vitro*. These effects have been shown in endothelial cells [248], liver cells [249] and erythrocytes [250], all of which are potential targets of oxidative stress in diabetes. However, caution must be exercised when extrapolating these results to human subjects since the bioavailability of AGE may be very different *in vivo*, as may the oxidative process that is being replicated in the laboratory.

AGE has also been found to have antioxidant properties in a small number of clinical studies. Dillon et al [243] compared the antioxidant action of AGE in 20 smokers and non smokers without diabetes. This study used 2 weeks treatment with 5ml/day of AGE and then had a 2 week washout period. In the smoking group, 2 weeks of treatment with AGE reduced urinary and plasma levels of the prostaglandin F2-isoprostane 8-iso-prostaglandin, a marker of lipid peroxidation, by 48% and 35% respectively. The urinary and plasma levels of F2-isoprostane 8-iso-prostaglandin were also reduced in the non-smoking group and the levels in both groups returned to baseline after a 2 week washout period. These subjects were otherwise healthy and not taking any medication.

1.5.5 Garlic and endothelial function

There is limited data examining the effect of garlic on endothelial function. Garlic has successfully improved nitric oxide mediated vasodilation in isolated rat pulmonary arteries [257] and in the isolated aortic rings in rats with streptozocin-induced diabetes [258]. Kim-Park et al [257] exposed isolated rat pulmonary arteries to garlic extract in experimental circumstances. This study found that garlic had the ability to protect against hypoxia induced vasoconstriction and the effects seemed to be endothelium-dependent. Baluchnejadmojarad et al [258] showed similar results in isolated aortic rings in a rodent diabetic model. However, these results should be considered with caution. Both these studies used isolated tissue which may react very differently *in vitro* and used hypoxic vasoconstriction as a marker of vascular reactivity which is a different process than that involved in diabetic vascular reactivity. Furthermore, Kim-Park's study treated the pulmonary arteries directly with large doses of garlic, a process which is unlikely to happen *in vivo*. Baluchnejadmojarad's study used a streptozocin-induced diabetes model and the garlic extract was given to the rats before aorta isolation, making this more physiological, although the garlic extract was given via the peritoneum which is not physiological. However, the streptozocin model is suitable for investigating the effects of hyperglycaemia only and may not suitably mimic the pathological processes in type 2 diabetes.

Not all rodent studies have been supportive of garlic's potential endothelial action. Sun et al [259] fed rats relatively large doses of raw garlic, boiled garlic and aged garlic and then investigated the vascular reactivity of isolated coronary arteries to vasoconstrictors or relaxants. This study only showed benefit from raw garlic and the authors suggest this may be due to the lack of allicin content in the other garlic preparations. However, it is known allicin

is not bioavailable in humans after ingestion of garlic. Many of the limitations mentioned previously are also applicable to this study.

Clinical studies using AGE to improve the vasomotor element of endothelial function have been limited in number, though AGE remains the most investigated garlic preparation. Weiss et al [260] studied flow mediated dilatation in the forearm after acute homocysteinaemia. After treatment with AGE, a 66% increase in forearm blood flow was detected.

Budoff et al [242] also studied the effect of AGE upon endothelial function in 65 subjects with moderate cardiovascular risk over 1 year. Williams et al [261] studied the effect of AGE on endothelial function in 15 subjects with coronary artery disease over 2 weeks. Both of these studies showed improvement in endothelial function following treatment with AGE.

1.5.6 Garlic and inflammation

Inflammation has been established as a key process in the development and progression of atherosclerosis. Normal endothelial cells resist leucocyte adhesion, however once inflamed (secondary to oxidative stress) endothelial cells express adhesion molecules (such as Vascular Cell Adhesion Molecule, VCAM-1 and Intercellular adhesion molecule, ICAM- 1). Intima-derived chemo attractants (such as Monocyte chemo attractant protein-1, MCP-1) then facilitate the movement of macrophages and T cells in to the intima. These cells stimulate a local inflammatory response with an increase in cytokines such as interleukin 6, CRP and TNF- α.

This inflammatory process stimulates proliferation of vascular smooth muscle cells and the production of proteases which progress the development of the atherosclerotic plaque and eventually can lead to its rupture [262].

Many of the molecules involved in this process have been used as biomarkers for atherosclerotic progression. Indeed, several of these markers (particularly CRP) have been shown to have strong predictive value for future coronary artery disease, even in apparently healthy individuals [263, 264, 265]. However, it is questioned whether these are markers of systemic inflammation or mediators of the atherosclerotic process [266] and it is uncertain whether reduction of these circulating molecules will reap clinical benefits. These molecules are not specific to vascular inflammation and circulate in plasma in response to many causes of inflammation [267]. CRP, for example, is mainly produced in the liver following cytokine stimulation and therefore is a systemic acute phase protein. *In vitro* evidence of the direct role of CRP has been inconsistent and has often used supraphysiological doses [268]. However, more recent *in vivo* studies have shown CRP to be produced more locally in the smooth muscle cells underlying atherosclerotic plaques [269] and endothelial cells of normal and diseased coronary arteries [270]. Furthermore, CRP levels have also been associated with fragility of atherosclerotic plaques [271].

Ide et al studied the effect of AGE on LDL oxidation but also on TNF-α-induced NF κB activation, a key precursor to the intracellular inflammatory process. Ide suggested endothelial cells cultured in S allylcysteine (a key antioxidant in AGE) had 60% less NF κβ activation than those without S allylcysteine [244].

Hui et al studied the anti-inflammatory effect of S-Allyl-L-cysteine Sulphoxides (ACSO), a water soluble garlic derived compound on the human umbilical vein endothelial cells. Hui and colleagues showed a reduction in several TNF-α mediated intracellular pathways [221].

Clinical studies have largely failed to replicate these *in vitro* results. Van doorn et al [273] studied the effect of garlic powder upon 90 overweight smokers at a dose of 2.1g/day over a period of 3 months. This study showed garlic powder had no effect on plasma CRP or TNF-α

levels in comparison to placebo. In the same study, Atorvastatin showed significant reductions in all plasma inflammatory markers. Williams et al [261] also showed no effect of AGE in their study in individuals with coronary artery disease. Budoff et al [242] did show an improvement is plasma CRP levels however this was using a combination of antioxidants including AGE.

1.5.7 Garlic and insulin resistance

There is very little evidence for employing garlic to treat insulin resistance. Jalal et al [274] used fructose fed rats as a model for insulin resistance and examined the effect of garlic extracts upon glucose handling as measured by glucose tolerance tests. This study suggests garlic extract could improve glucose handling in this rat model. However, this study used essentially laboratory prepared raw garlic extracts in high doses (500mg/kg) and administered them intravenously. The researchers used intraperitoneal glucose tolerance tests which have no human comparison.

Lui et al [275] studied the effect of garlic oil or diallyl trisulphide (organosulphur derivative of garlic) in streptozocin-induced diabetic rats on insulin secretion and insulin resistance as measured by HOMA-IR. This study found increased insulin production and reduced insulin resistance in this model after treatment with high doses of garlic oil and diallyl trisulphide. As mentioned previously, streptozocin induced diabetes is more synonymous with type 1 than type 2 diabetes and may be an inappropriate model to study insulin resistance.

Eidi et al [276] also used a streptozocin-induced rat diabetes model and administered a short term (72 hr) ethanol extraction of garlic. This study found a significant increase in plasma insulin levels after treatment with the garlic extract. Interestingly, this study also suggested that garlic extract was more effective at increasing insulin levels than glibenclamide, a

commonly used sulphonylurea. The same criticisms can be levelled at this model and both garlic extract and glibenclamide were administered in large doses.

1.5.8 Garlic and blood pressure

There have been numerous clinical studies which have employed various preparations of garlic to treat hypertension.

In brief, there has been a heterogenic effect; some have shown garlic to have successfully reduced blood pressure whilst others have shown no effect (Table 4). However, these studies have used 3 different preparations of garlic (only 1 used AGE) and for different durations of treatment of between 12 and 23 weeks and with different methodologies, thus forming conclusions on the results is problematic. A recent meta-analysis [277] incorporating 11 randomised control trials concluded that garlic reduced systolic blood pressure by an average of 8.36 mmHg and diastolic blood pressure by an average of 7.27 mmHg in hypertensive individuals, although this was not seen in normotensive individuals and none of the trials included patients with diabetes, nor did they include individuals on vasoactive medication.

Author	No of subjects	Study Design	Duration	Subjects	Type of Garlic	Duration & Dose	Mean BP Pre-intervention	Mean BP Post-intervention
Auer et al 1990 [363]	40	Parallel PC	12 wks	HT	Kwai garlic powder	600mg/day	171/102	152/89*
Alder et al 1997 [371]	50	Parallel PC	12 wks	NT	Kwai garlic powder	900mg/day	123/83	118/80*
Vorberg & Schneider1990 [364]	40	Parallel PC	16 wks	HT	Kwai garlic powder	900mg/day	144/91	138/87*
Keisewetter et al 1993 [363]	64	Parallel PC	12 wks	Unknown	Kwai garlic powder	800mg/day	SBP not reported/84	SBP not reported/81
Jain et al 1993 [366]	42	Parallel PC	12 wks	NT	Kwai garlic powder	900mg/day	129/82	130/81
Saradeth et al 1994 [367]	52	Parallel PC	15 wks	NT	Kwai garlic powder	600mg/day	125/80	127/82
Simons et al 1995 [368]	56	Crossover PC	12 wks	NT	Kwai garlic powder	900mg/day	127/80	119/76
Steiner et al 1996 [369]	82	Parallel PC	23wks	NT	Aged Garlic Extract	2400mg/day	134/84	126/82
Zhang et al 2000 [370]	27	Parallel PC	16wks	NT	Distilled Garlic Oil	12.3mg/day	117/72	113/68*

Table 4: Summary of studies investigating garlic's effect on blood pressure in non-diabetic subjects. NT = normotensive (<140/90), HT = hypertensive (>140/90), PC = Placebo controlled

1.5.9 Garlic and LDL Cholesterol

Active reduction of circulating atherogenic LDL cholesterol in high-risk individuals also reduces cardiovascular mortality. In patients with type 2 diabetes, a typical lipid profile is characterised by increased LDL cholesterol, low HDL cholesterol and high triglyceride concentrations.

In vitro studies have suggested AGE can inhibit cholesterol synthesis in hepatocytes, [278] including by inhibition of HMG-Co A reductase [279], a similar action to that of statins.

Clinical studies have also shown garlic to lower total cholesterol and triglycerides concentrations, though not all studies have been consistent (Table 5). A meta-analysis concluded that garlic does have modest cholesterol-reducing properties (total cholesterol reduced by 9.9%, LDL by 11.4%) in hypercholesterolaemic subjects [280], though the significant trials were smaller trials and the meta-analysis excluded some of the negative, longer trials. Moreover, these studies also used a variety of garlic preparations and methodologies, making it difficult to reliably interpret. Currently there is scanty evidence that garlic can reduce circulating LDL cholesterol in patients already taking LDL cholesterol-lowering medications; nor have the hypolipidaemic properties of garlic been studied in type 2 diabetic subjects.

Table 5: Summary of studies investigating garlic's effect on lipids in non-diabetic hyperlipidaemic subjects

Author	No of subjects	Study Design	Duration	Daily Dose	Subjects	Type of Garlic	Total Chol Pre-intervention (mmol/l)	Total Chol Post-intervention (mmol/l)	LDL conc pre garlic (mmol/l)	LDL conc post garlic (mmol/l)
Adler et al 1997 [371]	50	Parallel PC	12 wks	900mg	HL	GP	6.54	5.79*	4.39	3.77*
De Santos et al 1993 [372]	52	Parallel PC	26 wks	800mg	HL	GP	6.92	6.31*	4.75	4.30*
Jain et al 1993 [366]	42	Parallel PC	12 wks	900mg	HL	GP	6.8	6.4*	4.8	4.4*
Mader et al 1990 [373]	261	Parallel PC	16 wks	800mg	HL	GP	6.9	6.1*	Not reported	Not reported
Simons et al 1995 [368]	30	Crossover PC	12 wks	900mg	HL	GP	6.72	6.54	4.76	4.64
Neil et al 1996 [374]	115	Parallel PC	26 wks	900mg	HL	GP	6.96	6.91	4.96	4.94
Superko et al 2000 [375]	50	Parallel PC	12 wks	900mg	HL	GP	6.3	6.2	4.3	4.2
Turner et al 2004 [376]	75	Parallel PC	12 wks	920mg	NL	GP	4.9	5.2	3.3	3.3
Van Doorn et al 2006 [273]	90	Parallel PC	12 wks	2100mg	NL	GP	5.47	5.59	3.37	3.37

* Statistically significant (p>0.05), PC = Placebo controlled, HL – Hyperlipidaemic (TC>6), NL – Normolipidaemic (TC<6)

1.6 Aims & Hypothesis

The antioxidant effects of AGE coupled with the hypolipidaemic, anti platelet and antihypertensive properties would suggest that it has the potential to ameliorate oxidative stress, vascular inflammation and subsequently endothelial dysfunction. This triad of interlinked biochemical processes may underlie the complications of type 2 diabetes, particularly atherosclerosis. This Common Soil hypothesis may also be responsible for insulin resistance; key to the pathogenesis of type 2 diabetes itself.

To date much of the evidence to support garlic as a potential anti-diabetic agent has been in in-vitro and animal studies. There have been no clinical studies looking at the effect of garlic upon these novel cardiovascular markers in subjects with type 2 diabetes. Therefore, this study aims to test the hypotheses that;

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

If these hypotheses are proven it would support further large scale clinical studies to ascertain whether AGE could have a role in ameliorating cardiovascular disease in people with type 2 diabetes.

The secondary aim is to examine the relationship between insulin resistance, oxidative stress, endothelial function and vascular inflammation. Our hypothesis states that these processes are interdependent. (See figure 3)

Chapter 2: Methods

A randomised, double blind, placebo controlled study in 26 type 2 diabetic patients was undertaken to investigate the effect of AGE upon endothelial function, oxidative stress, vascular inflammation and a selection of metabolic parameters.

2.1 Subjects

26 type 2 diabetes subjects who were deemed to be at high cardiovascular risk (30% risk of a cardiovascular event within the next 10 years [281] were recruited. These subjects were identified using the Diabeta 3 electronic database of outpatients of the Diabetes Centre of Portsmouth Hospitals NHS Trust and those who had previously undertaken trials in the department. All subjects gave written informed consent. The Hampshire and Isle of Wight Research Ethics Committee gave their approval for this study (REC Ref No: 05/Q1704/79).

The inclusion criteria included type 2 diabetes patients aged between 18-70 years, who were not treated with insulin. Exclusion criteria included established cardio or cerebrovascular disease, treatment with insulin or Warfarin.

The presence of vascular disease was determined by obtaining a history from the patients and clinical examination. The Rose questionnaire was used at the first visit. The Rose questionnaire was developed and adopted by the WHO in 1962 [282]. It has been used extensively since and validated against clinical findings and thallium scans for detecting occult ischaemic heart disease [283, 284]. It was used in our exclusively white European population. A 12 lead ECG was also performed to investigate occult coronary artery disease.

2.2 Clinical Protocol

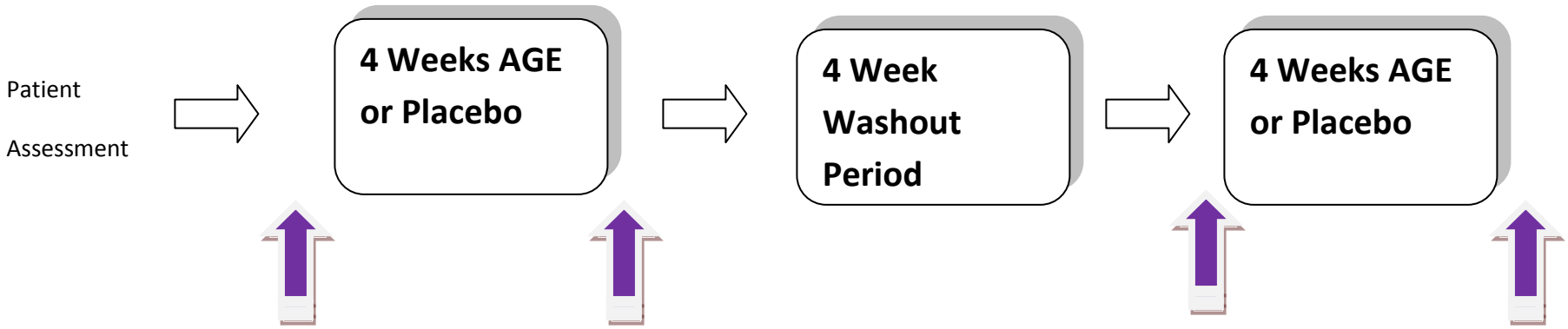
Once recruited, each subject underwent a direct clinical history and examination. Body weight was measured (without shoes) using an electronic balance (Welch Allyn, 52000 Series). Baseline blood pressure was measured by an automated Sphygmomanometer with the subject having been supine in quiet surroundings for at least 5 minutes.

All participants arrived in the Diabetes Department, Queen Alexandra Hospital by 9am in the morning after fasting overnight. A catheter was inserted into a superficial vein and 3 samples of 10 ml of blood were taken 5 minutes apart. Baseline fasting investigations included measurements of plasma lipids (total cholesterol, high density lipoprotein cholesterol and triglyceride) serum urea and serum electrolytes, liver function tests, INR. Glycaemic control was measured using HbA1c and fructosamine. Measurements were also made of insulin resistance, endothelial function, vascular inflammation and oxidative stress as described below.

Once baseline assessments had been concluded, the subjects were given either a commercial garlic preparation (Aged Garlic Extract; "Kyolic" Wakunaga Pharmaceuticals, Mission Viejo, CA, USA) or a placebo. Double blind, randomised allocation (randomization and blinding performed in hospital pharmacy) of the placebo or garlic treatment was undertaken. Subjects took 4 capsules per day (1200mg) for 4 weeks. There was then a 4 week washout period and then the subjects entered the crossover arm (see Figure 8).

The dose and duration of treatment was chosen based upon previous studies using AGE [242, 261]. Compliance levels were monitored with a tablet count at the end of each 4 week treatment period.

Figure 8. Diagram of Clinical Protocol



2.3 Metabolic markers

Metabolic markers including fasting glucose, insulin (measured by Radio-immuno Assay), HOMA-IR, fructosamine, lipid profile (total cholesterol, HDL-cholesterol and triglycerides) (colorimetric assay), liver function tests, urea and electrolytes were also measured at each visit. HbA1c was only measured at baseline visit since the study period was too short to detect any AGE-induced change in this measurement. HbA1c was measured by HPLC (Menarini Diagnostics, Wokingham, UK; intra-assay coefficient of variation (CV 1.5%). Plasma total cholesterol concentration was measured by esterase and oxidase conversion (Advia 1650, Bayer Diagnostics, Newbury, UK; CV<1.9%) and HDL-cholesterol and plasma triglyceride concentration by enzymatic determination (Advia 1650, Bayer Diagnostics, NEWBURY, UK; CV<1.7%)

2.4 Homeostasis Model Assessment

Calculation of the insulin sensitivity index or insulin resistance was carried out using the HOMA (Homeostasis Model Assessment) method. [285]

HOMA uses a mathematical model to estimate steady state beta cell function (%B) and insulin sensitivity (%S), as percentages of a normal reference population. These measures are based upon non-steady state estimates of beta cell function and insulin sensitivity derived from clinical stimulatory models such as the hyperinsulinaemic clamp, the hyperglycaemic clamp and the oral glucose tolerance test.

In 1976, Robert Turner and Rury Holman developed the concept that fasting plasma insulin and glucose concentrations were determined, in part, by a hepatic-beta cell feedback loop. They postulated that elevated fasting glucose levels reflected a compensatory mechanism that maintained fasting insulin concentrations when there was a reduced insulin secretory capacity,

and that fasting insulin concentrations were elevated in direct proportion to diminished insulin sensitivity. A mathematical feedback model based on these hypotheses was constructed to estimate the degrees of beta cell function and insulin sensitivity that would equate to the steady state plasma glucose and insulin levels observed in an individual.

In 1985, David Matthews et al published an expanded and more comprehensive structural model known as the Homeostasis Assessment Model (HOMA). This model took greater account of peripheral glucose uptake and could use fasting levels of insulin [285]. These gave approximate values of steady state beta cell function (%B) and introduced HOMA IR (insulin resistance) which is the reciprocal of %S (100/%S).

Jonathan Levy et al published an updated HOMA model (HOMA2) in 1998 which took account of variations in hepatic and peripheral glucose resistance, increases in the insulin secretion curve for plasma glucose concentrations above 10 mmol/L and the contribution of circulating proinsulin, which has a small biological action and secretion is increased in type 2 diabetes [286]. The model was recalibrated also to give %B and %S control values of 100% in normal young adults when using more modern assays for insulin.

In 2004, the HOMA Calculator was released. This provides quick and easy access to the HOMA2 model in the form of computer software. This is the model that was used in this project to derive estimates of insulin resistance in our subjects.

Plasma insulin and fasting plasma glucose were collected at the beginning of each visit of each of the subjects. These were repeated at 5 minute intervals to give a total of 3 pairs of results and the average of these was used to calculate insulin resistance. An example of the software

used is shown below.

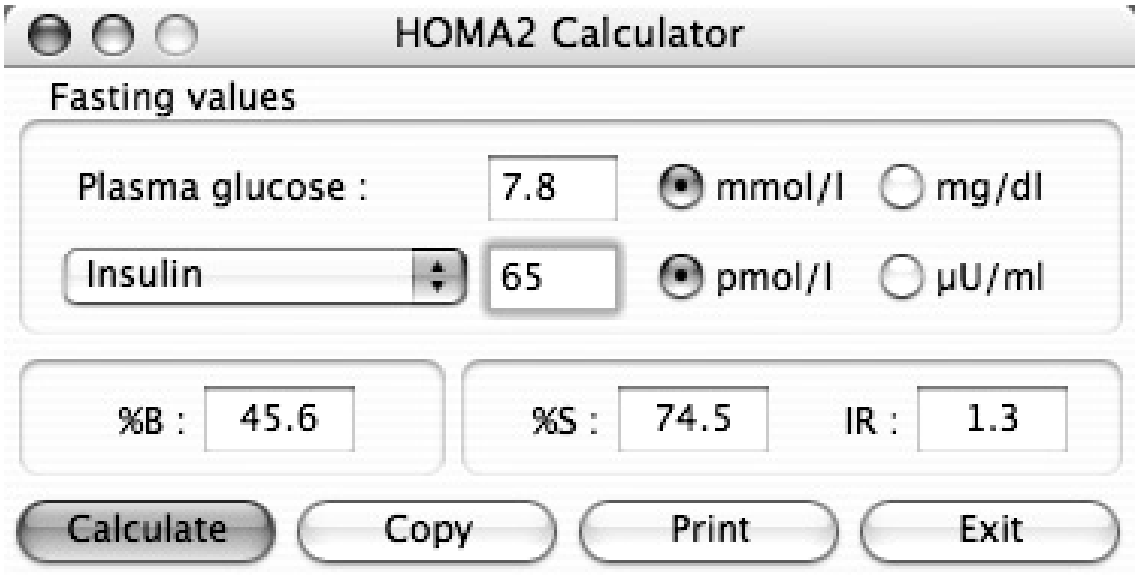


Figure 9: Screenshot of the HOMA IR calculator

2.5 Oxidative Stress Markers

Markers of oxidative stress and antioxidant defence included whole blood ratio of reduced and oxidized glutathione [GSH/GSSG] (enzymatic colourimetric assay), plasma total antioxidant status [TAOS] (enzymatic colorimetric assay) and plasma lipid hydroperoxides [LHP] (enzymatic colorimetric assay).

2.5.1 GSH/GSSG (Glutathione Ratio)

Background

Glutathione (GSH) is the principal thiol intracellular antioxidant and as such the relative amount of intracellular reduced and oxidised GSH is a measure of the cellular redox status. Thornalley et al, 1996 [77] and Jain et al 1994 [78] showed a negative correlation between reduced erythrocyte glutathione and diabetic complications suggesting antioxidant defences were depleted by chronic oxidative stress of type 2 diabetes. These studies also showed a negative correlation between the duration of diabetes and the levels of reduced glutathione. Thornalley postulated that an inherent low level of reduced glutathione in an individual may predispose them to diabetic complications, a phenomenon that is noted clinically. In diabetic patients, a significant increase in plasma free radical concentrations can contribute to a reduction in plasma GSH concentration and to declining blood GSH/GSSG ratios and this can effect insulin action [287].

Using euglycaemic clamp studies, Paolisso also found that supplementation of GSH lead to an improvement of whole body glucose disposal [287].

Technique

2 ml of blood was collected from patients in an EDTA (Ethylenediamine tetraacetic acid) bottle. This was then put into a plain bottle to which 1 ml 0.5mM EDTA/10% (w/v) SSA (Sulpho-salicylic acid) was added. This was further centrifuged at 2580x g (IEC centra- 3C, International Equipment Company, Milford, USA) for 5 minutes. The supernatant was then stored in the –85° C freezer in the Oncology Laboratory at Queen Alexandra Hospital, Portsmouth.

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 [288, 289].

GSH was assessed photometrically in a plate reader at 37° C in a GSH reductase / 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) recirculating assay essentially as described by Tietze (Tietze, 1969). The 100 µl assay well comprised of (final concentration): 70µl DTNB (0.6 mM); 10µl β-NADPH (0.5 mM); 10µl sample; 10µl GSH reductase (2.5 U/ml). Reagents were dissolved in sodium phosphate buffer (120mM, pH 7.4) +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 every 10 seconds at 405nm over 1 minute. For the selective measurement of GSSG (=2GSH), thiols were first derivatised (1 hour) with 2-vinylpyridine (2-VP) in the presence of triethanolamine (TEA) (2µl 2-VP and 6 µl TEA added to the 100µl sample). This procedure was sufficient to fully derivatise up to at least 15mM GSH.

The intra-assay %CV is <3, while the inter-assay CV is <10.

2.5.2 Plasma Lipid Hydroperoxides (LHP)

Background

Lipid hydroperoxides are formed when lipids are exposed to free radical activity and as such plasma levels have been used as a marker of oxidative stress. Both Nourooz-Zadeh et al [74] and Martin-Gallan et al [75] showed that LHP's are raised in those with diabetes, whether or not they have complications. Sugherini et al 2005 [290] found that there is a correlation between levels of plasma LHP and HbA1c. They combined LHP with a measure of total antioxidant status to give a "Redox Compensation Index" which was negatively correlated with glycosylated haemoglobin. The technique used was first described by Ruiz et al in 1997 [314].

Technique:

2 ml of blood collected from the patient was put in a heparinised tube. This was centrifuged at 2580 x g (IEC centra- 3C, International Equipment Company, Milford, USA) for 5 minutes. Supernatant plasma was extracted and then stored in the -85° C freezer.

700µl of buffer (124mmol/l Tris HCl/0.2mmol/l EDTA) was placed in a 1 ml microcuvette and the 200 µl of the sample added. This was then incubated for 5 minutes at room temperature. 50µl of 0.2mmol/l of NADPH, 10µl of glutathione peroxidase (16kU/l) and 100µl of reduced glutathione (4.25mmol/l) were then added. This was then incubated at 37°C for 5 minutes. The spectrophotometric absorbance at 340nm was measured. A further 10 minutes of incubation at 33°C was carried out after the addition of 10µl of butylated hydroxytoluene solution (20g/l) to stop lipid peroxidation. The spectrophotometric absorbance at 340nm was measured for a second time. The difference between the 2 absorption

measurements is proportional to the sample hydroperoxide content. These values were obtained from comparison with the standard curve. The standard curve was obtained by performing the process described above with standard dilutions (25 to 300µmol/l) of t-butyl hydroperoxide prepared from an aqueous solution of the pre-prepared 60mmol/l stock solution.

2.5.3 Plasma Total Antioxidant Status (TAOS)

Background

In 1999, a photometric microassay was developed by Laight et al [291] for the assessment of plasma total antioxidant status in plasma at physiological pH and temperature. Plasma TAOS, expressed as the ascorbate equivalent antioxidant concentration, was found to be significantly reduced in animal models treated with oxidant compounds for 7 days and thus inversely related to oxidant stress. It has since been used successfully in many clinical trials, including those with diabetes [292, 293, 294].

Technique

Two ml of blood was collected from the patient in an EDTA bottle. This was then centrifuged at 2580 x g (IEC centra- 3C, International Equipment Company, Milford, USA) for 5 minutes. The supernatant plasma was stored in a –85° C freezer.

TAOS was assessed by the ability of plasma to inhibit the peroxidase-mediated accumulation of $ABTS^{+}$ radical [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] [291].

The generation and detection of $ABTS^{+}$ was a modification of the spectrophotometric method described by Arnao et al [295] in the assessment of the total antioxidant status of food, adapted to become suitable for use with plasma by employing an inhibition assay with a fixed time point [296].

The reaction mixture consisted of 20µl ABTS (2mM), 10µl horseradish peroxidase (30mU/ml), 20µl H_2O_2 (0.1mM) and 50µl phosphate-buffered saline (pH 7.4), to make a total volume of 100µl in a 96-well plate. The reaction was initiated by the addition of H_2O_2 and conducted at 37° C. The increase in absorbance at 405 nm, reflecting the accumulation of $ABTS^+$, was determined in a Molecular Devices "Versamax" microplate reader. All determinations were made at least in duplicate.

For TAOS, the intra-assay %CV is <3, while the inter-assay CV is <10

2.6 Biochemical Markers of Inflammation

The marker used for endothelial inflammation was highly sensitive C-reactive protein (HsCRP) (Elisa).

2.6.1 Highly sensitive C-reactive protein (hsCRP)

Cardiophase hsCRP is a suspension of polystyrene particles coated with mouse monoclonal antibodies to hsCRP. These are diluted with the plasma sample (ratio 1:20) and allowed reach room temperature. The particles get aggregated when mixed with samples covered with CRP. When light is then passed through the sample it is scattered by the aggregates. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample.

The mixture of hsCRP and the sample were measured on the DADA BEHRING BN prospec system analyzer. The result was then analysed by comparison with a standard of known concentration. The assigned values of CRP were standardized against the international reference preparation BCR-CRM 470 [313]. The results from the analyzer were evaluated automatically and represented in mg/L. This work was undertaken in the biochemistry laboratory at St Thomas's Hospital, London.

2.7 Measurement of Endothelial Function

2.7.1 Digital Plethysmography

Background

It has been established that the Aortic Pulse Wave Velocity (PWV) is a marker of arterial stiffness and arterial ageing. Increased stiffness in the aorta and the large arteries transmits pulse pressure waves from the left ventricle more rapidly. This is a phenomenon found in ageing of the arteries and may be accelerated in hypertensive and diabetic individuals. Aortic

PWV is an independent predictor of cardiovascular mortality in a diabetic population, and may be more sensitive than systolic BP [297].

Dawber et al [298] established the link between the loss of the dicrotic notch in the digital volume pulse (DVP) and coronary artery disease whilst investigating the Framington Study Group. He postulated that the dicrotic notch was not due to the closure of the aortic valve as previously thought but represented the elasticity of the arterial tree. Digital volume pulse has a distinctive contour which Dawber divided into 4 classes (Fig. 8)

Table 6: Dawber's classification of digital volume pulse

CLASS		**CHARACTERISTIC**
1		A distinct notch is seen on the downward slope of the pulse wave
2		No notch develops, but line of descent becomes horizontal
3		No notch, but there is well-defined change to the angle of descent
4		No notch develops or no change occurs in angle of decent

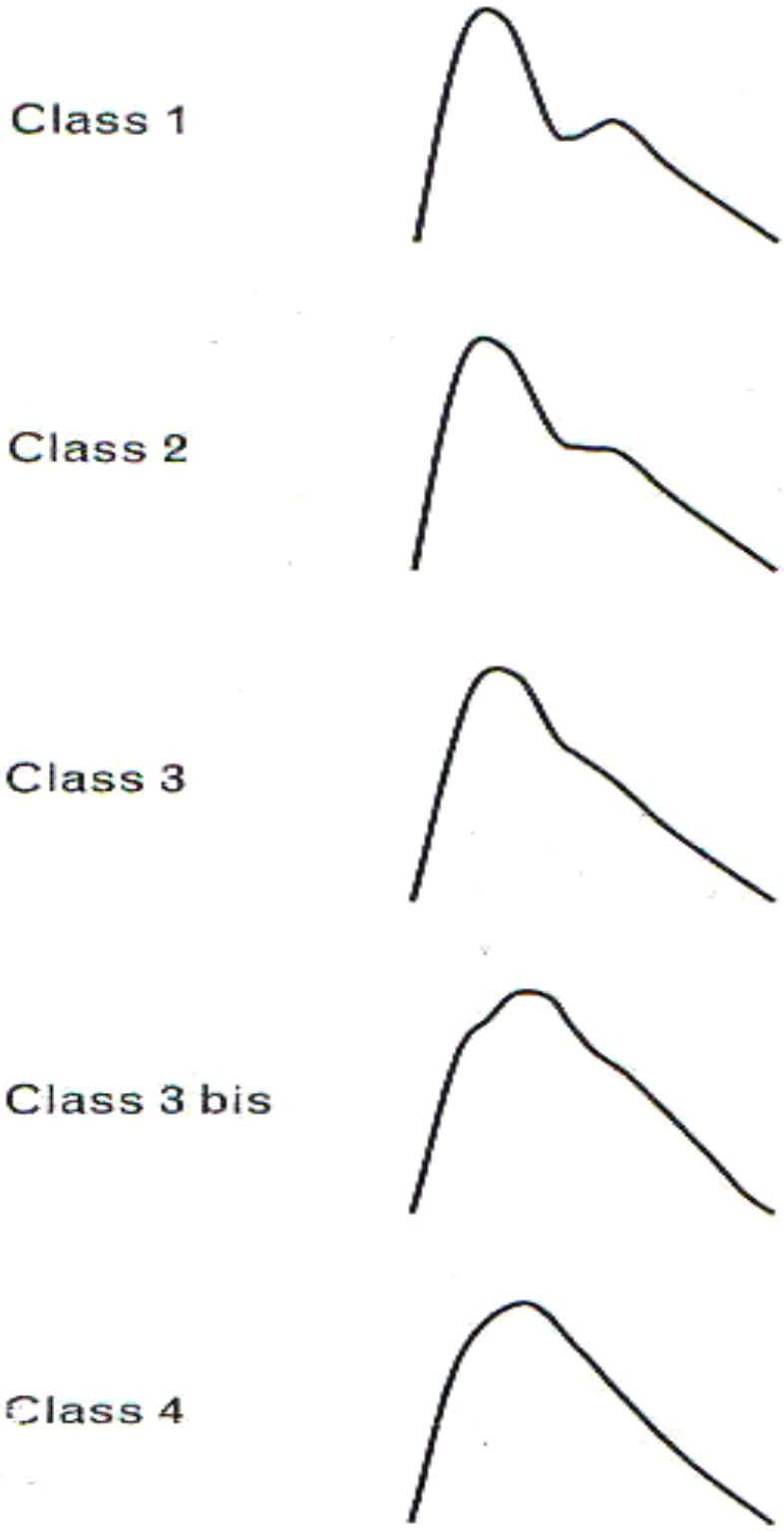


Figure 10. Diagrams of the typical digital pulse waveforms obtained (adapted from Dawber [298])

Class 1 was more common in young fit individuals whilst class 4 was more prevalent in those in older age groups or those with coronary artery disease. Class 4 was also more common in those with diabetes or hypertension.

The DVP contour has since been shown to reflect pressure wave changes seen in both the aorta and the carotid arteries [299, 300]. This suggests that the first peak is a component of the pressure created by contraction of the left ventricle. The distinctive second wave produced in the peripheral pulse represents a reflected, backward wave from the arterial tree of the trunk and limbs [301] and the relative velocity of this peak can be used as a surrogate for the elasticity of the resistance vessels [302]. The time between these first and second peaks in the digital volume pulse has been also been used as a surrogate marker of Aortic PWV [303].

The reflective index (RI) is defined as the ratio of the amplitude of the reflected wave to the first peak, expressed as a percentage.

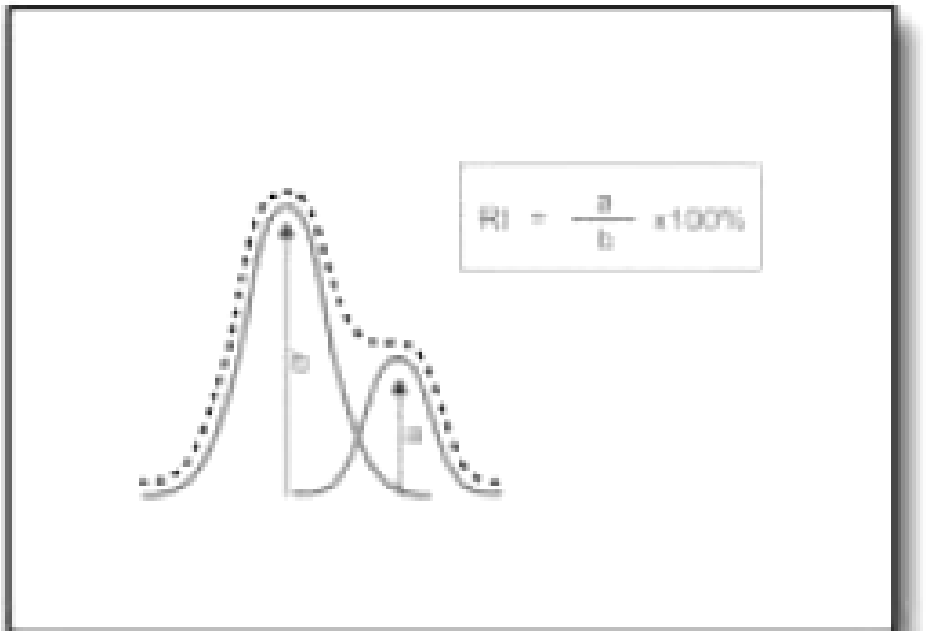


Figure 11. Calculation of Reflective Index (adapted from Micromedical Ltd)

This reflection index has been used to study the effects of vasoactive medications and has been shown to closely reflect pressure changes seen in the radial and aortic pulses. Chowiencyzk et al has also validated the use of the reflective index in diabetic and non diabetic populations [304].

DVP can be measured using photoplethysmography, which is transmission of infra-red light through the finger. The absorption of light is proportional to the volume of blood in the capillaries and thus is proportional to the digital pulse wave.

Endothelial independent vasodilators have been used to assess the effect of an intervention independent of any endothelial involvement. The most commonly used agent has been glyceryl trinitrate (GTN). GTN is a nitric oxide donor and acts independently of endothelial function. Endothelial dependent agents, such as salbutamol, stimulate the release of nitric oxide from the endothelium [305]. If these agents are used alongside each other, an effect of an intervention could be described as either endothelial dependent or independent. The RI has been shown to be sensitive to the use of both GTN and beta adrenergic agonists and therefore these could be used as a measurement of the vasodilator component of endothelial function. [306].

Technique

Measurements were made to determine the digital volume waveform [DVW] using the photoplethysmography apparatus (Micro Medical Pulse Trace, Rochester, Kent, UK). This technique was previously described by Chowienczyk 1999 [304].

Each subject had the probe attached to an index finger for 20 minutes, resting supine, before measurements were taken. Digital pulse wave readings were taken at baseline and the software calculated reflective index (RI). This was then repeated following administration of a

sublingual glyceryl trinitrate (GTN). GTN is an endothelium-independent vasodilator and thus acted as a control. These readings were then repeated following inhaled salbutamol (an endothelium dependent vasodilator). Three readings were taken at baseline and an average taken. 500mcg of sublingual GTN was then administered in tablet for. Readings were taken at 3 and 5 minutes. A washout period of 20 minutes was allowed after which another reading was taken to confirm the return to baseline. Inhaled Salbutamol (400 mcg) was administered using a standardised technique via a spacer device, and readings taken at 10, 12 and 15 minutes. An average of the readings was taken.

2.7.2 Urine Albumin Creatinine Ratio (ACR)

An early morning urine sample was taken at each visit. This was analysed in the biochemistry laboratory at Queen Alexandra Hospital, Portsmouth. There is a well established association between vasoactive endothelial dysfunction in those with diabetes and microalbuminuria [307-312].

Urine albumin was measured by radioimmunoassay (CV < 9%) and urine creatinine concentration by an end-point Jaffe reaction (CV <6%).

2.8 Statistical analysis

Statistical software Graphpad Instat 3 and XLStat 2007 were used for statistical analysis.

- The Kolmogorov-Smirnov test (KS test) was used to assess whether distributions were parametric or non-parametric.
- Repeated measures of ANOVA (Analysis of Variance) were used to compare 4 sets of values obtained. These were: value at baseline; value after AGE intervention; value at baseline (after washout period) and value after placebo. All combinations were examined but only post intervention values were clinically relevant.
- For parametric values, post-tests were undertaken using the Bonferroni method.
- Friedmans test with Dunn's post-test were used if the distribution was non-parametric.

For the purpose of this study, normally distributed data has been expressed as mean ± 1 standard deviation (SD) while the non-parametrically distributed data are shown as median ± interquartile ranges

Spearman rank correlation test was used to determine the associations between baseline measurements in each of the variables measured. A p value <0.05 was considered to be

significant. Multiple linear regression analysis was performed to account for confounding factors in any associations found.

Missing values were small in number (<5%) and where possible were repeated. The remaining missing values occurred randomly and were excluded from the analysis (casewise deletion).

Chapter 3: Results

3.1 Subjects

30 subjects were recruited and met the inclusion and exclusion criteria. 2 discontinued the study due to side effects of AGE (indigestion) and 2 due to other health reasons. Twenty six subjects successfully completed the study. Intention to treat analysis was not undertaken.

3.2 Baseline Characteristics (see Table 7)

The mean age of the participants was 61 ± 8 years and the mean duration of diabetes was 6±3 years. 17 were male and 9 female. 7 were current smokers. The mean BMI (body mass index) was 32.2±5.1 kg/m^2, meaning the majority of subjects were obese (BMI 30-35). 9 out of 26 subjects were taking an ACE inhibitor throughout the study, 25/26 were taking a statin, 16/26 were taking 75mg aspirin and 19/26 were taking metformin (see figure 12).

The baseline characteristics of the cohort were typical of patients found in local GP and diabetes outpatient clinics. 7 (26%) were current smokers and this percentage is in keeping with local smoking rates. Analysis of the BMI of the subjects showed that 24/26 (92%) of subjects were at least overweight (BMI>25). Of these 24 subjects, 33% were overweight (BMI 25-30 kg/m^2), 58% obese (BMI 30-40 kg/m^2) and 9% extremely obese (BMI >40 kg/m^2). Given that a high percentage of our cohort were taking statins, metformin, aspirin and/or anti hypertensive medications, baseline metabolic parameters were generally well controlled (see section 3.2.1)

None of the subjects were taking additional garlic supplements. Garlic in usual diet was not measured.

Table 7: Baseline Characteristics of Subjects

Subject no	Age (yrs)	Diabetes Duration (yrs)	Sex	Height (m)	Weight(Kg)	BMI(Kg/m^2)	Smokers	Statins	Metformin
8001	51	2	M	186.5	125.8	36.2	YES	YES	YES
8002	64	6	M	174	90.4	29.9	NO	YES	NO
8003	70	18	M	186	85.2	24.6	EX	TES	YES
8004	57	6	F	171	106.8	36.5	YES	YES	YES
8005	60	5	M	185	92	26.9	NO	YES	NO
8006	58	9	M	177.5	110.7	35.1	YES	YES	YES
8007	54	7	M	178	109.2	34.1	NO	YES	YES
8009	60	6	F	163	94.3	35.4	YES	YES	YES
8010	71	8	M	176.5	91.2	29.3	NO	YES	YES
8011	65	11	F	152	68.6	29.7	NO	YES	YES
8013	61	5	M	178	102.7	32.5	NO	YES	NO
8014	62	9	M	183.5	114.5	33.9	NO	YES	YES
8015	66	8	F	167.5	94.9	34	NO	YES	NO
8016	54	6	F	164.5	63.4	23.4	YES	NO	NO
8017	69	11	F	170.5	83.1	28.6	NO	YES	YES
8019	59	2	M	168.5	73.6	26.1	NO	YES	NO
8023	40	6	F	176	107.5	34.7	NO	YES	YES
8024	64	1	M	164.5	89.6	33.3	NO	YES	YES
8025	70	5	M	178	106.5	33.6	NO	YES	YES
8026	70	6	F	180	97.9	30.2	NO	YES	YES
8027	63	4	M	171	91.4	31.3	EX	YES	NO
8028	59	3	M	187	93.9	26.9	YES	YES	YES
8029	49	5	M	171	130.5	44.6	NO	YES	YES
8030	52	2	M	192	117.5	31.9	EX	YES	YES
8031	70	2	M	179	142.2	44.4	YES	YES	YES
8032	75	5	F	167	83.5	29.8	NO	YES	YES

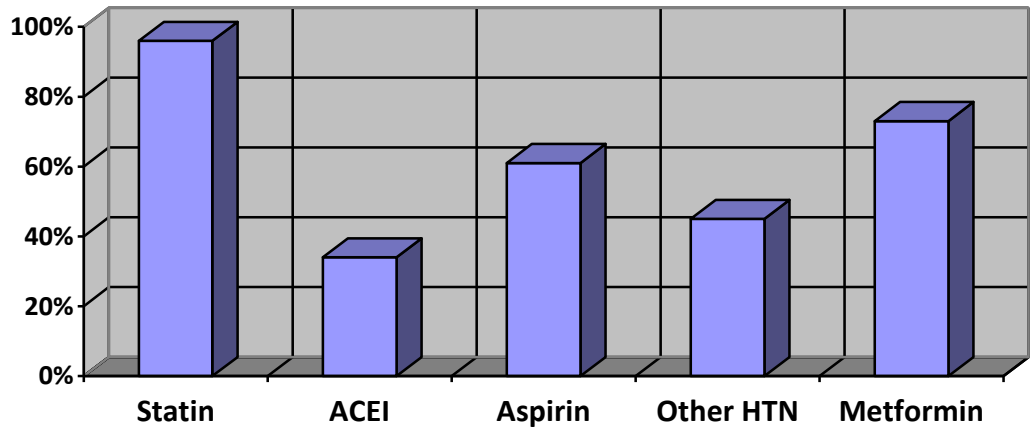


Figure 12. Percentage of subjects on vasoactive medication (HTN = anti-hypertensive medication)

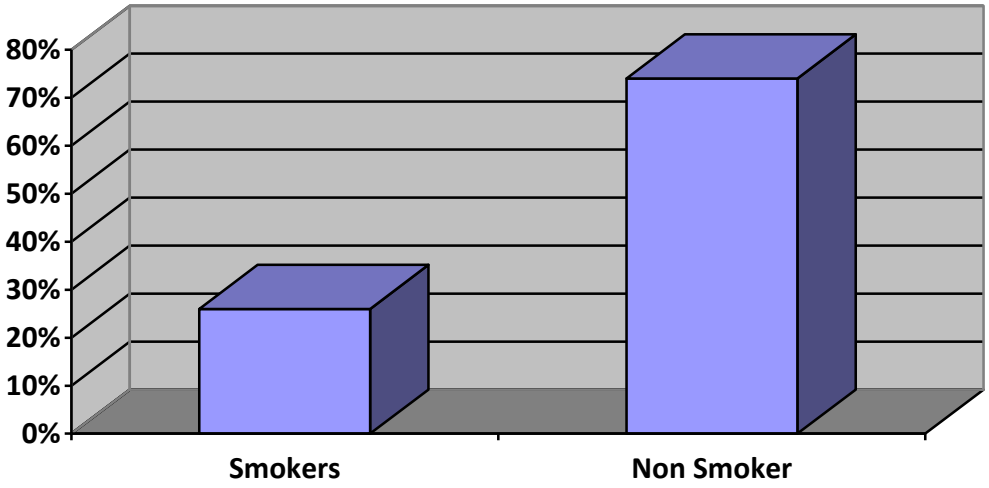


Figure 13. Percentage of active smokers amongst group

Table 8: Baseline Metabolic parameters of subjects

Subject no	HbA1c (%)	Total Cholesterol (mmol/L)	HDL (mmol/L)	Triglycerides (mmol/L)	Systolic BP(mmHg)	Diastolic BP (mmHg)
8001	6.7	4.25	0.84	1.36	146	92
8002	7	2.9	0.83	1.11	103	66
8003	6.4	4.53	1.33	0.66	136	75
8004	8.1	6.04	0.92	2.02	138	83
8005	5.5	5.42	1.91	1.45	151	86
8006	6.1	4.67	1.06	1.8	131	73
8007	8.6	4.09	0.9	2.23	122	77
8009	7.5	3.74	1.06	1.52	129	77
8010	6.6	3.16	0.87	1.18	126	63
8011	6.7	3.85	1	1.61	131	78
8013	5.6	4.05	1.23	1.32	118	73
8014	6.7	3.25	1.05	1.7	128	74
8015	6.6	4.37	1.3	1.27	112	69
8016	6.9	4.8	1.46	1.22	99	58
8017	6.4	3.89	1.21	1.13	144	66
8019	6	4.68	1.47	1.62	141	78
8023	8.4	5.69	0.94	8.24	149	91
8024	6.8	4.43	0.88	3.52	165	99
8025	7.2	3.72	0.69	3.88	144	74
8026	9.7	3.71	0.79	1.57	133	77
8027	5.9	4.29	0.98	1.44	124	70
8028	7.4	3.48	0.63	3.48	116	76
8029	9.5	3.89	0.87	2.73	132	78
8030	8.1	4.04	0.99	2.56	105	58
8031	8.8	3.54	0.68	1.1	120	67
8032	7.4	5.84	1.06	3.23	144	68

3.2.1 Baseline Metabolic Markers

The baseline metabolic markers of our group (see table 8) were close to the NICE recommendations for this patient group [315]. The glycaemic control amongst the subjects at baseline was near optimal with a mean HbA1c of 7.2±1.13% (NICE target<7.5%). Mean plasma total cholesterol concentration at baseline was 4.24±0.81mmol/l (Dept of Health target <5mmol/l). Mean HDL cholesterol concentration was 1.04±0.29mmol/l (NICE Target >1mmol/l) and mean plasma triglyceride concentration was 2.11±1.51mmol/l (NICE target<2mmol/l). Mean blood pressure at baseline was 130/75 ±16/10mmHg (NICE target 140/80mmHg).

Table 9: Baseline comparisons before and after washout period

Parameter	Baseline Pre-placebo	Baseline pre AGE	P value
Plasma total Cholesterol (mmol/l)	4.2±0.8	4.2±0.9	0.83
Plasma HDL Cholesterol (mmol/l)	1.0±0.3	1.0±0.3	0.24
Plasma triglycerides (mmol/l)	1.6 IQR 1.2	1.4 IQR 0.7	0.01[w]
Fructosamine (µmol/l)	284±46	274±33	0.48
Diastolic BP(mmHg)	74.8±9.8	74.7±7.5	0.92
Systolic BP(mmHg)	130.3±15.9	130.3±14.0	0.98
RI Change post Salbutamol	8.0 IQR 4.7	6.5 IQR 9.7	0.45[w]
Insulin resistance (HOMA-IR)	2.5±2.0	1.9 ±1.1	0.13
LHP(µM)	158.3±97.0	144.7±55.4	0.89[w]
GSH/GSSG	17.0 IQR 15.1	18.8 IQR 21.8	0.43[w]
Total Glutathione(µM)	698.0 ±193.7	690.2 ±177.9	0.81
TAOS (µM)	62.9 ±3.6	63.1±3.2	0.76
HsCRP (mg/l)	1.8 IQR 2.1	2.1 IQR 18.3	0.49[w]

[w]= Wilcoxon (non parametric)

There were no significant differences in the baselines prior to each of the treatment arm apart from triglycerides (Table 9). This would suggest the washout period was effective. The difference between the triglyceride baselines would not be clinically significant.

3.3 Effect of AGE and placebo upon measured outcomes

Metabolic markers

3.3.1 Weight Change

The mean change in weight following treatment with AGE was 0.5±1.1kg. 27% lost weight. After treatment with placebo the weight change was 0.1±1.3kg. 46% lost weight. This result was tested using a Student two tailed t-test and found not to be statistically significant (p=0.24). The data for the subjects is shown in table 10.

Table 10: Weight change after 4 weeks treatment (kg)

Subject no	Post Placebo	Post AGE
8001	0.7	0.9
8002	0.7	0.6
8003	-1.2	0.8
8004	-0.8	-1.6
8005	1	-0.5
8006	2.5	0
8007	0.6	1.4
8009	-0.4	1.7
8010	0.9	0.8
8011	0.4	0.3
8013	-0.1	1.6
8014	-2.6	0.4
8015	-1.3	0.4
8016	-1.8	1.2
8017	1.2	1.7
8019	0.3	-0.1
8023	-0.1	2.3
8024	0.8	-1
8025	1	0.6
8026	-1.3	-1.9
8027	1.8	1.2
8028	1.3	0.3
8029	-2.8	2.4
8030	1.9	-0.3
8031	-0.7	-0.5
8032	-0.3	0

3.3.2 Systolic Blood Pressure

Baseline mean systolic blood pressure (BP) in the treatment arm was 130.3±14.0mmHg. Following treatment with AGE it was 130.8±14.6mmHg.

In the placebo arm, baseline mean systolic BP was 130.3±15.9mmHg (n=26). Following treatment with placebo it was 131.6±17.5mmHg.

The data was distributed parametrically. Repeated measures ANOVA testing showed no statistically significant difference in either arm (p=0.94).

The data for the subjects is shown in table 11.

Table 11: Changes in Systolic BP (mmHg)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline pre-AGE	Post AGE
8001	146	137	125	134
8002	103	108	105	120
8003	136	140	138	143
8004	138	122	131	120
8005	151	138	149	164
8006	131	141	129	130
8007	122	120	130	116
8009	129	127	122	129
8010	126	148	131	147
8011	131	132	128	137
8013	118	114	127	127
8014	128	122	124	121
8015	112	111	104	120
8016	99	111	119	105
8017	144	140	117	120
8019	141	121	136	126
8023	149	145	132	148
8024	165	169	148	163
8025	144	164	146	118
8026	133	122	129	119
8027	124	132	147	134
8028	116	116	113	119
8029	132	125	132	130
8030	105	110	115	128
8031	120	137	152	131
8032	144	169	159	153

3.3.3 Diastolic blood pressure

In the treatment arm, baseline mean diastolic BP was 74.7±7.5mmHg. Following treatment with AGE it was 73.9±7.7mmHg. In the placebo arm, baseline mean diastolic BP was 74.8±9.8mmHg. After treatment with placebo it was 75.1±8.3mmHg.

KS test showed the data to be distributed parametrically. Repeated measures ANOVA testing showed no statistically significant difference in either arm (p=0.81). The data for the subjects is shown in table 12.

Table 12: Changes in Diastolic BP (mmHg)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	92	84	74	84
8002	66	70	68	77
8003	75	79	70	75
8004	83	68	76	73
8005	86	76	83	80
8006	73	85	77	78
8007	77	77	79	75
8009	77	80	79	79
8010	63	66	65	64
8011	78	77	76	77
8013	73	64	77	73
8014	74	76	77	80
8015	69	68	62	65
8016	58	62	66	52
8017	66	60	63	64
8019	78	70	66	71
8023	91	85	85	88
8024	99	97	86	85
8025	74	82	74	70
8026	77	74	75	80
8027	70	74	74	75
8028	76	76	77	73
8029	78	77	88	74
8030	58	67	65	64
8031	67	80	87	72
8032	68	79	73	74

3.3.4 Plasma Total Cholesterol

In the treatment arm, the baseline mean total plasma cholesterol was 4.22±0.92mmol/l. Following treatment with AGE the mean total plasma cholesterol was 4.22±0.79mmol/l.

In the placebo arm, the baseline mean total plasma cholesterol was 4.24±0.81mmol/l. Following treatment with placebo the mean total plasma cholesterol was 4.18±0.95mmol/l.

KS testing showed the data to be distributed parametrically. Repeated measures ANOVA testing showed no statistically significant difference in either arm ($p=0.96$)

The data for the subjects is shown in table 13.

Table13: Changes in Plasma Total Cholesterol (mmol/l)

Subject no	Baseline Pre-Placebo	Post placebo	Baseline Pre-AGE	Post AGE
8001	4.25	3.99	4.00	4.46
8002	2.90	3.26	2.69	3.29
8003	4.53	4.76	5.5	4.34
8004	6.04	5.71	5.65	5.32
8005	5.42	4.87	4.85	4.68
8006	4.67	4.04	3.98	4.00
8007	4.09	3.92	4.05	3.82
8009	3.74	3.63	5.09	3.51
8010	3.16	3.10	3.02	2.93
8011	3.85	3.68	4.39	4.76
8013	4.05	3.50	3.75	3.93
8014	3.25	3.28	3.01	3.47
8015	4.37	3.72	5.13	4.71
8016	4.80	4.60	5.09	5.44
8017	3.89	3.97	3.70	5.88
8019	4.68	5.48	4.70	5.07
8023	5.69	6.61	4.82	4.41
8024	4.43	4.33	4.14	4.11
8025	3.72	3.55	3.7	3.72
8026	3.71	3.43	3.5	3.67
8027	4.29	4.62	4.87	4.89
8028	3.48	3.06	2.55	2.94
8029	3.89	3.42	3.62	3.38
8030	4.04	3.86	3.68	4.16
8031	3.54	4.10	4.06	3.60
8032	5.84	6.27	6.17	5.36

3.3.5 Plasma HDL Cholesterol

In the treatment arm the baseline mean plasma HDL cholesterol was 1.01±0.28mmol/l. After treatment with AGE it was 1.01±0.27mmol/l.

In the placebo arm, the baseline mean plasma HDL cholesterol was 1.04±0.29mmol/l. Following treatment with placebo, the mean plasma HDL cholesterol was 1.00±0.28mmol/l.

KS showed the data to be distributed in a parametric fashion. Repeated measures ANOVA testing showed no statistically significant difference in either arm ($p=0.46$).

The data for the subjects is shown in table 14.

Table 14: Changes in HDL Cholesterol (mmol/l)

Subject no	Baseline Pre-placebo	Post placebo	Baseline Pre-AGE	Post AGE
8001	0.84	0.81	0.77	0.84
8002	0.83	0.76	0.83	0.82
8003	1.33	1.35	1.57	1.40
8004	0.92	0.90	0.93	0.90
8005	1.91	1.79	1.64	1.54
8006	1.06	0.95	0.86	0.88
8007	0.9	0.82	0.89	0.85
8009	1.06	1.27	1.32	
8010	0.87	0.83	0.93	0.89
8011	1.00	1.01	0.96	0.98
8013	1.23	1.00	1.22	
8014	1.05	1.13	0.98	1.00
8015	1.30	1.01	1.16	
8016	1.46	1.28	1.44	1.59
8017	1.21	1.33	1.23	1.29
8019	1.47	1.47	1.31	1.46
8023	0.94	1.13	0.99	0.91
8024	0.88	0.79	0.77	0.89
8025	0.69	0.68	0.63	0.71
8026	0.79	0.66	0.81	0.73
8027	0.98	1.01	1.14	1.28
8028	0.63	0.66	0.58	0.65
8029	0.87	0.76	0.88	1.02
8030	0.99	0.80	0.80	1.00
8031	0.68	0.93	0.77	0.73
8032	1.06	0.94	0.89	0.95

3.3.6 Plasma Triglyceride

In the treatment arm the baseline median plasma triglyceride was 1.43 IQR 0.73 mmol/l. Following treatment with AGE it was1.45 IQR 0.77 mmol/l.

In the placebo arm, the baseline mean plasma triglyceride was 1.59 IQR 1.19 mmol/l. Following treatment with placebo it was1.49 IQR 1.09 mmol/l.

KS test showed the data to have a non parametric distribution. Friedman repeated measures ANOVA testing showed statistically significant difference ($p=0.04$) but this was between baselines, suggesting any perceived change post treatment would be difficult to interpret.

The data for the subjects is shown in table 15.

Table 15: Changes in Plasma Triglyceride (mmol/l)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	1.36	1.54	1.11	1.12
8002	1.11	1.25	1.00	1.19
8003	0.66	1.21	0.63	0.60
8004	2.02	1.58	1.35	1.56
8005	1.45	1.19	1.03	0.74
8006	1.80	1.78	1.53	1.58
8007	2.23	1.96	1.77	1.99
8009	1.52	0.96	1.67	1.51
8010	1.18	0.94	0.93	0.89
8011	1.61	1.29	1.90	2.16
8013	1.32	1.00	1.11	1.39
8014	1.70	1.14	1.00	1.07
8015	1.27	0.80	0.92	1.03
8016	1.22	1.02	1.35	1.30
8017	1.13	1.13	1.29	1.18
8019	1.62	2.26	1.83	1.85
8023	8.24	13.00	5.68	4.32
8024	3.52	3.65	3.25	3.21
8025	3.88	3.33	3.27	3.87
8026	1.57	1.26	1.38	1.36
8027	1.44	1.83	1.37	1.44
8028	3.48	2.2	1.85	1.47
8029	2.73	2.27	1.48	1.53
8030	2.56	3.86	2.00	2.30
8031	1.10	1.38	1.70	1.20
8032	3.23	3.56	4.00	3.60

3.3.7 Fructosamine

In the treatment arm, the baseline mean fructosamine was 272±32µmol/l. Following treatment with AGE it was 270±33µmol/l.

In the placebo arm, the baseline mean fructosamine was 273±32µmol/l. Following treatment with placebo mean fructosamine was 270±33µmol/l.

KS testing showed the data was distributed parametrically. Repeated measures ANOVA testing showed no statistically significant difference in either arm (p=0.88).

The data for the subjects is shown in table 16.

Table16: Changes in Fructosamine (µmol/l)

Subject no	Baseline Pre-Placebo	Post placebo	Baseline Pre-AGE	Post AGE
8001	285	268	291	285
8002	335	281		356
8003	266	232	267	252
8004	336	347	323	350
8005	263	241	232	232
8006	279	281	244	241
8007	340	252	277	251
8009	301	304	281	282
8010	263	255	256	263
8011	264	253	272	260
8013	217	217	223	214
8014	253	266	341	274
8015	253	234	248	245
8016	280	264	285	273
8017	246	252	254	256
8019	244	252	242	247
8023	300	307	291	275
8024	258	266	231	259
8025	246	246	308	311
8026	368	343	332	353
8027	266	256	263	258
8028	277	260	261	254
8029	433	484	309	
8030	287	279	280	305
8031	276	306	299	271
8032	255	279	251	268

3.3.8 Insulin Resistance

Insulin resistance was measured using the HOMA- IR method as described previously. In the treatment arm, the baseline insulin resistance was 1.89 ±1.1 units. Following treatment with AGE insulin resistance was 1.7±0.9 units. Repeated measures ANOVA testing showed this result to not be statistically significant ($p = >0.05$)

In the placebo arm, the insulin resistance at baseline was 2.5±2.0 units whereas following treatment with placebo it was 2.0±1.1 units. Repeated measures ANOVA testing showed this result not to be statistically significant ($p = >0.05$)

KS test showed the data to be parametrically distributed. Repeated measures ANOVA testing showed this result to have borderline statistical significance difference ($P=0.05$, $n=26$). Post hoc testing with Bonferroni supported this finding as non significant.

The data for the subjects is shown in table 17.

Table17: Changes in Insulin Resistance

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	4.8	4.8	3.7	3.6
8002	1.6	1.6	1.5	1.2
8003	1.1	0.7	0.5	0.9
8004	2.3	1.9	1.7	2.3
8005	0.6	0.7	0.5	0.7
8006	2.2	1.6	1.9	2.3
8007	3.2	2.9	2.7	2.8
8009	9.7	1.2	1.3	2.3
8010	1.3	0.9	1.1	0.9
8011	2.7	2.9	2.7	2.2
8013	1.4	1.2	1.6	1.4
8014	1.3	2.1	1.6	1.6
8015	1.1	0.9	0.7	0.6
8017	0.7	0.6	0.5	0.5
8019		1	1	0.8
8023	5.1	2.5	2.5	2.4
8024	1.7	1.6	1.8	1.6
8025	1.9	2.2	2.2	1.8
8026	3.8	4.1	4.4	3
8027	0.9	0.8	0.8	1
8028	1.3	1.3	0.9	0.7
8029	4	2.6	3.7	3.2
8030	2.1	2.7	2.5	1.6
8031	1.9	2	2.1	2
8032	2.2	2.3	2.5	2

3.4 Markers of Endothelial Function

3.4.1 Digital Plethysmography

At baseline, the mean change in reflective index (RI) post GTN was 11±5.7%. Following treatment with AGE mean change in RI was 12±7.3%. In the placebo arm, at baseline the mean change in RI post GTN was 12.5±8.2% following treatment with placebo it was 11±8.2%.

KS test showed the data to be distributed in a parametric fashion. Repeated measures ANOVA testing showed no statistically significant difference in either arm (p=0.52).

The data for the subjects is shown in table 19.

At baseline, the mean change in RI post salbutamol was 6.5 IQR 8.3% and following treatment with AGE it was 6.5 IQR 10.3%. The mean change in RI at baseline post salbutamol was 8.0 IQR 5.0%.

KS testing showed the results to be distributed non-parametrically. A Freidman ANOVA test was used. This showed no statistical significant difference (p=0.95) in GTN or salbutamol responses following AGE or placebo.

The data for the subjects is shown in table 18.

Table 18: Reflective Index change post Salbutamol (%mean)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	8	4	1	1
8002	5	11	16	21
8003	4	0	1	13
8004	9	16	7	5
8005	14	16	11	15
8006	4	2	0	6
8007	9	3	6	3
8009	20	12	11	2
8010	8	2	6	12
8011	8	10	6	18
8013	0	1	1	1
8014	4	0	13	5
8015	12	13	27	29
8016	9	11	4	2
8017	6	14	2	7
8019	4	10	5	13
8023	18	9	11	10
8024	9	7	10	3
8025	0	9	12	7
8026	9	4	1	7
8027	7	11	7	6
8028	9	0	3	2
8029	19	17	4	10
8030	0	13	24	15
8031	6	1	11	2
8032	6	6	7	4

Table 19: Reflective Index change post GTN (% mean)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	30	15	9	19
8002	13	12	16	16
8003	6	6	13	9
8004	10	3	8	20
8005	10	12	2	4
8006	14	3	15	16
8007	23	25	10	16
8009	11	12	16	16
8010	1	7	10	8
8011	4	2	0	3
8013	13	10	10	2
8014	12	10	9	12
8015	10	7	8	21
8016	28	23	21	9
8017	7	0	9	7
8019	2	2	2	3
8023	31	27	16	28
8024	9	2	3	6
8025	9	17	12	15
8026	14	19	15	20
8027	0	9	5	8
8028	13	12	12	7
8029	15	19	19	19
8030	20	26	22	21
8031	14	3	9	12
8032	6	3	13	0

3.4.2 Urinary Albumin /Creatinine Ratio (ACR)

In the treatment arm, baseline median ACR was 0.55 IQR1.25. Following treatment with AGE the median ACR was 0.60 IQR1.03.

The baseline median ACR in the placebo arm was 0.80 IQR 0.88. After treatment with placebo it was 0.65 IQR1.23.

KS test showed the results to be distributed non- parametrically and therefore a Friedman Repeated measures ANOVA test was performed. This showed the result in neither arm to be statistically significant ($p=0.43$, $n=26$).

The data for the subjects is shown in table 20.

Table 20: Changes in Urinary Albumin/Creatinine ratio

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	10.2	0.7	0.6	0.6
8002	0.2	0.2	<2	<2
8003	1.6	2.7	2.6	2.4
8004	7.7	7.1	4.6	
8005	<2	0.3	<2	<2
8006	0.2	0.2	<2	<2
8007	0.3	0.2	0.6	0.2
8009		<2	1	1.2
8010		0.4	0.5	<2
8011	0.4	<2	0.5	<2
8013	0.1	<2	0.2	0.2
8014	1	0.5	0.4	0.4
8015	0.8	0.9	0.4	1.5
8016	0.7	1.2	0.9	0.9
8017	<2	<2	0.4	<2
8019	0.8	0.4	0.5	0.6
8023	0.8	0.6	0.5	0.6
8024	0.3	<2	0.3	0.4
8025	1.3	1.5	1.6	1.7
8026	0.4	0.6	0.5	0.5
8027	<2	0.3	0.4	0.5
8028	0.5	0.3	0.3	0.4
8029	5.3	4.3	4.3	1.9
8030	<2	0.4	<2	0.3
8031	1.5	2.3	1.8	1
8032	0.8	0.5	0.4	0.5

3.5 Markers of oxidative stress

3.5.1 Plasma Total Antioxidant Status (TAOS)

In the treatment arm, plasma TAOS (expressed as ascorbate equivalent antioxidant concentrations -AEAC) at baseline was 63.1±3.2µM. Following treatment with AGE plasma TAOS was 64.0±4.4µM.

In the placebo arm, plasma TAOS at baseline was 62.9 ±3.6µM. After treatment with placebo this was 63.6±5.6µM.

KS test showed the data to be parametrically distributed. Repeated measures ANOVA testing showed the result in neither arm to be statistically significant (p=0.57, n=25).

The data for the subjects is shown in table 21.

Table 21: Changes in TAOS (µM AEAC)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	63.0	70.1	66.2	71.9
8002	63.4	69.6	70.1	69.3
8003	67.7	69.4	70.9	72.9
8004	65.6	63.6	63.2	65.0
8005	68.6	67.3	62.2	71.2
8006	60.8	70.9	61.6	59.1
8007	54.9	69.7	63.8	69.4
8009	60.1	60.9	62.3	62.0
8010	63.1	61.1	62.5	66.7
8011	68.4	69.1	65.9	68.1
8013	63.3	72.6	61.3	63.2
8014	60.7	61.2	58.9	62.3
8015	61.4	60.7	63.5	61.1
8016	65.0	63.2	64.2	60.9
8017	55.9	57.0	59.6	57.7
8019	61.1	57.2	62.1	62.3
8023	57.6	50.0	55.9	57.9
8024	63.1	65.6	61.3	61.4
8025	66.3	64.4	60.8	66.9
8027	62.7	59.4	65.3	62.1
8028	66.5	59.8	66.5	62.0
8029	61.5	59.6	63.1	65.9
8030	61.1	60.3	62.7	61.6
8031	64.3	58.3	63.9	58.0
8032	67.0	68.8	60.8	63.5

3.5.2 Glutathione ratio

The median glutathione ratio (GSH/GSSG) at baseline in the treatment arm was 18.8 IQR 21.8. After treatment with AGE it was 22.8 IQR 25.1.

In the placebo arm, glutathione ratio at baseline was 17.0 IQR 15.1 and following treatment with placebo this was 20.6 IQR 22.5.

KS test showed these results to be non-parametric in distribution. Freidman repeated measure ANOVA testing showed this result was not statistically significant (p=0.63).

The data for the subjects is shown in table 22.

Table 22: Changes in Glutathione ratio (GSH/GSSG)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	7.1	43.5	19.9	22.8
8002	12.2	16.4	13.6	18.8
8003	39.1	35.9	32.1	15.5
8004	33.8	11.3	15.5	10.5
8005	11.2	36.9	10.1	8.5
8006	7.1	43.5	19.9	22.8
8007	21.4	10.2	7.1	5.2
8009	8.8	11.1	13.2	19.9
8010	62.1	9.6	93.2	35
8011	13.9	20.7	5.6	1.8
8013	16	31.4	59.7	19.1
8014	69.1	44.7	10.6	15
8015	27.7	26.7	38.5	50.9
8016	44.6	9.4	12	46.5
8017	19.7	19.9	8.3	9.7
8019	63.8	17.8	34.8	47.4
8023	18.7	13.9	15.6	27.8
8024	18.9	23.4	27.5	71.5
8025	17.7	16.9	46.2	29.4
8026	7.1	99.3	40.8	23
8027	11	12.9	17.7	77.1
8028	20.9	20.5	28.4	12.7
8029	5.7	31.8	4.4	40.7
8030	12.3	16.3	17.2	46.2
8031	10.8	44.4	20.7	38.6
8032	16.3	66.9	36	18.7

3.5.3 Whole blood glutathione

The mean total whole blood glutathione at baseline in the treatment arm was 690.2 ±177.9µM. Following treatment with garlic it was 725.8 ±224.5 µM.

In the placebo arm, at baseline the mean total whole blood glutathione was 698 ±193.7µM. following treatment with placebo it was 690.9 ±169.1µM.

KS testing showed these results to have a parametric distribution. Repeated measure ANOVA showed there to be no statistically significant difference in either arm. (p=0.67).

The data for the subjects is shown in table 23.

Table 23: Changes in total whole blood glutathione (μM)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	338.2	393.6	530.2	406.5
8002	539.3	791.5	434.2	771.2
8003	852.7	697.0	595.8	630.7
8004	575.9	487.9	475.5	472.1
8005	560.3	672.9	562.5	672.9
8006	416.6	486.3	507.9	537.8
8007	724.4	557.5	472.7	489.3
8009	387.5	414.6	574.2	592.5
8010	744.8	707.6	951.7	657.6
8011	593.8	731.7	471.8	343.8
8013	770.9	914.3	811.3	978.6
8014	876.0	768.8	780.9	704.7
8015	756.4	845.8	919.9	1059.4
8016	911.2	788.0	893.3	942.2
8017	553.0	432.6	423.9	549.3
8019	1143.8	762.6	833.6	939.6
8023	712.9	874.1	808.8	738.2
8024	898.3	729.6	779.4	1138.7
8025	603.1	597.1	820.5	611.1
8026	761.1	867.1	961.2	629.9
8027	535.7	496.0	601.5	801.7
8028	918.0	732.0	859.3	877.3
8029	531.9	552.3	546.9	619.9
8030	963.5	958.5	690.1	1209.9
8031	715.6	746.4	883.0	910.0
8032	763.6	949.6	755.5	587.7

3.5.4 Plasma Lipid Hydroperoxides (LHP)

At baseline, the mean plasma LHP in the treatment arm was 144.7±55.4 µM. Following treatment with AGE this was 134.19±41.07 µM.

In the placebo arm, mean plasma LHP at baseline was 158.3±97.0µM and following treatment with placebo it was 134.1±39.7 µM.

KS testing showed the data to be distributed parametrically. Repeated measure ANOVA testing of this result failed to show statistical significance in either arm ($p=0.41$, $n=24$).

The data for the subjects is shown in table 24.

Table 24: Changes in LHP (µM)

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	158.29	120.31	155.89	150.60
8002	281.85	123.68	115.50	108.77
8003	115.02	122.72	111.66	79.93
8004	303.00	160.70	235.70	182.33
8005	127.04	156.86	141.47	163.58
8006	112.14	123.68	113.1	131.37
8007	258.77	97.24	213.1	110.22
8009	106.85	119.35	119.35	110.7
8010	130.41	112.62	132.33	102.52
8011	107.81	126.56	143.87	133.29
8013	135.22	114.06	112.14	120.31
8014	114.54	90.02	108.77	127.04
8015	108.77	92.43	148.2	96.27
8016	167.91	157.33	323.2	154.45
8017	496.27	228.97	53.49	161.66
8019	176.56	103.97	139.06	72.24
8023	102.04	108.77	112.62	173.68
8026	153.97	144.35	228.97	122.24
8027	154.93	128.00	142.43	127.04
8028	22.24	104.93	116.47	127.52
8029	179.93	250.60	125.60	133.77
8030	133.77	130.89	102.52	278.97
8031	22.72	183.77	135.22	121.27
8032	128.97	117.91	141.95	130.95

3.6 Markers of vascular inflammation

3.6.1 Plasma high-sensitivity CRP

The baseline median plasma hsCRP in the treatment arm was 2.1 IQR1.9 mg/l. Following treatment with AGE this was 1.83 IQR 2.11mg/l.

The baseline median plasma hsCRP in the placebo arm was 1.8 IQR 2.3 mg/l. After treatment with placebo this was 2.0 IQR 1.8 mg/l.

KS test showed the results to be of a non parametric distribution and therefore a Friedman (non parametric) Repeated measures ANOVA test was performed. This showed the result in neither arm to be statistically significant ($p=0.90$, $n=26$).

The data for the subjects is shown in table 25.

Table 25: Changes in Plasma hsCRP

Subject no	Baseline Pre-Placebo	Post Placebo	Baseline Pre-AGE	Post AGE
8001	2.94	2.33	2.35	3.39
8002	0.16	0.22	0.17	0.21
8003	0.84	0.77	1.35	1.23
8004	6.34	10.70	9.00	41.90
8005	1.51	2.14	2.77	1.27
8006	6.36	2.17	1.81	3.19
8007	3.00	2.75	3.11	2.64
8009	1.18	1.07	0.91	1.14
8010	0.71	0.65	0.38	0.59
8011	0.62	0.62	0.68	1.02
8013	1.41	1.42	0.40	0.22
8014	1.32	1.66	2.72	2.17
8015	2.49	8.46	3.67	1.50
8016	1.23	1.02	2.51	1.30
8017	0.72	0.60	1.78	1.08
8019	0.47	1.21	0.64	0.72
8023	1.87	1.94	1.51	1.19
8024	8.58	2.07	4.44	2.57
8025	3.19	2.41	2.59	3.54
8026	3.07	17.40	2.59	2.38
8027	2.87	1.99	2.49	7.75
8028	3.27	0.92	0.83	3.72
8029	9.19	8.37	5.47	5.63
8030	0.41	3.46	0.30	1.43
8031	2.63	2.99	2.34	2.30
8032	1.68	2.05	1.80	2.44

3.7 Summary of results

Table 26: Summary of the effect of AGE and placebo on measured parameters

Marker	Mean Pre-Placebo	Mean post placebo	Mean Pre-AGE	Mean post AGE	P Value (ANOVA)
Weight (kg)	98.7±18.5	98.8±18.4	98.2±18.2	98.7±18.2	0.24
Systolic BP (mmHg)	130.3±15.9	131.6±17.5	130.3±14.0	130.8±14.6	0.94
Diastolic BP(mmHg)	74.8±9.8	75.1±8.3	74.7±7.5	73.9±7.7	0.81
Total Cholesterol (mmol/l)	4.2±0.8	4.2±0.9	4.2±0.9	4.2±0.8	0.96
Plasma HDL (mmol/l)	1.0±0.3	1.0±0.3	1.0±0.3	1.0±0.3	0.46
Plasma Triglycerides (mmol/l)	1.6 IQR 1.2	1.5 IQR 1.1	1.4 IQR 0.7	1.4 IQR 0.8	0.04*
Fructosamine (µmol/l)	284±46	270±33	274±33	270±33	0.88
RI post GTN (%)	12.5±8.2	11±8.2	11±5.7	12±7.3	0.52
RI post Salbutamol (%)	8.0 IQR 4.7	9.0 IQR 9.5	6.5 IQR 7.7	6.5 IQR 9.7	0.95
Insulin Resistance (HOMA-IR)	2.5±2.0	2.0±1.1	1.89 ±1.1	1.7±0.9	0.05*
A/C Ratio	0.8 IQR 1.6	0.6 IQR 1.6	0.5 IQR 1.55	0.9 IQR 1.5	0.43
HsCRP (mg/l)	1.8 IQR 2.1	2.0 IQR 1.6	2.0 IQR1.8	1.9 IQR 1.9	0.90
TAOS (µM)	62.9 ±3.6	63.6±5.6	63.1±3.2	64.0±4.4	0.57
GSH/GSSG Ratio	17 IQR 15.1	20.6 IQR22.15	18.8 IQR 21.2	22.8 IQR25.1	0.63
Total blood glutathione (µM)	698.9±193.7	690.9±169.1	690.2±177.9	725.8±224.5	0.67
Plasma LHP (µM)	158.3±97	134.2±29.7	144.7±55.4	134.19±41.1	0.41

* = non-significant in post hoc testing

3.8 Subset Analysis

Given previous studies had studied either only men [261] or found gender differences [265], we performed a gender subset analysis. This found no statistically or clinically significant differences between the baselines of men and women. The 2 values were compared using an unpaired t-test (See Table 27). Measured outcomes in both groups were analysed using repeated measures of ANOVA as previously mentioned and this delivered no significant results in either gender group (see Table 28).

Table 27: Comparison of baselines in men and women

Baseline	Mean Value Men	Mean Value Women	Comparison of baselines (p=)
Hba1c (%)	6.9±1.6	7.5±1.1	0.26
BMI (kg/m^2)	33.2±6.2	31.4±4.1	0.44
Diastolic Blood Pressure (mmHg)	75±10	75±9	0.97
Systolic Blood Pressure (mmHg)	129± 16	131± 16	0.86
Total Cholesterol (mmol/)	4.0± 0.6	4.7± 1.0	0.55
HDL	1.0 ±0.3	1.1 ±0.2	0.68
Triglycerides	1.9± 0.9	2.4± 1.1	0.99

Table 28: ANOVA analysis of men and women subsets

Variable Measured	ANOVA p value Men	ANOVA p value Women
LHP	0.65	0.51
HsCRP	0.74	0.21
TAOS	0.62	0.54
GSH/GSSG ratio	0.71	0.75
Total cholesterol	0.58	0.88
HDL	0.34	0.96
Triglycerides	0.02*	0.17
RI Salbutamol	0.69	0.79
Insulin Resistance	0.17	0.14

*****Triglycerides were only statistically significant as baselines were significantly different

3.9 Associations

For our secondary outcomes, we found baseline correlations between BMI (Body Mass Index) and Insulin Resistance (Spearman rank correlation P= 0.001, R =0.63), BMI and HsCRP (Spearman rank correlation P=0.004, R=0.54), BMI and TAOS (Spearman rank correlation P= 0.05, R=-0.39), BMI and GSH/GSSG ratio (Spearman rank correlation P=0.02, R= -0.44). All of these would be expected as it is well established that BMI is related to the above parameters.

Insulin Resistance and GSH/GSSG ratio were also found to have an association (Spearman rank correlation P= 0.03, R=-0.43). However, when multiple regression analysis was performed to account for the effect of BMI, this association was no longer present.

Endothelial function and CRP correlation showed a trend towards significance (P=0.08). All other correlations were found to be non-significant.

Correlations were taken from baseline measures as no positive post treatment changes had been obtained.

Table 29: Table of correlations

Parameters Correlated	R value (Spearman)	P value
BMI v Insulin Resistance	0.63	0.001*
GSH/GSSG Ratio v Insulin Resistance	-0.43	0.03*
Insulin Resistance v HsCRP	0.27	0.19
Insulin Resistance v RI (sal)	0.33	0.12
Insulin Resistance v TAOS	-0.25	0.24
RI (Sal) v TAOS	0.07	0.72
RI (Sal) v GSH/GSSG Ratio	-0.14	0.48
RI (Sal) v Tot Glutathione	0.27	0.17
RI (Sal) v GSH/GSSG ratio	-0.14	0.48
RI (Sal) v HsCRP	0.34	0.08
RI (Sal) v BMI	0.22	0.28
TAOS v HsCRP	0.0015	0.99
TAOS v BMI	-0.39	0.05*
GSH/GSSG Ratio v HsCRP	-0.3	0.14
GSH/GSSG Ratio v BMI	-0.44	0.02*
LHP v HsCRP	0.05	0.81
LHP v RI (Sal)	-0.02	0.9
LHP v BMI	-0.06	0.78
BMI v HsCRP	0.54	0.004*

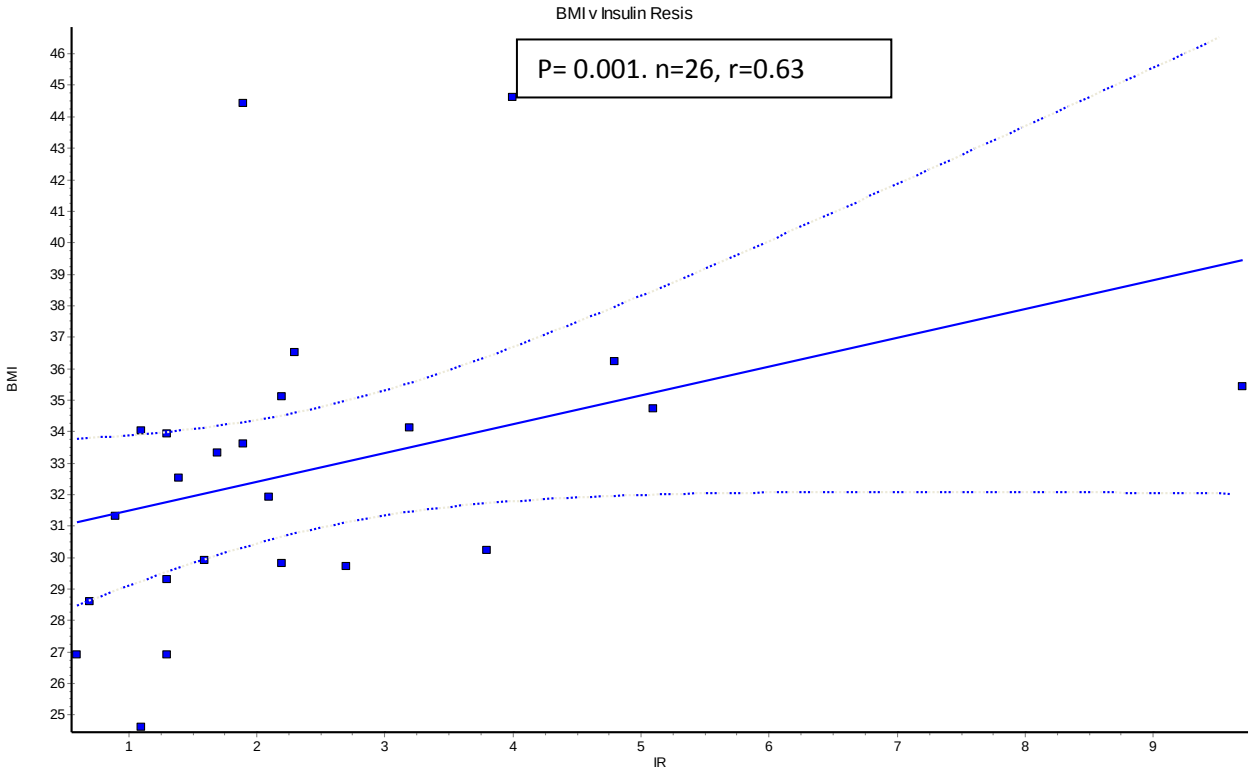


Figure 14. Correlation between BMI and Insulin Resistance

---- 95% confidence interval

Linear Regression

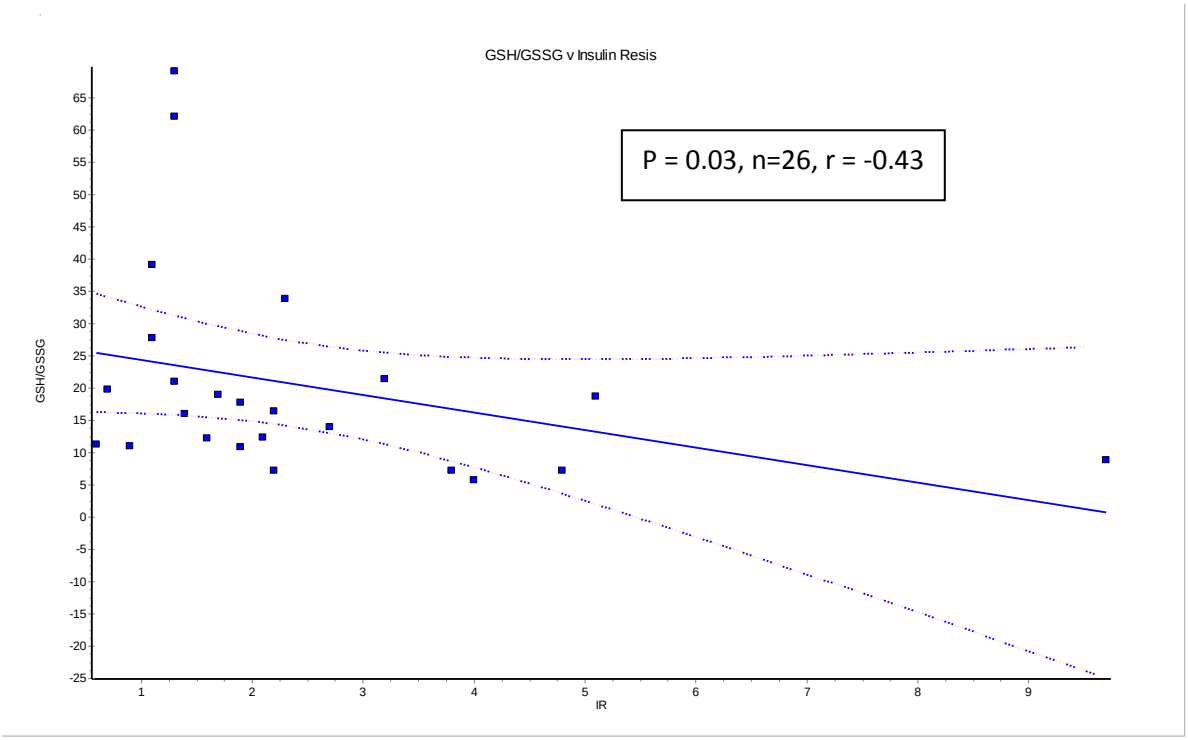


Figure 15. Correlation between GSH/GSSG ratio and Insulin Resistance

---- 95% confidence interval

Linear Regression

3.11 Adverse Events

Two subjects withdrew due to side effects from the medication, namely indigestion. Two subjects withdrew due to concurrent, unconnected illness. None of the data was used in the analysis.

Ten subjects complained of mild indigestion or an unpleasant after taste. Many of the subjects also noticed a garlic odour. Three subjects complained of nausea but these mild side effects did not affect compliance as assessed by tablet count.

Chapter 4: Discussion

As a primary outcome, this study tested the hypothesis that AGE may improve endothelial function, oxidative stress, vascular inflammation and insulin resistance in high risk cardiovascular subjects with type 2 diabetes (defined as >30% CV risk over 10 years). The study also examined the effect of AGE treatment on other metabolic risk factors for cardiovascular disease.

As a secondary outcome we aimed to study the associations between oxidative stress, vascular inflammation, endothelial function and insulin resistance to test the hypothesis that these biochemical processes are interlinked.

4.1 Previous clinical studies examining the effect of garlic upon metabolic parameters

Table 30: Characteristics of previous clinical studies using garlic to examine metabolic parameters

Author	Design	No of Subjects	Duration of treatment	Daily Dose	On Statins	On Anti - HTN medication	Raised Chol (>5mmol/l)	Raised BP (>140/90)	Subjects with DM
Present Study	CO, PC	24	4 weeks	1.2g AGE	96%	46%	11.5%	31%	100%
Williams et al [261]	CO, PC	15	2 weeks	2.4g AGE	100%	100%	0%	0%	0%
Budoff et al [242]	Parallel, PC	65	1 Year	250mg AGE	100%	97%	77%	46%	5%
Duda et al [265]	Long	70	30 days	Garlic oil	NS	100%	NS	100%	NS
Durak et al [320]	Parallel	11	6 months	Garlic extract (1ml/kg)	NS	NS	NS	NS	NS
Durak et al [321]	Long	23	4 months	Garlic extract (1ml/kg)	NS	NS	100%	NS	NS
Kosegolu et al [322]	Long	17	30 days	Garlic powder	0%	0%	0%	0%	0%
Weiss et al [260]	CO, PC	11	6 weeks	4ml AGE	0%	0%	0%	0%	0%
Steiner et al [332]	CO, PC	56	4-6 months	7.2g AGE	NS	NS	100%	NS	NS
McCreadle et al [333]	Parallel, PC	30	8 weeks	900mg AGE	0%	0%	100%	0%	0%
Zhang et al [335]	Parallel, PC	34	7yrs	800mg	N/S	NS	50%	NS	NS
Gardner et al [236]	Parallel, PC	192	6 months	1.8g	0%	0%	100%	0%	0%
Rahman et al [334]	Parallel, PC	23	13 weeks	5ml	0%	0%	0%	0%	0%
van Doorn [275]	Parallel, PC	84	3 months	2.1g	0%	0%	0%	0%	0%

Abbreviations: CO = Crossover, PC = Placebo controlled, HTN = hypertensive, NS = not stated, Long = longitudinal

The current study found no significant effect of 4 weeks treatment with 1200mg daily of Aged Garlic Extract on the metabolic parameters studied in our cohort of subjects. There were some associations at baseline between BMI and insulin resistance, BMI and HsCRP, BMI and TAOS, BMI and GSH/GSSG ratio. There was also an association between insulin resistance and GSH/GSSG ratio but when BMI was factored for, this association was no longer present.

To date few clinical studies have examined the effects of garlic preparations on oxidative stress, endothelial function or vascular inflammation. Most of the current evidence consists of *in vitro* and animal model studies. Previous clinical studies are summarised in table 27 and discussed below.

4.1.1 AGE and oxidative stress

Previously, Budoff et al [242] investigated the effect of AGE treatment upon markers of oxidative damage in subjects at moderate cardiovascular risk. This randomised, parallel, placebo controlled study of 65 individuals employed treatment with 'AGE plus' (AGE + Vitamin B6+Vitamin B12+folate+L-arginine). The markers of oxidation studied were oxidised phospholipids/apoprotein B ratio and malondialdehyde epitopes on apolipoprotein B-100. These are markers of oxidised LDL and represent oxidative damage and have a potential role in the pathophysiology of atherosclerosis. This study showed a statistically significant improvement in these markers after treatment with AGE plus. The strengths of this study lie in its long duration and relatively large numbers. Furthermore, it employed oxidative stress markers that have correlations to angiographically-determined coronary artery disease and cardiovascular disease prognosis [316]. The weaknesses included that the study used a mixture of antioxidant compounds and it is not clear which was the active ingredient. All of the participants were treated with statins and the majority were treated with antihypertensive

medication and therefore were not dissimilar to our cohort. Only 4% of the subjects had diabetes.

However, the findings of the Budoff study have not been consistently replicated. Williams et al [261] investigated the anti-oxidant effect of AGE on men with established coronary artery disease in a crossover study of 15 subjects. In this study 2.4g/day of AGE was used for a period of 2 weeks. None of the participants had diabetes. Plasma markers of oxidative stress were used (FRAP, Ox-LDL and peroxides). Ox-LDL and peroxides are markers of oxidative stress induced cell damage, whereas FRAP (Ferric reducing ability of plasma) is a marker of total antioxidant status. FRAP assays may not detect the actions of all antioxidants in the plasma, particularly thiols, and therefore may not have been the most suitable assay to study the effect of AGE [317]. 2 weeks treatment with AGE failed to have a significant impact upon these oxidative stress markers. These subjects did have known endothelial dysfunction at baseline but it is not known whether their oxidative stress markers were abnormal at baseline. Furthermore, this study did not use a control group. All the subjects in this study were treated with aspirin and statins which could confound any effect of AGE given these agents also have antioxidant effects.

Duda et al [265] studied the effect upon oxidative stress markers of a garlic oil extract in 70 patients with hypertension. This was an uncontrolled, longitudinal study which demonstrated a statistically significant reduction in markers of lipid oxidation after 30 days of treatment. The marker of oxidative stress used was lipid peroxidation products reacting with thiobarbituric acid (TBARS.) This study also looked at antioxidant potential using hemolysate total glutathione levels (GSH) and glutathione peroxidase activity. The study found a significant increase in GSH levels in women only and no significant change in glutathione peroxidase activity. The study also examined plasma levels of the antioxidant vitamins A, C and E, but

only levels of vitamin E improved significantly. 80% of the subjects in this study were treated with a combination of antihypertensives (mainly ACEI). The authors found no significant decrease in blood pressure in this study but did find a significant 9.2% reduction in plasma total cholesterol concentration. The authors concluded that the small changes in markers of oxidative stress seen have uncertain clinical implications. The marker of oxidative damage used (TBARS) employs thiobarbituric acid to detect malonaldehyde (MDA), a product of lipid peroxidation. This method has been criticised as lacking specificity [318]. Blood MDA is metabolised rapidly and may only represent 1% of total lipid peroxides [319]. Total GSH and glutathione peroxidise activity are both markers of single antioxidants and given the complex interaction between antioxidants may be too simplistic a measure of *in vivo* oxidant status [317].This study also examined the effect of garlic oil which has different antioxidant components than those found in AGE. It is unknown how many of the subjects had diabetes or were treated with a statin.

Durak et al [320] performed a clinical study in 11 subjects with atherosclerosis examining the effect of a garlic extract upon markers of oxidative stress. This study employed a control group of healthy subjects but no placebo was involved. The subjects were treated with a number of cardiac medications throughout the study but the baseline characteristics of the 2 groups were not described. The garlic extract used was created using a short term aqueous extraction process in the author's laboratory and, whereas the antioxidant capacity was standardised, the active ingredients were not established. This was given in large doses (1ml/kg) for a period of six months. The markers of oxidative stress used were erythrocyte and plasma MDA and a measurement of antioxidant enzymes though this was poorly described. The authors found that after 6 months of garlic extract treatment there was a reduction in plasma and erythrocyte MDA but there was no effect upon antioxidant enzyme activity. However, the published details of this study were insufficient to allow critical review.

A further study by Durak et al [321] investigated the effect of the same garlic extract on markers of oxidative stress in subjects with high plasma cholesterol concentration (>5.98mmol/l). This was an uncontrolled, longitudinal cohort study. The 23 subjects were further divided into hypertensive (>140/90mmHg) and a normotensive group. The extract was administered at 1ml/kg for 4 months. This study showed statistically significant changes in all the markers of oxidative stress investigated (Plasma MDA, oxidation resistance, antioxidant potential, nonenzymatic superoxide radical scavenger (NSSA) activity). The clinical relevance of these results is uncertain. It is unclear whether the subjects had diabetes or were treated with other medications.

Kosegolu et al [322] studied the effect of garlic powder upon plasma total antioxidant capacity (TAC) in 17 young (mean age 35), healthy volunteers. This was an uncontrolled, longitudinal cohort study using dried garlic powder for a period of 30 days. This study showed there to be a statistically significant increase in plasma TAC after treatment with garlic. Whereas this is not a widely used assay, it did measure total antioxidant capacity of the plasma and is based on a widely used measurement Total Equivalent Antioxidant Capacity [317]. However, this study used a very different subject group, a different garlic preparation and did not have a control arm or a placebo.

This study used a small dose of AGE for a period of 4 weeks. Our results are consistent with the study of Williams et al but the Budoff study suggests a larger dose for a much longer period of administration (>12 months) may be necessary to have an effect upon oxidative stress markers in a cohort treated with statins. The Dillon study [243] suggests that other markers of oxidative stress may change more rapidly but it may be that measurement of F2-isoprostane 8-iso-prostaglandin is more sensitive than other markers of oxidative stress.

4.1.2 AGE and endothelial function

Weiss et al [260] investigated the effects of AGE on flow mediated dilatation in the brachial artery after induced acute homocysteinaemia. Acute homocysteinaemia gives experimentally induced endothelial dysfunction by reducing bioavailable nitric oxide at the endothelium. This cross-over study of 11 healthy individuals found subjects treated with AGE had a 66% increase in flow mediated dilatation in the brachial artery, as measured by Doppler ultrasound, in comparison with the placebo-treated subjects. However, there was also a significant decrease in plasma homocysteine concentrations after treatment with AGE which has a direct endothelial effect. Furthermore, this study recruited a very different cohort to ours (relatively young, healthy individuals with no cardiac risk factors or diabetes) and small numbers.

Williams et al [261] investigated the effect of AGE upon endothelial function in men with established coronary artery disease in a crossover study of 15 subjects. In this study 2.4g/day of AGE was used for a period of 2 weeks. This study employed ultrasound and Doppler measured brachial artery flow mediated dilatation (FMD) as a measure of endothelial function and found a significant improvement following treatment with AGE over placebo. The limitations of this study include its small numbers and the relatively short duration of treatment. This study investigated non diabetic subjects and all of the subjects were treated with statins and aspirin.

Budoff et al [242] investigated the effect of AGE treatment on microvascular endothelial function in subjects at moderate cardiovascular risk. This randomised, placebo controlled study of 65 individuals used 1 year's treatment with AGE plus (AGE + Vitamin B6+Vitamin B12+folate+L-arginine) and measured endothelial function using digital thermal monitoring. This is a mechanism which measures temperature rebound in the hands after a period of occlusive hyperaemia (blood pressure cuff on forearm). These investigators found AGE

significantly increased temperature rebound. This study also discovered significantly reduced coronary artery calcium progression (a marker of atherosclerotic plaque progression) after treatment with AGE. Subjects were all treated with statins and 97% were treated with unspecified antihypertensive agents. Only 5% had diabetes. However, as the agent used was a mix of antioxidants, it is difficult to extrapolate these results to AGE used in isolation. The temperature rebound method of measurement of endothelial function lacks reproducibility and the skin's role as important thermoregulatory organ may mean that vasoreactivity in this organ is not synonymous with other capillary beds [323]. Moreover, the occlusive hyperaemia model for testing endothelial function may not be a purely nitric oxide induced phenomenon and there is a complex of mechanisms involved [324]. As a result, occlusive hyperaemia may be a poor surrogate marker for endothelial function.

In the present study, digital photoplethysmography was used as a marker of endothelial function and found no significant change after garlic or placebo treatment. This may be due to the dose and length of AGE treatment used, which is lower and shorter respectively than some of the studies mentioned above. It may also be that digital plethysmography is a less sensitive tool than FMD for measuring changes in vasomotor activity [325] and as a result we were unable to detect any change.

4.1.3 AGE and inflammation

There have been a small number of previous clinical studies examining the effect of AGE on circulating inflammatory markers. Williams et al [261] studied the inflammatory markers CRP, interleukin 6 and VCAM-1 and found that 2 week treatment with AGE had no significant effect.

CRP is a robust, stable biomarker, with a well standardised assay and an association with clinical outcomes [263, 264, 326, 327, 328]. The lack of effect in the Williams study may have been due to a relative short exposure to AGE. By contrast, Interleukin -6 has only a very short half life and therefore its measurement in plasma is technically difficult and may explain the negative result [326]. V-CAM 1 is thought to exhibit significant diurnal variation [329] and assays lack standardisation [268]. These may account for the failure to detect any change in this study.

Budoff et al [242] used AGE plus, a combination of agents, for a much longer period (12 months) and on more subjects (n=65) than the Williams group. Despite this, AGE was found to significantly reduce markers of oxidative stress but not a circulating inflammatory marker (hsCRP).

There is evidence that CRP exhibits diurnal variation (up to 34%) and only a small (non significant) seasonal variation [330]. The seasonal variation may have been playing a part in the Budoff study but is less likely to be important in this study and the Williams study given the short treatment times. In this study and the Williams study all subjects were sampled in the early morning. No mention of sampling times was made in the Budoff study and this could partly explain the lack of effect.

In respect to the ability of AGE to reduce markers of inflammation, this study supports these previous findings in that AGE had no significant effect upon circulating CRP, probably the most reproducible marker of inflammation. Both the present study and the Williams study could be criticised for a relatively short duration of treatment, though this was addressed in the Budoff study. However, it must be recognised that all the studies involving CRP and AGE have used small numbers (n =15-65) and therefore may lack the power to allow reliable interpretation.

4.1.4 AGE and insulin resistance

Currently, the effect of AGE or other garlic preparations upon insulin resistance has not been studied in clinical studies. The present study suggested that there was no effect on insulin resistance in our subjects after 4 weeks of treatment with AGE.

If oxidative stress and inflammation are integrally linked to insulin resistance as discussed in the introduction chapter then the antioxidant capacity of AGE may have had an effect upon endothelial function in the tissues involved in glucose disposal and thus have a positive effect upon insulin resistance.

4.2 AGE and traditional risk factors for cardiovascular disease

4.2.1 Blood pressure

There have been few studies investigating the hypotensive effects of AGE. AGE has been used to reduce blood pressure in hypertensive rodent models but not in diabetic models [331, 235]. In clinical studies, Steiner et al [332] found that 26 weeks treatment with 7.2g daily of AGE reduced blood pressure in normotensive individuals by 5.5%. Of note, this was a placebo controlled, crossover study of 41 individuals which had crossover arms of different durations (4 months and 6 months) and had a poor compliance rate in the AGE group (<80%). This study was also designed as a study to investigate cholesterol reduction rather than hypertension. It used very large doses and had a high dropout rate (21%) which left it with relatively small numbers.

Further studies using AGE on BP have all shown no effect [261, 333]. McCreadle et al [333] administered 900mg AGE for 8 weeks but had a very different cohort; studying the effects in hypercholesterolaemic children. It is difficult to extrapolate these results to adults.

4.2.2 Lipid Metabolism

Whilst there is in-vitro evidence to suggest AGE may have cholesterol lowering properties, clinical studies have largely shown no significant statistical or clinical effect (see introduction chapter). The present study showed no significant change in total cholesterol, HDL or triglycerides over the 4 week study period.

In previous studies, Steiner et al [332] demonstrated a modest 7% reduction in total cholesterol concentrations (0.4-0.52mmol/l) after 7.2g AGE supplementation for 6 months. Unlike the present study, the subjects in this study were not on statins, did not have diabetes and were hypercholesterolaemic.

A more recent study by Budoff [242] used an antioxidant combination including AGE and was of long duration (1 year). This study found a significant reduction (8.8%) in total cholesterol and this was in addition to statin therapy. As mentioned previously, this study employed a combination of antioxidants therefore it is difficult to attribute this effect to AGE alone.

Further studies have failed to replicate this effect on lipid metabolism and have shown no effect of AGE treatment over placebo [236, 261, 334, 335].

Zhang et al [335] performed a small study (n=12 in each arm) for a long period (7 yrs) but used a very different cohort to the present study (rural Chinese subjects with precancerous gastric lesions) and investigated a mix of garlic supplements and therefore it is difficult to interpret these results.

Gardner et al [236] studied several different preparations of garlic in 192 individuals without hypercholesterolaemia for 6 months. There were 48 subjects in the AGE group but none had diabetes or hypertension.

The Rahman Study [334] was a cohort study and not placebo controlled. Moreover, it was carried out in 23 young, healthy normocholesterolaemic individuals using 5ml of AGE over 13 weeks. This makes extrapolation of these results to those patients/subjects with disease states difficult.

Williams et al [261] also failed to show any effect of AGE on normocholesterolaemic individuals. A similar study by van Doorn et al [273] compared garlic powder, atorvastatin (an established HMG-CoA reductase inhibitor) and placebo, and found that garlic and placebo had no significant effect on plasma lipids after 3 months whereas atorvastatin reduced total plasma cholesterol by 37%. This would suggest any modest hypocholesterolaemic effect of AGE would be dwarfed by co-administration of a statin. It may be that the relatively small numbers used in this study did not allow detection of the small reduction seen in cholesterol levels by AGE in addition to statins. Furthermore, both Steiner and Budoff used longer treatment durations (6 months and 1 year respectively) suggesting longer treatment with garlic may be necessary to reduce cholesterol, particularly when used in addition to statins therapy.

4.3 Associations

As no significant findings were obtained following treatment with AGE, associations were studied at baseline. No correlation was found between markers of oxidative stress, inflammation, endothelial function and insulin resistance. There was no correlation between HbA1c and oxidative stress markers, inflammatory markers or endothelial function.

Despite significant pathophysiological evidence suggesting a link between oxidative stress, inflammation, endothelial function and insulin resistance, clinical studies investigating correlations between these processes have led to mixed findings.

Song et al [377] studied the relationship between oxidative stress markers, DNA damage and insulin resistance in 283 patients with normal glucose tolerance, impaired glucose tolerance and type 2 diabetes. The group found a positive correlation between insulin resistance and markers of oxidative damage and an inverse relationship between insulin resistance and anti-oxidant defence.

Rizzo et al [378] studied 13 obese men with impaired fasting glucose, but not diabetes, before and after 4 weeks administration of combined vitamin E and C. This study found an independent inverse correlation between plasma levels of Vitamin E and oxidative stress markers (plasma [8-] isoprostane levels and TNF-alpha levels). However, no correlation was found with other inflammatory markers or with glucose disposal.

De Matia et al [379] studied the effects of GSH infusion on glucose handling in 10 healthy subjects and 10 subjects with type 2 diabetes. This study found an increase in glucose handling and intraerythrocyte GSH/GSSG levels in both groups and only found a correlation when both groups' data were pooled.

McSorley et al [380] studied 28 healthy, young subjects who were the offspring of individuals with type 2 diabetes. This study found a link between insulin resistance and vasoactive endothelial function. They also studied the effect of vitamin E on these parameters but found no effect.

Kar et al [293] found treatment with the antioxidant Grape Seed Extract (GSE) improved markers of glycaemic control, inflammation and oxidative stress. This was an *in vivo* study examining a very similar cohort to the present study using a similar methodology. Kar et al however found failed to find any correlations between markers of oxidative stress, insulin resistance, inflammation and endothelial function.

Many of the above studies were small studies and associations may not have been detectable with these numbers and the markers used. In the present study, the lack of treatment effect made the study of associations limited. The only significant associations were linked to BMI at baseline. This may suggest adipose tissue could be a potential source of the inflammation and oxidative stress in our patient group. Adipose tissue has been well established as a potential source of a variety of cytokines and cellular components of the inflammatory process [397-401]. However, as yet this remains an association rather than a causative effect.

Associations were calculated using the spearman rank correlation and in cross-over studies it can be argued that this method does not take account of the periodicity (i.e the order in which the treatments were administered). Nor does this method take account of any carry-over effect, ie an assessment of the adequacy of the wash out period. A statistically more robust method may have been to use linear mixed modelling. Although the numbers used in the current study would not have allowed linear mixed modelling to detect any change due to periodicity or carry over [396].

4.4 Limitations of present study

4.4.1 Cohort

In the present study, the baseline oxidative stress markers and vascular inflammation were assumed to have been raised in the diabetic population examined [381,382]. However, a control group without diabetes was not employed, nor have previous studies using these markers established a normal level in healthy individuals.

Our cohort of diabetes patient had reasonably well controlled metabolic parameters which met many of the NICE treatment guidelines [315]. Many of our subjects continued to take diabetic and vascular medications (statins [273], ACEI [336], Aspirin [318] and metformin [265]) throughout the study, all of which have been shown to have anti-inflammatory effects. This suggests the subjects may have had relatively normal levels of oxidative stress and vascular inflammation at baseline and therefore any effect of AGE would have been difficult to detect. Moreover, any effect of the AGE treatment may have been attenuated by concurrent medication use.

The subjects in the present study were also treated for only 4 weeks with AGE, which is similar to previous studies. It could be that this is insufficient time for oxidative stress markers to change. However, a longer study [242] showed significant changes in markers of lipid oxidation but no changes in HsCRP.

Although, the numbers of subjects studied was relatively small, this study was still the largest of its kind investigating oxidative stress markers in subjects with type 2 diabetes. However, our power calculations suggest these small numbers may mean that the study was insufficiently powered to detect any changes using the markers we used in a group with near optimal metabolic control therefore this study could only be used as a pilot study.

The garlic extract used was not palatable for some subjects and the side effects of indigestion and "repeating" was relatively common. We performed a tablet count to assess compliance, however this may not be reliable and it could be that compliance was lower than we assumed.

The trial was designed to be double blind and indeed the placebo and the garlic tablet looked and smelled the same due to the addition of a drop of liquid AGE to the placebo container. Anecdotally, many of the subjects mentioned they could tell the capsules apart as the garlic capsules left a taste and smell of garlic after administration. The subjects were not asked to guess which tablets they were taking so comparison of these results to the unblinded results does not allow any indication of how successful the blinding had been.

We did not assess dietary garlic intake amongst our subjects. This is consistent with previous studies and in line with the manufacturer's advice. It is thought that the concentration effect of the extraction process 1200mg/day AGE would give the same antioxidant content as 7 cloves of raw garlic (12g dry weight), which would overwhelm any effect of dietary garlic (manufacturers correspondence and [335]. However, our subjects verbally confirmed that they were not taking garlic supplements in their diets.

4.4.2 Methodology

This study was a double blind, randomised cross over trial with four weeks of treatment/placebo and then 2 weeks washout followed by 4 weeks of treatment/placebo. This method allowed the subjects to act as their own controls and reduces inter-individual discrepancies. It was assumed that the baseline endothelial function, vascular inflammation and oxidative stress were all abnormal in our diabetic subjects in keeping with previous studies [243, 304, 327]. However, in this study the baselines did return to previous baseline after the washout period in all subjects.

This study used a Bonferroni method for ad hoc analysis of the variables studied. Bonferroni is a widespread method used in many scientific papers to add a further level of scrutiny to the potential of a chance finding (type 1 error) in those investigations of multiple variables [445]. However, Bonferroni method has been criticised in clinical trials as fundamentally examining the wrong question. Bonferroni will only tell us whether there is a statistical difference in the variables but will not tell us which variables differ [446]. The results also depend on how many variables have been examined in each study, which is often not clinically relevant [445,447]. The bonferroni method also is likely to increase type 2 errors [446] (the likelihood of falsely accepting the null hypothesis) which could mean clinically relevant changes are rejected. Use of the bonferroni method for statistical analysis in this study could have been too conservative to detect a meaningful clinical outcome.

4.3.3 Endothelial function

The present study employed digital photoplethysmography measurement of the pulse wave contour as a marker of the vasomotor aspect of endothelial function. Digital photoplethysmography studies the resistance vessels of the microcirculation and thus the blood flow. It uses easily available pharmacological stimulants to distinguish between endothelial effects and smooth muscle effects on vascular tone. This method has the advantages of non-invasive, pain-free measurement of the digital pulse wave contour allowing it to be used in larger numbers of subjects. It is also a relatively inexpensive method and is operator independent [337]. The method has also been studied in diabetic subjects [304] and has been shown to correlate with flow mediated dilatation studies [338]. However, some have criticised the techniques' reproducibility [339] and it lacks association with clinical end points [323]. Flow mediated dilatation studies probably remain the best method of non-invasively assessing vasomotor endothelial function, both in reproducibility [339] and in correlation with

clinical end points [340]. However, this method is expensive, is dependent upon the skill of the operator and requires lengthy training in its use [323, 341].

Microalbuminuria describes small amounts of protein loss in the urine. It is defined as 30-300mg of albumin in the urine over 24 hours. A more practical, spot test of urinary Albumin/Creatine ratio of <2 has also been validated for clinical use [342]. The relationship between endothelial function and microalbuminuria is a complex one and not fully understood [308] but it is thought to be due to protein leakage through dysfunctional renal glomerular capillaries and as such is viewed as a reflection of systemic endothelial dysfunction.

Both endothelial function and microalbuminuria have been proven to predict cardiovascular outcome [343-345]. Many studies have shown an association between vasoactive endothelial dysfunction in those with diabetes and microalbuminuria [307-312]. Microalbuminuria has been shown to be correlated with endothelial dysfunction and plasma hsCRP levels in type 2 diabetes [307] and with insulin resistance [312]. Reduction of microalbuminuria with ACEI has also been shown to improve cardiovascular outcomes independently of effects on blood pressure [346], although whether this is through amelioration of endothelial dysfunction is unclear [347, 348].

Urinary Albumin Creatinine Ratio was utilised in the present study as a measurement of microalbuminuria. At baseline, only 4 of our subjects exhibited microalbuminuria and therefore it was unsurprising no difference was detected following treatment with AGE. Furthermore, baseline changes in reflective index following administration of salbutamol in our patients were in the range 6.5-8%. This is much closer to the control group (5.9%) cited by Cheowiencyzk's et al [304] rather than the diabetes group (11.5%), suggesting our cohort did not have significantly deranged endothelial function pre-treatment.

4.3.4 Oxidative stress markers

Measurement of plasma markers of oxidative stress failed to detect a significant difference between AGE and placebo treatment. Direct measurement of oxidative stress in plasma is very difficult given the extreme reactivity of the free radicals and therefore markers of oxidant status or oxidative damage are used. Measurement of specific antioxidant levels can be misleading as there is a complex interaction between all antioxidants in the system [317]. For example, when vitamin E is oxidised it is regenerated by both vitamin C and GSH. Therefore, a marker of total antioxidant status would give a more biologically significant result [349]. We used TAOS and whole blood GSH/GSSG ratio, both measures of total antioxidant status. We also used plasma lipid hydroperoxides (LHPs) as a marker of oxidant damage to give a marker of overall oxidative stress.

Plasma TAOS, whole blood GSH/GSSG ratio and LHP's have all been used as markers of oxidative stress in previous studies. [350-353]. Plasma TAOS has been associated with cardiovascular outcomes [354]. However, metformin [355], ACEI [356], statins [273] and aspirin [357] have all been suggested to reduce oxidant stress which may confound interpretation.

4.3.5 Circulating markers of inflammation

There was no improvement in hsCRP following treatment with AGE or placebo. Both diabetes and obesity have been proven to be states of low grade inflammation [320-322], though the source of this inflammatory load remains uncertain [346, 347]. HsCRP has been used in

numerous previous studies as a marker of inflammation. It has been proven to be robust [358], stable [359] and predictive of future vascular events [360, 361, 362].

The baseline plasma hsCRP in the present study was lower than similar studies [383, 384] (Jupiter study baseline 4.2mg/l our baseline 2.07mg/l and Korean study 3.0mg/l). The Jupiter study did also not include any subjects with diabetes and only 16% were treated with aspirin [383]. Furthermore, the group treated with Rosuvastatin in the Jupiter trial had a post-treatment plasma HsCRP of 2.2 mg/l; still higher than the pre-treatment baseline in the present study.

Metformin has been linked to a reduction in hsCRP [385]. However, a more recent study in patients with similar baseline characteristics and treatments as our subjects, found no effect of metformin on circulating markers of inflammation [386]. Statins [247] and aspirin [357] have been associated with reductions in circulating hsCRP levels and it could be that the low baseline measurements of hsCRP were due to the actions of these medications. Furthermore, in a head to head study of a statin and garlic powder in an overweight population of smokers, (high inflammatory load), the statin significantly reduced hsCRP whereas garlic had no effect [247]. It is also possible that the cohort in the present study simply were a "low inflammation" group due to current therapy, reflecting their well controlled metabolic baseline state despite the presence of both diabetes and obesity (mean BMI =32.2kg/m^2).

4.3.6 Measurement of insulin resistance

Insulin resistance is the cornerstone pathophysiology in type 2 diabetes and describes a state where there is a reduced biological effect for a given concentration of insulin. Insulin resistance is a complex interaction of many dynamic biochemical and physiological processes and therefore direct measurement *in vivo* is difficult. The gold standard test for insulin resistance is widely accepted as the euglycaemic glucose clamp which measures direct whole body glucose disposal at a given level of insulinaemia under steady state conditions [387, 388]. However, the use of clamps is costly and time consuming and there are some doubts about the intersubject variability (up to 46% in type 2 diabetes) [389]. This makes the use of this technique difficult for larger studies.

To overcome the practical difficulties of direct measurement of insulin resistance, several mathematical models have been derived to give surrogate indices. The Homeostasis Assessment Model (HOMA) is a mathematical model which allows values for insulin sensitivity/resistance to be obtained if simultaneous fasting plasma glucose and fasting insulin/C-peptide are known. Since insulin secretion is pulsatile, this method uses the mean of three results at 5-min intervals (0, 5, and 10-min samples) [285].

HOMA modelling has been shown to be an appropriate method for assessing changes in insulin resistance with time in individuals, is robust and correlates well with euglycaemic clamp studies [387, 390]. This method is also inexpensive and simple to use [388], making it feasible in many circumstances.

HOMA is a surrogate measure of insulin resistance and therefore has limitations. It is also a measure of basal insulin resistance which is largely determined by hepatic insulin resistance. Our hypothesis may suggest that skeletal muscle glucose handling is more likely to be affected by endothelial dysfunction; therefore measuring basal insulin resistance may mean potential

changes are not being detected. Measuring stimulated insulin resistance may have given a better indication of skeletal muscle capillary bed recruitment.

4.4 AGE as a therapeutic agent

This study employed Aged Garlic Extract as a preparation of garlic because its contents are standardised [233]; it has proven safety data and has been used extensively in similar clinical studies [238, 239,242, 243, 260, 261, 332, 369]. It also has extensive in-vitro data in comparison with other preparations and it has been suggested that AGE may have more potent anti-oxidant potential than other garlic preparations [247- manufacturers report].

However, much of the research examining AGE has been conducted in the manufacturer's laboratories and by employees of the company and the results published in journal supplements (non peer reviewed) edited by company employees [391] and this has the potential to introduce bias.

The bioavailability of AGE thiosulphates has been questioned [392] and the results of clinical studies upon markers of inflammation and oxidative stress have been mixed [242, 243, 261]. Therefore, the present results are not inconsistent with previous findings. The present study was also of a relatively limited duration and there have been studies using larger doses and longer durations [242] but with similar results.

4.5 Conclusion and future direction

Treatment of our subjects with AGE showed no significant effect upon markers of oxidative stress, endothelial function, inflammatory markers or insulin resistance in type 2 diabetes in comparison with placebo. The potential reasons for these findings have been discussed. In particular, a baseline of near optimal metabolic control and concurrent administration of

vasoactive medications in our cohort could have been responsible for our inability to detect a response with AGE.

Future consideration should give rise to the use of a different subject cohort with the addition of inclusion criteria such as those with poor metabolic control at baseline. Exclusion criteria could include those on vasoactive medications, which would most likely represent those patients quite early in their diabetes diagnosis and who therefore had not been previously treated with other cardiovascular modifying medication.

Consideration should also be given to increasing the treatment period of AGE given that the evidence would suggest the use of a longer period of exposure to AGE may be necessary, particularly in those taking other cardiovascular modifying medications.

Furthermore, consideration should also be given to a longitudinal study looking at the effect of AGE supplementation upon clinical outcomes in high risk cardiovascular subjects. This would determine whether the changes in surrogate cardiovascular risk markers seen in relatively short studies elsewhere reflect changes in clinical outcome.

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