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**NON-TRANSFERRIN-BOUND IRON AND PROTEIN GLYCATION
IN TYPE 2 DIABETES**

By

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Abstract

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Background and Methods

The involvement of iron in the risk for, and complications of, type 2 diabetes has generated substantial interest over the past 15 years, initially sparked by an association with raised serum ferritin, and the observation that people with iron overload diseases frequently develop diabetes. Considerable advances have since been made in understanding the effect glucose has on molecules, cells, and tissues; and the role that oxidative stress plays in the development of the pathologies of long-term diabetes. Poorly liganded iron is potentially both a contributor to, and consequence of, these complications.

In vitro experiments with glucose-incubated transferrin by earlier workers have demonstrated loss of function with increasing glycation, leading to the suggestion that the failure of this key iron-binding protein may contribute to diabetic pathology, via the presence of redox active non-transferrin-bound iron (NTBI). *In vitro* glycated transferrin is examined here by ultrafiltration, to assess loss of function and possible oxidative fragmentation. Mass spectrometry is used to identify a range of amino acid glycation sites on *in vitro* glycated transferrin for the first time. Finally, several groups have previously measured NTBI in people with diabetes, finding little agreement in results. NTBI is measured here in a cohort of people with type 2 diabetes, using a new adaptation of earlier NTBI assays. NTBI is also assessed in pre-dialysis chronic kidney disease (CKD) stages I to III for the first time.

Results and Conclusions

Experiments with glycated transferrin *in vitro* demonstrate oxidative fragmentation, explaining the loss of function reported by earlier groups. *In vitro* glycated transferrin examined by mass spectrometry reveals a substantial number and range of amino acids subject to glycation. Comparison with *in vivo* glycated transferrin suggests that many of the *in vitro* glycation sites are not glycated *in vivo*, and that there are many oxidized methionine residues which are potential artefacts, or likely to be repaired by methionine sulphoxide reductases *in vivo*. A study of people with type 2 diabetes finds no direct association between NTBI and protein glycation. Unexpected correlations between NTBI and LDL, and LDL and haemoglobin with increasing protein glycation, are reported for the first time. NTBI is suggested to be iron sourced from haemoglobin or haem, from erythrocyte haemolysis prior to sample collection. In people with pre-dialysis CKD stages I to III no significant difference in NTBI level compared to controls is seen, or correlations with markers of renal function. No link between NTBI and kidney function at this stage of disease is indicated.

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Dedication

This work is lovingly dedicated to the memory of my father,
and to John.

“Iron sharpeneth iron; so man sharpeneth the countenance of his friend”

Proverbs 27:17

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the candidate been registered for any other University award without prior agreement of the Graduate Committee.

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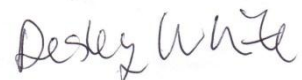
Mass spectrometry of transferrin was carried out by Dr Joanne Connolly and Dr Gushinder Atwal at Waters UK, Manchester, and Dr Kate Heesom at The University of Bristol Proteomics Facility. The *in vivo* study of people with type 2 diabetes was undertaken in collaboration The Diabetes and Vascular Medicine Research Centre, University of Exeter, led by Prof Angela Shore. The specific work carried out by the candidate is described within the text.

The courses and conferences attended, presentations made, and publications, are given as an appendix.

During the four year PhD period of study the candidate was also employed as Dietitian to the Diabetes and Vascular Medicine Research Centre, University of Exeter; and as a Lecturer of Dietetics at Plymouth University.

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Signed



Date

13th August 2012

Chapter 1. Introduction and Background

“A faint magenta tinge, due to the greater exposure of the reduced iron bearing clay, is apt to show where the glaze runs thin on the edge of bowls...This ‘life’ in the old glazes is due in large measure to the presence of elements in the raw material which the old potters either did not know how, or did not desire, to eliminate. The most common was a small percentage of iron.”

Bernard Leach

“A Potter’s Book”

Introduction

The evolutionarily successful *Homo sapiens* has built a world where, for some, the modern environment predisposes for an energetically unbalanced life-style. The consequence of this are metabolic diseases characterised by obesity, inflammation, high blood pressure, and cardiovascular risk; and globally the prevalence of one consequence - type 2 diabetes - is reaching epidemic levels. It is paradoxical that as a species gifted in adaptability we are suffering at our own hands from our success, and that the influence of modern spatial and nutritional environments are frequently greater than an individual’s capacity for beneficial change.

Much research activity has focused on defining dietary risks for type 2 diabetes (in addition to a surfeit of kilocalories). Other research aims to define the processes that drive development, and the later complications of, type 2 diabetes. These complications are life-threatening – heart disease, stroke, renal disease, blindness, and neuropathies. Oxidative stress-related damage, from errant oxygen and nitrogen species, is likely to be involved in at least the pathologies of advanced

disease, and possibly the disease itself; and in biology it seems, where oxidative stress is found, the abundant element iron will soon be suspected of involvement.

A substantial literature exists for the many aspects of iron and risk for diabetes; and iron and links to its later complications. This thesis aims to identify the links for both of these which have the firmest evidence base, and for the former, suggest a scenario by which some of the threads are drawn together. For the latter, experimental work has been undertaken that clarifies some effects reported *in vitro* by other groups, but suggests they may not be as relevant to the *in vivo* reality. Finally, in collaboration with The Diabetes and Vascular Medicine Research Group in Exeter, a cross-sectional investigation is made of the presence of reactive non-transferrin-bound iron in type 2 diabetes, its association with pathologies, and with a range of physiological parameters.

Background

1.1 Type 2 diabetes

1.1.1 Prevalence and impact of diabetes

Globally, the burden of all forms of diabetes now stands at 347 million people, with increases in age standardized prevalence from 8.3% of men and 7.5% of women in 1980, to 9.8% of men and 9.2% of women in 2008. The highest levels are seen in Oceania, approaching 16% of the population (Danaei et al 2011). Within the UK Danaei et al find prevalence to be generally stable, with 7.8% of men and 5.7% of women affected, compared with 8.1% of men and 5.7% of women in 1980. However our population is growing and increasingly elderly, and actual numbers have risen from 1.4 million in 1996 to 2.6 million in 2009. By 2025, the number is

estimated to be 4 million people (Quality and Outcomes Framework 2009, Diabetes UK 2010).

In the UK around £9 billion a year (10% of the NHS budget) is spent on diabetes, including the cost of one in five coronary heart disease, renal, and foot admissions (DH 2006, NDST 2005). Of sufferers, 90% will have type 2 diabetes, which will be up to six times more prevalent in people of South Asian descent, and up to three times more prevalent in people of African and African-Caribbean origin, compared to Caucasians (DH 2007, DH 2001).

Type 2 diabetes has long been associated with obesity and dyslipidaemia (Gordon et al 1977), with obesity being the most critical risk factor (Kahn et al 2006). In UK children, rising levels of obesity are leading to a rapid increase in prevalence of type 2 diabetes, from an estimated 0.21 per 100,000 (0.00021%) in 2000 (Ehtisham et al 2004), to 3.0 per 100,000 (0.003%) in England for 2009 (RCPCH 2009). Rates are set to continue rising as obesity levels increase, from 11% of boys and 12% of girls aged 2 to 15 years in 1995, to 16% of boys and 15% of girls in 2009 (HSCIC 2010)

1.1.2 The pathophysiology of diabetes

In essence, diabetes is a cellular resistance to the action of insulin, and/or the inability of pancreatic β -cells to produce insulin. As insulin is required for the uptake of glucose from plasma its insufficiency results in high plasma glucose levels, and dependence for energy metabolism on fats and protein. In adipocytes and myocytes glucose uptake follows insulin-receptor binding, which stimulates translocation of, mainly, GLUT4 storage vesicles within the cell to the cell surface for

glucose transportation into the cell. Over time, persistently raised blood glucose levels lead to the complications of advanced diabetes.

Type 1 diabetes is a genetically acquired disease, affecting the HLA region of chromosome 6, but also requiring an unknown environmental trigger or triggers. It is an autoimmune disease causing the inflammatory destruction of β -cells.

Type 2 diabetes also has a genetic component, but is not HLA-related. Obesity is a considerable risk factor for type 2 diabetes, and onset of disease can be delayed by lifestyle changes leading to weight loss (Klein et al 2004). The disease progresses from obesity, to insulin resistance, hyperinsulinaemia (with normal glucose tolerance), diminished insulin secretion, to type 2 diabetes. Approximately 90% of diagnosed patients have an islet cell defect, and increased peripheral insulin resistance (Watkins 2003).

A higher risk of disease is seen with abdominal fat distribution, reflecting the link between adipocyte release of non-esterified fatty acids, which induces insulin resistance, and inflammatory cytokine release, which is involved in islet cell damage. A feedback system modulates insulin expression in response to insulin sensitivity, which increasingly fails as type 2 diabetes develops (Kahn et al 2006).

Risk factors for type 2 diabetes are:

- Age ≥ 45 years
- Overweight (BMI $> 25 \text{ kg/m}^2$, but may vary with ethnicity)
- Family history of diabetes
- Habitual physical inactivity
- Ethnicity

- Previously identified impaired fasting glucose or impaired glucose tolerance
- History of gestational diabetes, or delivery of a baby weighing > 4 kg
- Hypertension ($\geq 140/90$ mmHg in adults)
- Dyslipidaemia HDL cholesterol 0.91 mmol/l and/or a triglyceride level ≥ 2.82 mmol/l
- Polycystic ovary syndrome
- History of vascular disease

(Klein et al 2004)

1.1.2.1 The metabolic syndrome

Suggested to affect ~25% of Western adults (Reaven 2002), the metabolic syndrome describes a cluster of conditions associated with obesity, namely, hypertension, dyslipidemia, insulin resistance, non-alcoholic fatty liver disease (NAFLD), and chronic, low-grade inflammation, and which gives an increased risk for type 2 diabetes and cardiovascular disease (Hotamisligil 2006, Achike et al 2011). Shared pathogenic factors are likely between metabolic syndrome conditions and NAFLD, and a review of population studies for the USA, Italy, and Japan suggests that approximately 22% of lean, and 76% of obese individuals have prevalent NAFLD, and 10% to 25% of these may have the progression of this, non-alcoholic steato-hepatitis (NASH) and fibrosis or cirrhosis (Farrell & Larter 2006).

1.1.2.2 WHO criteria for the diagnosis of diabetes

1. Symptoms of diabetes plus random venous plasma glucose ≥ 11.1 mmol/l.

The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss

2. Fasting plasma glucose ≥ 7.0 mmol/l or whole blood ≥ 6.1 mmol/l
3. Two hour plasma glucose ≥ 11.1 mmol/l during oral glucose tolerance test using 75 g glucose load

In the absence of symptoms these criteria should be confirmed by repeating testing on a different day. If the fasting or random values are not diagnostic, the 2 hour value post-glucose load should be used.

1.1.2.3 Diagnostic fasting plasma glucose values

Normal: < 5.6 mmol/l

Impaired fasting glycaemia: ≥ 5.6 and < 6.1 mmol/l; 2-h post glucose load < 6.7

Impaired glucose tolerance: < 6.1 mmol/l; 2-h post glucose load ≥ 6.7 and < 10.0

Diabetes (provisional diagnosis): ≥ 6.1 mmol/l; 2-h post glucose load ≥ 10.0

(préci of Alberti et al 1999, WHO)

1.1.2.4 Complications of advanced diabetes

Chronic exposure to high glycaemia leads to hypertension, microvascular complications affecting the eyes, kidneys, and nerves, and macrovascular diseases including coronary artery disease, strokes, and peripheral vascular disease. A prospective study of 3642 type 2 diabetes patients followed for 10 years (Stratton et al 2000) found that, after controlling for confounding factors, for every 1% (1 mmol/mol) reduction in HbA_{1c}:

- Microvascular complications decreased by 37%

- All micro and macrovascular endpoints relating to diabetes decreased by 21%
- Deaths related to diabetes decreased by 21%
- Myocardial infarctions decreased by 14%

The links between hyperglycaemia and the complications of diabetes are complex and multifactorial, and orchestrated by the combination of inflammation and oxidative stress. The main mechanisms involved are given in outline over the following pages.

1.1.2.5 Vascular disease

In the UK, cardiovascular disease (CVD) accounts for around 33% of all deaths in the general population (Scarborough et al 2010), and type 2 diabetes gives an additional risk for CVD of two to fourfold compared with normal glycaemic control (Wood et al 2005).

Vascular disease results from the effects of hyperglycaemia, insulin resistance, and free fatty acids on the endothelial layer between the bloodstream and the smooth muscle cells of the vessel wall (Creager et al 2003). Central to this pathology is a reduction in the bioavailability of nitric oxide (NO) produced by endothelial NO synthase, and required for vasodilation and degradation of oxygen-derived free radicals. NO level reflects the balance between NO production and inactivation by reactive oxygen species (ROS). In the vascular wall, NO binds to Fe²⁺-haem groups in the enzyme guanylate cyclase in smooth muscle cells, activating them to produce more cyclic GMP, and lowering intracellular Ca²⁺. This relaxes muscle and dilates

blood vessels. Most NO is removed from the vascular system following reaction with haemoglobin (Halliwell & Gutteridge 2007). NO also protects blood vessels by controlling proinflammatory transcription factor nuclear factor kappa β (NF- $\kappa\beta$), and subsequently reducing leukocyte adhesion molecules, chemokines and cytokines, all involved in the development of atherosclerotic plaques via monocyte and smooth muscle cell migration into the intima layer, and the formation of macrophage foam cells within.

Increased circulating free fatty acids from adipose tissue, and reduced uptake by myocytes, induce very-low-density lipoprotein (VLDL) production and cholesterol ester synthesis by the liver, and a decrease in high density lipoprotein (HDL), resulting in dyslipidaemia and increased atherosclerosis risk (Adiels et al 2005). Dietary fats also contribute to the lipid profile, and inflammation. The balance of the types of polyunsaturated long chain fatty acids (LC-PUFA) consumed influences inflammation, as dietary *n*-3 LC-PUFA can moderate the production of inflammatory eicosanoids, cytokines, ROS, and adhesion molecules, if sufficient levels are consumed. Essentially, *n*-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are metabolised to anti-inflammatory 3-, and 5-series eicosanoids, and inflammatory *n*-6 PUFA linoleic acid and arachidonic acid are metabolised to pro-inflammatory 2-, and 4-series eicosanoids, by a shared pool of cell membrane-based cyclooxygenase (COX) and lipoxygenase (LOX) processes, therefore the relative concentrations of the different PUFAs determine the type of eicosanoids produced (Calder 2006).

In type 2 diabetes, risk for myocardial infarction (MI) in people who have not previously had an MI is 20.2%, similar to that of someone without diabetes who *has* previously had an MI (18.8%) (Haffner et al 1998). Haffner et al suggest lipid profile as being a possible reason, although their diabetes groups had significantly less LDL than the non-diabetes groups. Blood pressure was higher in the diabetes groups. Carotid artery intimal wall thickness in diabetes and non-diabetes, and with and without coronary artery disease, suggested that the diabetes subjects with coronary artery disease had accelerated atherosclerosis compared with the other groups.

1.1.2.6 Retinopathy

Diabetes-related damage to the retina is caused by microvascular occlusion, breakdown and leakage. Small haemorrhages occur as dots and blots on the retina. Exudates of lipid deposits can build up around the haemorrhage, and retinal oedema may occur. Ischaemia may lead to the formation of new vessels, which are more prone to haemorrhage. In type 2 diabetes blindness is mainly caused by macular exudate build up, retinal oedema, and ischaemia.

1.1.2.7 Peripheral neuropathies

Damage to distal peripheral nerves, mainly in the legs and feet, is common in type 2 diabetes. Sensory loss may lead to small injuries being unnoticed, which can become ulcerated and septic, and resistant to treatment.

1.1.2.8 Autonomic neuropathies

These include diarrhoea, gastroparesis, postural hypotension, tachycardia, erectile dysfunction, and respiratory attack (discussed by Watkins 2003).

1.1.2.9 Diabetic nephropathy

Diabetic nephropathy develops in around 40% of people with diabetes, and is associated with increased cardiovascular mortality. Genetic susceptibility increases likelihood of disease. Renal function impairment progresses from microalbuminuria to macroalbuminuria, with decreasing glomerular filtration rate, and increasing serum creatinine and hypertension. See Appendix 1.i for UK clinical classification of stages.

Diabetic nephropathy occurs as glomerulosclerosis, with a thickening of the glomerular basement membrane, diffuse mesangial (smooth muscle cells surrounding the glomerular capillaries) sclerosis, hyaline arteriosclerosis (a build-up of proteinaceous material within the extracellular matrix, causing thickening of arteriolar walls). Nodular mesangial expansion (Kimmelstiel-Wilson nodules) is seen in 40 to 50% of patients with proteinuria (Gross et al 2005). Basement membrane thickening and mesangial expansion can be seen in all stages of albuminuria, and severity of lesions is related to glomerular filtration rate and urine albumin excretion, diabetes duration, glycaemic control, and genetic factors (Gross et al 2005). Reduced perfusion of the kidney results in secondary, renovascular hypertension.

1.1.3 Treatment of type 2 diabetes

The NICE Guidelines for the treatment of type 2 diabetes (2008) recommend that, because the disease is a progressive long-term condition that can largely be self-managed, and successful management can achieve optimal outcomes, all patients should be offered a structured educational program about the disease at the time of diagnosis. Nutritional advice should be central to management, with the aims of stabilising blood sugar levels, weight loss, lowering hypertension, and optimising cardiovascular risk. Emphasis should be on a healthy balanced diet, with low glycaemic index carbohydrates, fruit, vegetables, whole grains and pulses; low-fat dairy protein and oily fish; and controlling intakes of high fat and high salt foods, and saturated and trans fat acids. Use of foods manufactured specifically for people with diabetes should be discouraged. Individual recommendations for lifestyle modification should be given, including weight loss and physical activity advice.

HbA_{1c} should be monitored, and individual goals set at or above 48 mmol/mol (6.5%).

Oral glucose control therapies include metformin, that reduces the rate of gluconeogenesis by the liver (Hundal et al 2000), and can stimulate cell glucose uptake in the presence of a high level of free fatty acids (Adnitt et al 1973); and sulphonylureas, that stimulate the pancreas to secrete insulin, for example gliclazide. Insulin analogues include the fast-acting lispro, and aspart; long-acting detemir, and glargine; and mixed types for example NovoMix 30, and Humulin 30/70. Insulin sensitizers may be used, which bind to cell peroxisome proliferator-activated receptors (PPARs) that increase cell sensitivity to insulin, and can inhibit

inflammatory cytokine expression. The safety of some of these has been questioned in recent years, and use of one glitazone (Rosiglitazone) has been suspended in the UK.

Statins are used to treat dyslipidaemia by blocking a rate-limiting step in hepatic cholesterol synthesis. Acetyl salicylic acid (aspirin) is used to prevent myocardial infarction and strokes by inhibiting thromboxane production and blood clotting. Hypertension may be treated by renin-angiotensin system blockade with angiotensin-converting-enzyme (ACE) inhibitors such as lisinopril, and ramipril; and angiotensin II type I receptor blockers (ARB) such as ibesartan, and valsartan.

1.2 Oxidative stress

The many metabolic pathways and cellular and molecular processes of the body produce a constant stream of oxidant species, which are held in check by endogenous and exogenous antioxidants. This is a delicate balance, as some degree of oxidation is necessary, for instance, for the regulation of some cellular processes by oxidation and reduction, and cross-talk for phosphorylatory cell signalling. Sies (1991) describes oxidative stress as “a disturbance in the prooxidant-antioxidant balance in favour of the former, potentially leading to damage”. Whilst oxidative *challenge* may be a part of the normal cellular environment, oxidative *stress* requires a combination of factors to be present. For example, a lack of dietary antioxidant availability alone may not constitute oxidative stress, but combined with increased hydrogen peroxide formation and a lack of glutathione, oxidative damage may occur (Sies 1999).

The free radical species involved have been defined as “any species capable of independent existence that contains one or more unpaired electrons” (Halliwell & Gutteridge 2007). These are reactive oxygen species (ROS), such as superoxide (O_2^-), the highly reactive hydroxyl (HO^\cdot), carbonate (CO_3^{2-}), carbon dioxide (CO_2), peroxy (RO_2^\cdot), and alkoxy (RO^\cdot). Whilst it is a ROS, hydrogen peroxide (H_2O_2) is a non-radical species. Reactive nitrogen species (RNS) are also produced, such as nitric oxide (NO^\cdot), and nitrate (NO_3^\cdot). Sulphur-based free radicals thiyl (RS^\cdot), and perthiyl (RSS^\cdot) also occur, and single hydrogen (H^\cdot), and chlorine atoms (Cl^\cdot). Transition metal ions are also free radicals by this definition, and may donate or accept electrons depending on their electron state.

The effects of oxidative stress on cells are numerous. Many cells respond to mild oxidative stress by increasing proliferation, or by adapting defence systems to protect against damage. Oxidative cell injury can damage lipids, DNA, protein, and carbohydrate. Following injury, the cell repairs or replaces damaged molecules, or survives with persistent damage, or DNA damage may trigger cell death by apoptosis or necrosis. On DNA, free radical damage can produce base modifications, particularly to guanine, and double strand breaks that are difficult to repair and can lead to insertions and deletions from the DNA sequence, and chromosomal translocations (Halliwell & Gutteridge 2007).

Lipid peroxidation occurs by addition of a reaction species, or more usually by abstraction of H from methylene group ($-CH_2-$) by a reactive species. In either case a carbon radical results ($^\cdot CH$ or C^\cdot). The most likely fate of this in aerobic conditions is for the carbon radical to combine with O_2 inside the membrane, forming a

peroxyl radical (ROO^\cdot or RO_2^\cdot). Peroxyl radicals can then abstract H^\cdot from an adjacent fatty acid side chain, in the propagation stage of lipid peroxidation. New peroxyl radicals are formed and so the chain reaction of lipid peroxidation continues. The RO_2^\cdot combines with the H^\cdot that it abstracts to form a lipid hydroperoxide (or lipid peroxide) ROOH . Consequently a single initiation event has the potential to generate multiple peroxide molecules by a chain reaction (Halliwell & Gutteridge 2007).

Evidence of damage by RS occurring *in vivo* includes, on lipids, presence in tissues and body fluids of specific end-products of peroxidation, for example isoprostanes, isofurans, HNE-adducts, with levels increasing during oxidative stress. On proteins, increased carbonyls and other amino acid modifications are seen, low levels of which are detected in healthy animal tissues and body fluids. Nitrotyrosines, products of attack by RNS on tyrosine, have been detected in many normal body fluids at low levels, and at higher levels in diseased tissues. On DNA, low levels of oxidative base damage products present in DNA isolated from all aerobic cells. Levels often increase in animals with cancer or chronic inflammatory diseases, or subjected to oxidative stress by toxins. Some base damage products are excreted in urine, in part from DNA repair processes.

Within the vascular system, NO (itself a radical) is a source of oxygen radicals which may react with superoxide to form peroxynitrite, decaying to become the hydroxyl radical. Loss of nitric oxide by reaction with superoxide is thought to be involved in the development of hypertension, and therefore linked to risk for CVD and renal disease (Bender 2002). NO can also scavenge free radicals, such as the hydroxyl

radical, is a powerful inhibitor of lipid peroxidation, and also binds to ferrous iron – decreasing reactivity with hydrogen peroxide and thus hydroxyl radical production.

Many antioxidant systems exist to control reactive species and maintain homeostasis. Reactive species can be removed by a specific agent, for example the cellular enzymes superoxide dismutase (SOD), and catalases, that detoxify superoxide and hydrogen peroxide respectively; or they may repair other antioxidants, for instance oxidized glutathione may be reduced by glutathione peroxidases. Lipid membrane-based antioxidants, including tocopherols and carotenes, and some phenolic compounds intercept chain reactions of electron loss, preventing greater damage occurring. Antioxidants in the aqueous phase include ascorbate, glutathione, urate, and selenium. Many antioxidants work indirectly, for example plasma metal-binding proteins transferrin and albumin, prevent transition metal catalysed development of HO[•].

1.2.1 The role of oxidative stress in diabetes

1.2.1.1 Introduction

Whilst oxidative stress is generally believed to be involved in both the development, and subsequent complications of diabetes, reviewers have in the past been reticent to attribute redox toxicity to specific events, as many of the processes involved were not fully elucidated. Scott & King, in a 2004 review, found it unlikely that oxidative stress causes initiation of microvascular pathologies, although it may well accelerate the processes involved in their development. Halliwell & Gutteridge (2007) write that oxidative stress *is* thought to contribute to atherosclerosis, and *possibly* also to renal damage, neuropathy, cataract, and

retinopathy. They conclude that, in diabetes, oxidative stress stems from raised plasma glucose, with the development of atherosclerosis appearing to be linked to lipid oxidation and formation of non-enzymatic glycation products, and the development of neuropathy possibly via oxidative stress as a result of decreasing nerve glutathione levels.

Our understanding of the mechanisms linking oxidative stress, hyperglycaemia, and diabetes continues to grow, and a number of well-supported scenarios have been proposed. Tissue and cell types shown to be at particular risk include:

In the development of type 2 diabetes:

- Pancreatic β -cells, which are at increased risk of oxidative damage due to particularly low antioxidant levels (Grankvist et al 1981, Lenzen et al 1996).

In the development of complications:

- Endothelial, neural and kidney mesangial cells, which have insulin-independent glucose uptake and are unable to reduce glucose influx during hyperglycaemia (Kaiser et al 1993).
- Tissues at risk from protein glycation and therefore oxidative stress, including long-lived vascular and nonvascular collagen and lens crystalline, and low density lipoprotein (LDL) (Ulrich & Cerami 2001).
- Neurons, retinal, endothelial, and vascular cells that contain aldose reductase and can switch to polyol pathway metabolism in hyperglycaemia (Lorenzi 2007).

Some authors place great emphasis on the importance of oxidative damage in diabetes, and it has been suggested as a link between the various pathologies involved. Nishikawa et al (2000), and Brownlee (2005), propose that overproduction of superoxide by mitochondria results from increased substrate availability for ATP production, leading to electron 'leakage' from the electron transport chain. How this leads to cell deterioration is described under 'Mitochondrial source of oxidative stress in diabetes'.

The importance of oxidative stress in vascular disease is well supported, and roles include the overproduction of ROS by vascular cells, which then mediate inflammation signalling pathways, and oxidize LDL (reviewed in Madamanchi et al 2005). The oxidation of LDL is believed to be integral to the pathogenesis of atherosclerosis, with an extensive literature, yet as Steinberg (2009) points out, a number of questions remain unanswered. Where does LDL oxidation occur – intravascularly, or within the endothelium? And why have large scale clinical trials with antioxidants generally failed to improve cardiovascular outcomes? Halliwell and Gutteridge (2007) suggest part of the answer this last question lies with the experimental design of many of the large scale studies that have been done. Rather than categorise subjects into groups depending on physiological level of the antioxidant in question, and intervene and test the deficient group alongside a Control group, as would occur for example in an anti-hypertension medication, instead blanket supplementation is given to all, regardless of need. Consequently, when the results are pooled for analysis the results of those who have benefited from supplementation are diluted by those who have not, and perhaps erased by

those for whom unnecessary supplementation has had a detrimental effect on the measured endpoint.

1.2.1.2 Mitochondrial sources of oxidative stress in diabetes: The Brownlee Hypothesis

Proposed as a unifying link for diabetic complications (Brownlee 2005), Brownlee's group report that hyperglycaemia in cells that are unable to limit glucose intake in conditions of extracellular hyperglycaemia, leads to increased availability of electron donors NADH and FADH₂ for oxidative phosphorylation within the electron transport chain (ETC). The voltage gradient across the mitochondrial membrane subsequently increases until a critical threshold is reached, and electron transfer within complex III of the electron transport chain is blocked, causing electrons to be donated from coenzyme Q to molecular oxygen, generating superoxide. This would normally be converted to H₂O₂ by MnSOD, but the mitochondrial antioxidant capacity is apparently insufficient in these conditions. This group also found that insulin resistance led to increased free fatty acid flux from adipocytes into arterial endothelial cells, leading to β -oxidation of fatty acids, with the same result - increasing NADH and FADH₂, and electron leakage from the ETC.

Brownlee suggests that increased mitochondrial superoxide causes DNA strand breaks in the nucleus, activating poly-(ADP-ribose) polymerase (PARP) a repair enzyme that makes polymers of ADP-ribose. PARP utilises NAD⁺ to make ADP-ribose, which accumulates on nuclear proteins including the key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH). GAPDH activity is inhibited, increasing levels of all upstream glycolytic intermediates, with the following effects:

- Increased glyceraldehyde-3 phosphate activates AGE formation as the major intracellular AGE precursor methylglyoxal is formed from glyceraldehyde-3 phosphate.
- Increased glyceraldehyde-3 phosphate also activates protein kinase C (PKC), by increasing formation of diacylglycerol (DAG), a precursor of PKC. This activates a signalling cascade leading to effects including reduced vasodilation, increased vasoconstriction, up-regulated NF- κ B and inflammatory responses, and increased ROS formation (Brownlee 2005, Scott & King 2004).
- Increased glucose causes activation of the polyol pathway, with subsequent reduction of NADPH level limiting GSH formation, lowering cell antioxidant status.
- Increased fructose-6-phosphate causes activation of the hexosamine pathway, leading to production of N-acetyl glucosamine, which can attach to transcription factors, causing modified gene expression, and resulting in dysfunction in cells such as glomerular cells and cardiomyocytes. Activation of the hexosamine pathway is also found to induce oxidative stress and deterioration of pancreatic β -cell function (Kaneto et al 2001).

The presence of methylglyoxal (MGO) and glyoxyl (GO) is known to cause mitochondrial deterioration, and Rabbani & Thornalley (2008), and Morcos et al (2008) have produced work that shows that protection from glycation of mitochondrial proteins by MGO and GO prevents oxidative and nitrosative protein damage and extends life in nematodes. However, there is a gap in Brownlee's

hypothesis, in that it does not account for how mitochondrial superoxide – a fairly unreactive ROS - damages nuclear DNA. To speculate, trace amounts of labile cellular iron, perhaps originating from ferritin or hemosiderin, would be immensely damaging in the presence of increased levels of superoxide from high intracellular glucose as described above. Such a combination would be capable of destroying β -cells, and would explain the loss of insulin production in secondary diabetes of iron overload conditions, where iron is known to be stored in the pancreas (Rahier et al 1987).

1.2.1.3 Oxidative stress, β -cells, and the development of type 2 diabetes

Pancreatic β -cells are at increased risk of oxidative damage due to particularly low antioxidant levels (Grankvist et al 1981, Lenzen et al 1996). Tiedge et al (1997) for instance found that insulin-producing cells in rats produced less catalase, glutathione, and glutathione peroxidase than other tissues, and when challenged by high glucose, high oxygen, and heat shock treatment, they were unable to respond by up-regulation of antioxidants. Lenzen (1997) proposes that this increased sensitivity to reactive species allows β -cells to integrate their role of insulin biosynthesis and exocytosis with a metabolic requirement for glucose, and initiation of glucose-induced insulin secretion. The cell needs to ensure sufficient glucose for its own energy needs, whilst enabling signal generation for insulin secretion (a function of glucose metabolism). In order to sense glucose, and produce insulin, the β -cell needs an environment rich in glucose and oxygen but this increases opportunity for ROS activity. If the redox balance of the cell changes,

and ROS or RNS are over-produced, the β -cell is unable to protect itself and is ultimately vulnerable to cell death.

1.2.2 Oxidative stress in the development of diabetes complications

1.2.2.1 Cell types at particular risk from hyperglycaemia

Glucose entry into some cell types (including endothelial, and some neural and kidney cells) is independent of insulin action, and utilises transporter proteins in a concentration-dependent facilitated diffusion process. The GLUT4 transporter, found in insulin-sensitive skeletal and cardiac muscle and brown and white fat, is down-regulated by high levels of circulating glucose (Klip et al 1994). Other transporters, for instance GLUT1, are ubiquitously expressed throughout the body, in insulin-dependent and non-dependent cells, including erythrocytes. GLUT1 activity is enhanced by raised extracellular glucose concentrations, by either, increased activity, increased expression, or translocation from intracellular sites to the plasma membrane (Heilig et al 1995). The inability to decrease glucose uptake when appropriate means that intracellular glucose concentrations may approach extracellular levels in hyperglycaemia, with subsequent effects on polyol pathway activation and possible mitochondrial dysfunction. Pancreatic β -cells utilise GLUT2 transporters which have the capacity to increase in proportion to extra and intracellular glucose, and so do not become rate limiting in tissues for which it is the predominant transporter (Kahn 1992).

1.2.2.2 The polyol pathway

In conditions of high glycaemia, in tissues that contain the enzyme aldose reductase, surplus cellular glucose causes activation of the polyol pathway of

glucose metabolism, and inhibition of the pentose-phosphate pathway. Tissues affected include epithelium, aortic endothelium and smooth muscle cells, lens and retina, Schwann cells of peripheral nerves, and a number of kidney cell types (Kern et al 1981). The pentose-phosphate pathway is the major cellular source of nicotinamide adenine dinucleotide phosphate (NADPH), which required for reducing and repairing glutathione (GSH), a vital antioxidant for cell protection. Glucose is reduced to sorbitol by aldose reductase (AR) using NADPH as a cofactor, leading to further NADPH depletion in addition to loss of production. In the second step, sorbitol is converted to fructose via NAD^+ reduction to NADH. The overall effects of increased glucose metabolism via the polyol pathway at the expense of the pentose phosphate pathway are to cause cellular depletion of NADPH and NAD^+ , enhancing reactive oxygen species accumulation and subsequent oxidative damage, due to insufficient NADPH availability for reducing glutathione (GSH) level. GSH depletion therefore impairs cell redox balance, increasing vulnerability to oxidative stress (Bravi et al 1997).

1.3 Protein glycation and advanced glycaemic end product (AGE) formation

Nomenclature

'Glycation' refers to the reaction between glucose or other sugars and amino groups of proteins, whether enzymatically catalysed or formed via a Schiff's base and Amadori rearrangement. 'Glycosylation' refers specifically to an enzymatically catalysed reaction (IUPAC-IUB 1984).

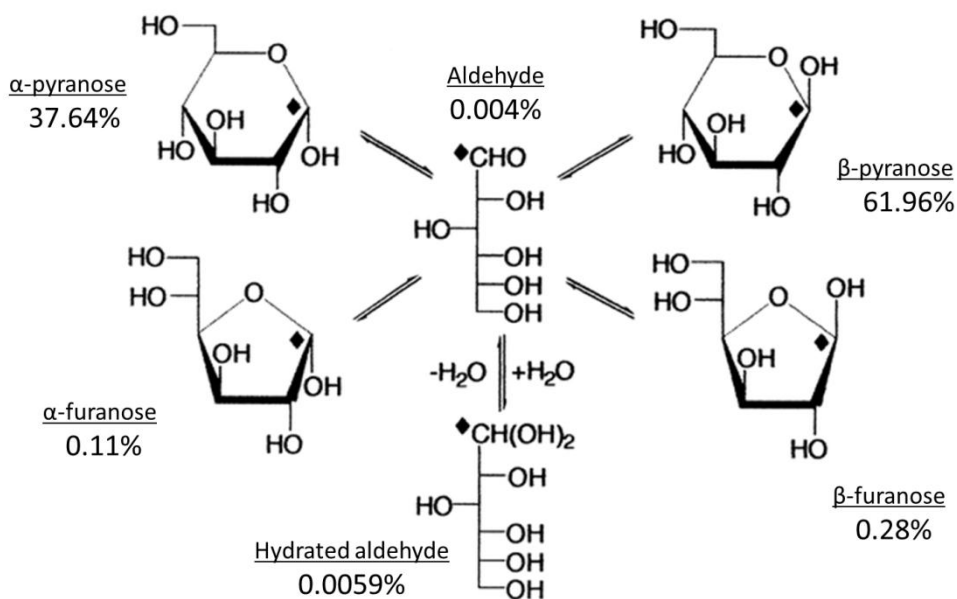


Figure 1. Isomers of glucose, % occurring in aqueous solution

Reprinted and adapted with permission from Zhu, Y. Zajicek, J. Serianni, A. (2001) Acyclic forms of [1-¹³C]aldohexoses in aqueous solution: quantitation by ¹³C NMR and deuterium isotope effects on tautomeric equilibria. **Journal of Organic Chemistry** 66 (19): 6244 – 6251. Copyright 2001 American Chemical Society.

In aqueous solution, glucose exists almost entirely in the pyranose (ring) form, with less than 0.40% in the furanose ring form, and less than 0.01% in the open-chain aldehyde form (Bubb et al 2003)(Figure 1). Acyclic glucose is transient and reactive, due to its carbonyl (C=O) double bond, and a reaction with the open-chain form results in a ring-form converting to take its place, with the four ring forms transforming into each other via the open-chain, until equilibrium is reached (Robyt 1998). Reactions involving the ring-form can include the replacement of OH groups at C-2 and C-6 by hydrogen or amine groups; however the C1 carbonyl of the chain-form is the most active.

Carbonyl groups on sugars such as glucose and fructose react with the $-NH_2$ amine group on proteins, DNA, and lipoproteins, to form glycated proteins. This non-enzymatic glycation is the first stage of the Maillard reaction, also responsible for browning reactions between carbohydrates and amines in cooked food. Changes are reversible in the initial stages, forming Schiff bases followed by Amadori products, which are then oxidised by reactive oxygen and nitrogen species to become irreversible advanced glycation endproducts (AGE). Glycation rate increases with glucose concentration, and AGE formation is dependent on the presence of reducing sugars and reactive oxygen species, in fact, ROS have been described as 'fixatives of glycation' (Halliwell & Gutteridge 2007, Ahmed & Thornalley 2007, Forbes 2008). For diagrams of AGE compounds and their routes of formation see Ahmed (2005), and Ahmed & Thornalley (2007).

The development of AGE products is a normal part of ageing, but is accelerated in diabetes (Ulrich & Cerami 2001). They form over months and years, and are present until the protein is ultimately degraded. HbA_{1c} , the commonly used measure of glycaemia over the previous 6 - 12 week red blood cell life-span, measures glucose Amadori product on haemoglobin beta-chain. Tissues at particular risk are those that are long lived, such as vascular wall tissue, nonvascular connective tissue such as tendon collagen, and lens crystallin. Skin collagen for example has a half-life of 15 years, and cartilage collagen has an estimated half-life of 117 years (Verzija et al 2000). Lens crystallin may last for the entire life time. Low density lipoproteins are also at risk of glycation, as both the amino groups and the lipid groups are potential sites.

There are two main routes for glucose to attach to proteins (Hunt et al 1988): covalent Amadori attachment, and autoxidative glycosylation – transition metal catalysed oxidation of glucose producing hydroxyl radicals and ketoaldehydes. The processes are not clear cut however, and Amadori products can also oxidise, becoming either carboxymethyllysine (CML) or pentosidine (for review see Ahmed & Thornalley 2007). CML is also produced via lipid peroxidation (Fu et al 1996), and this is thought to be important in the atherosclerotic complications of diabetes (Singh et al 2001). Glycated proteins are themselves redox active, and can catalyse lipid peroxidation (Fu et al 1996).

1.3.1 Functional effects of AGE products

AGE products and their precursors are heterogeneous in nature, and have many varied deleterious effects. Immunohistochemistry of human, non-diabetic, post-mortem and biopsy tissue (Ling et al 1989) has identified AGE (carboxymethyllysine (CML), and fluorolink (a fluorescent cross-linked AGE structure), distributed widely throughout the body. AGE are evident intracellularly, in the cytosol and compartments, including the nuclei, mitochondria, golgi complexes, and cell membrane, in the extracellular matrix, within numerous blood vessel endothelial cells, in blood cells, and in various organs including the heart.

Much of the evidence of links with pathologies is indirect, and relies on *in vitro* work. Intracellularly, AGE precursors can bind to and modify gene transcription proteins, impacting on protein synthesis (Brownlee 2005), and bind to fibroblast growth factors, reducing mitogenic activity (Goldin et al 2006). Finding that *in vitro* culture of β -cells with AGE reduced β -cell function, Tajiri et al (1997) suggested an

alteration of transcription factors would be compatible with the effects seen on protein synthesis and insulin mRNA. Within the mitochondria, *in vitro* treatment of vascular smooth muscle cells with methylglyoxal (MGO) – an α -oxoaldehyde AGE precursor – has been found to impair ATP synthesis and MnSOD activity, whilst increasing mitochondrial ROS production (Wang et al 2009). Electron transport chain impairment can result from glycated proteins causing electron ‘leakage’ and superoxide formation (Halliwell & Gutteridge (2007), and Brownlee’s hypothesis may describe only one route to mitochondrial impairment and ROS formation in diabetes.

In cells that utilise the polyol pathway, glucose is metabolised to sorbitol, and subsequently to fructose. The rate of AGE production is enhanced in the presence of fructose and its derivatives, compared with glucose *in vivo* (Goldin et al 2006), and this is thought to contribute to diabetic retinopathy development (Lorenzi 2007).

Shangrai & O’Brien (2004) have demonstrated that Amadori dicarbonyl glyoxal (GO) was cytotoxic to hepatocytes, via lipid peroxidation of the mitochondrial membrane. GSH oxidation, and ROS formation were also evident, and damage could be prevented by ROS scavengers. Human vascular endothelial cells, when exposed to 50 - 5000 μ M GO *in vitro*, produced inflammatory cytokine activation, swelling, and decreased cell density (Yamawaki & Hara 2008). For comparison, plasma GO levels in 20 healthy controls are: (mean \pm SD) $12.5 \pm 0.5 \mu\text{g/l}$, and methylglyoxal (MGO), $8.5 \pm 0.5 \mu\text{g/l}$. Plasma levels in 12 patients with chronic renal failure receiving peritoneal dialysis were: GO, $22.6 \pm 3.0 \mu\text{g/l}$, and MGO, 17.5 ± 6.9

$\mu\text{g/l}$ (Lapolla et al 2005) ($1 \mu\text{M}$ glyoxal = $58.04 \mu\text{g/l}$). The involvement of GO, MGO, and other AGE within endothelial cells in atherosclerosis are reviewed by Basta et al (2004), and include receptor-mediated inflammatory responses to RAGE binding, increased membrane permeability, and increased intracellular oxidative stress.

AGE precursors may diffuse out of the cell, interact with nearby molecules, and affect cell surface receptors and signalling (Monnier et al 2008), for instance by binding to plasma proteins such as albumin, which then bind to and activate AGE receptors (RAGE), triggering inflammatory responses involved in vascular pathology (Brownlee 2005).

In the extracellular matrix, evidence is clearer for the pathological effects of AGE, and is reviewed by Monnier (2008). For example, CML in skin collagen predicts the risk of future diabetes-related pathologies (Genuth et al 2005). Vascular basement membrane collagen is modified by MGO, and impairs endothelial cell survival by detachment of cells and apoptosis (Dobler et al 2006). Dobler et al glycated human type IV collagen under physiological conditions, identified the arginine residues involved in the protein-protein interaction with integrin, and described how the modified arginine (Arg390) would be unable to form a hydrogen bond with the integrin His258. Prevention of MGO formation was achieved with $500 \mu\text{M}$ aminoguanidine. Cross-linking occurs between glycated proteins, most importantly forming glucosepane. Cross-linking leads to tissue matrix stiffening and decreasing elasticity, leading to damage to the lens, within the collagen layer of blood vessel walls, and to the kidney basement membrane (Ulrich & Cerami 2001, Halliwell & Gutteridge 2007). AGE cross-link with collagen. Through cross-linking and redox

effects, AGE and ALE (advanced lipoxidation end-products) are also involved in the progression of diabetic nephropathy, generalised vascular disease, atherosclerosis, diabetic retinopathy, and neuropathy. In the eye lens, cumulative modification of crystallin proteins by glycation, oxidation, deamidation and other changes cause crystalline aggregation, which gradually lead to cataract formation and blindness (reviewed by Sharma & Santhoshkumar 2009).

In plasma, AGE products on LDL have pro-oxidant effects, acting as free radicals by accepting a hydrogen atom from LDL, leading to LDL peroxidation and subsequent involvement with macrophages and the atherosclerotic process (Ulrich & Cerami 2001). Bucala et al (1994) demonstrated that AGE-modified LDL, at the same degree of modification as seen in *in vivo* diabetes, in mice, had impaired plasma clearance due to non-recognition by tissue LDL receptors, leading to increased plasma LDL, dyslipidaemia, and the potential for increased time within the subendothelium, macrophage take-up, and foam cell formation in the arterial wall. In addition to this, and the inflammatory response by AGE receptors to AGE on albumin, AGE formation on LDL reduces NO production (Goldin 2006), decreasing vasodilation whilst matrix cross-linking causes vascular stiffness, adding to increasing cardiovascular risk.

In plasma proteins, Miyata et al (2000) have observed that AGE and ALE accumulate in uraemic renal patients with and without diabetes, and propose that increased oxidative stress plays a role in uraemia by enhancing formation of reactive carbonyl compounds (RCO), which then react with protein residues to form AGE and ALE. Thornalley (2005) suggests that the degradation of glycated proteins and the

formation of “glycation free adducts” via cellular proteolysis leads to the build-up of MGO-derived hydroimidazolones, which are subsequently filtered by the glomerular and reabsorbed or excreted. Two studies by Thornalley’s group examined the fate of proteolytic degradation products in disease. Ahmed et al (2005) found profound increases in plasma concentration and urine excretion of glycation free adducts in 21 type 1 diabetes patients with normal creatinine clearance, compared with 12 healthy controls. Over a range of glycation adduct types, plasma concentrations were increased by four- to ten-fold, and urinary excretion levels increased by two- to fifteen-fold. Plasma protein CML adducts correlated with HbA_{1c}. The authors conclude that the increases are not due to changes in protein turnover but to changes in protein modification, as glycation of albumin has no effect on half-life, and little difference is seen in red blood cell life-span in diabetes (although Cohen et al, 2008, found the average life span of diabetic red blood cell is 56 days, compared to 60 days for controls). In nephropathy, glycation free adducts are insufficiently cleared from plasma (Agalou et al 2005). In people with mild chronic renal failure, Agalou et al found plasma glycation free adducts increased up to 5 times that of healthy controls, and correlated with the decline in renal clearance. Amounts in peritoneal dialysis patients were up to 18 times, and haemodialysis up to 40 times greater. Many glycation-free adducts are known to be uraemic toxins, including CML, MGO, GO, fructoselysine, and pentosidine (Vanholder et al 2003).

Comparing intra- and extracellular effects, Johansen et al (2006) collated protein glycation data from the literature, and used their findings to predict, from the glycation potential of the protein primary structure, and the physiological role of

the protein, a scale of the impact of glycation in different functional realms. Overall, the intracellular environment was more prone to glycation than the extracellular, with the regulation of protein kinase activity, and transferase activity, the most affected realms. Whether this translates to actual effects on health is not known. Extracellular matrix proteins were less prone to glycation, as predicted by the model, and this is borne out by the findings of Zhang et al (2011), who found more glycated peptides from erythrocytes than human plasma. Johansen et al suggest this is due to evolutionary pressure disfavouring extracellular protein glycation, as extracellular matrix proteins such as collagen have a long half-life, and thus being exposed to glucose over a longer period have a greater need to deter glycation.

1.4 Iron in the body

1.4.1 Characteristics of iron binding

Iron has a co-ordination number of 6, therefore it is capable of six bonds within its 4s, 4p, and 4d orbital levels, irrespective of its ionic state. Bonds between atoms of iron and other molecular species (or ligands) are of a dative covalent nature, where a co-ordination bond is formed between a donor and an acceptor (the iron), of a shared electron pair. A unidentate ligand has one bond with the iron, bidentate, tridentate etc. ligands have two or three bonds. A chelate is a ligand with at least two bonding positions (IUPAC 1997). Table 1 lists the main plasma-iron ligands in humans, their size, and typical concentration.

1.4.2 Role, uptake, transport, and storage

The transitional nature of atomic iron, due to its incompletely filled 3d orbital shell, makes it an extremely useful physiological ligand and catalyst. For oxygen carrying, ferrous (Fe^{2+}) iron is bound to four nitrogen ions at the centre of haem, and a dioxygen molecule. Four haem structures make up haemoglobin, with between 200 and 300 million molecules of haemoglobin per red blood cell. Oxygen storage in muscle is facilitated by myoglobin, which also contains a haem group. In the electron transport chain, iron cytochromes alternate between the ionic states of ferric (Fe^{3+}) and ferrous iron, transferring electrons along the chain in the generation of ATP. Iron is also utilised as the active site in many enzymes, including cell lipid membrane lipoxygenases (for full review see Crichton 2009).

In healthy adults, approximately 1 - 2mg of iron per day is absorbed, equal to average losses via menstruation and sloughing of cells. In the modern diet, 0 - 2mg per day is consumed via haem iron (from meat, fish, and poultry), and 12 - 18mg of iron per day is available via non-haem iron (vegetables, pulses, fortified cereals) (Hunt 2005). (See section 1.4.2, and Appendix 1.iii for more information).

Non-haem dietary iron is in the ferric form. An intestinal haem carrier protein (HCP1) been identified by Shayeghi et al (2005), although this is thought to be a folate transporter (Qiu et al 2006). HCP1 is highly expressed in the duodenum, and post-transcriptionally regulated in response to body iron stores. HCP1 mRNA increased in response to hypoxia, but it was unclear whether this was a direct effect on HCP1 mRNA. Following translocation across the brush border membrane, haem is then degraded by haem oxygenase (HO) yielding ferrous iron. Non-haem iron absorption is better established, requiring conversion to ferrous iron by reductase

enzyme duodenal cytochrome b (Dcytb) prior to uptake by divalent metal transporter-1 (DMT-1) in gut enterocyte cells (McKie et al 2001).

Within the cell, ferrous iron is either oxidised and bound to ferritin, or transported across the basolateral membrane by ferroportin. Haem iron also joins this cellular pool. Release of iron to plasma by ferroportin is regulated by hepcidin, which binds to ferroportin leading to its internalisation and degradation within the cell. Hepcidin therefore regulates iron uptake by the body, as ferritin bound iron within the cell is (if not required) is lost when the enterocyte is sloughed from the villi tip. Once across (or prior to crossing - Evans & White unpublished work) the basolateral membrane, ferrous iron is oxidised to ferric iron by hephaestin and bound to transferrin, for transportation within the bloodstream. Ceruloplasmin (a homologue of hephaestin) performs this role in complex with lactoferrin. Two binding sites are available per molecule, and in healthy people, 20-45% of available transferrin binding sites are normally filled. The iron is then stored in cells bound to ferritin, around 4000 atoms per molecule. More ferritin is produced in situations of excess iron. Normally, 75% of absorbed iron is bound to haemoglobin (~80-90% Fairweather-Tait 2001), and 10-20% is recycled through erythropoiesis, and stored in bone marrow (Dooley & Worwood 2000). A healthy male body may typically hold around 4g iron in total, a female around 3g. Serum ferritin levels have been found to correlate with iron stored in bone marrow in healthy people, and 1 µg/l serum ferritin is equivalent to approximately 8 mg of stored iron (Walters et al 1973, in Aggett et al 2010).

Iron in excess of physiological requirements is stored by ferritin, which is most abundant in the liver and muscle, but ferritin-bound iron may be also be deposited in the pancreas, pituitary gland, and joints (Rahier et al 1987). When iron absorption exceeds storage and transporter capacity non-transferrin-bound iron (NTBI) results. NTBI is potentially toxic as it is vulnerable to free radical reactions, and subsequent oxidative damage to lipids, proteins and DNA (Gutteridge et al 1981).

Other than by blood loss or foetal growth, no regulatory method exists for the body to reduce iron stores once it has passed through the gut epithelia, therefore regulation of ferroportin and hepcidin production is closely maintained in response to need, as is the regulation of transport and storage proteins.

Table 1. Plasma iron ligands, size and typical concentrations

Ligand	Molecular mass	Plasma level
Albumin ¹	66 kDa	35 – 50 g/L
Transferrin ²	80 kDa	2.07 - 3.10 g/L
Phosphates ²	< 0.05 KDa	25 - 45 mg/L (~1000 µM)
Citrate ²	< 0.2 kDa	~18mg/L (100 µM)
Haemoglobin ²	68 kDa	0.3 - 8.9 mg/L
Pyruvate ²	0.046 KDa	~2.8 mg/L (~60 µM)
Lactoferrin ³	80 kDa	1 mg/L
Acetate ⁴	0.06 kDa	< 1.2 mg/L (< 20 µM)
Ferritin ⁵	450 kDa	10 - 447 µg/L

¹Roche et al 2008, ² Lentner 1984, ³Farnaud & Evans 2003, ⁴Skutches et al 1979, ⁵Knovich et al 2008

1.4.3 Dietary iron, adult recommendations and sources

In the UK, the recommended nutrient intake of iron for males 19 plus years is 8.7 mg/day; for females 19 to 50 years, 14.8 mg/day; females 50 plus years, 8.7 mg/day (DH 1991). In healthy adults, approximately 1 to 2mg of iron per day is absorbed, equal to average losses via menstruation and sloughing of cells. In the modern diet, 0 to 2mg per day is consumed via haem iron (from meat, fish, and poultry), and 12 to 18mg of iron per day is available via non-haem iron (fortified cereals, pulses, vegetables) (Hunt 2005). The biggest dietary contributors of iron in the UK are cereals and cereal products (44 to 55%), and meat and meat products (13 to 19%) (NDNS 2008/9), giving 10 to 15% haem iron (absorbed at 20 to 50% efficiency), and 85 to 100% non-haem iron (absorbed at 0.1 to 35% efficiency) (Dainty et al 2003, Hunt 2005). High consumers of red and processed meat (> 90 g/day cooked weight) should consider reducing their intakes to the adult population average of about 70 g/day (SACN 2010).

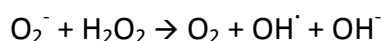
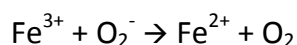
Rates of absorption of both haem and non-haem iron varies depending on the composition of the rest of the diet, and on the iron status of the individual, although this variation is more pronounced for non-haem iron (Hallberg et al 1997). Non-haem iron is more highly adjustable to requirement (Cook 1990, Hunt 2005), although Hallberg et al have demonstrated that, in a mixed diet over 5 days, approximately 40% of both types were absorbed by iron depleted people, but as total absorbed iron decreased the ratio of haem to non-haem altered, becoming at the lowest levels 20:5%, and 15:2.5%. This work showed that although haem iron is absorbed more efficiently than non-haem iron, it is not possible for high meat intakes to lead to accumulating iron stores in healthy people, as absorption was significantly related to ferritin status. They also found that 50% of ferritin variation was unrelated to iron stores, and may therefore be due to other roles, such as up-regulation in response to inflammation. It is not clear why non-haem iron is more adjustable to demand, however there appear to be differences in the mechanism

for absorption into gut enterocytes, and haem iron is thought to be absorbed as an intact porphyrin structure, possibly via endocytosis (Hunt 2005).

Various dietary components are known to influence iron absorption, for instance alcohol, ascorbic acid, and haem iron enhance non-haem iron absorption, and unidentified factor(s) in meat, poultry, and fish enhance haem iron absorption, proposed to be peptide fragments by Storcksdieck et al (2007). Phytic acid, calcium, iron-binding polyphenols, soy protein and egg can inhibit non-haem absorption (Hunt 2005). Other influences on enhanced absorption include erythropoiesis, pregnancy, hypoxia, and inflammation.

1.4.4 Non-transferrin-bound iron

The redox active nature of iron requires that it is tightly controlled by the body, to avoid unwanted reactions, and generation of ROS. In particular, the production of the hydroxyl radical is dangerous, and can occur via the following sequences:



In cells, a constant stream of superoxide is produced by mitochondria during ATP production. Superoxide and hydrogen peroxide are also produced during the respiratory burst prior to phagocytosis (Dahlgren & Karlsson 1999). Ferric iron (for example from ferritin) can then accept an electron from the superoxide, producing ferrous iron and oxygen. The Fenton reaction, between ferrous iron and hydrogen peroxide, can then produce the hydroxyl radical (Halliwell & Gutteridge 2007), which can participate in oxidative damage to lipid membranes, proteins, DNA, and lipoproteins, as described earlier. Even trace amounts of redox available iron (or

copper) can produce a range of damaging effects to the functioning of proteins, cells, and tissues, therefore numerous mechanisms exist for the safe transport and storage of iron. The interruption or disruption of these can result in the appearance of irregular and toxic “non-transferrin-bound iron”.

Non-transferrin-bound iron (NTBI) is a generic term for any plasma iron that is redox available, irrespective of its source or form. Redox inactive forms may also occur. It was initially observed in patients with iron overload and transferrin saturation, by Hershko et al (1978), is seen following intravenous and oral iron supplementation, and exists in numerous physiological conditions (see Appendix 1.ii for a compilation of relevant studies, and see 2009 for a review). NTBI can appear without transferrin saturation, although its existence may be transitory. Its chemical nature is not fully defined, and there are thought to be a number of isoforms, which vary with disease state. Studies in mice demonstrate at least three different fractions of NTBI (Simpson et al 1992), with a range of molecular masses from less than 10 kDa, to 150 kDa or greater, relating to the plasma component with which it is (loosely) bound. Suggested ligands include citrate, the similarly structured Heidi, albumin, albumin-citrate, acetate, pyruvate, and phosphates (Hider 2002, Evans et al 2008, Silva & Hider 2009). Different NTBI species may be taken up by cells by a variety of mechanisms, possibly accounting for the differences seen in organ iron loading and distribution in various iron overload syndromes (Evans et al 2008).

Despite its known existence in many pathological conditions, there is little consensus on physiological levels of NTBI within different diseases, and a lack of

standardised assay methodology (Jacobs et al 2005). Different assays may be measuring iron attached to different ligands. These factors, and the difficulties inherent in collecting and quantifying reactive iron without interference from safely transferrin-bound iron, have prevented NTBI measurement becoming a useful clinical tool.

1.4.4.1 Non-transferrin-bound iron and type 2 diabetes

Levels of non-transferrin-bound iron in type 2 diabetes have been investigated by several groups, using different methods and reporting a range of results. A discussion of these forms part of the Background to Chapter 4.

1.4.5 Iron as a risk factor for type 2 diabetes

Genetic iron overload disease, haemochromatosis, is a risk factor for secondary diabetes (McClain et al 2006) due to impaired insulin secretion, as a consequence of pancreatic-iron deposition (Rahier et al 1987). This has led to a great deal of investigation of iron status as a risk for diabetes, although the presence of increased pancreatic iron has not been demonstrated in non-haemochromatosis type 2 diabetes, and the iron marker of choice is serum ferritin, which is up-regulated in inflammation, and clinically more useful for measuring iron deficiency (Knovich et al 2008). Two lines of research have fuelled these studies: physiological iron status and risk for disease, and dietary iron intake and risk for disease. The latter is discussed in section 1.5.

Evidence for a link to risk for diabetes was first put forward by Salonen et al (1998), reporting positive serum ferritin correlations with serum insulin and blood glucose, without controlling for inflammation. Similar findings are reported by Ford &

Cogswell (1999), Fumeron et al (2006), and Sharifi et al (2008), using C-reactive protein (CRP), and Jehn et al (2006), who use an inflammation score to control for inflammation. Using CRP alone does not fully account for inflammation, (Rajpathak et al 2009, DePalma et al 2010, and see Table 28), and the results of studies controlled thus may still include inflammation as a factor in the level of risk seen. Jiang et al (2004b) followed 32,826 females for one year, comparing the 698 who developed diabetes with matched controls, using both serum ferritin and soluble transferrin receptor (sTfR) to ferritin ratio to assess iron status. The sTfR:ferritin ratio relies on increases of STfR seen during iron-deficient erythropoiesis (Skikne 2008). Using serum ferritin with multivariate adjustments including for CRP they found an increased relative risk for type 2 diabetes in the highest quintile of serum ferritin to be 2.61 (95% CI 1.68 to 4.07), $P < 0.001$ for trend. Occupation of the lowest quintile for sTfR:ferritin ratio gave a relative risk for diabetes of 2.40 (95% CI 1.55 to 3.71), $P = 0.02$ for trend.

Fernandez-Real et al (2005) found a protective effect of repeated venesection has been used as on risk for diabetes in a small study of 21 regular blood donors, however, other studies disagree. Zheng et al (2007) found no significant difference in risk between frequent donors (> 8 per 2 years) and infrequent donors (1-2 per 2 years). The results of the largest study of this kind (Jiang et al 2004b) do not support a link between blood donation and risk for type 2 diabetes, following 33,541 men for 12 years. The highest quintile of donation (≥ 30 in 30 years) gave a relative risk of 1.12 (95% CI 0.78 to 1.61), $P = 0.70$ for trend.

In summary, increased iron status, as measured by serum ferritin, and sTfR:ferritin ratio, has a positive association with risk for type 2 diabetes. This risk does not appear to be reduced by regular blood donation, and the adaptability of the gut to adjust iron absorption to physiological need may lead the body to respond to venesection by absorbing more iron.

As an anabolic peptide, insulin stimulates the cellular uptake of various nutrients besides glucose, including iron (Davis et al 1986). Davis et al demonstrated up-regulation of cell-surface transferrin receptors (TfR) in adipocytes in response to insulin. This would imply a reduced take-up of iron by insulin sensitive cells where insulin is lacking, perhaps raising plasma levels and predisposing non-insulin sensitive cells (such as endothelial, and some neural and kidney cells) to receiving the iron. Transferrin receptor regulation is also stimulated by hypoxia, via hypoxia-inducible factor (HIF-1) (Tacchini et al 1999), and the case could be made for a further source of iron uptake by cells, where intracellular hyperglycaemia leads to an increased cytosolic ratio of NADH to NAD⁺, and subsequent cell “pseudohypoxia” (Williamson et al 1993). Hypoxic responses might then promote TfR expression and increased iron uptake.

1.4.5.1 Iron and the metabolic syndrome

Using data from 6,044 adults contributing to the Third National Health and Nutrition Examination Survey (NHANES III), Jehn et al (2004) have found a positive association between serum ferritin quartile and prevalence of the metabolic syndrome. Multiple regression analysis including CRP as a variable, for post-menopausal women, found an OR for the metabolic syndrome in the highest

quartile of serum ferritin ($> 168 \mu\text{g/l}$) compared to the lowest ($\leq 54 \mu\text{g/l}$), of 2.7 (95% CI 1.7 to 4.1, $P < 0.001$ for trend). For men, the highest quartile of serum ferritin ($> 231 \mu\text{g/l}$) compared to the lowest ($\leq 86 \mu\text{g/l}$), gave an OR of 1.6 (95% CI 0.9 to 2.7, $P < 0.11$ for trend). The results for men is non-significant, and reflects the OR for the third quartile of serum ferritin (146 to 231 $\mu\text{g/l}$), of 1.6 (95% CI 0.8 to 3.2), the same as the fourth quartile but with a larger confidence interval, suggesting that the association between ferritin and the metabolic syndrome for men is weak.

1.4.5.2 Insulin resistance-associated hepatic iron overload

Hepatic iron loading, associated with overweight, dyslipidemia, hypertension, and glucose intolerance, and separate from genetic iron-overload disease, was first identified by Moirand et al (1997). Termed 'insulin resistance hepatic iron overload' (IR HIO), or 'dysmetabolic iron overload disease' (DIOS), it is seen in about a third of patients with NAFLD and the metabolic syndrome (Dongiovanni et al 2011).

IR HIO is associated with raised serum ferritin, independently of CRP, and increased liver iron, seen in periportal hepatocytes and less frequently in sinusoidal Kupffer cells (Moirand et al 1997). Altered iron regulation is seen, in association with subclinical inflammation (Dongiovanni et al 2011), and increased hepatic hepcidin expression correlates positively with hepatic iron concentration, whilst transferrin receptor mediated iron uptake is not associated with iron accumulation in NAFLD (Aigner & Datz 2008). Altered iron homeostasis may also be linked to low copper bioavailability, as copper is required for ceruloplasmin and hephaestin ferrioxidase activity (Aigner & Datz 2008). Aiger et al (2008) found an inverse relationship

between iron and copper stores in patients with NAFLD. Whether or not this is dietary in origin is unknown, but dietary sources of copper are broad, and include legumes, potatoes, nuts and seeds, beef, cereal grains including breads and breakfast cereals, and poultry (Ma & Betts 2000). Data from the 2008/2009 UK National Diet and Nutrition Survey find UK adults to have sufficient copper intakes (NDNS 2008/2009).

Whilst increased stored liver-iron is thought to cause insulin resistance, the mechanisms by which it does so are undefined (Dongiovanni et al 2011). In a review of insulin resistance and liver disease, Bugianesi et al (2005) give peripheral muscle rather than the liver as the primary site of insulin resistance in NAFLD, with the most likely mechanism to be cytokine-induced serine rather than tyrosine phosphorylation of insulin receptor substrate, IRS-1. Bugianesi et al suggest the likely progression of peripheral insulin resistance to be hyperinsulinemia, increased lipolysis, influx of fatty acids to the liver, and increased liver triglycerides, followed by hepatic steatosis and hepatic insulin resistance. The major source of intrahepatic fat, contributing to fatty liver disease, is plasma free fatty acids from adipose tissue (Donnelly et al 2005, in Bugianesi et al 2005), and therefore it appears that, following inflammation, peripheral insulin resistance and hyperinsulinemia drives adipocyte lipolysis, which causes hepatic fat deposits, and subsequent liver disease.

Hepcidin upregulation as a result of chronic inflammation might be suggested to lead to less hepatocyte-iron build up, via less enterally absorbed dietary iron, however Aiger & Datz (2008) propose that hepcidin upregulation is a consequence

of hepatocyte-iron accumulation, as iron is a stimulus for hepcidin production, and persistently high hepcidin levels would induce iron deficiency, which is not seen in biopsies of patients with NAFLD. Iron depletion therapy has been shown to increase peripheral and hepatic insulin sensitivity, increase pancreatic insulin sensitivity, reduce HbA_{1c}, improve liver function tests, and ameliorate low-grade inflammation, in patients with IR HIO, by numerous studies (see reviews by Aigner & Datz 2008, and Dongiovanni et al 2011).

As around a third of NAFLD and metabolic syndrome patients will have IR HIO, and these conditions are strong risk carriers for diabetes, a proportion of people with type 2 diabetes could be expected to have hepatic iron overload. Very few studies have measured liver iron in type 2 diabetes, however. Dineen et al (1994) finds, in 15 diabetes patients, and 17 age-matched controls, no significant difference in mean hepatic iron (1,303 vs 1,349 µg Fe/g dry weight of liver; $P = 0.87$, in diabetes vs controls consecutively). To compare with other conditions, Moirand et al (1997) found, in 65 IR HIO patients median liver iron concentrations of 4,760 µg Fe/g dry weight of liver, and in 65 controls with haemochromatosis, matched for a range of serum ferritin levels from 398 to 1,691 µg/l, 2,016 µg Fe/g dry weight of liver. Liver iron content of healthy controls ($n = 20$) was found to be 636.1 ± 203.4 µg Fe/g dry weight of liver by Aiger et al (2008). Given the low numbers of participants in Dineen et al's (1994) study, it is possible that they did not include any patients with hepatic-iron overload, and it may be the case that a particular genotype or types predisposes for liver-iron-loading. George et al (1997) found that heterozygosity for the haemochromatosis gene mutation Cys282Tyr was associated with increased hepatic iron, but not serum ferritin, in 16 non-alcoholic

steatohepatitis patients (NASH) with the mutation, compared to 35 NASH patients without the genotype.

1.5 The link between dietary iron and risk for type 2 diabetes

1.5.1 The historical perspective: Dietary adaptation theory of diabetes

Humans evolved from a plant-eating ancestor living 2 to 4 million years ago. The adaptation to hunting, and a diversely mixed diet of animals in addition to plants, appears to be linked to global cooling and ice ages, and the expansion of drier open savannah in place of forests (Brand Miller & Colagiuri 1994). What the diet of early man consisted of has been revealed by a number of methods, including comparative studies of the diets of hunter-gatherer societies today. Work by Cordain et al (2000) looking at current hunter-gatherers finds a reliance on meat, fish, and other tissues for between a third to two thirds of their diet, preferring to obtain at least half of their energy from animal foods when possible. In some cultures protein intakes reach 86% of total energy intake. Evidence from fossil isotope and tooth enamel studies indicate large quantities of grazing animal meat in ancient diets (reviewed in Mann 2000), and comparison of gut physiology with other mammals also suggests that meat contributed a significant amount to the diet of early hominids (approximately 1.8 million years ago). Diets would have varied depending on environment, season, and latitude; and carbohydrate intake would have consisted of foods such as fibrous vegetable roots and tubers, berries, honey, and fruits when available (Brand Miller & Colagiuri 1994; Leach et al 2006).

As man evolved the human brain size increased threefold over a period of 3.5 million years (Mann 2000), to represent 2% of total body weight, approximately 1.3

to 1.4 kg. This would have required long chain polyunsaturated fatty acids (PUFA), particularly arachidonic acid and docosahexaenoic acid (DHA) for its composition (whole brain is 10 to 12% lipid, McIlwain & Bachelard 1985), and increased energy intake for brain metabolism (nine times higher per kg than human tissue as a whole). O'Dea (1991) and Wendorf & Goldfine (1991) have proposed that insulin resistance would have been of benefit to people faced with periods of food scarcity, conserving what glucose was available for brain and reproductive functions. Insulin resistance in tissues such as muscle would have preserved glucose for these vital functions, and as a survival advantage, insulin resistance may have been genetically selected for throughout human evolution. Periods of starvation would have also led to a bottleneck effect in populations, where survival only by those with the best insulin resistance would have ensured the genotype was passed on to future generations.

Mechanisms involved in the initiation of insulin resistance by nutrients in plasma have been demonstrated by a number of groups. Postprandial levels of amino acids, particularly branched chain, interfere with insulin signalling in animals (Patti et al 1998), and in humans (Krebs et al 2002), by inhibiting the early stages of post-insulin receptor activity, preventing phosphorylation of insulin receptor substrates (IRS) and subsequent protein kinase C and other kinase sub-units, consequently interfering with GLUT4 translocation to the cell membrane (reviewed by Zierath et al 2000). This cell signalling pathway is also inhibited by plasma free fatty acids (Dresner et al 1999, Griffen et al 1999), and up-regulated by exercise and hypoxia (Zierath et al 2000).

Protein initiated insulin resistance would have benefited hunter-gathers, where low glucose intakes would be the norm, and a protein rich meal could inhibit muscle glucose uptake, conserving available glucose for the brain, and allowing peripheral cells to metabolise lipids and protein for energy. For modern man this advantage has become a disadvantage. Brand Miller & Colagiuri (1994) propose that cultures that adopted agriculture early, such as the Near East, Mediterranean and Europe, have been adapting to dietary change over a 12,000 year period, consequently eating less meat, of altered fat type, and relying on domesticated animals and cultivated grains. Brand Miller & Colagiuri suggest that these people now have less type 2 diabetes than cultures such as the American Pima Indian, Australian Aboriginal, and Inuit peoples, who have only recently adopted Western lifestyles, and have rates of diabetes several times higher than European populations. This period of time is known to be sufficient to alter gene frequency, they argue, as adult expression of lactase has developed in European people over the same period. In support of this, Mann (2000) report that when urbanised diabetic Australian Aborigines return temporarily to their traditional diet, their condition improves.

In the western world in recent centuries, changes in the quality of dietary carbohydrate as a result of the industrial revolution and the milling of grains, as well as the introduction of potatoes to our diets, has led to meals of higher glycaemic load, requiring higher postprandial insulin levels. Brand Miller & Colagiuri suggest that the modern diet has a higher carbohydrate intake, of a different quality, than the body has evolved to cope with, and that one physiological effect of this is the requirement of pancreatic β -cells to produce increased volumes of

insulin, beyond the capacity required in the past. Many other aspects of lifestyle have also changed of course, and many factors influence the body's progression towards diabetes. We live longer, and have decreased energy expenditure, increased portion sizes, increased energy intake from fats, and changed intake of fatty acid types. A lower ratio of n-3 to n-6 PUFA in the modern diet for instance promotes an inflammatory cellular environment (Calder 2006).

Clearly type 2 diabetes is a disease of lifestyle and environment at odds with the human body as it has evolved to be, and a review of how dietary iron fits into this picture is relevant, given the reports of a positive association of haem iron intake and risk (Rajpathak et al 2006; Lee et al 2004; Jiang et al 2004, for example), and the growing evidence claiming disordered iron homeostasis in the diabetic condition.

1.5.2 Dietary haem iron intakes – ancient and modern

Considering whether there is any relevance to links between changes in iron intakes and development of insulin resistance, it is evident that historically, humans would have had a plentiful supply of iron from diets rich in muscle tissue from meat and fish. Why then has haem iron intake been so positively associated with risk for type 2 diabetes? It seems unlikely to be due to elemental iron itself, considering the high levels consumed in ancient diets, and the body's ability to regulate dietary absorption of both haem and non-haem iron to match requirement (Hallberg et al 1997). Hallberg et al's work showed that whilst haem iron is more efficiently absorbed, high intakes do not lead to accumulating iron stores in healthy people.

Although absorption of both types are influenced by iron status, the effect on non-haem iron is greater.

One explanation may relate to other factors present in the source of haem iron rather than the iron itself. Cordain et al (2002) in their ethnographical study of 229 hunter-gatherer societies, found that of the 55 to 65% of daily energy from animal foods (haem iron providers), half would be derived from aquatic animals, and half from terrestrial animals. This would provide a fatty acid profile significantly different from that seen in the typical modern diet, with a saturated fat content of 7% energy compared with between 9 and 19% today (Salmeron 2001), and an n-3 to n-6 ratio of 1:1.5, compared with >1:15 today (Simopoulos 2006). In support of this, Hu et al (2001) in a review of cohort studies of dietary fat, carbohydrate, and risk for diabetes, found that higher intakes of PUFA, and possibly n-3 PUFA, could be beneficial, but there was no general consensus on proportions of total fat or carbohydrate as a risk factor for diabetes. Substituting a high saturated fat diet for a high monounsaturated fat diet has been shown to improve insulin sensitivity in 162 healthy men and women over a 3 month period, by Vessby et al (2001), however they failed to show any benefit in fat type substitution for individuals with a high fat intake (>37%), or with the addition of n-3 fatty acids. A review by Riccardi et al (2004) also found consistently positive associations between saturated fat and hyperinsulinaemia, though again, n-3 supplementation had no effect on insulin action. On the other hand, Steyn et al (2004) do report two epidemiological studies that found an inverse relationship between fish intake and glucose tolerance, and positive links between total fat, and saturated fat and diabetes risk. They recommend total fat intakes not exceeding 30%, and saturated fat not exceeding

7% total energy intake as beneficial to diabetes risk. They did not include iron or meat intakes as part of their review.

1.5.3 Review of cohort studies linking haem iron intake and risk for diabetes

A closer look at the studies that have reported haem iron intakes to be positively associated with risk for type 2 diabetes finds no clear picture of any risk associated solely with red meat over other sources of haem iron. For instance, Lee et al (2004) found multivariate adjusted relative risk (RR) for diabetes increased from 1.0 to 1.28 across quintiles of haem iron intake, and from 1.0 to 1.2 for red meat only, in 35,698 postmenopausal females over 11 years (The Iowa Women's Health Study). Jiang et al (2004b) followed 38,394 men for 12 years, finding relative risk increased from 1.0 to 1.28 over the quintile range for total haem iron intake (in support of Lee et al), however no increased risk was found to be associated with haem iron from other sources. Rajpathak et al (2006) followed 85,031 females for 20 years (The Nurses' Health Study), finding relative risk increased from 1.0 to 1.28 over the quintiles for total haem iron intake, but that red meat and other haem iron providers both independently raised risk, agreeing with Lee et al. It should be noted that Rajpathak et al, and Jiang et al, in their dietary analysis interpret red meat intake as being solely haem iron, but in fact it has a significant proportion of non-haem iron (from ferritin and mitochondria). For example in beef muscle meat 64% of total iron is in the haem form (Valenzuela et al 2009). Other studies have used a factor of 0.6 (Monsen et al 1978) to calculate non-haem iron in meat. Incorporating this attributes greater weight to a link with haem iron by Rajpathak et al, and Jiang et al.

On balance, it is not clear from these studies whether it is haem iron, as red meat, or total haem iron (red meat, poultry and fish), which increases risk for diabetes. Jiang et al also calculated RR for total iron, which over the quintiles increased from 1.0 to 1.16. This suggests that the iron content per se is less influential than dietary haem iron intake and risk for diabetes, and that other factors related to red meat, poultry and fish are affecting this.

An obvious possibility might be saturated fat - common in red meat products - but data analysis included adjustment for saturated fat by Jiang et al, animal fat by Lee et al, and polyunsaturated fat to saturated fat ratio by Rajpathak et al, ruling this possibility out. Two other factors have been proposed by Schulze et al (2003) as potential risk enhancers in relation to haem iron intake: dietary nitrites, and dietary advanced glycation end products (AGE). Both of these are found in high levels in processed meat products, and a number of cohort studies have found processed meat intake to be a stronger indicator of diabetes risk than total red meat (Schulze et al 2003; Song et al 2004), or dietary fat intake (van Dam et al 2002). It may be queried that these studies of processed meat intakes may be reflecting a less healthy life-style generally, however results were reported following adjustment for variables such as exercise, smoking, and intakes of alcohol; total energy; fibre; magnesium; and a range of fat types.

1.5.4 Processed meats, nitrites and dietary advanced glycation end products

Looking at haem iron intake from a different perspective, there is strong evidence for a link between processed red meat intake and incident type 2 diabetes. Using data from The Nurses' Health Study II, Schulze et al followed-up 91,246 young and

middle-aged females over 8 years, and analysed the data for processed meat intake. They found total red meat increased RR, from 1.00 to 1.58 for intakes of < 1/week to \geq 5/week, but increased processed meat intake to be more strongly associated with risk for diabetes, with RR increasing from 1.0 to 1.91 for intakes of < 1/week to \geq 5/week. They found no independent associations attached to fish intake, but poultry intakes decreased RR from 1.00 to 0.78 for intakes of < 1/week to \geq 5/week. Data were adjusted for BMI, and dietary fatty acids and cholesterol. This work has been updated (Pan et al 2011), consolidating the findings, and distinguishing between total and unprocessed red meat, leading to an estimation that substitution of 100g/d unprocessed, or 50 g/d processed red meat, for one serving of nuts, low fat dairy, and whole grains per day would reduce risk for diabetes by 16 to 35%.

Song et al (2004) evaluated 37,309 females aged 45 or older, finding red meat increased RR for type 2 diabetes, from 1.00 to 1.29 for intakes of < 1/week to \geq 5/week, and increased processed meat intake to increase RR from 1.0 to 1.43 for intakes of < 1/week to \geq 5/week. Only processed meat remained significantly associated with risk when all other nutrient factors had been adjusted for, with bacon and hot dogs having the highest association with risk compared with a mixed group of other processed meats. Confirmation of this comes from a meta-analysis of 20 studies investigating red and processed meat, and risk for coronary heart disease, stroke, or type 2 diabetes (Micha et al 2010), which found that unprocessed red meat was not associated with CHD, diabetes or stroke, but processed red meat was associated with CHD (RR per 50g/d, 1.42, 95% CI 1.07 to 1.89), and diabetes (RR 1.19, 95% CI 1.11 to 1.27), but not stroke. Further to these,

Lajous et al (2012) looked at unprocessed and processed red meat intake and incidence of type 2 diabetes in a cohort of 66,118 healthy French women, finding an association with processed meat only (hazard ratio for one serving/day, 1.29, 95% CI 1.14 to 1.45).

Aune et al (2009) performed a systematic review and meta-analysis of 12 cohort studies assessing meat consumption and risk of type 2 diabetes. Their findings had a large degree of heterogeneity, however overall results suggest that red meat consumption does increase the risk of type 2 diabetes. They did not include the large scale dietary iron intake studies of Jiang et al (2004b), and Rajpathak et al (2006) in their analysis. For total meat consumption, RR of high vs low intake is 1.17 (95% CI 0.92 to 1.48); red meat consumption, RR of high vs low intake 1.21 (95% CI 1.07 to 1.38); and processed meat consumption RR of high vs low intake 1.41 (95% CI 1.25 to 1.60). Assessed intake frequencies varied between studies, but were typically servings equal to or more than two a week versus less than one a week, or two to five servings a week versus less than 1 a month. Red meat portion size was set at 120 g; processed meat portion size was set at 50 g.

Processed meat clearly gives the greatest risk, and Song et al, and Schulze et al, propose dietary nitrites, and/or dietary advanced glycation end products, as possible reasons for the increased risk attached to processed meats. Nitrites are commonly used to preserve meats, and are transformed in the gut by reaction with amines and amides to become N-nitroso compounds nitrosamines and nitrosamides. Nitrites have been linked to the development of type 1 diabetes (Akerblom et al 2002), although the mechanism involved is unclear.

1.5.5 Dietary AGE and type 2 diabetes risk

The term 'advanced glycation end product' refers to a heterogeneous group of compounds known to have pro-oxidant and pro-inflammatory effects in the body (Uribarri et al 2005). AGE occur following reaction between carbonyl groups on sugars and amine groups on proteins, DNA, and lipoproteins, to form glycated proteins. This non-enzymatic glycation is the first stage of the Maillard reaction, also responsible for browning reactions between carbohydrates and amines in cooked food. Proteins can also be modified by lipid peroxidation, becoming advanced lipoxidation end products (ALE). Within the literature discussing dietary AGE, the term 'AGE' is often used as a generic term for any non-enzymatic protein modification, i.e. for reactions involving either carbohydrate or lipids.

1.5.6 AGE in meat

For the role of dietary AGEs in diabetes risk, support for earlier work by Schulze's group (van Dam et al 2002), is put forward by Peppas et al (2002), who propose that dietary glycoxidation products are the link between animal fats; meat; and diabetes; as the highest levels of AGE and ALE (advanced lipoxidation endproducts) are seen in animal products high in protein and fat, with industrially processed animal products such as frankfurters and bacon containing large amounts. Formation is increased by higher cooking temperatures and method of cooking, and meats cooked with a high dry heat such as grilling, frying, and roasting have the highest AGE values (Uribarri et al 2005). (Tables of AGE levels in common foods are available, see Goldberg et al 2004). The label 'AGE' for these products is misleading, as they refer to ϵ N-(carboxymethyl), and ϵ N-(carboxyethyl)lysine (CML and CEL), downstream products of glycation that can occur between lipids and

protein, independently of the Amadori compound fructolysine (Fu et al 1996). Consequently, the presence of glucose (or fructose) is not essential for their formation, and meats and cheese for example can have high levels, despite having very little or no intrinsic carbohydrate content. One common route of formation is from PUFA, where oxidation of a double bond causes fatty acid cleavage and formation of a 4-hydroxy-2-nonenal (4-HNE) moiety, which can then bind to a protein lysine residue, forming a protein carbonyl (Estevez 2011).

The proposal of AGE as the link between animal fats, meat, and diabetes can be extended to explain the positive association between haem iron intake and risk, with perhaps an involvement by N-nitroso compounds. It also raises the question of whether or not the formation of AGE products within these foods is enhanced by the presence of elemental iron, as shown to be the case in vitro with copper, LDL, and PUFA formation of CML (Fu et al 1996), and suggested within the body by Stadtman & Levine (2000); or whether some other factor(s) present in haem is influential, alongside the effects of heat during processing. The mechanical process of mincing meat for example breaks down tissue, exposing myoglobin and haem, and oxidation of the ferrous iron to ferric occurs when the reducing environment of the tissue surrounding the exposed myoglobin is exhausted. With storage, unsaturated fatty acids oxidize and produce free radicals that can oxidize the myoglobin (Claus 2007). Protection against meat protein oxidation by dietary antioxidants is able to compensate for high levels of PUFA in pasture fed lamb, compared with grain fed (Sante-Lhoutellier et al 2007). The potential for haem iron, and ferric iron from ferritin, to catalyse reactions has received some investigation by the meat industry, and myoglobin is found to be a good predictive marker for

protein carbonyl formation, however, relative contributions from different iron forms have not been determined (Estevez 2011). Several studies have compared the production of thiobarbituric acid reactive substances (TBARS) (a marker of lipid peroxidation) on stored raw and cooked muscle meats. Levels were low in chicken and pork compared with beef, and all increased with cooking, with myoglobin content best explaining the difference (Rhee & Ziprin 2007), later agreed with by Peiretti et al (2012). Again, no iron 'factor' has been drawn, by which the contribution of iron to lipid oxidation can be estimated.

When cooking red meat, incidentally, the darkening of colour is due to denaturing of the globin and oxidized haem iron, forming a hemichrome (Claus 2007).

1.5.7 Dietary AGE in the body

Approximately 10% of dietary advanced glycation end products are absorbed, directly proportional to the amount consumed (Koschinsky et al 1997), and a growing body of evidence describes their deleterious effects on the body. For example, Uribarri et al (2007) found that a single AGE drink led to a significant increase in serum AGE, and significantly decreased flow-mediated dilation of the brachial artery in both diabetic and healthy subjects, at 90 minutes post administration. The authors suggest that elevated circulating AGE may alter vascular endothelium response by reducing cellular nitric oxide production, or inhibiting nitric oxide activity. Vlassara et al (2008) have linked AGE intake to increased oxidative stress and inflammation, and reductions in dietary AGE to improved flow-mediated vasodilation in diabetes patients. A high AGE meal compared to a low AGE meal results in a marked increase in circulating markers of

endothelial function (VCAM-1 and E-selectin) in type 2 diabetes patients (Stirban et al 2008). Dietary AGE products have also been found to activate cell membrane AGE receptors (RAGE), triggering MAP kinase activation of pathways for inflammation, cell proliferation, and tumour growth (Zill et al 2003).

1.5.8 AGE and insulin

Of direct relevance to risk for diabetes, evidence that AGE can cause changes that predispose for diabetes has come from a number of studies from Vlassara's group, using the insulin resistant mouse model db/db⁽⁺⁺⁾. Dietary AGE restriction whilst maintaining other nutrient values (including kcals) improves insulin resistance, with the mice fed a low AGE diet demonstrating a doubling in glucose uptake by abdominal tissue compared with those receiving a high AGE diet (Hofmann et al 2002). Sandu et al (2005) found that high fat and high AGE diets led to increased visceral fat, plasma 8-isoprostane, plasma glucose and plasma insulin; altered pancreatic islet structure, with fewer insulin and glucagon producing cells; and increased diabetes. Less adiponectin (the mediator for transcription factor peroxisome proliferator-activated receptor γ) was also apparent.

Impaired insulin secretion in type 2 diabetes is thought to be due to cell function impairment and cell loss by apoptosis (Cunha et al 2008). β -cell apoptosis is thought to result from endoplasmic reticulum (ER) stress (Xu et al 2005), which can be induced by high glucose and free fatty acids in type 2 diabetes, and inflammatory cytokines in type 1 diabetes (Kharroubi et al 2004) (although Cunha et al propose the effect on ER in type 2 to be primarily lipotoxic rather than glucotoxic). Functionally, the cell signalling pathways that result in insulin

regulation in β -cells are not fully elucidated, but are known to involve transcription factor pancreatic duodenal homeobox factor-1 (Pdx-1), with a possible negative feedback inhibitory action by protein kinase CK2 (Meng et al 2010).

Sandu et al, above, did not speculate on the mechanism connecting AGE to insulin resistance, however later work (Zhao et al 2009) has described a link, whereby AGE inhibit ATP production in islet β -cells, subsequently inhibiting insulin secretion. In db/db mice, Zhao et al used glycated bovine serum albumin (AGE[CML]-BSA; 50 mg/ml BSA incubated in 0.5M glucose, at 37°C, for 8 weeks), administered intra-peritoneum for 2 weeks. Serum insulin, glucose tolerance, and insulin tolerance were all significantly impaired in a dose-dependent manner with AGE-BSA. Few morphological changes in islet structure were seen, but the isolated cells dose-dependently contained significantly less insulin. Fresh islets were isolated from healthy mice and treated with AGE-BSA for 48 hours. Inducible nitric oxide synthase (iNOS) mRNA was induced in a dose-dependent manner, accompanied by nitric oxide (NO) release in parallel. NO is thought to regulate mitochondrial oxygen consumption by inhibiting cytochrome c oxidase (the final enzyme in the electron transport chain) activity (Cooper & Guilivi 2007), therefore Zhao et al treated cells with aminoguanidine, which selectively inhibits iNOS, and found that cytochrome c oxidase activity was restored. Inhibition of iNOS also restored ATP production and insulin secretion, leading the authors to conclude that AGE inhibits insulin secretion via iNOS up-regulation (via cell membrane AGE receptor signalling cascade and nuclear factor- κ B activation) and NO up-regulation; inhibiting cytochrome c oxidase-dependent ATP production. ATP production is stimulated by cellular glucose, and increase in the ratio of ATP to ADP closes ATP-dependent K^+ channels

to depolarize cell membranes prior to insulin secretion. If ATP production is interrupted insulin secretion cannot take place.

1.5.9 Summary

It is known that diet contributes to the bodily pool of AGE and ALE products, and, as is described elsewhere in this thesis, endogenous AGE and ALE products (and perhaps redox active iron), are co-involved in the complications associated with diabetes. It can be seen from the literature that the emphasis on iron intake alone as accounting for the positive association between haem iron intake and risk for diabetes (Jiang et al 2004, and Rajpathak et al 2006) does not do justice to the real picture. A more detailed analysis of the haem iron intake of these earlier cohort studies, to include dietary AGE (ALE form) intake via processed meats and cooking methods, would clarify this. Although man has evolved with the capacity for large amounts of red meat (by modern Western standards) to safely contribute to our diet, the combination with dietary AGE from other components of the diet may well explain the apparent link with diabetes, and whole diet AGE intakes should be included as a factor when regression analyses are performed on red meat intakes.

In conclusion, from consideration of the studies discussed here, processed meat intake is associated with increased risk for type 2 diabetes, more than red meat, haem iron, or elemental iron. Meta-analysis by Micha et al (2010) finds that relative risk for diabetes per 50 g/day processed red meat is 1.19, with no significant increase with red meat.

Aune et al (2009) found processed red meat intake gave a RR of 1.57 per 50 g/day. The link may very plausibly be ALE products produced by food processing, with

meat PUFA content and heat facilitating their composition. A mechanism has been described that defines the link between dietary AGE(CML)-products and insulin loss (Zhao et al 2009). High red meat intakes may also increase risk for type 2 diabetes, to a lesser degree. The level of contribution from iron to dietary protein oxidation and AGE is not known, but experimental laboratory testing of this should be relatively straight-forward to carry-out, in meats with varying lipid contents, and between red meat and other dietary AGE.

1.6 Inflammation: BMI, and central adiposity

Conversely to the situation suggested in type 2 diabetes, obesity is generally associated with depleted iron stores. This has been suggested to be linked to hepcidin – the “master regulator” of iron metabolism, itself regulated by iron levels, inflammation, and erythropoietic activity. Inflammation, from cytokine release by adipocytes, leads to increased hepcidin production, which then down-regulates iron absorption by the gut (McClung & Karl 2009). Ruivard et al (2009) have suggested a possible 3-step response by the body to weight gain, whereby initial increased iron availability and hepatic iron loading is followed by visceral adiposity, inflammation, and hepcidin up-regulation, leading to the iron deficiency associated with obesity (Yanoff et al 2007).

1.7 Iron involvement in AGE development and the pathologies of advanced diabetes

Based on the evidence for AGE development in the long-term pathologies of diabetes, the role of reactive oxygen species in AGE development as “fixatives of glycation” (Halliwell & Gutteridge 2007), and the potential for iron to participate in

the generation of oxidative stress, and glucose autoxidation, poorly liganded iron has come under investigation for involvement in diabetic pathology.

Metal catalysed oxidation in diabetes was first proposed by Wolff & Dean (1987), following on from the work of Thornalley et al (1984) who identified that monosaccharides could oxidize haemoglobin to methaemoglobin (Hb-Fe³⁺), at pH 7.4 and 37°C. Wolff & Dean found that the metal chelator DETAPAC (10 µM to 1 mM) inhibited the formation of glucose attachment to bovine serum albumin (10 mg/ml BSA in 25 mM glucose in a 100 mM potassium phosphate buffer). However, the effect was biphasic, suggesting two routes of formation, and the addition of Cu²⁺ did not increase the reaction, suggesting saturation of the reactive component to the authors. The optimum inhibition of glucose attachment was 45%, achieved with 100 µM DETAPAC, in a system with no added Cu²⁺. The authors do not say where the metal had come from to catalyse glycation, presumably the source was trace levels in the buffer used, as reported by Halliwell & Gutteridge (2007).

Later *in vitro* studies on rat collagen supported a role for Fe²⁺ and Cu²⁺ in catalysis of the formation of glycated protein and cross-linking, which could be inhibited by the metal chelator diethylenetriaminepentaacetic acid (DTPA) (Chace et al 1991), and shown to involve hydrogen peroxide as the reaction could be inhibited by catalase. Sajithlal et al (1998) used rat tail collagen incubated in 250 mM glucose and 5 to 500 µM Cu²⁺ for one week. AGE formation was estimated by ELISA, and found to be concentration-dependent with copper. The reaction could be inhibited by a range of free radical scavengers, suggesting the oxidation of glucose to be involved, and aminoguanidine, suggesting carbonyls or dicarbonyls to be involved. Xiao et al

(2007) incubated rat tail collagen in 250 mM glucose for four weeks, with Fe²⁺ ranging from 0 to 300 μM. AGE-collagen was then incubated with human umbilical vein endothelial cells (HUVECS) and primary human monocytes for up to 60 hours, resulting in increasing losses of cell viability at 60 hours of 5.3% of total cells in HUVECS, and 8.6% in monocytes, demonstrating that the AGE-collagen was cytotoxic.

Further evidence for the role of metals in AGE formation comes from Miyata et al (2002), who found that angiotensin II receptor (AIIIR) antagonists (olmesartan, candesartan, irbesartan, losartan, and valsartan) lower *in vitro* formation of AGE, and have metal-chelating activity which accounted for their results. They found that aminoguanidine traps AGE precursors reactive carbonyl and dicarbonyl compounds, and that the AIIIR antagonists inhibit and block hydroxyl and carbon radicals mostly at the pre-Amadori stages.

1.8 Redox-active iron as a consequence of AGE-products

The studies above demonstrate how AGE development may be initiated and maintained by available redox-active iron, or copper. However, they have not speculated on the origin of such metals in the body. A number of studies have shown that glycation of key iron-binding proteins damages their structure and impairs their function, and thus sources of reactive iron in diabetes are presented that may contribute to the complications of advanced diabetes. Redox active iron apparently has the potential to be both the cause and the effect of glycation.

1.8.1 Haem proteins

Gutteridge (1986) first demonstrated that iron can be released from haemoglobin by H_2O_2 , and promote oxidative damage via the Fenton reaction. Subsequently, Khoo et al (1994) reported that glycation of haemoglobin A_{1c} causes altered electrophoretic motility due to being more negatively charged, and that deoxygenated haemoglobin can be glycated twice as rapidly as oxygenated. Khoo et al argued that if the conformational state of haemoglobin plays a part in the formation of HbA_{1c} , then its enzymatic properties may also be modified, which they demonstrated by reaction with H_2O_2 and 5-aminosalicylic acid. The peroxidase activity of glycated haemoglobin was lessened compared to non-glycated, in the pH range 4.5 to 6.0. Kar & Chakraborti (2001) confirmed this, and, since the presence of iron in the haem moiety is obligatory for peroxidase activity, examined the effect of H_2O_2 on iron release in HbA_0 and HbA_{1c} isolated from blood samples of diabetes patients. They found a greater, dose-dependent, loss of iron with glycated haemoglobin. Reaction with H_2O_2 and nitroblutetrazolium produced greater levels of ferric-form methaemoglobin (a promoter of superoxide formation and erythrocyte membrane damage), with HbA_{1c} compared with HbA_0 , and, when reacted with H_2O_2 , HbA_{1c} also produced more lipid peroxidation (measured by malondialdehyde), which could be prevented by catalase, and metal chelation, confirming the sources of the oxidation. Similar results were also reported for myoglobin (Roy et al 2004), although myoglobin glycation is not found significantly within muscle. Sen et al (2005) later identified structural alterations in HbA_{1c} suggested to represent an unfolding of α -helices and exposure of tryptophan

residues. They propose steric alterations to cause a weakened haem-globin linkage, providing a vulnerable target for H_2O_2 degradation and iron release.

In unglycated haemoglobin, degradation of haem was shown to be dependent on ferrous haemoglobin and not ferric methaemoglobin, by Nagababu & Rifkind (2000), as degradation could be inhibited by compounds that reacted with ferryl haemoglobin, and electron paramagnetic resonance showed that reaction between ferrylhaemoglobin and H_2O_2 involved a one-electron oxidation to superoxide. H_2O_2 reacting with methaemoglobin undergoes a two-electron oxidation, producing oxygen. Using spectrophotometry to assess the effects of glycation of methaemoglobin and metmyoglobin on haem proteins, Cussimano et al (2003) found that the Soret band of maximum absorption for haem (410 nm) was completely destroyed, which did not occur with the haem group of cytochrome *c*. Cussimano et al used 1 M glucose in 0.4 M phosphate buffer with 0.02% azide, over 20 days at 37°C, however they did not use an iron chelation agent such as Chelex 100, therefore, if trace levels of metals were present in the buffer then initial low levels of protein oxidation damage may have escalated following any initial haem release during the incubation process, producing the level of damage seen. They did not find the haem group in cytochrome *c* to be damaged, which has a ferric iron, although a rapid reduction to ferrocytochrome took place. Cussimano et al suggest the reason for the lack of degradation in cytochrome *c* may be due to inaccessibility of the haem iron to H_2O_2 produced during glycation, as a methionine-sulphur ligand is specific to cytochrome *c* at the sixth coordination position of the iron. Alternatively, they propose the cytochrome *c* haem group to be more securely attached due to its covalent bond with globin, unlike in haemoglobin and

methaemoglobin, where the bonds are reversible and dissociate easily. Cussimano et al raise the implications for haem degradation in red blood cells of diabetes sufferers, but point out the extensive intracellular antioxidant capacity of catalase and glutathione peroxidase. They suggest the extracellular matrix, with no established antioxidant enzymes, as being more vulnerable to the oxidative threat from free iron originating from degraded haemoglobin.

1.8.2 Haptoglobin

About 10% of red blood cells are haemolysed intravascularly (Garby & Noyes 1959, in Ashleh & Levy 2005), degrading to free, prooxidant haemoglobin. This is dealt with in the body by haptoglobin binding, producing a stable, high affinity complex, which is subsequently recognised and taken-up by the CD163 macrophage scavenger receptor (reviewed in Zuwala-Jagiello et al 2006). CD163 is recycled, the haem is degraded to biliverdin and bilirubin, and the iron is either stored as ferritin or transported out of the cell by ferroportin (Carter & Worwood 2007).

Haptoglobin comprises two α -chains, and two β -chains, and is coded for by two distinct genes, Hp1, and Hp2. Whilst the β -chains are identical to each, the α -chains are longer in Hp2, and can form a variety of cyclic multimers. Three genotypes therefore exist: Hp1-1 (86 kDa); Hp2-1 (86 to 300 kDa); and Hp2-2 (170 to 900 kDa) (Ashley & Levy 2005, Langlois & Delanghe 1996). Genotypes vary globally, with frequency of Hp1 ranging from around 0.07 in India, to over 0.7 in parts of West Africa and South America. The mean Hp1 frequency in Europe is 0.38 (Carter & Worwood 2007).

In diabetes, haptoglobin genotype is a determinant of risk for micro- and macrovascular complications, with Hp1-1 being resistant to diabetic retinopathy, nephropathy, and cardiovascular disease, Hp2-1 having an intermediate risk, and Hp2-2 having greatest risk, with a five-fold increase in risk for cardiovascular disease compared with Hp1-1 (Melamed-Frank et al 2001). In the healthy population, evidence for differences in risk for these and other diseases due to genotype is not clear, and normally a 400 molar excess of haptoglobin to free haemoglobin results in no significant effect on function of the haptoglobin types (Ashleh & Levy 2005, Carter & Worwood 2007). Ashleh & Levy suggest a molecular basis for risk in diabetes linked to an increased clearance rate of Hp1-1-haemoglobin complexes by CD163 receptors, and a subsequently increased production of anti-inflammatory IL-10 compared with Hp2-2-Hb. The smaller Hp1-1 can more easily enter the sub-endothelial space, the site of vascular injury, to scavenge free haemoglobin; and additionally, they found that the oxidative effect of glycated haemoglobin on LDL is greater than that of unglycated haemoglobin, and is not completely blocked by binding to haptoglobin. Finally, they also report finding a profound lack of monocytes expressing CD163 receptors in people with diabetes.

Ashleh & Levy's theory that Hp2-2 size prevents it accessing free haemoglobin in the sub-endothelial space implies that the same must also be occurring in people without diabetes, for whom no increased vascular risk is established. The difference in vascular risk may therefore be explained by either: more haemoglobin being present as a result of increased red blood cell degradation (diabetic erythrocytes on average exist for four days less than non-diabetic, Cohen et al

2008), increased LDL oxidation by glycated haemoglobin, or increased LDL oxidation by haem from degraded glycated haemoglobin, or a combination of these.

In order to gauge the effect Hp genotype might have on vascular risk in diabetes compared to non-diabetes, a very rough comparison can be made between rates of CVD in populations with low versus high rates of Hp2 genotype. The Indian urban population has a high rate of diabetes (Gupta et al 2007), and the highest global Hp2 rate (~ 0.93). CVD accounts for 52.9% of deaths in the diabetic population, and 24.2% of deaths in the non-diabetic population (Mohan et al 2006). In London, where the (European) Hp2 rate is 0.62 (a moderate global rate), in type 2 diabetes the rate of cardiovascular mortality is 63.5%, with around 33% of deaths caused by CVD in the general population (Morrish et al 2001, Scarborough et al 2010). In both countries, CVD deaths in diabetes are approximately double those seen in the general population, which, if Hp only has an influence on diabetic CVD risk, implies that there are other factors at play.

A direct comparison of all UK deaths between 1985 and 1986 of people with type 2 diabetes, between South Asian born people (Indian Hp2 rate ~ 0.93), and African Caribbean born people (African Hp2 rate ~ 0.3 , the global lowest), with diabetic people born in England and Wales (Hp2 rate 0.62), was carried out by Chaturvedi & Fuller (1996). They did not include Hp genotype data. They found highly significant and very highly significant increases in CVD and stroke in both immigrant ethnic populations, compared to people born in England and Wales. When population rates of Hp are considered, no consistent effect by Hp rate is indicated. For CHD

mortality however, a very highly significantly increased risk was seen in the South Asian population, with no difference in men and only a small increase in women for CHD death by the African Caribbean group, in some agreement with the increased risk suggested by Hp genotype. The African Caribbean group however might have been expected to have a decreased risk compared to the indigenous population, by Hp genotype rate alone, which was not seen. These results echo the general UK population trends, with CHD being highest in South Asian people, and stroke being highest in African Caribbean people (Scarborough et al 2010). Hp genotype does not therefore explain the different cardiovascular risks seen, and many factors could influence this, such as diet, apolipoprotein and other genotypes, parental health and epigenetic changes, and healthcare prior to and age at migration. A comparison between ethnic populations with and without diabetes, and correcting for different factors, would provide more information on the causes of CVD, and the contribution from Hp genotype in different populations.

In search of a link between Hp genotype, haemoglobin clearance, and atherosclerosis, two studies have studied iron deposition in plaques by Hp genotype. Moreno et al (2008) looked at 189 aortic plaques from 21 male type 2 diabetic, and 16 male control cadavers, comparing Hp1-1 and Hp2-1 against Hp2-2, and measuring iron as being present or not by Perl's stain. They found that in both diabetes, and non-diabetes, Hp2-2 resulted in a larger number of plaques with iron, with the effect being greater in diabetes. In non-diabetes, Hp1-1 and Hp2-1, 11.1% of plaques had iron, with Hp2-2 being 26.8%; in diabetes Hp1-1 and Hp2-1, 11.8% of plaques had iron, with Hp2-2 being 46.2%.

More recently, Lioupis et al (2011) measured hemosiderin-iron deposits in carotid atherosclerotic plaques from subjects undergoing surgical plaque removal operations. Single plaques from each subject were used, 27 with diabetes (diabetes type unspecified), comparing Hp1-1 and Hp2-1 (n = 16), with Hp2-2 (n = 11); and 43 non-diabetic plaques, Hp1-1 and Hp2-1 (n = 20), Hp2-2 (n = 23). Iron was measured in every plaque from the diabetes Hp2-2 group, unlike the other three groups. Within the diabetes group, they found a highly significant increase in Perl's iron stain in the Hp2-2 group ($p = 0.008$), and a non-significant increase in Hp2-2 compared with Hp1-1 and Hp2-1 in the non-diabetes group ($p = 0.197$). No other tests were performed on the plaque material, and so it is not possible to say if the presence of iron correlated with oxidative stress measures, or plasma malondialdehyde or antioxidants. Plaque iron correlated with serum homocysteine ($r = 0.354, p < 0.05$), and it would be interesting to know if plaque iron or serum homocysteine correlated with plaque length or depth, as homocysteine is known to autoxidize in the presence of ceruloplasmin, and thus iron too, and in the presence of human serum, and is injurious to endothelia (Starkebaum & Harlan 1986). The presence of homocysteine in the vascular extracellular matrix would be particularly prooxidant if NTBI was present. It seems perplexing that the Hp2-2 plaques had more iron, if the problem with Hp2-2 is thought to be its lack of monocyte recognition and take-up, and so this appears to support Ashleh & Levy's theory that the larger Hp2-2 is unable to access haemoglobin in the sub-endothelial space, leaving it vulnerable to plaque incorporation. Alternatively the Hp2-2-glycated haemoglobin complex might enter the plaque independently of macrophages, from

a raised plasma pool due to lack of monocyte scavenging. Inflammatory responses to Hp2-2-glycated haemoglobin would also play a role in plaque development.

It can be seen from the two studies above that Hp2-2 clearly leads to plaque iron development in diabetes *and* non-diabetes, with greater effect evident in diabetes. Although neither group looked at the oxidative nature of the plaque contents, Lapenna et al (2007) have done this, in a study that compared serum ferritin with low molecular weight iron, and lipid peroxidation products, in 38 plaques from 38 carotid artery surgical plaque removals. Following plaque homogenation, iron was measured by centrifugation and ultrafiltration through 10,000 kDa filters, then by spectrophotometric reaction to ferene. Lipid peroxidation was measured by Fox assay, and spectrofluorescence. Plaques were categorised into groups by stenosis (less than or greater than 90%); and by contralateral stenosis (occurrence or not). Plaque iron and lipid peroxidation measures were all significantly higher in the > 90% stenosis and contralateral stenosis groups. A significant correlation was seen between plaque iron and the oxidation measures in the > 90% stenosis group, which was non-significantly associated in the < 90% group. Serum ferritin significantly correlated with plaque iron throughout. Lapenna et al demonstrated that plaque iron was present, associated with redox active plaque tissue, and more concentrated in more severe atherosclerosis.

This suggests that the iron seen in non-diabetic Hp2-2 plaques will be redox active, the effect of which might be to further lipid peroxidation, inflammation, and plaque expansion, and, that iron might contribute to cardiovascular risk in non-diabetic

Hp2-2 genotype, although the effect of free glycated haemoglobin in the sub-endothelial space is evidently stronger.

1.8.3 Transferrin

Glycation of transferrin *in vitro* has been shown to decrease its ability to bind ferric iron (Fujimoto et al 1995, Van Campenhout 2004 for example), and this is discussed in depth in Chapters 2 and 3.

1.8.4 Albumin

Human serum albumin represents around 60% of the plasma proteome, and estimates for rate of glycation range from 1% normally to 10% in diabetes (Peters 1996). Garlick & Mazur (1983) reported 10 – 12% normally glycated. *In vivo* glycated albumin reduces its ability to bind fatty acids and bilirubin, and demonstrates conformational changes, shown by a reduction in tryptophan fluorescence at 295 nm suggesting the sole tryptophan residue had become more hydrophobic, and presumably more buried due to the protein contracting (Shaklai et al 1984). Qian et al (1998) investigated the ability of glycated albumin to bind iron and copper *in vitro*. They found a pronounced binding affinity for both metals, compared with controls, which maintained redox activity *in situ*. The mechanism for this was not clear, but did not involve carbonyls or aldehydes, the authors argue, as reduction of the glycated proteins with borohydride did not affect binding.

The number of iron atoms capable of being bound by albumin is not agreed, but may be one (Xu et al 2008), two or three (Løvstad 1993), or nine (Loban et al 1997). Albumin-iron-citrate ternary complexes can be formed (Løvstad 1993), and a high

affinity copper-binding site exists at the N-terminal peptide Asp-Ala-His (Roche 2008).

Silva & Hider (2009) used mass spectrometry to measure the influence of oxidation and glycation of human serum albumin on iron binding. Both modifications increased iron binding, with glycation having the stronger effect. Albumin glycation of up to 16% of available primary amine groups led to 99.8% of iron being bound from a 1 μ M solution, compared with 0.6% by normal albumin. At 50 μ M iron concentration, 96.1% was bound by glycated albumin, compared with 4.8% by normal. Unlike Qian et al however, Silva & Hider found the glycated albumin-iron was resistant to interaction with other compounds. They pointed out that physiological levels of citrate should be used when incubating albumin with glucose, which were not present in Qian et al's experiment, as the citrate acts as a counter ion in the ternary complex. It is not clear whether this accounts for the difference in findings for the two groups.

Silva & Hider (2009) suggest that the sites for iron-liganding on glycated albumin may be the carboxylic acid end groups on AGE-products carboxymethyllysine (CML) and carboxyethyllysine (CEL), which lie at the distal end of the residue to the protein backbone. If this is the case, other CML- or CEL-glycated proteins might also acquire a capacity for iron binding, if glycation isn't destructive to the protein as appears to be the case for haem-proteins. It is not known if citrate forms a bond or bonds with the CML-iron; if coordination is provided by neighbouring CML hydroxyl groups; or if the iron is subsequently redox available. CML are redox active species (Fu et al 1996) and the proximity of the iron ion to the carbonyl group of CML may

further the risk of oxidative reactions. The proteolysis of glycated proteins produces free glycation adducts which can be measured intra- and extracellularly, as discussed earlier, and the inclusion of iron with these degraded species, which are exposed to vascular endothelia and subject to renal filtration, provides a mechanism by which iron could be damaging to these tissues.

Iron and AGE - summary

Copious literature exists hinting at the potential for iron to be involved in diabetic pathology via the instigation and the effects of AGE products. Evidence for a direct link between iron and AGE development relies on their inhibition by chelators *in vitro*. Evidence for the involvement of glycation in development of redox active iron *in vivo* is more circumstantial. Haptoglobin Hp2-2 genotype is associated with risk for vascular complications in diabetes, and the presence of iron in carotid artery plaques, probably by poor sub-endothelial scavenging, and subsequently increased LDL oxidation by glycated haemoglobin, presumably also in the sub-endothelia. Glycated haemoglobin is unstable *in vitro*, and a potential source of haem in plasma. Glycated albumin binds more iron than unglycated albumin, and degrades to an unstable uraemic toxin, and other proteins may do the same.

It is not certain whether the increased cardiovascular risk seen in type 2 diabetes is completely accounted for by Hp genotype, but it is evidently an important factor, and might perhaps occlude the risk from other sources of reactive iron. It is unknown if Hp genotype influences other diabetes pathologies, and this would be useful research to do.

1.9 Iron and diabetes summary

Mechanisms exist which could lead from high iron status to risk for diabetes, if surplus dietary iron is absorbed and subsequently deposited in the pancreas. A meta-analysis of red meat intakes finds an increased relative risk (RR) for type 2 diabetes of 1.20 per 120 g/day, with processed red meat intake giving a higher RR of 1.57 per 50 g/day (Aune et al 2009). A different meta-analysis finds RR per 50 g/day processed red meat of 1.19, and no significant increase with red meat (Micha et al 2010). As described in section 1.5, dietary iron, when combined with advanced lipoxidation endproducts, could lead to β -cell loss and type 2 diabetes. Pancreatic iron, in the presence of increased levels of superoxide, as proposed by Brownlee and others, might drive β -cell damage. Measurement of pancreatic iron in people with and prior to type 2 diabetes but without iron overload disease would inform this.

Increased physiological iron status, as measured by serum ferritin, and sTfR:ferritin ratio, finds a positive association with risk for type 2 diabetes, but this does not appear to be reduced by regular blood donation. Studies that assess physiological iron status and risk for diabetes by using serum ferritin alone, and which control for inflammation with CRP, may be inadequately controlling for inflammation.

In the pathologies of advanced diabetes many possible routes exist for iron to be involved, via oxidative damage and development of AGE, as described above. Iron-binding proteins are altered by glycation, and may consequently be impaired in their various functions, resulting in plasma and sub-endothelial redox active non-transferrin-bound iron. Such iron forms are capable of contributing to endothelial, and renal diabetic pathologies, and possibly others, although direct evidence of

their involvement is elusive. The following chapters are concerned with iron in established diabetes and its possible association with pathology. The potential source of one form of non-transferrin-bound plasma iron, from glycated transferrin, is explored in depth. The measurement of (probably another form of) NTBI is made in people with type 2 diabetes, and compared with controls. Assessment of diabetic pathologies are reported, and their association with NTBI, alongside measures of antioxidant status and lipid peroxidation.

1.10 Aims of this project

- To investigate the effects of *in vitro* glycation of transferrin, and, using ultrafiltration, determine whether loss of transferrin-iron-binding capacity is due to protein fragmentation.
- Using two mass spectrometry systems as a model only, to evaluate potential differences between *in vitro* and *in vivo* glycated transferrin, in position and extent of glucose modifications, which may be relevant to the protein fragmentation seen *in vitro* or other loss of function.
- To evaluate non-transferrin-bound iron in people with type 2 diabetes and controls, and to assess its association with haemoglobin glycation, lipid peroxidation, antioxidant status, and other iron parameters.
- To compare non-transferrin-bound iron in people with and without diabetes, and with and without cardiovascular and renal disease

Chapter 2. *In vitro* study of glycated transferrin

2.1 Aim

To investigate the effects of *in vitro* glycation of transferrin, and, using ultrafiltration, whether loss of transferrin-iron-binding capacity is due to protein fragmentation.

2.2 Background

The observation by Austen et al in 1987 that, like other plasma proteins, transferrin became glycated *in vivo*, coupled with an interest in the role of iron in diabetes, has led several groups to investigate transferrin glycation *in vitro*. The interest has been in the effect glycation might have on transferrin-iron-binding, with the speculation that a functional loss may contribute to diabetes complications such as cardiovascular disease, via a reduction in antioxidant capacity, and the potential occurrence of redox-active plasma iron (Fujimoto et al 1994, Van Campenhout et al 2003, Goodarzi et al 2010). In 1987 Austen et al had observed in 5 people with diabetes (with HbA_{1c} between 12% and 19.7%), that transferrin was 5.2% glycated on average, compared with 1% in a single control (HbA_{1c} 4.3%). This was done by separating glycated and unglycated plasma protein fractions on a Glycogel B affinity column, then quantifying them by electrophoresis and densitometry.

Three groups have previously carried out *in vitro* glycation experiments with transferrin (Table 2).

Table 2. In vitro glycation of transferrin by other groups

<p>Fujimoto et al (1994)</p> <p>Bovine holotransferrin (hTf) & apotransferrin (aTf) (Wako Pure Chemicals)</p>	<p>Incubation method 10 mg/ml protein in 0 or 1 M glucose, 50 mM phosphate buffer pH 7.4, 37°C for 10 days. hTf also received 9 mM & 34 mM incubations</p> <p>Glycation (mean±SD nmol HMF equivalent/mg protein, n=3) 0 M: Not reported 1 M: hTf, 17.43±4.02; aTf, 20.4±3.42 9 mM: hTf, 0.6±0.1 34 mM: hTf, 2.9±0.6 (<i>P</i><0.01 compared with 9 mM)</p>	<p>Glycation assessment method Thiobarbituric acid reaction using 5-hydroxymethyl-2-furfural (HMF) as a standard compound</p> <p>Unsaturated iron binding capacity (ng/mg) aTf: 0 M, 1013±82.6 1 M, 480±82.5 (<i>P</i><0.001) hTf: 0 M, 25±8.2 1 M, 0.0</p>
<p>Van Campenhout et al (2003)</p> <p>Human apotransferrin (Sigma)</p>	<p>Incubation method 5 mg/ml protein in 0, 5.6, 13.9, 22.2, 33.3, or 1000 mM glucose, 0.1 M sodium phosphate buffer pH 7.4, 37 °C for 14 days</p> <p>Glycation (mean±SD, μmol fructosamine/g aTf, n=8-26) 0 mM, 1.1±0.7 5.6 mM, 4.9±3.4*** 13.9 mM, 6.2±1.0*** 22.2 mM, 9.6±1.5*** 33.3 mM, 13.4±3.0*** 1000 mM, 157.4±57.8*** (***<i>P</i><0.001, vs 0 mM)</p>	<p>Glycation assessment method Fructosamine measured by reaction with nitrotetrazolium-blue (Roche kit 109893), with fructosamine standard</p> <p>Total iron binding capacity (mean±SD, μg/g aTf, n=8-26) 0 mM, 0.726±0.089 5.6 mM, 0.696±0.083 13.9 mM, 0.672±0.069** 22.2 mM, 0.667±0.056* 33.3 mM, 0.585±0.079*** 1000 mM, 0.011±0.010* (*<i>P</i><0.05; ** <i>P</i><0.01; ***<i>P</i><0.001, vs 0 mM)</p>
<p>Goodharzi et al (2010)</p> <p>Human apotransferrin (Sigma-Aldrich)</p>	<p>Incubation method 5 mg/ml protein in 0, 50, 100, 150, 200 mM glucose in 0.1 M PBS, pH 7.4, at RT for 10 & 20 d</p> <p>Glycation (μmol HMF/mg aTf) 0 mM: 10 d, 5.06±1.10 20 d, 6.08±1.09* 50 mM: 10 d, 20.95±1.17 20 d, 25.02±1.26* 100 mM: 10 d, 28.64±1.54 20 d, 34.04±1.16* 150 mM: 10 d, 39.04±1.10 20 d, 45.10±1.21* 200 mM: 10 d, 51.04±1.32 20 d, 58.02±1.17* (*<i>P</i>=0.029 vs 10 d)</p>	<p>Glycation assessment method Thiobarbituric acid reaction using HMF as a standard compound</p> <p>Antioxidant capacity (μM vitamin C equivalence) 0 mM: 10 d, 43.85±1.2 20 d, 41.79±1.19* 50 mM: 10 d, 39.90±1.21 20 d, 36.90±1.13* 100 mM: 10 d, 34.76±1.23 20 d, 30.72±1.17* 150 mM: 10 d, 28.82±1.19 20 d, 23.79±1.25* 200 mM: 10 d, 21.66±1.12 20 d, 15.83±1.18* (*<i>P</i>=0.029 vs 10 d) Results are from 2 triplicate experiments</p>

Key: PBS: Phosphate buffered saline; RT: Room temperature; HMF: 5-hydroxymethyl-2-furfural

Protein glycation *in vitro* is essentially straightforward to achieve, and a range of glucose concentrations up to 1 M have been used, with protein incubations of between 10 and 20 days, at 37°C (Fujimoto et al 1994, Van Campenhout et al 2003) or room temperature (Goodarzi et al 2010) (Table 2). At the end of this period samples are buffer exchanged through Sephadex G-25 columns, or by dialysis against glucose-free buffer. Degree of glycation was assessed by these groups by measurement of fructosamine, a stable product of the Schiff base reaction and Amadori rearrangement, or alternatively, 5-hydroxymethyl-2-furfural (HMF), which is related to available lysine.

All three groups found positive associations between glucose concentration, degree of glycation, and reduction in function, with significant differences between glucose concentration and controls, and between different concentrations, or increased incubation time. Fujimoto et al used denaturing electrophoresis to further examine the samples, finding that substantial fragmentation of the protein could be seen only in the glycated holotransferrin sample. This is interesting because they reported a higher level of glycation in the apo-sample compared to holo (which might be expected due to the iron-free form having a more open structure). They also tested the capacity of the different samples to generate superoxide and the hydroxyl radical, in a system with phenylalanine and hypoxanthine-xanthine oxidase, finding a significant increase in activity with the holotransferrin compared to apotransferrin and both controls. These findings all suggest that oxidative damage is occurring in the presence of holotransferrin and that the iron bound to the glycated holotransferrin is redox-available, and therefore the iron-binding ability of transferrin is impaired by glycation.

Van Campenhout et al (2004) supported this by studying the rate of transferrin-iron-binding in solution, using spectrophotometry at 450 nm. Lyophilised human serum transferrin was incubated in SPB with a range of glucose concentrations from 0 to 33.3 mM, which, following buffer exchange, were incubated with iron-nitrilotriacetic acid solutions calculated to achieve 32%, 64%, and 96% of total iron-binding capacity. Glycation impaired binding at all levels of iron-loading, compared with freshly made-up and SPB with no added glucose, although they conclude that glycation facilitates the initial binding to iron, but that when high amounts of iron are present iron-binding is impaired. They referred to the work of Qian et al (1998) to propose that the resulting chelate was more loosely bound, however whilst Qian et al did find this to be the case with *in vitro* studies of albumin, gelatin, and elastin, they did not study glycated transferrin, and the nature of the two specific iron bonds in transferrin and lactoferrin are entirely different to the non-specific binding seen in other proteins (Aisen et al 1967, Roche et al 2008).

Van Campenhout et al (2004) hypothesised that, as glycation of apotransferrin *in vitro* decreased its total iron-binding capacity, in addition to its capacity to prevent lipid peroxidation (Van Campenhout et al 2003), the increased lipid peroxidation in diabetes might be due to poor iron-binding by glycated transferrin.

To add to these findings, Goodarzi et al (2010) found that transferrin antioxidant capacity reduced with glycation when tested against the thermal decomposition of 2,2'-AZO bis-dihydrochloride (AAPH), and AAPH-induced lipid peroxidation of erythrocytes.

In order to gain further insight into the effects of glycation on transferrin, and to confirm Fujimoto et al's 1994 suggestion that protein fragmentation was taking place, transferrin glycation studies were undertaken as part of this PhD work. Protein fragmentation was measured by ultrafiltration through Amicon Ultra filter units (Merk-Millipore), which uniquely can be reversed to recover the retentate. This is a novel approach to this question, and no other report of ultrafiltration for this purpose can be found in the protein oxidation literature.

2.3 Experimental methods

2.3.1 Equipment list

Burkard Koolspin 6S refrigerated centrifuge BS-CAT/006D/K, serial number 81140

CamSpec M302 UV/vis spectrophotometer

Beckman Microfuge 11

Thorn Electrim 10W heat mat

Mettler Toledo AB 104 balance

Eppendorff pipettes, calibrated prior to start of experiment

Merke-Millipore Amicon Ultra filter units 30 kDa & 10 kDa

Bio-Rad Mini-Protean II electrophoresis system, with a Bio-Rad PowerPac

2.3.2 Laboratory solutions

0.1 M Sodium phosphate buffer (SPB) pH 7.4, to make 500 ml:

Na_2HPO_4 14.505 g in 405 ml Milli-Q H_2O

NaH_2PO_4 1.482 g in 95 ml Milli-Q H_2O

pH to 7.4 with 1 M HCl

1 M glucose solution:

D-glucose (Sigma-Aldrich) 0.9008 g in 5 ml Milli-Q H_2O

Dilute as necessary in SPB

Protein precipitation solution:

0.6 M trichloroacetic acid (TCA) and 0.4 M thioglycolic acid, made up in 1 M HCl

(To 9.803 g TCA add a small volume 1 M HCl. Add 3.76 ml 98% thioglycolic acid. Make up to 100 ml with 1 M HCl)

Iron chromogen:

0.5 mM 3-(2-pyridyl-5,6-bis-[2-(5-furyl sulphonic acid)]-1,2,4-triazine (ferene)

in 1.5 M sodium acetate (20.412 g sodium acetate trihydrate in 100 ml di H_2O)

Iron Saturating Solution:

100 μ M ferric chloride solution in 5 mM HCl.

Made up from a 1:10 dilution of stock ferric chloride solution 1 mM in 50 mM HCl

2.3.3 Laboratory protocols

Protein assay (Lowry et al 1959)

Solutions:

Lowry A: 2% Na_2CO_3 in 0.1 M NaOH

Lowry B: 1% CuSO_4 in diH_2O

Lowry C: 2% sodium potassium tartrate ($\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$)

Lowry stock reagent:

49 ml Lowry A

0.5 ml Lowry B

0.5 ml Lowry C

Folin's reagent: Phenol reagent – 2N (Folin-Ciocalteu reagent). Dilute 1:1 in diH_2O before use.

Procedure:

- 1) To 50 μ l sample, bovine serum albumin (BSA) standards and blank add 1 ml Lowry stock reagent
- 2) Incubate at room temperature for 30 minutes
- 3) Add 100 μ l of diluted Folin's reagent to each tube
- 4) Incubate at room temperature for 30 minutes
- 5) Read in a spectrophotometer at 595 nm

The coefficient of variance for the Lowry protein assay, compiled from repeated standards, is given in Table 3, shown in Figure 2.

Table 3. Lowry protein assay, compilation of bovine serum albumin (BSA) standards

BSA μg/ml	Mean (AU)	SD	n =	CV
100	0.044	0.012	37	0.26
250	0.117	0.021	40	0.18
500	0.243	0.040	40	0.17
1000	0.347	0.050	40	0.14

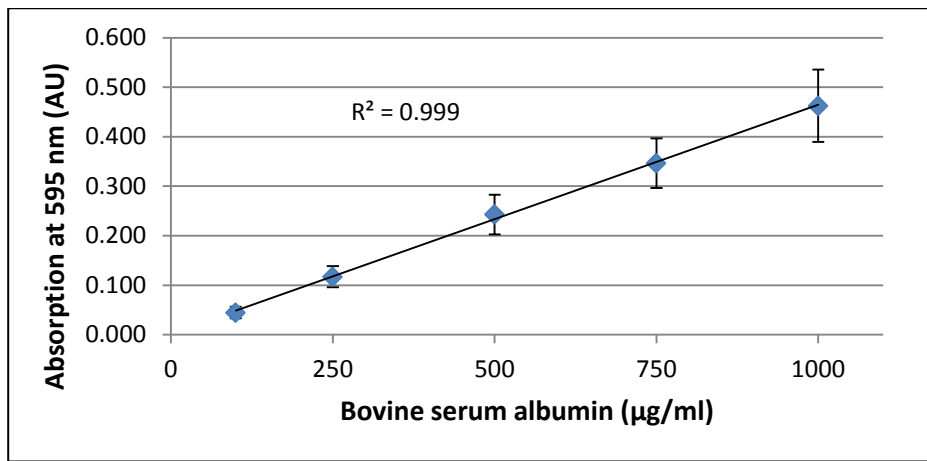


Figure 2. Lowry protein assay

Lowry protein assay, compilation of standards, mean & SD (n = 37 to 40, see Table 3).

2.3.4 *In vitro* glycation of transferrin

Human apo-transferrin (Sigma T4382-100 mg, 98% purity, 049K15511), 8 mg, was dissolved in 200 µl of freshly made sodium phosphate buffer (SPB) pH 7.4. Four 50 µl aliquots of this were added to glucose solutions plus SPB to give 1 ml of 0, 500, 750, and 1000 mM glucose concentration. Protein concentrations were therefore 2 mg/ml. Glucose concentrations were chosen to correspond with those used by earlier groups, and give an amplified degree of glycation for detection of effects.

SPB was made with Milli-Q HPLC grade water, and all plastic tubes and tips were autoclaved and stored in sealed containers prior to use. Tubes were incubated at 37°C, on a heat mat, in a closed, insulated, incubation box, for 14 days. Tubes were vortexed at the start and on Day 7. The experiment was carried out between January and August 2011. The temperature within the incubation box was tested by thermometer at the start, and mid-way through the project, and found to be 37°C on both occasions.

Further filtration studies used Sigma apo-transferrin T4382-100 mg, 98% purity, lot # 031M19551V.

2.3.5 Buffer exchange

On Day 15 samples were removed from the incubation box and placed on ice, prior to buffer exchange into glucose-free SPB. Buffer exchange was through a 5 ml Sephadex G-25 column, made-up in the lab. This volume was found to give the optimum separation of protein from glucose, with 1 ml samples. Tests using bovine serum albumin (BSA) had established that a mean of 86% of a 1 mg/ml protein solution would be recovered in the first three 500 μ l collections, following the 1 ml run-on from application of a 1 ml sample. The recovery of protein following buffer exchange, and the separation of protein from glucose are shown in Figure 3 and Figure 4.

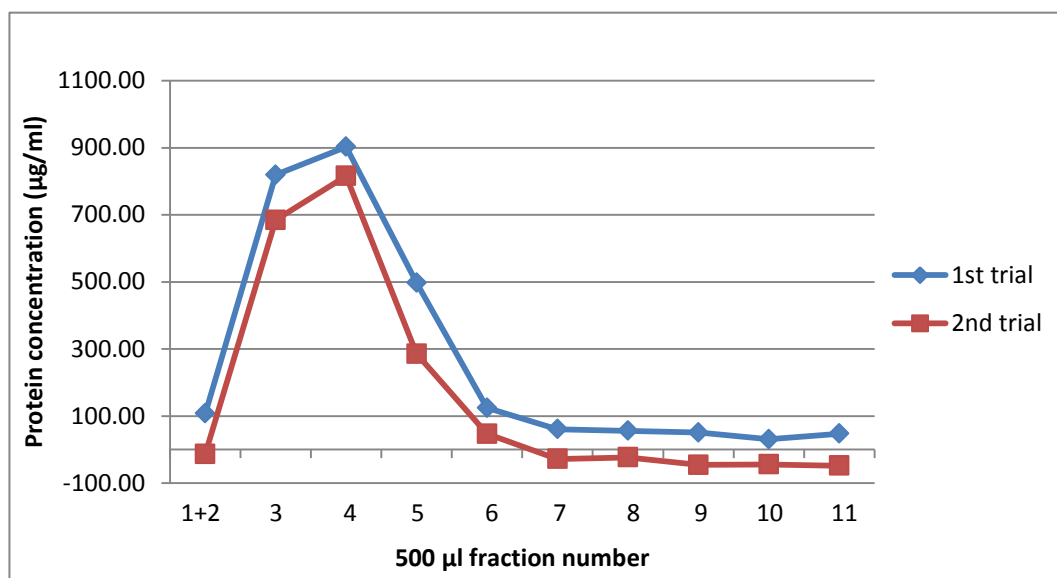


Figure 3. The recovery of bovine serum albumin

Recovery of serum ferritin following application of 1 mg/ml of protein through a 5 ml Sephadex G-25 column, in the development of the glucose-buffer exchange process.

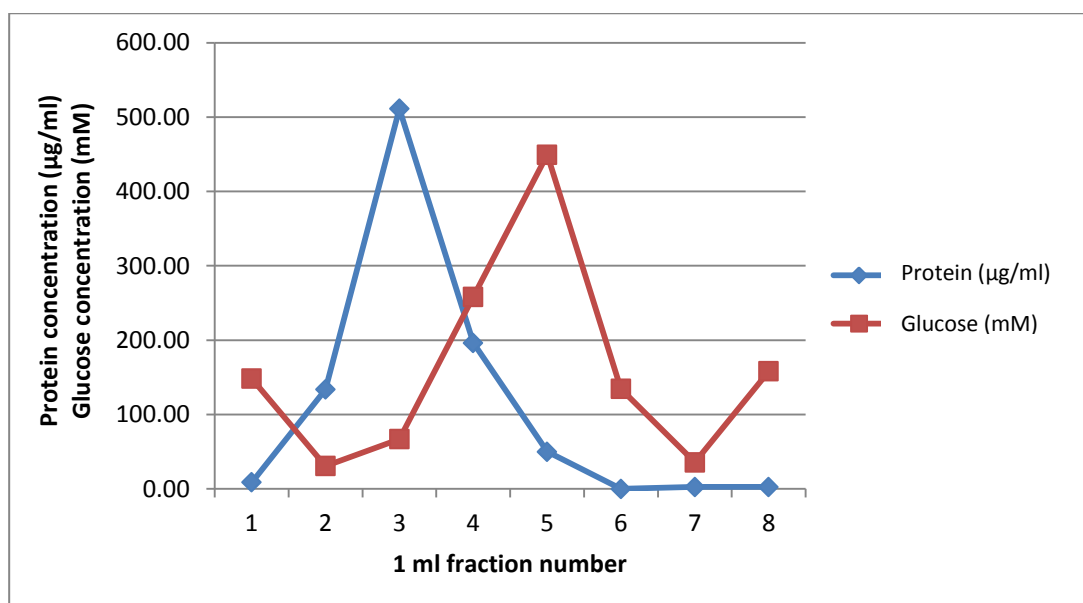


Figure 4. The separation of bovine serum albumin

The separation of bovine serum albumin from glucose, following application of 1 mg/ml of protein in 1 M glucose in 0.1 M SPB buffer, through a 5 ml Sephadex G-25 column, in the development of the glucose-buffer exchange process.

2.3.6 Assessment of glycation

Glycation is a condensation reaction between the acyclic glucose hydroxyl (OH) group and the protein primary amine (NH₂). Degree of glycation was assessed by measurement of NH₂ groups.

Following buffer exchange into 0.1 M SPB, protein concentration was measured by the method of Lowry et al (1951). A solution of fresh apotransferrin was made up in SPB (1 mg/ml), to act as a further control. Degree of glycation was measured by trinitrobenzene sulphonic acid (TNBSA) assay (also known as picrylsulphonic acid), which reacts with NH₂ groups at pH 8, to form adducts detectable at 335 nm. TNBSA is specific for primary amine groups (Satake et al 1959). Solutions of L-lysine were used as standards for quantification, and results are reported as lysine equivalent NH₂ groups (µM/mg/ml). The assay was a scaled-down and adapted

version of that used by Silva & Hider (2009), described by Satake et al (1959), and Cabot & Tainturier (1996):

To 25 μl of glycated apotransferrin, and L-lysine standards and blank, the following were added: 25 μl 4% Na_2HCO_3 ; 25 μl 0.1% TNBSA. The sample was incubated at 37°C for 2 hours, then the following were added: 25 μl SDS, 12.5 μl 1 M HCl, and 787.5 μl diH_2O , to make a final volume of 900 μl . The absorbance of the resulting solution was read immediately, at 335 nm.

Table 4. TNBSA assay of NH_2 groups, compilation of L-lysine standards, read at 335 nm, absorbance units (AU)

Lysine μM	Mean (AU)	SD	n =	CV
10	0.015	0.016	3	1.05
25	0.016	0.01	11	0.64
50	0.032	0.009	15	0.29
100	0.055	0.01	18	0.19
250	0.12	0.023	19	0.19
500	0.242	0.029	14	0.12
750	0.37	0.023	6	0.06

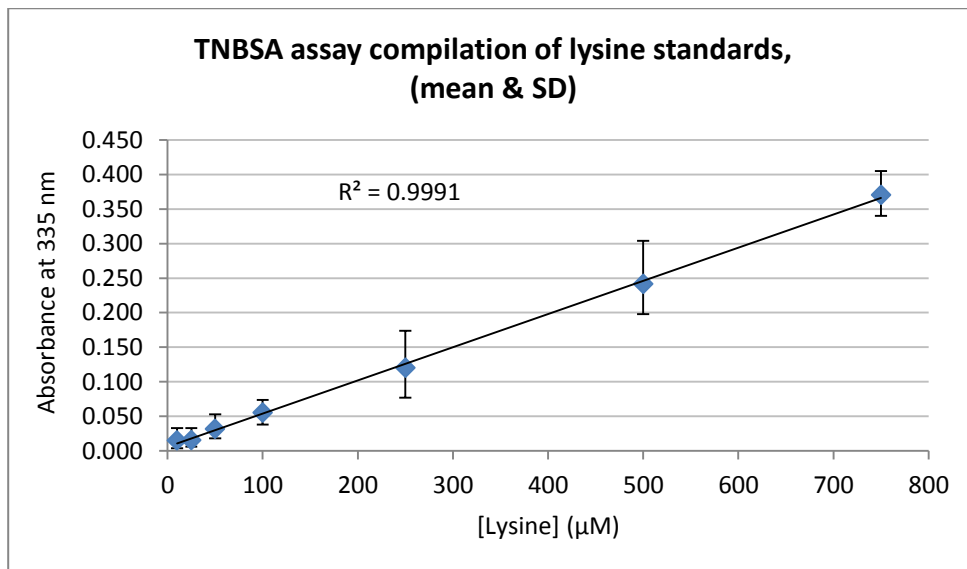


Figure 5. Compilation of L-lysine standards

Compilation of L-lysine standards, showing NH_2 groups by TNBSA assay (mean & SD, $n = 3$ to 19, see Table 4).

2.3.7 Measurement of total iron

(Adapted from Bothwell et al 1971).

To 350 μl glycated transferrin sample, iron standards (10 to 100 μM), and SPB blank, was added 350 μl protein precipitation solution. This was mixed thoroughly for 1 minute, and then heated at 56°C for 15 minutes in a water bath. Samples were then centrifuged at 1,000 x g for 5 minutes, and 350 μl of the resulting supernatant was removed, and added to 350 μl of 0.5 mM ferene solution. After incubation for 5 minutes at room temperature absorbance was read at 593 nm.

2.3.8 Measurement of total iron-binding capacity

(Method of Ramsay 1957).

To 350 μl samples of the protein solutions ($n = 2$) was added 350 μl of iron saturating solution. This was mixed and left for 5 minutes at room temperature. To this was added 35 mg light magnesium carbonate, in the form of $\text{Mg}(\text{OH})_2 \cdot 3\text{H}_2\text{O}$.

This was agitated frequently and thoroughly for 40 minutes, and then centrifuged at 1,000 x g for 5 minutes. The supernatant was removed and re-centrifuged at 1,000 x g for 5 minutes. The resulting supernatant was removed and analysed for iron as in the measurement of total iron methodology.

2.3.9 Measurement of glucose

Glucose separation was determined by the dinitrosalicylic colorimetric method of Miller (1959). The compilation of results for glucose standards is given in Table 5, and shown in Figure 6.

Table 5. Glucose assay, compilation of standards read at 575 nm, absorbance units (AU)

	Glucose concentration (mM)				
	100	200	500	750	1000
n =	5	6	7	3	2
Absorbance at 575 (AU, mean)	0.04	0.19	0.35	0.58	0.83
Absorbance at 575 (SD)	0.02	0.040	0.03	0.03	0.00

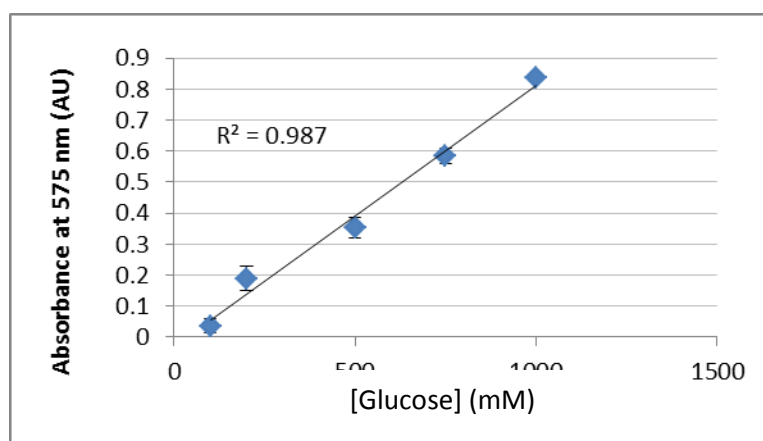


Figure 6. Glucose assay, compilation of standards

Glucose assay, compilation of standards, mean and SD ($r = 0.993$, $n =$ range 2 to 7, see Table 5).

2.3.10 Measurement of glucose in samples, post buffer exchange

To assess the adequacy of the buffer exchange process for the removal of glucose from the buffer containing the glycated transferrin, the method of Miller (1959)

was used to measure glucose. Results are given as glucose (mM). A lyophilized apo-human transferrin samples was freshly dissolved in SPB, and included as a control. Results for glucose concentration in SPB following buffere exchange are given in Table 6 and Figure 7.

Table 6. Glucose in glycated transferrin solutions post buffer exchange

	Glucose concentration (mM)				
	Fresh	0	500	750	1000
n =	1	3	3	3	3
Glucose (mM, mean)	97.2	122.2	127.6	135.7	112.4
Glucose (mM, \pm range)	0	20.6	13.0	35.9	5.9

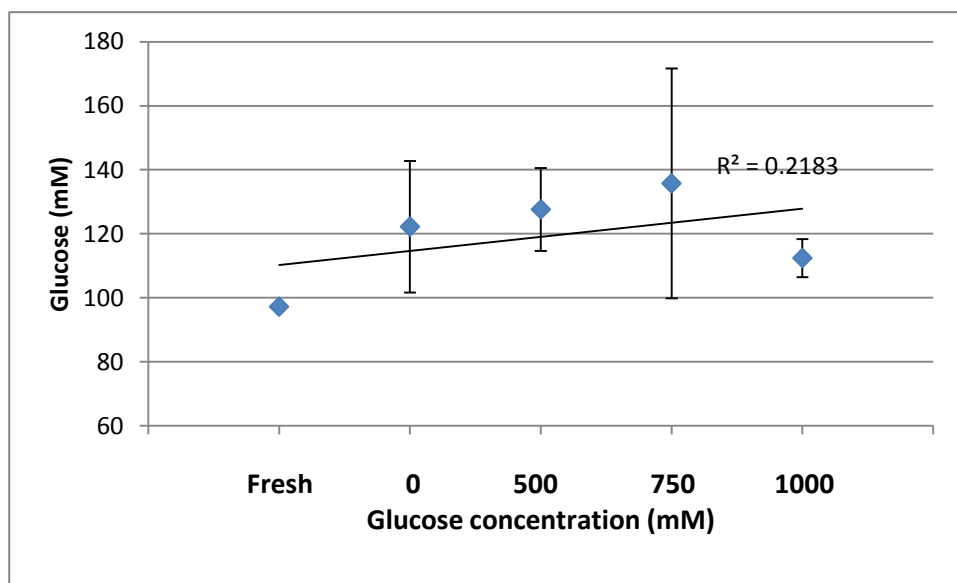


Figure 7. Glucose present in glycated transferrin solution

Glucose present in glycated transferrin solution post buffer exchange, Batches A to C, mean \pm range ($r = 0.467$, $n =$ range 1 to 3, see Table 6).

The samples that were tested for glucose post buffer exchange were all found to have mean glucose present in excess of 101.6 mM. Transferrin freshly made-up in

SPB, and transferrin incubated in SPB with no added glucose, which could be expected to have no glucose, had levels ranging from 97.2 to 142.8 mM.

A positive trend with glucose incubation level was seen ($r = 0.467$), suggesting that the buffer exchange process assay may be able to measure glucose adducts. There may be contamination from the buffer exchange process, if the 1.5 ml protein collection also collected part of the glucose elution, however as the fresh sample (which did not undergo buffer exchange) also had glucose present this would not appear to be the case. The compilation of standards shows little variation in results at 100 μM glucose (Figure 6), and therefore the assay appears to be reliable, suggesting that whilst a positive trend for glucose may be associated with glucose incubation concentration, some other factor may be causing interference with the assay.

2.3.11 Assessment of iron-binding by spectrophotometry

Four incubated samples of transferrin (0, 500, 750, 1000 mM glucose) were compared to freshly dissolved transferrin. Each 900 μl sample was adjusted by the addition of SPB to have ~ 500 $\mu\text{g}/\text{ml}$ protein, dissolved in SPB pH 7.4. Each received 50 μM (50 μl of 1mM) iron:nitrilotriacetic acid (Fe:NTA), and 1.5 mM (50 μl of 30 mM) sodium bicarbonate (NaHCO_3). Fe:NTA and NaHCO_3 were added after the first reading at 0 minutes. All samples were read concurrently, in a seven position spectrophotometer, optical pathway 1 cm. Quartz cuvettes were used for the ultraviolet measurements. Absorption readings were taken against a blank SPB sample containing 50 μl Fe:NTA and 50 μl NaHCO_3 set at zero the start of every run of 6 readings.

2.3.12 Ultrafiltration

Following buffer exchange, protein quantification, and NH₂ group quantification, protein samples were placed on ice, in a refrigerator, overnight. The following day three aliquots of transferrin solution, volumes varying depending on transferrin concentration, were taken from each of the five transferrin samples (0, 500, 750, 1000 mM glucose and Fresh), and placed in Eppendorf tubes. SPB was added to a final volume of 450 µl volumes with an approximately equal protein concentration in each. 50 µl of each was then taken for protein quantification.

Tubes were designated Run 1, 2, or 3, and samples for Runs 2 and 3 were placed on ice until required. All runs within a batch were performed on the same day.

Each of the 5 samples for Run 1 received 50 µM (50 µl of 500 µM) iron atomic absorption standard solution (25 µl of 990 µg/ml Fe in 1 wt. % HCl, added to 861 µl distilled H₂O) (gift from R. Evans), plus 50 µl of 500 µM (50 µM) NaHCO₃. Samples were incubated at room temperature for 30 minutes. The pH of the protein-iron solutions were measured on the first day of filtration, and were found to range from pH 7.43 to pH 7.48.

Millipore Amicon Ultra filter units 500 µl, 30 kDa (Merke Millipore), were pre-washed in 500 µl dH₂O, spun at 6,000 x g for 10 minutes at 4 °C, and the effluent discarded. Filters were then inverted and spun for a further 5 minutes at 2,500 g to empty them, before being placed inside clean, dry, individually weighed filtrate collection tubes. Samples were centrifuged at 13,000 x g for 30 minutes, at 4 °C. The filtrate tubes were collected, and the retentate was recovered by centrifuging into a second set of weighed collection tubes, at 6,000 x g for 10 minutes, at 4 °C.

Filtrate tubes, and retentate tubes, were re-weighed, the volume of solution in each calculated, and adjusted to 500 μl by the addition of SPB. Protein quantifications were repeated, and expressed as $\mu\text{g}/500 \mu\text{l}$.

Filter units were reused for each of the 3 runs on the day, keeping filters to the same glucose concentration sample type each time, before being discarded.

Iron in each fraction was measured by the method of Bothwell et al (1971), see section 2.3.7 for method.

This procedure was repeated for each run. Four separate incubation batches (A to D) were tested by this method, giving 12 runs at each glucose incubation level in total. Samples were treated individually for statistical purposes.

2.3.13 Electrophoresis

Denaturing, SDS-urea gel electrophoresis was carried out following the method of Blackshear (1984), in Bollag et al (1996), with 8.4% acrylamide gel, and 6 M urea. Samples consisted of 20 μg protein, in buffer containing 10 mM dithiothreitol and 6 M urea. Gels were run at 170 V over 45 minutes, and stained for 1 hour with coomassie blue, and destained overnight.

2.3.14 Statistical analysis

Statistical analyses were performed in Excel (Microsoft 2010). Differences were tested by unpaired, two-tailed Student t-test for equal variance (unless stated), with significance set at $P \leq 0.05$ in all cases. Excel can be used without reservation for this method (Dale 2001). Correlations were by Pearson's correlation test, with two-tailed P -value significance set at ≤ 0.05 . Significance testing was not used for small sample size ($n = 5$), non-parametric, correlations.

2.4 Results

2.4.1 Iron-binding by spectrophotometry

To investigate whether *in vitro* glycation of transferrin has an effect on iron-binding, a time course of spectrophotometry at 470 nm was undertaken. The rate of transferrin-iron-binding can be followed by spectrophotometry at 470 nm, as the bond between a single iron atom, two tyrosine ligands, one aspartate, one histidine, and a carbonate anion (Bailey et al 1988) produces a red colour, with an absorption maximum at 470 nm. Earlier work by Van Campenhout et al (2004), has shown that the increase in absorbance at 450 nm over 90 minutes, when *in vitro* glycated transferrin and iron are incubated together, is lessened with increasing level of glycation.

See section 2.3.11 for method.

2.4.1.1 Glycation of samples

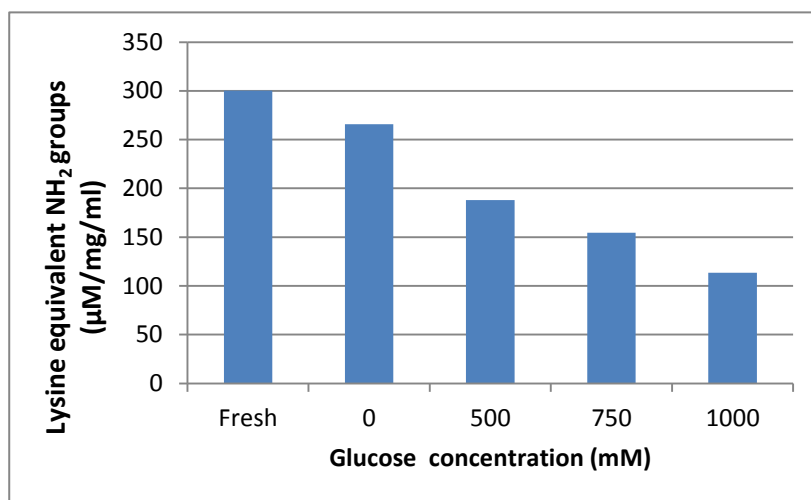


Figure 8. Glycation of samples

Glycation of samples used to measure the time course of iron-binding by spectrophotometry at 470 nm, shown by lysine equivalent NH₂ groups (n = 1)

2.4.1.2 Results

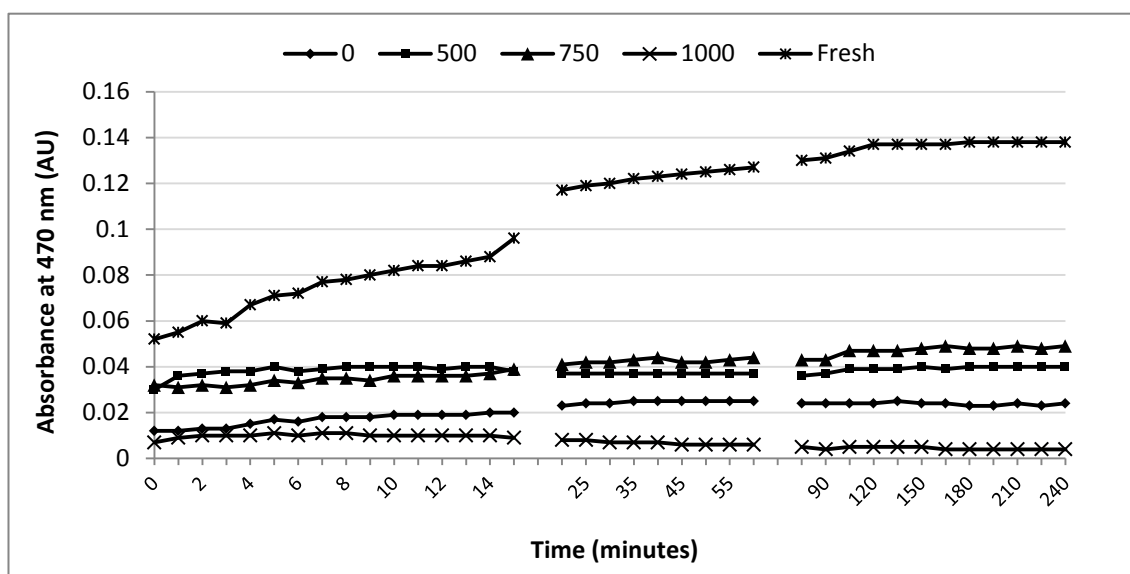


Figure 9. The time course of transferrin-iron-binding

The timecourse of transferrin-iron binding with *in vitro* glycosylated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and freshly made-up transferrin in SPB, with the addition of 50 μ M iron:nitrilotriacetic acid, and 50 μ M sodium bicarbonate.

Protein can be quantified by absorption at 280 nm, and transferrin saturation can be monitored by 280 nm/470 nm (Evans & Williams 1978). Fully saturated human serum transferrin has a value of 23.5. Transferrin saturation by 280 nm/470 nm is shown in Table 7.

Table 7. Transferrin saturation by 280 nm/470 nm

	Glucose concentration (mM)				
	Fresh	0	500	750	1000
280 nm	1.546	1.04	1.164	1.157	1.094
470 nm at Start	0.052	0.012	0.03	0.032	0.007
280 nm/470 nm	29.7	86.7	38.8	36.2	156.3
470 nm at 4 hr	0.138	0.024	0.04	0.049	0.004
280 nm/470 nm	11.2	43.3	29.1	23.6	273.5
Delta Start - 4 hr	0.086	0.012	0.01	0.017	0.0033

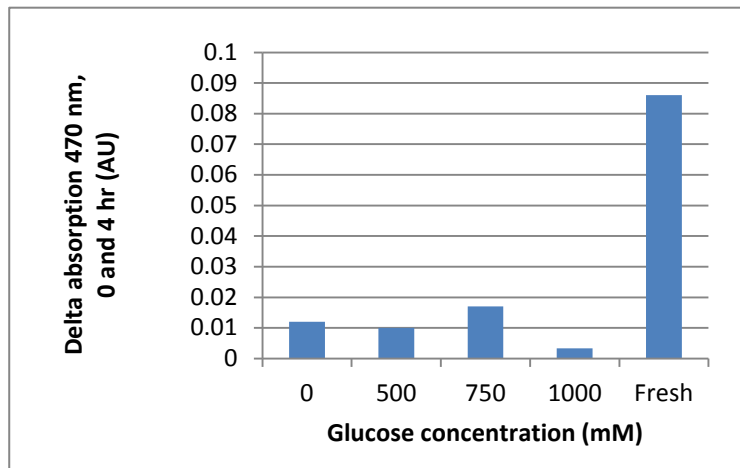


Figure 10. The change in absorption at 470 nm

The change in absorption at 470 nm, between 0 and 4 hours, following transferrin-iron-binding with *in vitro* glycosylated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly dissolved in SPB, with the addition of 50 μ M iron:nitrilotriacetic acid, and 50 μ M sodium bicarbonate.

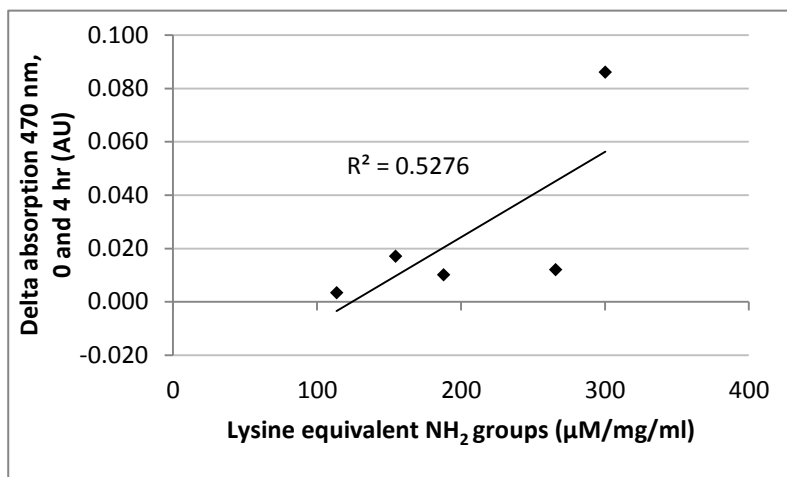


Figure 11. Glycation vs the change in absorption at 470 nm

Lysine equivalent NH₂ groups vs the change in absorption at 470 nm, between 0 and 4 hours, following transferrin-iron-binding with *in vitro* glycosylated transferrin (n = 5, r = 0.726).

2.4.1.3 Discussion and Conclusion

Inhibition of iron-binding capacity is clearly seen in all the incubated samples, in comparison to the fresh sample. Used as a measure of iron-binding, the change in absorbance over the 4 hours shows a general positive correlation between degree of glycation and iron-binding function ($r = 0.785$) (Figure 11), however, as can be seen from the difference between freshly dissolved transferrin and transferrin pre-incubated in SPB with no glucose (Figure 10), the process of incubation, independently of glucose concentration, is damaging to the protein.

2.4.2 Total iron-binding capacity

Incubated and fresh transferrin was tested for total iron-binding capacity (TIBC) by the method of Ramsay (1957), see section 2.3.8. Protein concentrations ranged from 103.5 to 177.0 $\mu\text{g}/350 \mu\text{l}$ SPB, and samples were run in duplicate (Table 8).

It was not possible to calculate transferrin saturation (total iron/TIBC x 100) by the method normally used *in vivo* (Beilby et al 1992), as total iron in this case is a universal 50 μM .

Table 8. Total iron-binding capacity (TIBC) of *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB. Glycation assessed by picrylsuphonic acid assay of NH_2 groups, measured against L-lysine standards

	Glucose concentration (mM)				
	Fresh	0	500	750	1000
Lysine equivalent NH_2 groups ($\mu\text{M}/\text{mg}/\text{ml}$)	186.2	213.8	69.3	44.7	16.6
Protein, mean ($\mu\text{g}/350 \mu\text{l}$, $n = 2$)	176.0	111.6	127.7	142.0	158.5
TIBC, mean ($\mu\text{M}/\text{mg}$, $n = 2$)	81.43	84.44	42.25	25.24	21.53
TIBC range ($\mu\text{M}/\text{mg}$)	13.6	68.2	62.7	9.1	12.4

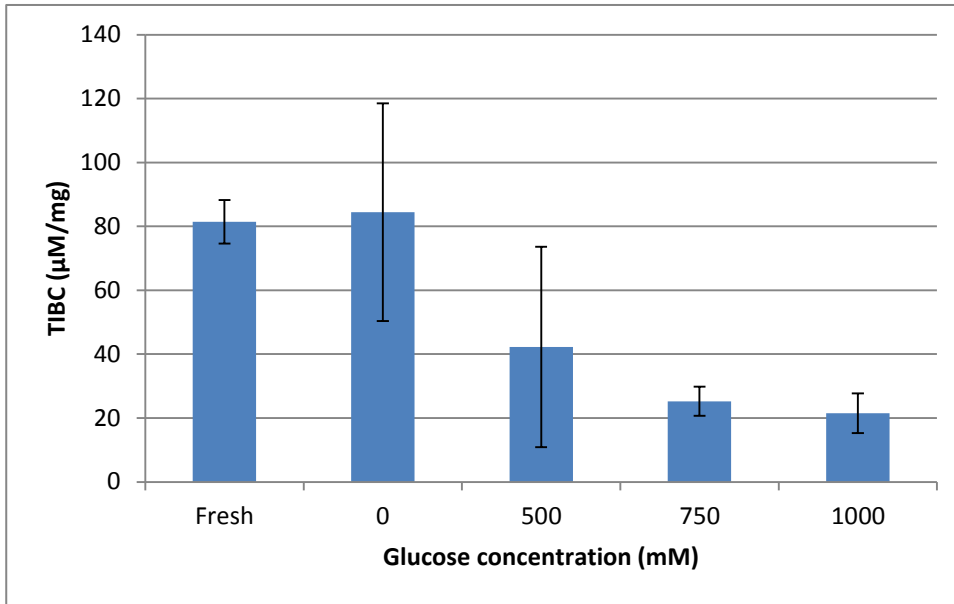


Figure 12. Total iron-binding capacity of *in vitro* glycated transferrin

Total iron-binding capacity of *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB (μM/mg, mean and range, n = 2).

Mean TIBC is seen to decrease with degree of glycation (Figure 12). No difference in mean TIBC is apparent between freshly dissolved transferrin and transferrin pre-incubated in SPB with no glucose (Figure 12).

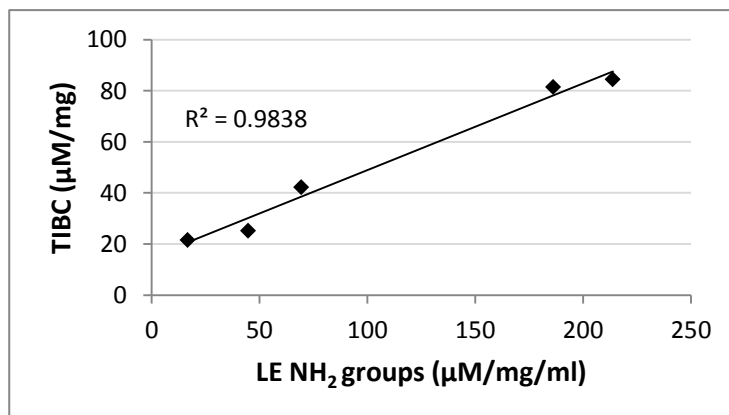


Figure 13. Glycation of *in vitro* glycated transferrin

Glycation of *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, shown by lysine equivalent (LE) NH₂ groups vs total iron-binding capacity (n = 5, r = 0.992).

A clear decrease in total iron-binding capacity can be seen with increasing glycation, as measured by lysine equivalent NH₂ groups. TIBC is closely related to glycation (Figure 13), in agreement with Van Campenhout et al (2003).

2.4.3 Filtration studies

To investigate whether the loss of iron-binding ability in glycated transferrin is due to protein fragility and fragmentation, further batches of glycated transferrin were tested by filtration through Millipore Amicon Ultra 30 kDa filter units. As transferrin has a molecular mass of ~ 80 kDa no intact protein should pass through this size of filter. See section 2.3.12 for procedures.

2.4.3.1 Glycation of samples

Table 9. Glycation of *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, shown by lysine equivalent (LE) NH₂ groups (μM/mg/ml), batches A to D

LE NH ₂ groups (μM/mg/ml)	Glucose concentration (mM)				
	Fresh	0	500	750	1000
Batch A	234.15	236.13	125.54	94.85	111.52
Batch B	323.90	284.04	203.15	191.89	181.32
Batch C	207.19	196.38	125.70	55.44	90.45
Batch D	251.34	248.77	147.20	75.36	92.35
Mean	254.15	241.33	150.40	104.39	118.91
SD	49.93	36.18	36.61	60.51	42.68
Mean % of Fresh	100	94.96	59.18	41.07	46.79
"% glycation"	0	5.04	40.82	58.93	53.21

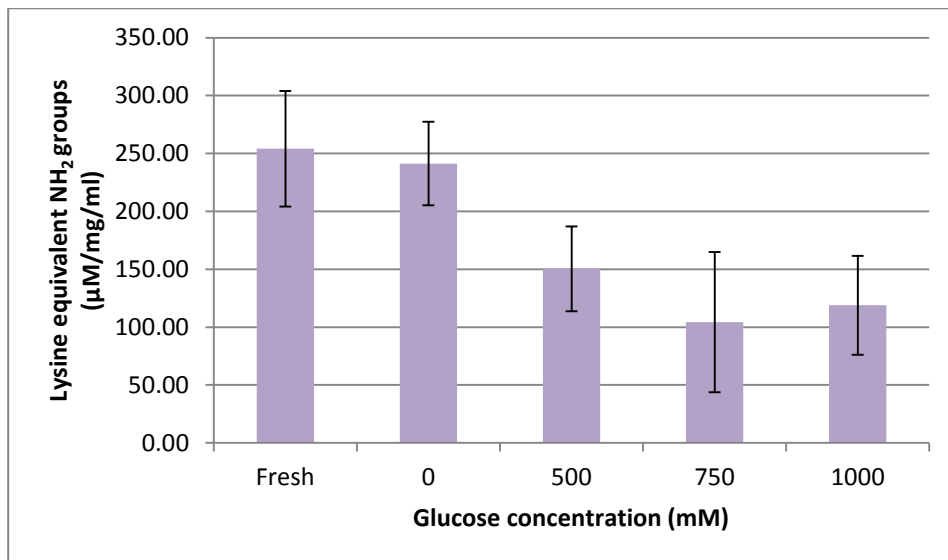


Figure 14. Glycation of *in vitro* glycosylated transferrin

Glycation of *in vitro* glycosylated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, shown by lysine equivalent NH₂ groups (µM/mg/ml), batches A to D (mean and SD).

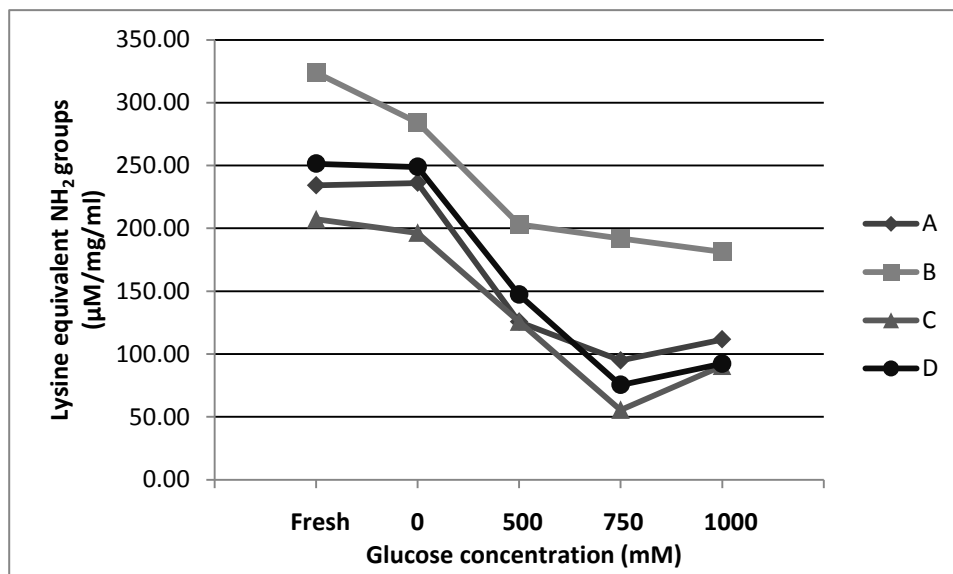


Figure 15. Glycation of *in vitro* glycosylated transferrin

Glycation of *in vitro* glycosylated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, shown by lysine equivalent NH₂ groups (µM/mg/ml), batches A to D, individual levels.

Following buffer exchange, the amount of glycated sample produced in each of the four incubated batches varied. Runs were adjusted to have an equal protein concentration within batches. Mean values per batch, and within batch standard deviation, were as follows:

Batch A, 274.3 $\mu\text{g}/400 \mu\text{l}$ (SD 30.8)

Batch B, 606.8 $\mu\text{g}/400 \mu\text{l}$ (SD 59.5)

Batch C, 345.7 $\mu\text{g}/400 \mu\text{l}$ (SD 29.7)

Batch D, 225.1 $\mu\text{g}/400 \mu\text{l}$ (SD 8.7)

Mean protein concentration for all 60 samples was 363.0 $\mu\text{g}/400 \mu\text{l}$ (SD 20.8).

2.4.3.2 Results, filtrate protein

Protein concentrations varied between batches and a range of protein concentrations were used in the experiment. To aid comparison between batches, results for protein are reported as percentages of the pre-filtration protein concentration.

Increasing mean levels of protein were seen in the filtrate with increasing glucose concentration, ranging from 0.17 % pre-filtration for freshly made-up transferrin, to 3.55 % pre-filtration for 1000 mM glucose. Significant differences were seen between 0 and 500 mM ($P = 0.043$), and 0 and 1000 mM ($P = 0.027$) (Table 10). A non-significant correlation was seen between glycation as measured by Lysine equivalent NH_2 groups and filtrate protein ($r = -0.301$) (Figure 17).

Table 10. Protein in filtrate, % of amount pre-filtration, batches A to D

		Glucose concentration (mM)				
(n = 12)		Fresh	0	500	750	1000
	Mean	0.17	0.36	1.82	2.53	3.55
	SD	0.44	1.26	1.98	3.56	4.49
	<i>P</i>		0.626	0.043*	0.060	0.027*
			<i>P</i> vs Fresh	<i>P</i> vs 0	<i>P</i> vs 0	<i>P</i> vs 0

SD: Standard deviation

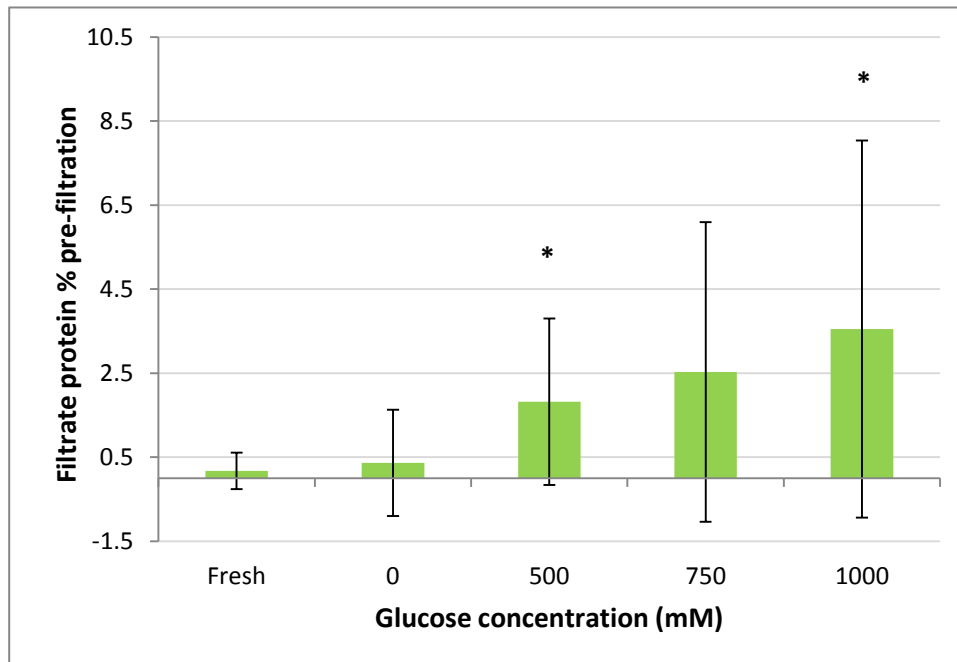


Figure 16. Protein in filtrate

Protein in filtrate, % of amount pre-filtration, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D, mean & SD (n = 12, 0 vs 500 mM, *P* = 0.043; 0 vs 1000 mM, *P* = 0.027).

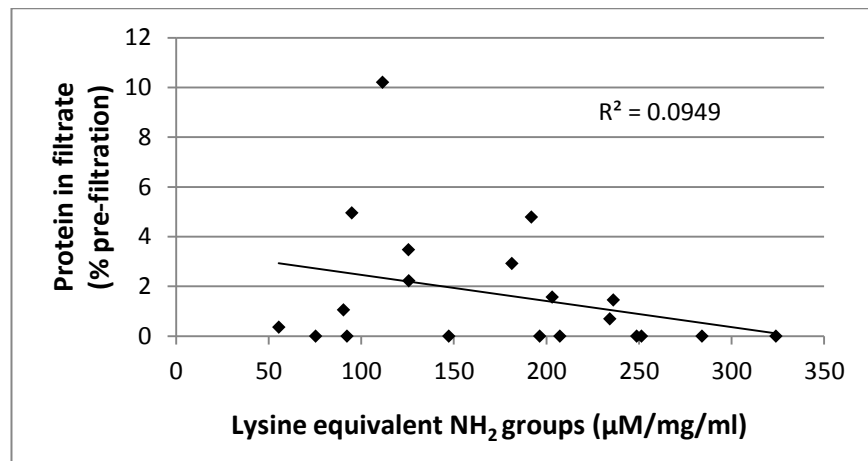


Figure 17. Glycation versus filtrate protein

Glycation versus filtrate protein, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D (n = 20, r = - 0.301, P = > 0.05).

2.4.3.3 Results, retentate protein

Decreased mean levels of protein were seen in the retentate in the glycated samples, ranging from 94.09 % pre-filtration for freshly made-up transferrin, to 77.66 % pre-filtration for 1000 mM glucose. Highly significant differences were seen between 0 and 750 mM ($P = 0.002$), and 0 and 1000 mM ($P = 0.001$) (Table 11, Figure 18). A highly significant correlation was seen between glycation as measured by Lysine equivalent NH₂ groups and retentate protein ($r = 0.610$, $P = 0.01$) (Figure 19).

Table 11. Protein in retentate, % of amount pre-filtration, Batches A to D

Batch	Run	Glucose concentration (mM)				
		Fresh	0	500	750	1000
A	1	90.08	89.13	85.01	81.28	71.17
	2	81.17	75.09	91.41	70.26	89.99
	3	98.20	115.60	84.11	110.24	87.79
B	1	107.43	97.17	75.73	68.98	62.72
	2	107.36	92.63	76.14	71.64	74.85
	3	97.22	96.86	86.04	59.52	75.96
C	1	94.58	125.00	75.15	63.86	74.36
	2	90.49	91.51	67.36	77.79	73.52
	3	86.96	93.14	106.71	60.93	80.03
D	1	82.36	80.94	84.72	81.04	83.78
	2	98.35	96.21	93.06	75.68	74.71
	3	94.92	84.59	90.29	83.17	83.00
	Mean	94.09	94.82	84.64	75.37	77.66
	SD	8.40	13.80	10.40	13.60	7.60
	<i>P</i>		0.877	0.053	0.002**	0.001**
			<i>P</i> vs Fresh	<i>P</i> vs 0	<i>P</i> vs 0	<i>P</i> vs 0

SD: Standard deviation

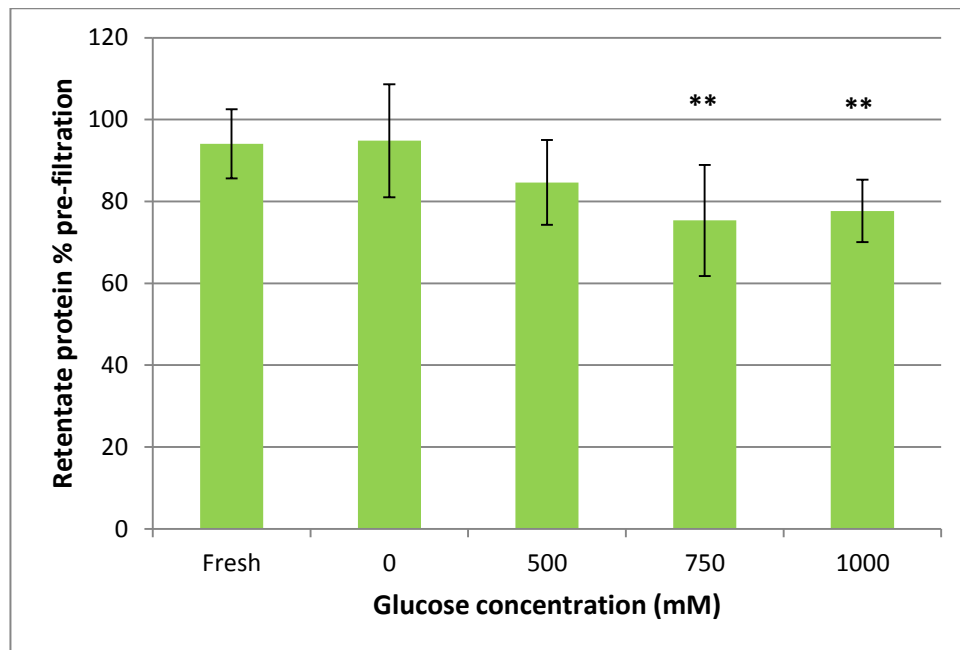


Figure 18. Protein in retentate

Protein in retentate, % of amount pre-filtration, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D, mean & SD (n = 12, 0 vs 750 mM $P = 0.002$; 0 vs 1000 mM $P = 0.001$).

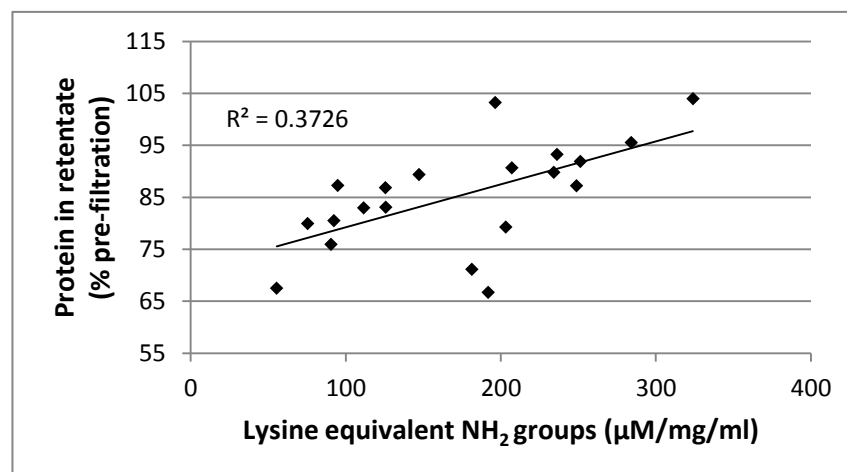


Figure 19. Glycation versus retentate protein

Glycation versus retentate protein, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D (n = 20, $r = 0.610^{**}$, $P < 0.01$).

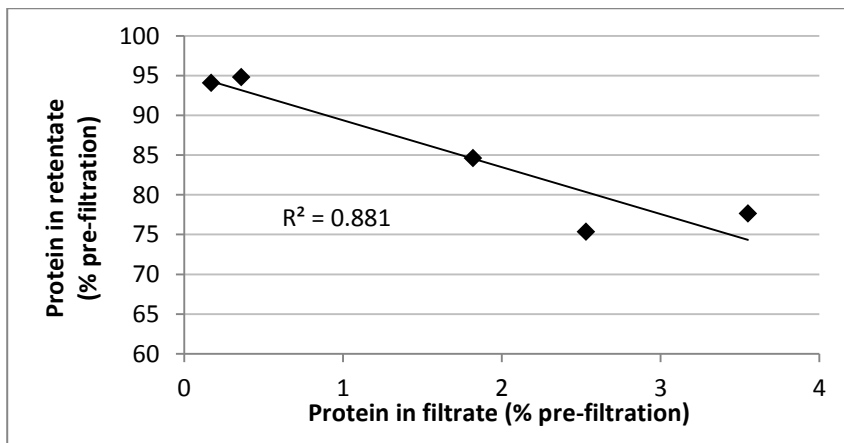


Figure 20. Correlation between filtered and retained protein

Correlation between filtered and retained protein, *in vitro* glycosylated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D, mean values (n = 5, r = - 0.939).

2.4.3.4 Results, filtrate iron

The concentration of iron used in the experiment (50 µl of a 500 µM iron solution) is in excess of the iron-binding capacity of the transferrin present, calculated as follows:

Transferrin 300 µg (80 kDa), $300 \times 10^{-6} \text{ (g)} / 80 \times 10^3 \text{ (g in 1 mol)} =$

$$3.75 \times 10^{-9} \text{ mols}$$

Iron 50 µl of a 500 µM iron solution, $50 \times 10^{-6} \times 500 \times 10^{-6} = 25,000 \times 10^{-12}$ or

$$25 \times 10^{-9} \text{ mols}$$

300 µg of transferrin will bind 7.5×10^{-9} mols of iron, therefore, to bind all 25×10^{-9} mols iron in the solution, 1 mg of transferrin would be needed.

Increased mean levels of iron were seen in the filtrate with the glycosylated samples, ranging from 37.1 µM for freshly prepared transferrin, to 46.1 µM for 500 mM glucose. Significant difference was only seen between 0 and 500 mM ($P = 0.041$)

(Table 12). A significant correlation was seen between glycation as measured by Lysine equivalent NH₂ groups and filtrate iron ($r = -0.459$, $P < 0.05$) (Figure 21).

Table 12. Iron in filtrate, batches A to D (μM)

		Glucose concentration (mM)				
(n = 12)		Fresh	0	500	750	1000
	Mean	37.1	40.5	46.1	43.1	45.5
	SD	6.5	5.7	6.9	7.9	7.8
	<i>P</i>		0.189	0.041*	0.362	0.084
			<i>P</i> vs Fresh	<i>P</i> vs 0	<i>P</i> vs 0	<i>P</i> vs 0

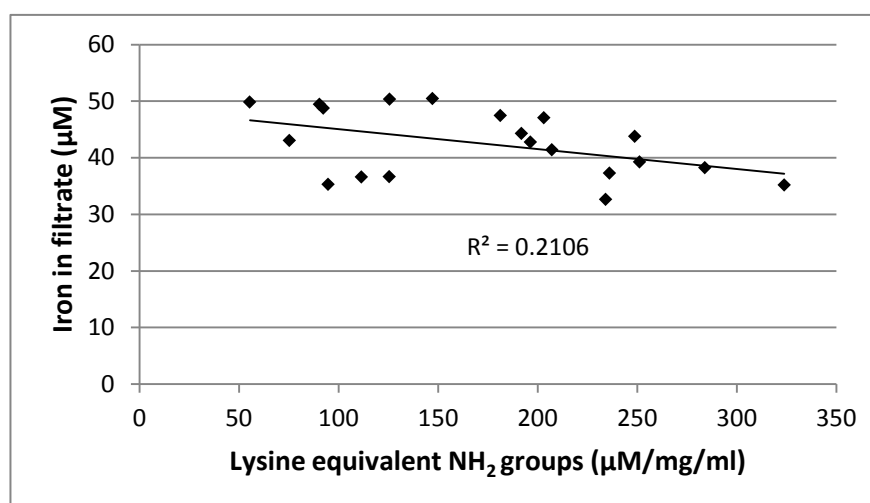


Figure 21. Glycation versus filtrate iron

Glycation versus filtrate iron, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D (n = 20, $r = -0.459^*$, $P < 0.05$).

2.4.3.5 Results, iron in retentate

Decreasing mean levels of iron were seen in the retentate with an increase in glucose concentration, ranging from 20.23 μM for Fresh, to 9.97 μM for 1000 mM glucose. A highly significant difference was seen between 0 and 500 mM ($P =$

0.0011), and very highly significant differences were seen between 0 and 750 mM ($P = 0.0006$), and 0 and 1000 mM glucose ($P = 0.0002$) (Table 13). A very highly significant correlation was seen between glycation as measured by Lysine equivalent NH_2 groups and retentate iron ($r = 0.793$, $P < 0.001$) (Figure 23). A non-significant correlation is seen between mean values for filtered and retained iron, ($n = 5$, $r = -0.950$, Figure 24). A near significant correlation is seen between filtrate protein and filtrate iron, batches A to D, when all samples are included individually ($r = -0.253$, non-parametric significance at 0.255, Figure 25).

Table 13. Iron in retentate, batches A to D (μM)

		Glucose concentration (mM)				
(n = 12)		Fresh	0	500	750	1000
	Mean	20.23	17.21	10.71	10.63	9.97
	SD	7.24	4.60	3.84	3.31	2.59
	<i>P</i>		0.2359	0.0011**	0.0006***	0.0002***
			<i>P</i> vs Fresh	<i>P</i> vs 0	<i>P</i> vs 0	<i>P</i> vs 0

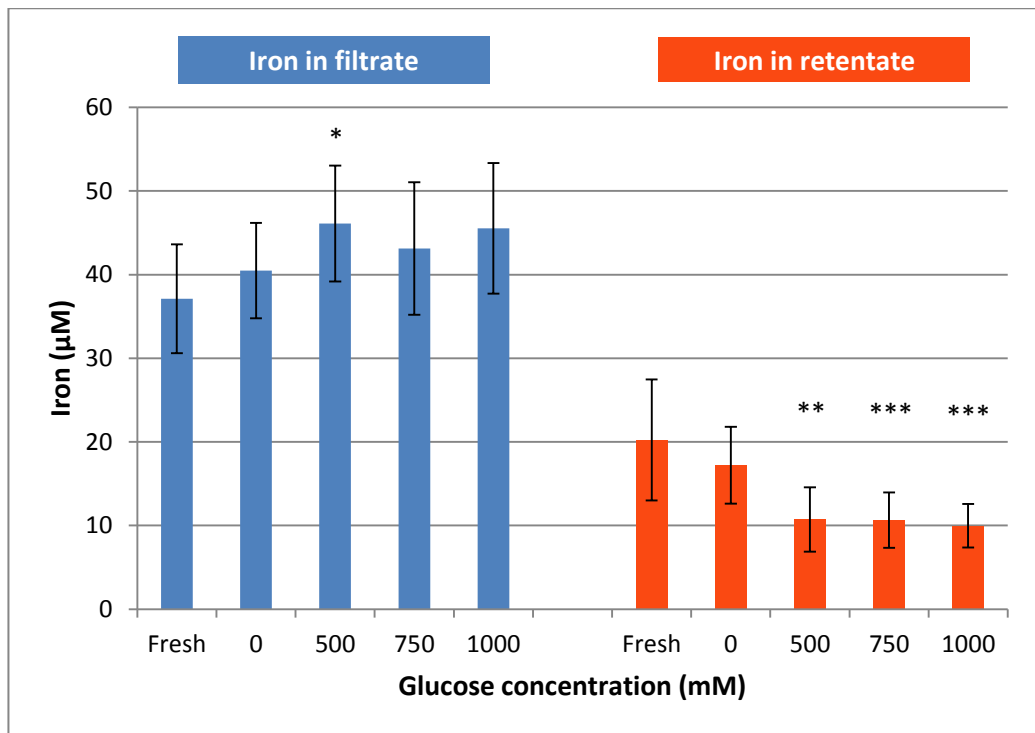


Figure 22. Iron in filtrate and retentate

Measurement of iron in filtrate and retentate following glucose incubation, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D, mean & SD (n = 12). Significance shown in comparison with 0 mM in each phase, see Table 13 for *P* values.

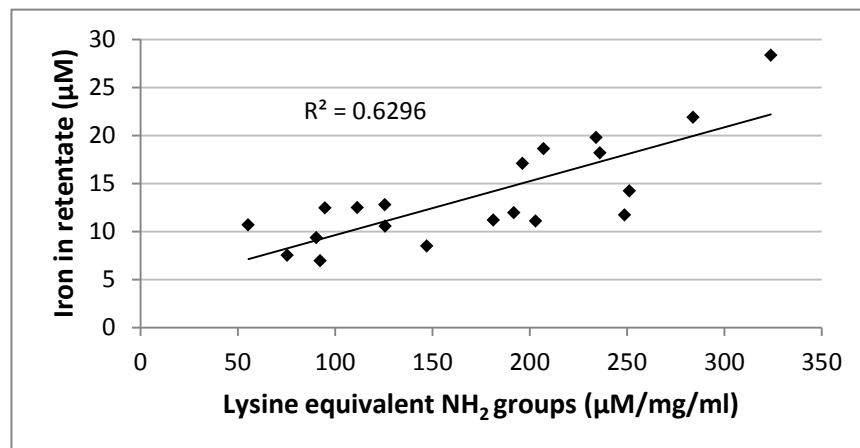


Figure 23. Glycation versus retentate iron

Glycation versus retentate iron, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D (n = 20, r = 0.793***, *P* < 0.001).

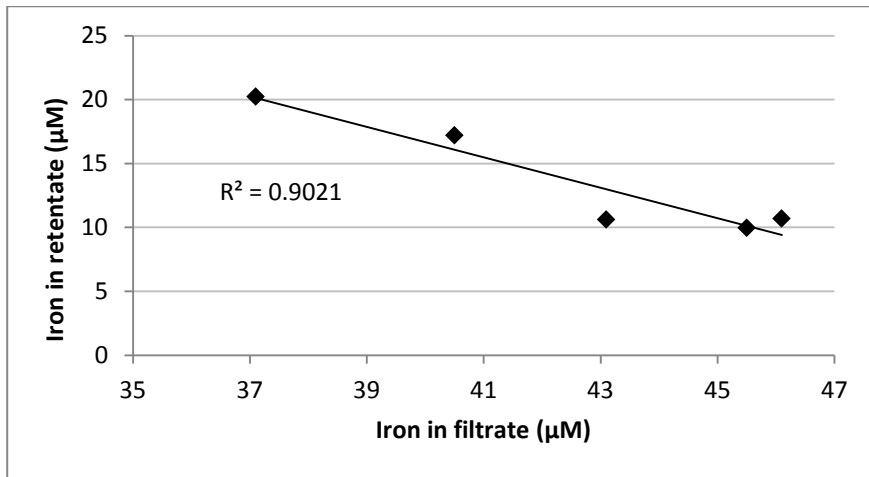


Figure 24. Correlation between filtered and retained iron

Correlation between filtered and retained iron, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D, mean values (n = 5, r = -0.950)

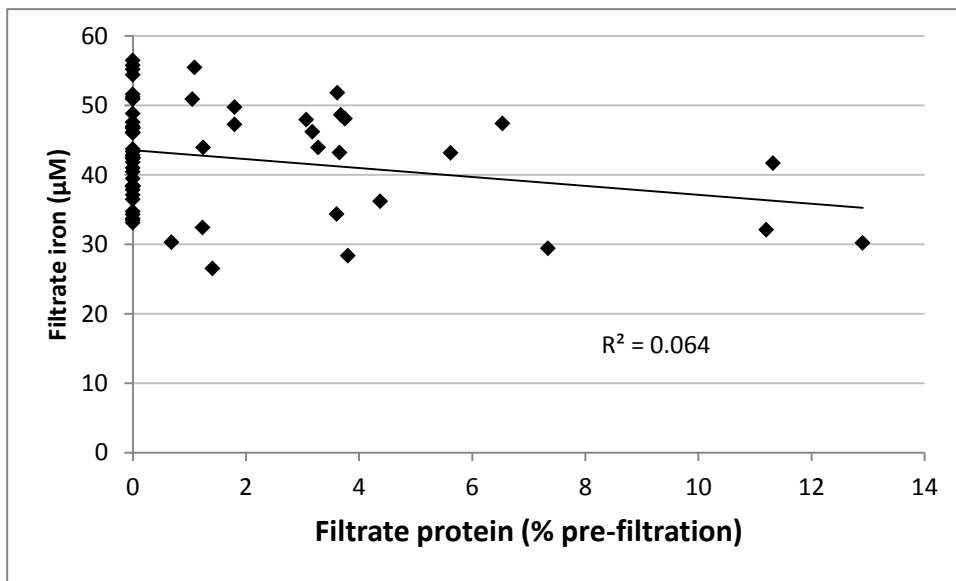


Figure 25. Correlation between filtrate protein and filtrate iron

Correlation between filtrate protein and filtrate iron, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D (n = 60, r = - 0.253, non-parametric significance at 0.255)

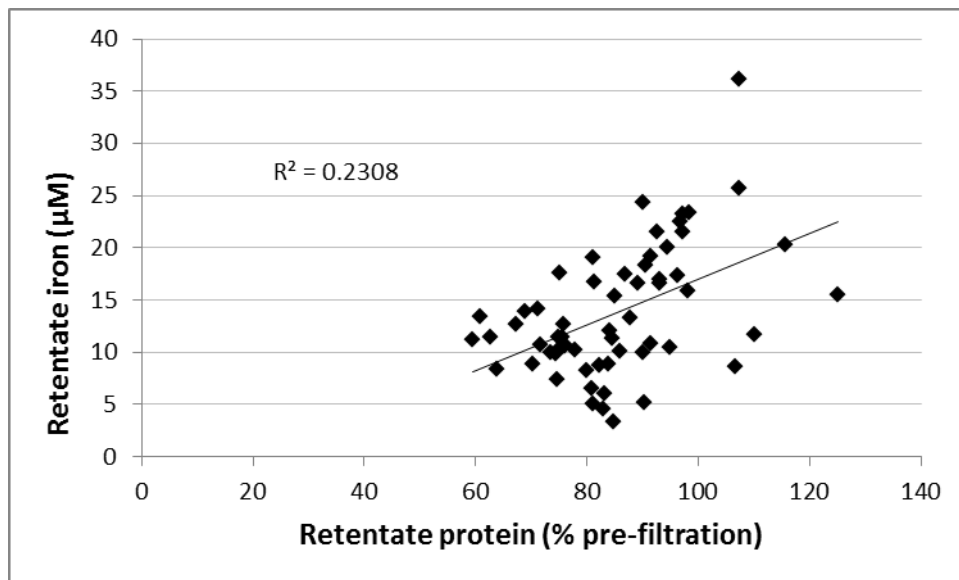


Figure 26. Correlation between retentate protein and retentate iron

Correlation between retentate protein and retentate iron, *in vitro* glycated transferrin (0, 500, 750, 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, batches A to D (n = 60, $r = 0.480^{***}$, $P < 0.001$).

2.4.3.6 Discussion

Filtration was used to determine whether *in vitro* transferrin glycation and loss of transferrin-iron-binding was linked to protein fragmentation, as suggested by Fujimoto et al (1995). Glycation was established by loss of NH_2 groups, and was found to correlate very strongly with iron loss during filtration, and protein fragmentation, by the methods used here. Significant differences were seen in results between incubation with and without glucose, but not between incubation without glucose and freshly made-up protein, indicating that, unlike the findings for absorption at 470 nm, the glucose was causing the damage to the protein, rather than incubation *per se*.

The strengths of the relationships between filtered and retained protein ($r = 0.939$) and iron ($r = 0.950$), and the similarity of these correlation coefficients, demonstrate the parallelism in the results for these two measures, as do the

correlations between filtrate protein and iron ($r = - 0.253$, Figure 25), and retentate protein and iron ($r = 0.480^{***}$, Figure 26). Whilst correlation does not signify cause, these results strongly suggest that loss of function in *in vitro* glycated transferrin is due to protein fragmentation.

2.5 Further studies

2.5.1 Electrophoresis

SDS-urea gel electrophoresis was used to examine the effect of the *in vitro* glycation process on transferrin in comparison with lyophilized apo-transferrin freshly dissolved in SPB, and transferrin incubated for 14 days at 37°C in glucose-free SPB. Pre-filtration apo-transferrin (pre-30 minute iron incubation), and 30 kDa retentate samples were used (post-30 minute iron incubation). Insufficient quantities of filtrate protein were available to study.

2.5.1.1 Result, electrophoresis

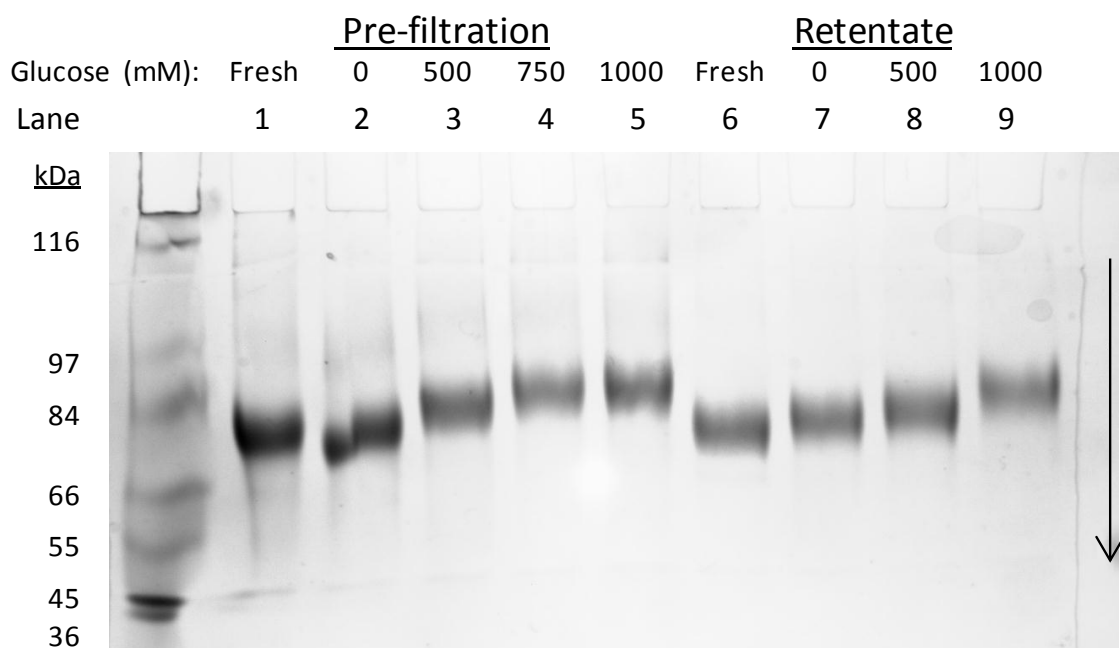


Figure 27. SDS urea gel electrophoresis of transferrin

SDS, 6 M urea gel electrophoresis of *in vitro* glycosylated transferrin. Lanes 1 to 5: human serum apo-transferrin incubated in 0, 500, 750, and 1000 mM glucose concentration, and freshly dissolved lyophilized apo-transferrin in SPB, pre-filtration, and pre-30 minute iron incubation. Lanes 6 to 9: human serum apo-transferrin incubated in 0, 500, and 1000 mM glucose concentration, and freshly dissolved apo-transferrin in SPB, retentate samples following 30 minute iron incubation and filtration through 30 kDa filters.

Some disruption to the uniformity of protein size is suggested by the blurred edges of the glycosylated, and 0 M retentate samples. This is particularly evident in the 1000 mM retentate sample. Pre-filtration glycosylated samples show some blurring of the protein band. In both pre-filtration and retentate, degree of glycosylation is seen to decrease mobility through the gel.

2.5.2 25 – 250 mM Glucose incubation

Incubation and filtration were carried out at lower glucose concentrations, approaching high blood glucose levels *in vivo*, which are 10 to 33.3 mmol/l plus. Two batches of *in vitro* glycated transferrin were produced using the method described in section 2.3.4, using glucose concentrations of 25, 100, and 250 mM in SPB, and SPB with no glucose. Three ultrafiltration runs were performed from each batch, making six runs in total. Due to the small sample size of six, non-parametric statistical analyses were carried out, using IBM SPSS version 19, for two-tailed, unpaired, median tests between groups (Table 14).

2.5.2.1 Results, 25 – 250 mM glucose incubation

Transferrin glycation following incubation in 25 to 250 mM glucose, measured by loss of NH₂ groups, occurs in a stepwise manner in keeping with that seen following incubation with higher concentrations of glucose, as shown in section 2.4.3.1. No statistical differences were seen between groups when comparing incubated transferrin samples, for filtrate and retentate protein and iron (Table 14, Figure 30, Figure 31, Figure 32, Figure 33). Surprisingly high filtrate protein concentrations were seen for freshly dissolved lyophilized transferrin, and for transferrin incubated in SPB without glucose (Figure 30), although values for the 25 to 250 μM samples increase with glycation in the expected manner (Figure 30). No difference is seen in median levels for retentate protein across the groups (Figure 31). Results for filtrate and retentate iron for freshly dissolved lyophilized transferrin, and for transferrin incubated in SPB without glucose, show a surprisingly high level of iron in the filtrate (Figure 32), with a wide range of values (Figure 33). Results for filtrate

and retentate iron for the 25 to 250 μM samples change with glycation following a trend opposite to that seen in the higher glucose incubation concentrations (section 2.4.3.4 and section 2.4.3.5).

Table 14. Filtration results, transferrin glycation at 25 to 250 mM glucose incubation

	Glucose incubation (mM)					
	Fresh	0	25	100	250	<i>P</i> <i>0 to 250</i>
LE NH ₂ groups, mean ($\mu\text{M}/\text{mg}/\text{ml}$) (mean, <i>n</i> = 2)	262.5	245.8	200.1	186.4	140.2	
Protein pre-filtration, mean ($\mu\text{g}/400 \mu\text{l}$) (<i>n</i> = 6)	471.2	452.4	459.7	444.0	423.3	
SD	247.3	221.8	225.1	208.0	186.5	
Filtrate protein (% pre-filtration) (median, <i>n</i> =6)	3.21	4.88	2.11	2.30	3.41	0.083
Range (% pre-filtration)	1.2 to 7.4	0.7 to 6.7	1.1 to 3.7	1.0 to 3.4	2.1 to 5.8	
Retentate protein (% pre-filtration) (median, <i>n</i> =6)	80.45	81.07	81.65	81.14	84.71	1.000
Range (% pre-filtration)	72.1 to 92.0	55.1 to 98.7	66.8 to 92.6	76.3 to 93.6	72.9 to 96.3	
Filtrate iron (μM) (median, <i>n</i> = 6)	28.36	30.78	27.95	30.14	35.84	0.261
Range (μM)	26.8 to 35.8	21.6 to 37.5	21.8 to 37.0	24.4 to 42.3	20.7 to 42.0	
Retentate iron (μM) (median, <i>n</i> = 6)	16.91	15.14	15.60	14.08	12.47	0.083
Range (μM)	14.5 to 22.0	10.4 to 16.6	12.8 to 18.8	12.3 to 17.4	10.8 to 14.5	

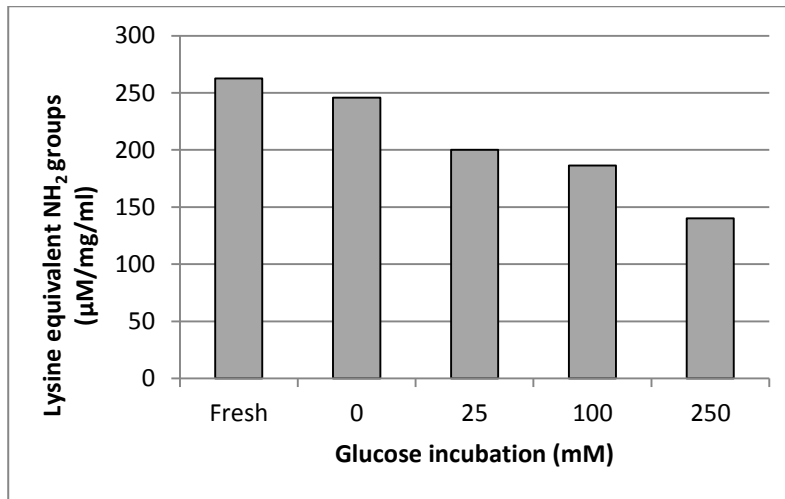


Figure 28. Lysine equivalent NH₂ groups, 25 to 250 mM glucose incubation

Lysine equivalent NH₂ groups following *in vitro* glycation of transferrin (0, 25, 100, and 250 mM glucose pre-incubation prior to buffer exchange into SPB), and transferrin freshly made-up in SPB, mean of 2 (µM/mg/ml).

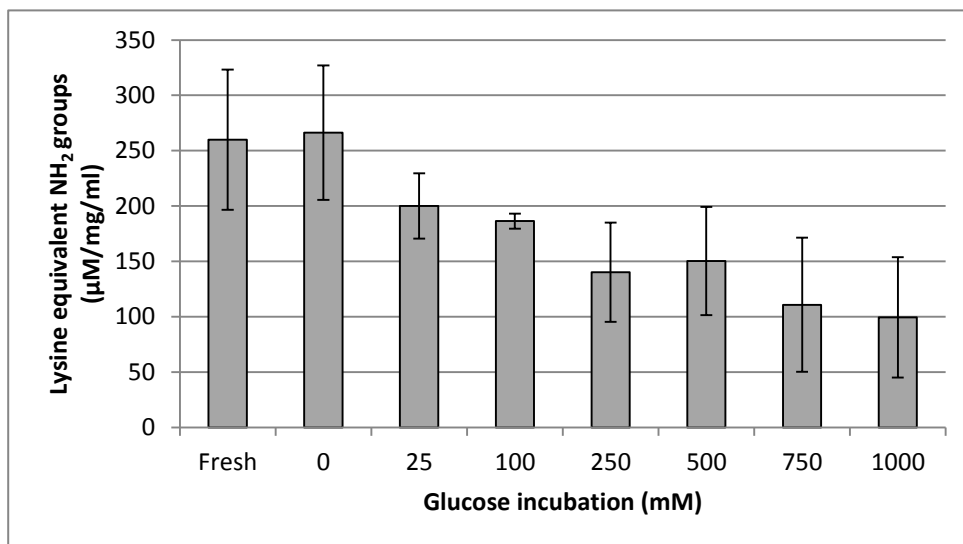


Figure 29. Lysine equivalent NH₂ groups, 25 to 1000 mM glucose incubation

Lysine equivalent NH₂ groups following *in vitro* glycation of transferrin (0, 25, 100, 250, 500, 750, and 1000 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, mean and SD (µM/mg/ml) (Fresh & 0 mM, n = 18; 25 to 250 mM, n = 2; 500 to 1000 mM, n = 12).

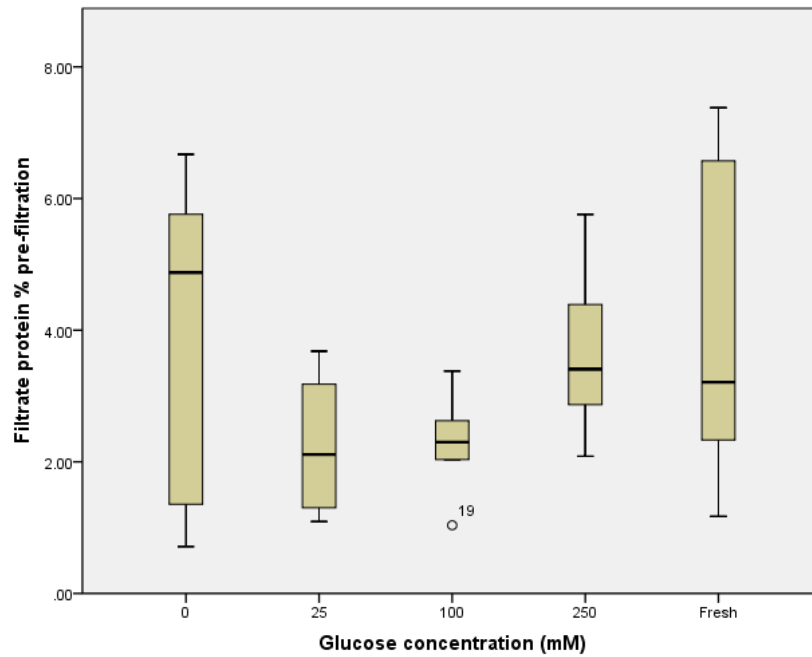


Figure 30. Filtrate protein, 25 to 250 mM glucose incubation

Filtrate protein, % pre-filtration, following *in vitro* glycation of transferrin (0, 25, 100, and 250 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, and ultrafiltration (n = 6, $P = 0.083$ between 0, 25, 100 and 250 mM).

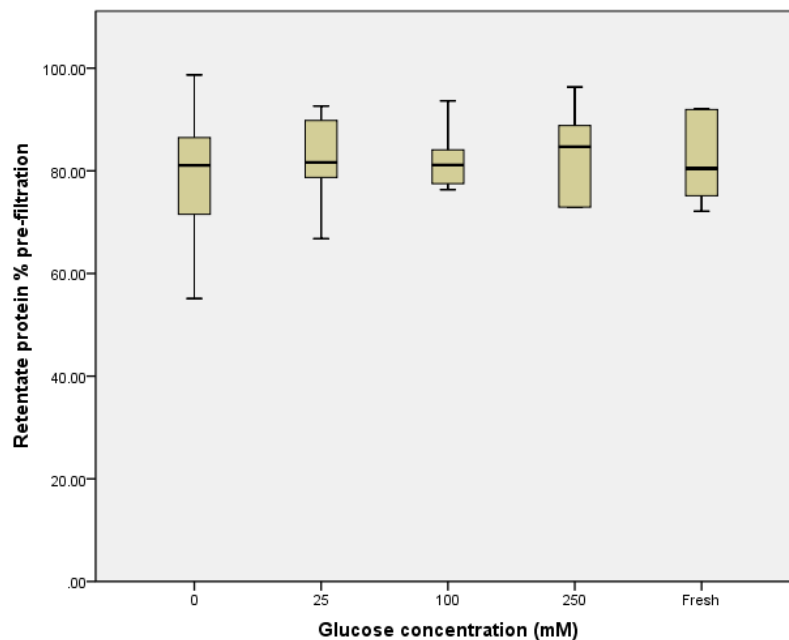


Figure 31. Retentate protein, 25 to 250 mM glucose incubation

Retentate protein, % pre-filtration, following *in vitro* glycation of transferrin (0, 25, 100, and 250 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, and ultrafiltration (n = 6, $P = 1.000$ between 0, 25, 100 and 250 mM).

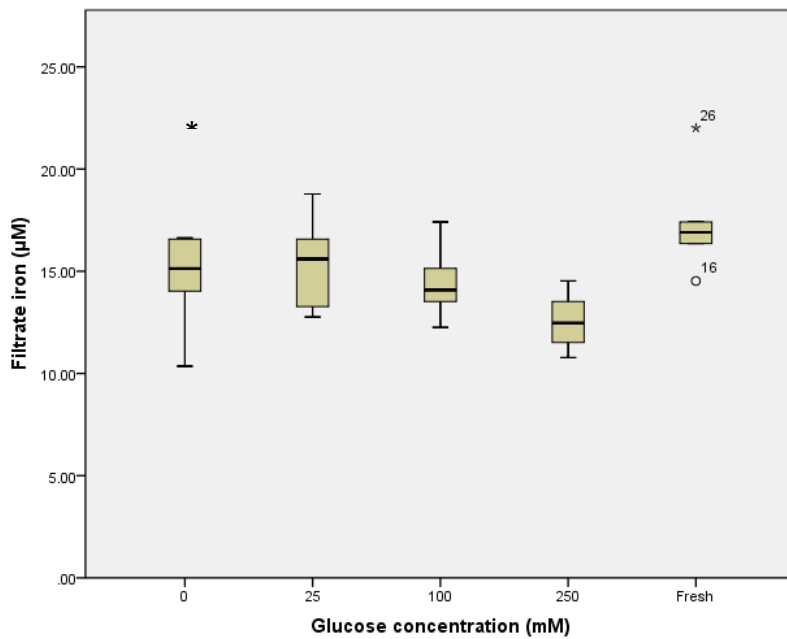


Figure 32. Filtrate iron, 25 to 250 mM glucose incubation

Filtrate iron following *in vitro* glycation of transferrin (0, 25, 100, and 250 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, and ultrafiltration (n = 6, P = 0.261 between 0, 25, 100 and 250 mM).

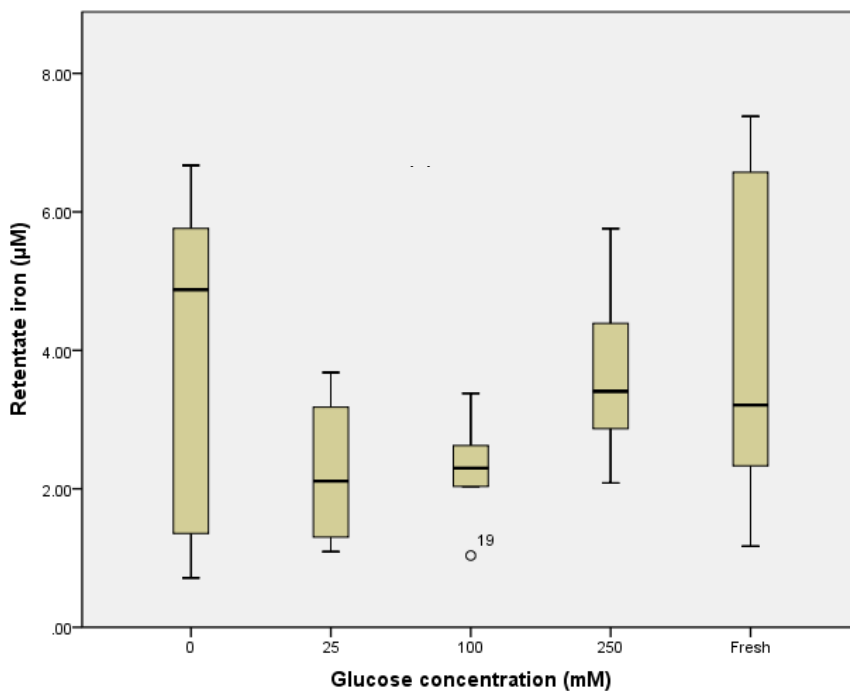


Figure 33. Retentate iron, 25 to 250 mM glucose incubation

Retentate iron following *in vitro* glycation of transferrin (0, 25, 100, and 250 mM glucose pre-incubation prior to buffer exchange into SPB) and transferrin freshly made-up in SPB, and ultrafiltration (n = 6, P = 0.083 between 0, 25, 100 and 250 mM).

2.5.2.2 Discussion, 25 to 250 mM Glucose incubation

When lyophilized human apo-transferrin is incubated with lower, physiological, glucose concentrations, glycation is seen to occur following the trend seen at higher glucose incubation concentrations (Figure 29). However, no significant differences are seen in apparent protein fragmentation or iron retention by the transferrin during ultrafiltration, with trends occurring in an opposite direction to that seen in higher glucose concentrations, therefore no effect on transferrin oxidative fragmentation or iron-binding ability is suggested at lower glucose concentrations.

2.6 Discussion

The aim of these experiments has been to investigate the effects of *in vitro* glycation of transferrin, and confirm or refute the role of protein fragmentation as a reason for loss of iron-binding capacity in this context. These results show support for the glycation-fragmentation theory; however a degree of oxidative damage may be occurring during the experiment itself cannot be ruled out.

Some discrepancy in the data exists between the filtration results and the iron-binding spectrophotometry findings, in the effect of the 14 day incubation in the absence of glucose. Most of the data suggests that these samples suffered some inhibition of function when compared to fresh transferrin, but in general maintained reasonable iron-binding capacity; however when iron-binding was measured by spectrophotometry at 470 nm, very little binding capacity in the sample incubated in the absence of glucose was found (Figure 10). This test

differed from the filtration studies in two ways. 1) An iron-nitrilotriacetic acid compound was used for iron-loading; however, this seems unlikely to account for the difference as the Fresh sample worked well. 2) Samples were frozen at -80°C for 12 days prior to testing by spectrophotometry, whereas samples for filtration were kept overnight on ice prior to use. This suggests that comparatively mild oxidative damage may occur to in the absence of glucose during the 14 day incubation which provokes more destructive structural damage during freezing and thawing.

Oxidative damage in the absence of glucose may have occurred during incubation from trace amounts of iron contaminating the SPB, known to be present in other common laboratory reagents and buffers (Halliwell & Gutteridge 2007). If iron contamination from SPB is occurring, passing the samples through Chelex 100 resin prior to the addition of glucose might have removed it. However, this was decided against because Chelex had not been used by the earlier groups, and the study sought to explain their findings. In fact Qian et al (1998) had not used it so that trace levels of iron might promote protein glycation during incubation. Microbial contamination during the Chelex process was also considered to be a risk.

Despite efforts to work aseptically, microbial contamination of the samples may have occurred. Low concentrations of sodium azide have been used to avoid this (e.g. Hunt et al 1993), however it was not used, as being a reducing agent sodium azide would disrupt the chemical balance of this experiment.

The reactions between glucose and protein, under the umbrella term of Maillard reactions, and including Schiff's base formation and Amadori rearrangements,

involve structural changes to protein and the development of fluorophores, similar to those seen in tissue in diabetes (Monnier et al 1984). However, these alone are not thought to be sufficient to cause structural damage such as fragmentation to the protein (Hunt et al 1993). The involvement of reactive oxidant species is required, and a number of mechanisms have been outlined which lead to the formation of the hydroxyl radical (OH·), capable of this degree of damage (Wolff & Dean 1986, Hunt et al 1993, Dean et al 1997):

(a) Glycooxidation – the transition metal-catalysed degradation of Amadori products. The two hydroxyl (OH) groups in the protein enediol (two OH group separated by a C=C double bond) are vulnerable to oxidation by available copper or iron, to form a protein dicarbonyl and H₂O₂. Further metal catalysed oxidation of H₂O₂ produces OH·

(b) Autoxidation – the “Wolff Pathway”, involving oxidation of free glucose in solution by transition metals, also producing H₂O₂, OH·, and reactive dicarbonyls, prior to attachment to protein.

As described in section 1.3, glucose in solution exists almost entirely in the pyranose form, with less than 0.01% in the reactive, open-chain aldehyde form. As the percentage of chain-form glucose is low, reactions such as amine glycation are slow. However, in neutral and alkaline conditions, following Schiff base formation, and prior to the Amadori reaction, cleavage of 2 and 3 carbon sugar moieties can occur. These are thought to contain reactive carbonyl species, as they dramatically increase Maillard browning (Hayashi & Namiki 1986). In the presence of iron these

would increase the availability of carbon compounds for autoxidation via the Wolff Pathway, glycation, and consequently glycooxidation and dicarbonyl occurrence.

Independently of glucose, for protein fragmentation to occur the presence of O₂ is required. In anaerobic conditions protein-crosslinking will occur (Dean et al 1997). Using human serum albumin (HSA) Wolff & Dean (1986) found that only OH· and O₂ was capable of cleaving protein directly, via development of carbonyl groups, although other radical species were capable of enhancing susceptibility to enzymatic cleavage. In the presence of Fe³⁺ or Cu²⁺, and H₂O₂, fragmentation could also occur, which could be inhibited by metal chelation.

In support of glycation fragmentation of protein, other authors have reported this finding. Islam et al (1995) found *in vitro* glycation of ceruloplasmin led to fragmentation, releasing its copper ion, which subsequently promoted OH· production via Fenton chemistry. Copper was identified following ultrafiltration through 5 kDa filters. The reaction could be inhibited with catalase and EDTA, implicating the involvement of H₂O₂ and copper. Earlier studies with Cu,Zn-superoxide dismutase had demonstrated a similar pattern, whereby initial cleaving into two fractions could be prevented by catalase, and subsequent random smaller fragmentations could be prevented by EDTA and catalase. EDTA could not prevent the initial fragmentation, which happened following glycation at one specific peptide bond (Ookawara et al 1992).

Hunt et al (1993), working with HSA, found that glycation was a prerequisite for oxidation and subsequent fragmentation *in vitro*, unglycated protein was resistant to fragmentation, and copper increased oxidation and fragmentation,

which could be inhibited by EDTA. Based on studies in the same report, they also proposed that glycation of LDL enhances copper-catalysed lipid peroxidation.

All these *in vitro* examples of glycation-fragmentation involve metal-catalysed oxidation. Apart from trace iron possibly contaminating the SPB used in the filtration studies reported in this thesis, iron was added 30 minutes prior to ultrafiltration. In retrospect this period could allow OH \cdot development and protein oxidation to occur, relative to degree of glycation, perhaps producing the reported results. On the other hand, the results for total iron binding capacity reported here, and in other studies, are lessened with glycation but have a much shorter iron incubation time of 5 minutes.

2.7 Conclusion

These studies demonstrate the fragmentation of glycated transferrin by protein oxidation and the potential for damage by the combination of glucose, iron, and oxidation. However, drawing direct comparisons between *in vitro* and *in vivo* conditions cannot be presumed, due to the high concentrations of glucose and redox available iron used, and the presence in plasma of many antioxidants.

Chapter 3. Mass spectrometry of glycated transferrin

“Nature, in conformity with her usual benevolence, has limited the power of iron.”

Pliny the Elder

(23 to 79 AD)

3.1 Aim

Using two mass spectrometry systems, to indicate potential differences between *in vitro* and *in vivo* glycated transferrin, in position and extent of glucose modifications which may be relevant to the protein fragmentation seen *in vitro* or other loss of function.

3.2 Background

3.2.1 Total iron binding capacity in type 2 diabetes

Impaired iron-binding by transferrin is evident in *in vitro* glycated protein, as shown in the previous chapter. *In vivo*, assessment of transferrin performance is problematic. Total iron binding capacity (TIBC) has been used by two diabetes studies (Van Campenhout et al 2006a, Ganesh et al 2011); however TIBC is not a specific measure of transferrin capacity, as described below. With the same subjects, Van Campenhout et al have also used iron-binding antioxidant capacity (IB-AC) (Van Campenhout et al 2006b), an assessment of antioxidant protection by plasma against lipid peroxidation, generated by iron and ascorbate, and measured by TBARS (Gutteridge & Quinlan 1992). The two studies found show slight non-significant reductions in TIBC in diabetes compared with healthy controls (Table

15). Ganesh et al (2011) carried out their study in India, and an attenuated iron status can be seen in both groups compared to the European study.

Table 15. Selected iron parameters in two diabetes studies

	Van Campenhout et al 2006a		Ganesh et al 2011	
	Controls (n = 91)	Diabetes (Type 1, n = 40; Type 2, n = 67)	Controls (n = 30)	Diabetes Type 2 (n = 30)
Age, years (range)	46 (26 - 77)	58 (19 - 84) ^{###1}	41.83 ± 6.70	43.57 ± 9.49 ^{NS}
M/F	54/37	54/53	12/18	11/19
HbA _{1c} (%)	5.3 ± 0.3	7.4 ± 1.1 ^{###1}	-	-
Type 1 diabetes		7.7 ± 0.8		
Type 2 diabetes		7.2 ± 1.2 [#]		
Fasting bd sugar (mM)	4.8 ± 0.5	8.9 ± 3.7 ^{###1}	6.05 ± 1.18	14.09 ± 1.33 ^{###1}
Type 1 diabetes		10.8 ± 4.1		
Type 2 diabetes		7.8 ± 3.0 ^{###2}		
Serum iron (µM)	19 ± 7	16 ± 6 [*]	13.0 ± 4.28	14.8 ± 4.67 ^{NS}
TIBC (µM)	68 ± 9	67 ± 11	69.37 ± 14.50	65.45 ± 15.00 ^{NS}
Type 1 diabetes		63 ± 9		
Type 2 diabetes		69 ± 12 [#]		
Transferrin (g/l)	2.65 ± 0.44	2.56 ± 0.43 ^{***}	1.33 ± 0.24	1.26 ± 0.30 ^{NS}
Type 1 diabetes		2.38 ± 0.44		
Type 2 diabetes		2.67 ± 0.38 ^{***}		
TIBC/g transferrin			-	-
Mean (µM/g/l)	25.7	26.2		
Type 1 diabetes		26.5		
Type 2 diabetes		25.8		
TSAT (%)	25 ± 15	27 ± 11 ^{NS}	20.87 ± 1.17	20.50 ± 0.77 ^{NS}
Tf glyc (µM fruc/g Tf)	0.79 ± 1.09	1.39 ± 1.12 ^{**}	-	-
Hs-CRP (mg/dl)	0.06	0.34 ^{**}	-	-
median (range)	(0.02 – 1.43)	(0.02 – 1.90)		
Type 1 diabetes		0.12 (0.02 – 1.78)		
Type 2 diabetes		0.41 (0.02 – 1.66) ^{##}		
Iron-binding antioxidant capacity (% inhibition relative to control with no plasma, ± SD)	88.3 ± 6	83.6 ± 7 [*]		
Type 1 diabetes		82.9 ± 7 [*]		
Type 2 diabetes		84.0 ± 5 [*]		

Results expressed as mean ± SD except where stated. TIBC: Total iron binding capacity; TSAT: Transferrin saturation; Tf glyc: Transferrin glycation (µM fructosamine/g transferrin); Hs-CRP: High sensitivity C-reactive protein.
^{*}denotes $P < 0.05$ compared with control, within gender; ^{**} $P < 0.005$ comparing diabetes to controls within gender; ^{***} $P = 0.001$ between diabetes (all types) and controls, and types 1 and 2 diabetes, within gender; [#] $P = 0.098$ between type, within gender; ^{##} $P < 0.05$ between types; ^{###1} $P < 0.0005$ between diabetes and controls, within gender; ^{###2} $P < 0.0005$ between types 1 and 2, within gender.

Van Campenhout et al found that TIBC differed significantly between type 1 and type 2 diabetes, 63 ± 9 and 69 ± 12 (μM) (mean \pm SD) respectively; as did transferrin concentration: 2.38 ± 0.44 and 2.67 ± 0.38 (g/l); and transferrin glycation: 1.95 ± 1.02 and 1.06 ± 1.04 (μM fruc/g Tf), respectively. No significant difference was seen in serum iron. Expressing their TIBC in μM per gram transferrin per litre finds that mean TIBC for type 1 and type 2 diabetes are very similar (type 1, $26.5 \mu\text{M/g/l}$; type 2, $25.8 \mu\text{M/g/l}$), showing that the lessened TIBC in diabetes type 1 can be explained by the presence of less transferrin. This is interesting because significantly more inflammation is seen in the type 2 group (Table 15), and as transferrin is a reverse acute-phase protein *less* transferrin might be expected. The authors speculate that oxidative damage might explain this loss, although they found no correlation between transferrin glycation and transferrin concentration in their study group. Oxidative damage independent of glycation is not considered. They also suggest transferrin loss via urine in diabetic nephropathy as a route, but no renal markers are reported.

Looking at diabetes (all types) versus controls, Van Campenhout et al found mean TIBC/g transferrin/l to be $26.2 \mu\text{M/g/l}$, compared to controls: $25.7 \mu\text{M/g/l}$, with slightly less transferrin being present in diabetes. As Hs-CRP was highly significantly increased in the diabetes group, this might account for the reduced concentration of transferrin seen overall, except that within group results or types 1 and 2 do not support this, as described above. As, *in vivo*, TIBC assessment involves the precipitation of all serum iron-binding proteins, including ferritin (and albumin), TIBC may be confounded by inflammation, although increased ferritin in inflammation is presumably more likely to be in the apo form, and albumin is a

reverse acute-phase protein. The authors used linear regression analysis to determine the main parameters affecting TIBC, and found that transferrin concentration determined 60% of the variance, with less than 5% determined by serum iron, but do not mention Hs-CRP.

Ganesh et al (2011) report a non-significant reduction in serum transferrin concentration in their type 2 diabetes group, however they calculate transferrin concentration by the simple method of multiplying TIBC by 0.7. Their results are low compared to normal (2.5 g/l Lentner 1984), and do not agree with their given method, and so cannot be relied upon.

The significant decrease in serum iron in Van Campenhout et al's (2006b) diabetes group compared with controls might also be explained by inflammation, being due to hepcidin up-regulation reducing dietary iron absorption and cell efflux. However it is interesting that the same relationship is not seen between the two Indian groups, who are known to have a population-level low iron status. In fact, the diabetes group have a higher serum iron level. Ganesh et al don't report HbA_{1c}, only fasting blood sugar: 6.05 ± 1.18 mM control, and 14.09 ± 1.33 mM for type 2 diabetes, and so presumably a similar degree of inflammation would exist. In iron-poor bodies less transferrin-bound plasma iron would result in less transferrin receptor-induced pro-hepcidin transcription. Perhaps this hepcidin regulation pathway may be more influential than the inflammation driven JAK/STAT pathway, as there would be no physiological need for inflammatory hepcidin up-regulation.

Despite the serum iron picture, TIBC loss is more evident in the diabetes group in Ganesh et al's study, though not significantly. The diabetes group have more serum

iron, and less TIBC, yet the TSAT for the two groups are very similar. This might be explained by inflammation reducing transferrin production, but as shown in Van Campenhout et al more hs-CRP in their type 2 group did not equate with less transferrin.

In summary, Van Campenhout et al's (2006b) results are interesting not for the lack of TIBC in diabetes, but the lack of transferrin per se, in diabetes compared with controls, and in type 1 diabetes compared with type 2. A number of other studies have measured serum transferrin levels and diabetes. Memisogullari & Bakan (2004) found, in controls (n = 21): 2.57 ± 0.30 g/l; type 2 diabetes without complications: 2.28 ± 0.32 g/l (n= 29, $P < 0.05$ compared with controls); type 2 diabetes with complications: 2.08 ± 0.40 g/l (n = 21, $P < 0.001$ compared with controls). Telci et al (2000) also found less iron in type 2 diabetes compared with controls, although both levels were unusually high: 3.85 ± 0.65 g/l (n = 84) versus 4.59 ± 0.79 g/l (n = 61, $P < 0.05$).

The reason for loss of serum transferrin in diabetes may well be renal filtration loss, as urinary transferrin excretion has consistently been shown to be increased in diabetes (O'Donnell et al 1991, Konen et al 1993, Narita et al 2004 & 2006, for example), and in fact is recommended as a gold standard marker of diabetic renal damage, more sensitive than urine albumin (Matheson et al 2010). Narita et al (2004) found, for example, mean transferrin excretion in 61 type 2 diabetes to be 110 ng/min (range 21 to 620), compared with 69 ng/min (20 to 230) in 17 controls. For the same subjects, albumin losses were: 3.9 μ g/min (0.5 to 9.6); and 2.9 μ g/min (1.1 to 8.2) respectively. As a more sensitive marker of renal damage, urine

transferrin can also indicate early glomerular damage before urine albumin (Bernard et al 1988). These results may be explained by difference in shape, as transferrin is elongated, 55 by 28 Å; and albumin is more spherical, 36 Å, therefore transferrin may pass more easily through the glomerular membrane pores (Howard et al 1991). This is supported by Narita et al (2004) who found good correlations between different urinary proteins with a radius of less than 55 Å, in diabetes patients with normal urine albumin, and no increase in the larger α_2 -macroglobulin (molecular radius 88 Å). The glomerular filter is thought to consist of two populations of pores, the majority of which have a radius of 29 to 31 Å, with a small number of 80 to 90 Å radii pores. Additional sporadic defects or “shunts” are suggested to occur in the glomerular capillary wall which can allow very large proteins or red blood cells through (Bakoush et al 2002).

Van Campenhout et al (2006b) assessed plasma iron-binding antioxidant capacity, finding a significant reduction in diabetes compared with controls, but not within diabetes types (Table 15). Regression analysis found that lessened transferrin, ceruloplasmin, and albumin did not fully account for this, and that their influence was lower in diabetes than in controls, explaining 4.2 and 6.3% of variance in type 1 and type 2 respectively, and 13.9% in controls. IB-AC improved with transferrin concentration in controls ($r = 0.3$, $n = 100$), but not with diabetes (type 1: $r = 0.13$, $n = 40$; type 2: $r = -0.07$, $n = 67$). Transferrin glycation did not account for this, and the authors suggest the presence of another, unidentified, factor associated with diabetes which either impaired iron binding or promoted iron-catalysed lipid peroxidation.

To summarise from the small number of studies which have included TIBC in diabetes, reduction of TIBC in diabetes appears to be linked to reduced levels of transferrin rather than loss of function. Although transferrin is a reverse acute-phase protein, this does not seem to be due to inflammation, but is linked to protein losses via renal damage. This being the case other proteins of a similar size or smaller may also be expected to be reduced, as has been reported by Narita et al (2004). The link with diabetes therefore is AGE-related basement membrane damage as described in Chapter 1, and so, as Narita et al (2006) found urinary transferrin, IgG, and ceruloplasmin to be equally able to predict microalbuminuria, we might assume transferrin losses in diabetes to be no different from losses of any other similar size protein.

Whether or not protein glycation and subsequent oxidative cleavage plays any part in increased renal protein loss is unknown, however as Van Campenhout et al (2006b) found no link between glycation and loss of plasma IB-AC *in vivo*, perhaps any indirect functional effects of transferrin glycation, such as from a (mechanistically undefined) risk for increased NTBI, may not be important in the plasma phase.

3.2.2 Glycated proteins *in vivo*

Efforts to identify and quantify glycated proteins *in vivo*, in people with diabetes, have been on-going for a number of decades, with methods based on affinity gel chromatography to separate glycated sites. Most early work centred on haemoglobin and the use of HbA_{1c}, however albumin has also long been recognised as a useful marker of blood glucose due to it representing 60% of total plasma

proteins, and having a shorter half-life, ranging from 12 to 19 days for glycated albumin (Yatscoff et al 1984, Roohk et al 2008), compared with 120 days for haemoglobin. Estimates for clinical levels of glycated albumin range from 0.6% to 8.6% for healthy people, and 1.4% to 16.59% in diabetes (Roohk et al 2008). Garlick & Mazur (1983) determined the major site of albumin glycation as being lysine-525.

Glycated transferrin *in vivo* has been observed and comparatively quantified, by Van Campenhout et al (2006a), Austen et al (1987, Table 16), and Jaleel et al (2005). Austen et al started with whole plasma, separated glycated from non-glycated protein using Glycogel B affinity columns, and then identified protein bands after electrophoresis. They found most glycation on gamma-globulins, albumin, beta-globulins, and complement C3. Transferrin was comparatively much less glycated (5.2% compared with 19.8% IgG). Jaleel et al used electrophoresis to separate proteins, and Western blotting to identify those proteins that reacted with Amadori antibody, followed by mass spectrometry to identify the protein, in eight type 2 diabetes patients. They identified 11 plasma proteins that showed a significant difference in glycation compared with controls, including albumin, fibrin, Ig heavy chain constant, transferrin, and Ig light chain. Intensive 10 day insulin treatment was able to significantly reduce this glycation. Band intensity for transferrin was ~40 arbitrary units (AU) in controls, compared with ~75 AU in diabetes, falling to ~53 AU after insulin. Other proteins identified as glycated and improved by insulin included hemopexin and vitamin D binding protein.

Table 16. Characteristics of glycated proteins identified by Austen et al (1987)

	Glycation (%)	Half-life (days)	Lysine mole (%)
Immunoglobulin G	19.8	24	7
Albumin	16	15 - 19	9.5
Complement C3	11.1	2 - 5	7.1
Fibrinogen	5.9	2.5	6.7
Transferrin	5.2	7	8.7
Haptoglobin	2.2	2	10.5
Alpha-1-antitrypsin	1.4	4	8.9

Study of *in vivo* glycation of other proteins has reported the following observations:

Affinity chromatography was used by Danze et al (1987) to observe a good correlation between glycated haemoglobin and glycated albumin, with a smaller correlation between glycated haemoglobin and glycated immunoglobulin G (IgG). The main site of IgG glycation was found to be the Fab fragment.

Calvo et al (1988) reported a 400% increase in glycated high density lipoprotein (HDL) in diabetes patients, and a positive correlation with blood glucose level. Whilst all the HDL apolipoproteins were glycated, Apo A-1 represented 80% of the total. They later showed, in rats, that glycated Apo A-1 had a diminished association with HDL, and that, *in vitro*, glycated Apo A-1 had a reduced capacity to activate lecithin:cholesterol acyltransferase, necessary for reverse cholesterol transportation to the liver (Calvo et al 1993). In further work on Apo A-1, Lapolla et al (2008) compared 10 people with diabetes, 10 with nephropathy but not diabetes, receiving peritoneal dialysis, and 10 healthy controls. Looking exclusively in the 30 kDa region following electrophoresis, they found glycated apolipoprotein A-

1 to be as prevalent as unglycated Apo A-1 in the diabetes and nephropathy patients, and that the protein was over-expressed in comparison to controls. The glycated peptide sequences indicated that the preferential site for glycation was the ϵ -amino group of lysine, and single and double glycations were seen repeatedly to three particular peptides. In the same 30 kDa region, retinol binding protein was also highlighted as increasingly glucose-modified in these subjects.

More recently the use of mass spectrometry has added depth to this literature, and in particular ion-trap Orbitrap devices (Thermo Fischer) have provided a more detailed analysis of the specific amino acids involved. Working with reconstituted whole human plasma, Priego-Capote et al (2010) used 24 hour, 37°C incubation with 30 mM isotopically labelled glucose, boronate affinity chromatography, and Orbitrap mass spectrometry to identify glycated proteins and lysine sites, and estimate glycation quantity. They looked at lysine glycation only, and found 35 different proteins to be glycated, with 113 sites in total, 35 of which were on albumin. Eleven lysine glycation sites were found on transferrin, seven on Apo A-1, and six on haptoglobin β -chain. Transferrin sites were Lys 252, 278, 315, 359, 384, 508, 515, 553, 564, 588, and 676 (the numbering includes the 19 residue signal peptide).

An extensive study of glycated peptides in diabetes and control plasma and erythrocytes was carried out by Zhang et al (2011), using plasma samples from 238 individuals with normal glucose tolerance, 97 individuals with impaired glucose tolerance, and 25 individuals with type 2 diabetes, with electron transfer dissociation, on an LQT-XL Orbitrap mass spectrometer. Again, lysine glycations

only were counted. Pooled whole diabetes plasma identified 799 glycated proteins, and a further 380 following immunodepletion of the top 12 most abundant proteins. 952 erythrocyte membrane proteins were glycated, and 853 from the cytosol. Ten glycated plasma and erythrocyte membrane proteins were found to be consistently up-regulated in diabetes, including clusterin, hemopexin, and spectrin alpha and beta chain.

3.2.3 Glycation sites on proteins

The occurrence of glycated proteins *in vivo* is primarily linked to half-life, as shown by Table 16. Glycation has been proposed to be a flag for protein identification by AGE receptors for proteolysis, however as mentioned earlier, glycated albumin half-life is unchanged (Johnson 1991).

The siting of Schiff and Amadori adducts within protein structures has been the subject of a great deal of study. In general terms, reaction sites need to be solvent-accessible, and not buried within the protein. Positively charged amino acids lysine and arginine are the least hydrophobic, and charged groups are almost never buried (Lesk 2003), therefore they will be more exposed. Schiff's bases commonly occur on the ϵ -amino group of lysine residues, and [within the guanidinium group of] arginine (Ahmed & Thornalley 2003), or on N-terminal α -amino groups of other amino acids. Reaction is dependent on their nucleophilicity (Watkins et al 1985), and arginine and lysine are both basic in nature at pH 7.4. Stable, post-Amadori, AGE products have also been found on histidine side-chains (which has a positively charged group within its side-chain) of sarco(endo)plasmic reticulum Ca^{2+} -ATPase (Bidasee et al 2004). Glycated haemoglobin HbA_{1c} is the product of a reaction on

the β -chain N-terminal valine (Acharyna & Sussman 1983). Valine residues were found to be more at risk of modification, and the presence of an adjacent acidic amino acid in the primary or tertiary structure is thought to be influential, (Watkins et al 1985, Johansen et al 2006).

A number of larger scale data analyses have described the environment needed for glycation to occur. Johansen et al (2006) found that for lysine glycation at neutral pH, acidic residue glutamic acid, and lysine, commonly occur C-terminally of the glycated lysine, whereas basic lysine, and neutral valine and leucine residues occur more frequently N-terminally. The influential region lay within 11 amino acids either side of the glycation. In disagreement with Acharyna & Sussman (1983), Johansen et al found no evidence for histidine involvement in promotion of lysine glycation, in the primary structure, however catalysis through three dimensional proximity could not be ruled out.

In the most comprehensive investigation to date Zhang et al (2011), catalogued the amino acid at every position 30 residues either side of the glycated lysine, along the primary sequence of peptides from 3742 human plasma and erythrocyte proteins. They found that position + 1 (C-terminally) alanine and valine were always in the top 4 occurring, for all sample types (Plasma/RBC/*in vivo*/*in vitro*). *In vivo*, hydrophobic, short chain or uncharged side chain amino acids such as alanine, valine, leucine, and serine occurred most frequently, with leucine, serine, and glutamic acid occurring most frequently *in vitro*. In agreement with Johansen et al (2006), histidine was not in evidence significantly in the primary sequence proximal to glycation. Also identified were lysine residues that became glycated *in vitro*, that

did not occur *in vivo*, for example the peptide corresponding to Lys500 in HSA, perhaps due to the protein unfolding during incubation. Zhang et al (2011) identified leucine, glutamic acid, and serine, as occurring most frequently N-terminally of glycation, from *in vitro* glycated human plasma proteins.

Both of these interesting studies are limited by consideration only of lysine glycation, and proximities within the primary sequence only, and there may be much more to learn by considering other glycated residues, and the three dimensional environment surrounding them. Also, it might be useful to take into account the rate of occurrence of a particular amino acid within that protein, as those that appear more frequently glycated may occur more frequently anyway, for example, human serum transferrin has 58 lysine compared to 26 arginine residues.

Gallet et al (2000) predicted which amino acid sequences within a protein were likely to be receptor-binding domains. Their predictions are based on plotting the mean alpha-helical hydrophobic moment against the mean hydrophobicity within a 5-residue window, for each amino acid along the protein chain (Eisenberg et al 1984). Likely interaction sites are highlighted by the plot, and are of relevance to glycation as it is the protein surface-seeking regions that are predicted. The 80,000 proteins in the SwissProt database were analysed, finding the most frequently occurring receptor-binding regions to be arginine (19.6%), lysine (12.5%), glutamic acid (8.9%), serine (7.0%), and aspartic acid (6.9%). When compared with gross rate of occurrence in the database, arginine was found to occur 3.8 times more frequently in a binding domain than would be suggested by random distribution.

Results for all residues, reported as times more frequently occurring than random distribution would suggest, were: arginine (3.8), lysine (2.1), glutamine (1.5), glutamic acid (1.4), aspartic acid (1.3), asparagine (1.3), histidine (1.2), serine (1.0), threonine (0.9), tyrosine (0.9), proline (0.8), cysteine (0.8), glycine (0.6), methionine (0.6), alanine (0.5), tryptophan (0.5), leucine (0.4), valine (0.3), phenylalanine (0.3), isoleucine (0.2). Based on this study, the investigation of glycation on arginine could be expected to provide a rich source of data.

3.2.4 Summary of Background and Aims

In summary, glycated transferrin *in vitro* is clearly damaged by glucose incubation in a concentration-dependent manner, and the lack of iron-binding capacity in diabetes may be explainable by protein losses via the kidney, due to AGE-related tissue damage. It is not known whether the glycation state, or oxidation, of the transferrin plays any role in this. Mass spectrometry and other methods have been able to describe much about protein glycation generally, but very little has been done specifically on human serum transferrin. In an attempt to compare, for the first time, *in vitro* with *in vivo* glycated transferrin, outsourced mass spectrometry was undertaken. Work was very generously carried-out by the laboratories of Waters UK (Manchester), and The University of Bristol Proteomics Facility. Although only single samples in each of three cases are analysed (one healthy control, one type 2 diabetes, one *in vitro* glycated transferrin sample), it is hoped that they might give an indication of where glycation is occurring, how the siting of glycation adducts may differ between *in vitro* and *in vivo glycation*, whether these

differences could account for the damage seen *in vitro*, and whether or not further studies would be justified.

3.3 Experimental methods (in brief)

3.3.1 *In vivo* samples

Following consent, and local and regional ethical approvals, blood samples were given by one healthy adult male, and one adult male with type 2 diabetes (HbA_{1c} 6.2%). Whole undepleted serum was lyophilized and briefly stored at -80°C prior to dilution to 1 µg/µl. 100 µl of each sample was reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. Single 2 µl injections of 1.56 µg protein were used, in a Waters NanoAQCUIITY UPLC plus SYNAPT G2 mass spectrometer. Peptide identification was by BiopharmaLynx software, with a minimum fragment ion match per peptide of 3, minimum fragment ion match per protein of 7; minimum peptide match per protein of 1. Work was carried out by Dr Joanne Connolly, and Dr Gushinder Atwal at Waters UK, Manchester, in July 2010.

3.3.2 *In vitro* sample

Human apo-transferrin (Sigma Aldrich, 98% purity) was incubated in 100 mM glucose solution in sodium phosphate buffer, pH 7.4, at 37° C, for 15 days. Following buffer exchange, protein was assessed for glycation by picrylsulphonic acid assay of remaining NH₂ groups, measured against standard solutions of L-lysine. Fresh, 0 mM, and 100 mM incubations had consecutively 336, 305, and 191 µM/mg/ml lysine equivalence. The lyophilized sample digest was treated as above. A single injection of 1 µg protein in 2 µl of solution was used, on a Thermo Scientific LTQ Orbitrap Velos mass spectrometer. Modified amino acid and peptide

identification was by Proteome Discoverer software. Glycation on Cys, Lys, Asn, Arg, Thr, Trp, Tyr, and the N-terminus were allowed as variable modifications. Separate searches were done for oxidation on Met, Cys, Asp, Phe, Lys, His, Trp, Asn, Arg, Tyr, & C-terminal Gly. High and medium confidence scores were counted. Work was carried out by Dr Kate Heesom, at The University of Bristol Proteomics Facility, in July 2011.

3.4 Results

3.4.1 Results *in vivo*

The Waters mass spectrometer identifies glycated peptides only, not individual residues. In the healthy control 249 transferrin peptides in total were identified (Table 18), giving 96% protein coverage, average length 11.2 amino acids (SD 4.6). Of these, 87 peptides (34.9%) had a single glycation, average length 10.6 (SD 3.8), 32 (36.8%) from the N-lobe, 55 (63.4%) from the C-lobe (Table 17).

Table 17. Rate of glycation per lobe, all samples

	<i>In vivo</i>		<i>In vitro</i> (100 mM Glucose)		
	Control	Diabetes	Total	Top 4 [#]	Less top 4
Peptide count	249	261	1136	-	-
Glycated peptide count (% of total for type)	87 (34.9)	103 (39.5)	240 (21.1)	92 (8.10)	148 (13.03)
N-Lobe glycation count (% of glycated total) (Less top 4, % glyc total)	32 (36.8) -	32 (31.1) -	144 (60) -	92 (38.3) -	52 (21.7) (35.2)
C-Lobe glycation count (% of glycated total) (Less top 4, % glyc total)	55 (63.4) -	71 (68.9) -	96 (40) -	-	96 (40) (64.8)

[#] N-lobe peptides associated with Met309, Lys312, Tyr314, and Tyr317

Table 19. Regulated proteins, Waters

Unique and regulated proteins by Waters mass spectrometry, Control and Type 2 diabetes				
Control unique		Type 2 diabetes unique		
Accession	Description	Unique	Accession	Description
P01623	Ig kappa chain V III region WOL OS Homo sapiens PE 1 SV 1	Control	P04206	Ig kappa chain V III region GOL OS Homo sapiens PE 1 SV 1
P01717	Ig lambda chain V IV region HiI OS Homo sapiens PE 1 SV 1	Control		
P04208	Ig lambda chain V I region WAH OS Homo sapiens PE 1 SV 1	Control		
Down-regulated Diabetes to Control		Up-regulated Diabetes to Control		
Accession	Description	D:C_Ratio	Accession	Description
P01861	Ig gamma 4 chain C region OS Homo sapiens GN IGHG4 PE 1 SV 1	0.14	P01777	Ig heavy chain V III region TEI OS Homo sapiens PE 1 SV 1
P01860	Ig gamma 3 chain C region OS Homo sapiens GN IGHG3 PE 1 SV 2	0.18	P02749	Beta 2 glycoprotein 1 OS Homo sapiens GN APOH PE 1 SV 3
P02763	Alpha 1 acid glycoprotein 1 OS Homo sapiens GN ORM1 PE 1 SV 1	0.38	P00738	Haptoglobin OS Homo sapiens GN HP PE 1 SV 1
P01766	Ig heavy chain V III region BRO OS Homo sapiens PE 1 SV 1	0.43	P00739	Haptoglobin related protein OS Homo sapiens GN HPR PE 1 SV 2
P01764	Ig heavy chain V III region VH26 OS Homo sapiens PE 1 SV 1	0.44	P20742	Pregnancy zone protein OS Homo sapiens GN PZP PE 1 SV 4
P01621	Ig kappa chain V III region NG9 Fragment OS Homo sapiens PE 1 SV 1	0.45	POC0L4	Complement C4 A OS Homo sapiens GN C4A PE 1 SV 1
P01876	Ig alpha 1 chain C region OS Homo sapiens GN IGHA1 PE 1 SV 2	0.47	P27169	Serum paraoxonase arylesterase 1 OS Homo sapiens GN PON1 PE 1 SV 2
P01591	Immunoglobulin J chain OS Homo sapiens GN IGJ PE 1 SV 4	0.48	P00751	Complement factor B OS Homo sapiens GN CFB PE 1 SV 2
P01617	Ig kappa chain V II region TEW OS Homo sapiens PE 1 SV 1	0.49	P00734	Prothrombin OS Homo sapiens GN F2 PE 1 SV 2
P01620	Ig kappa chain V III region SIE OS Homo sapiens PE 1 SV 1	0.50	P01023	Alpha 2 macroglobulin OS Homo sapiens GN A2M PE 1 SV 2
P06396	Gelsolin OS Homo sapiens GN GSN PE 1 SV 1	0.53	P68871	Hemoglobin subunit beta OS Homo sapiens GN HBB PE 1 SV 2
P01857	Ig gamma 1 chain C region OS Homo sapiens GN IGHG1 PE 1 SV 1	0.53		
POC0L5	Complement C4 B OS Homo sapiens GN C4B PE 1 SV 1	0.53		
P04220	Ig mu heavy chain disease protein OS Homo sapiens PE 1 SV 1	0.54		
P01842	Ig lambda chain C regions OS Homo sapiens GN IGLC1 PE 1 SV 1	0.54		
P01834	Ig kappa chain C region OS Homo sapiens GN IGKC PE 1 SV 1	0.55		
P02649	Apolipoprotein E OS Homo sapiens GN APOE PE 1 SV 1	0.59		
P01877	Ig alpha 2 chain C region OS Homo sapiens GN IGHA2 PE 1 SV 3	0.64		
P02655	Apolipoprotein C II OS Homo sapiens GN APOC2 PE 1 SV 1	0.69		
P01871	Ig mu chain C region OS Homo sapiens GN IGHM PE 1 SV 3	0.72		
P05090	Apolipoprotein D OS Homo sapiens GN APOD PE 1 SV 1	0.73		
P01019	Angiotensinogen OS Homo sapiens GN AGT PE 1 SV 1	0.74		
O43866	CD5 antigen like OS Homo sapiens GN CD5L PE 1 SV 1	0.76		
P02656	Apolipoprotein C III OS Homo sapiens GN APOC3 PE 1 SV 1	0.76		
P02747	Complement C1q subcomponent subunit C OS Homo sapiens GN C1QC PE 1 SV 3	0.78		
Q96PD5	N acetylmuramoyl L alanine amidase OS Homo sapiens GN PGLYRP2 PE 1 SV 1	0.79		
P04217	Alpha 1B glycoprotein OS Homo sapiens GN A1BG PE 1 SV 3	0.79		

In the type 2 diabetes sample 261 peptides were identified, with 100% protein coverage, average length 12.7 amino acids (SD 6.3). 103 (39.5%) of these were glycosylated, average length 12.9 (SD 6.3), 32 (31.1%) from the N-lobe, 71 (68.9%) from the C-lobe. Areas of highest modification were peptides Cys19 to Lys27, Ser415 to Lys433, Cys523 to Lys527, and Asn546 to Lys599 (Table 18). No double glycosylations were reported in either case.

The total number of proteins confidently identified in the control and diabetes samples were 143 and 124, respectively. 117 proteins were identified in at least 2 out of 3 repeated sample injections, 3 of these were unique to the control, 1 protein was unique to the diabetes subject. All 4 unique proteins were regions of immunoglobulin light chain kappa and lambda. Expression differed in 38 proteins, 11 of these were up-regulated in diabetes versus control, 27 were down-regulated in diabetes versus control. 75 proteins were unregulated (Table 19).

3.4.2 Results *in vitro*

In the *in vitro* glycosylated sample, 1136 transferrin peptides were identified (Table 20), average length 15.8 amino acids, of which 240 (21.1%) had a single glycosylation, 60% from the N-lobe, and 40% from the C-lobe (Table 17). 38.3% of the glucose modifications were to four amino acids (Met309, Lys312, Tyr314, and Tyr317, referred to as “the top 4”), sited close to the C-terminal end of the N-lobe in an area free of glycosylation in the human serum samples. No doubly glycosylated peptides were reported. 823 oxidized residues were found, 49.3% of which were methionine (Table 23). The most frequently occurring oxidized residue was Met389 (99), followed by Met382 (94), which between them made-up 23.5% of the oxidations

(Table 21). Forty seven peptides had both glycation and oxidation simultaneously, 66% of these from two α -helices near the C-terminal end of the N-lobe, Met309 to Arg324. Other peptides with simultaneous glycation and oxidation are: Ile366 to Lys401, Lys103 to Arg113, Leu497 to Arg522 (Table 20).

Twenty amino acids were prone to both glycation and oxidation non-simultaneously, of particular note are Met313, Tyr314, Tyr317, and Asn383 (Table 22). Two of the top 4 glycated residues were also subject to non-simultaneous oxidation (Tyr314 and Tyr317), with adjacent Met313 also being prone to oxidation.

Table 20. *In vitro* modified peptides, Orbitrap

All modified peptides, by Orbitrap							
Start	End	Glycated peptide	Count	Start	End	Oxidized peptide	Count
24	41	dHMKSVIPSDGSPVAcVK	17	8	18	WcAVSEhEATK	13
42	50	KASyLDcIR	3	51	88	AIAANEADAVTL DAGLVYDAYLAPNNLkPVVAEFYGSK	6
51	88	AIAANEADAVTL DAGLVYDAYLAPNNLkPVVAEFYGSK	7	89	102	EDpQTFYAVAVVK	5
103	113	KDSGFQmNQLR	6	103	113	KDSGFQmNQLR	5
197	217	DGAGDVAFVkhSTIFENLANK	1	104	113	DSGFQmNQLR	47
207	217	HStIFENLANK	3	125	143	SAGWnIPIGLLYcDLPEPR	14
221	232	dQYELLCdDNTR	2	197	206	DGAGDVAFVK	1
233	254	kPVDEYKdChLAQVPSHTVVAR	7	200	206	GDVAFVK	2
240	254	dChLAQVPSHTVVAR	1	207	217	HSTIFENLANK	6
255	276	sMGGKEDLIWELLNQAQEHFGK	2	221	232	dQYELLCdDNTR	1
279	291	sKEFQLFSSPHGK	2	233	254	KPVDEYKdChLAQVPSHTVVAR	2
309	324	MDAKMyLGIEYVTAIR	91	240	254	dChLAQVPSHTVVAR	2
313	324	mYLGIEYVTAIR	2	255	276	SMGGKEDLIWELLNQAQEHFGK	18
355	380	cDEwSVNSVGKIEcVSAETTEDcIAK	8	281	291	EfQLFSSPHGK	2
366	380	iEcVSAETTEDcIAK	2	313	324	mYLGIEYVTAIR	16
366	401	iEcVSAETtEDcIAKImNGEADAMSLDGGFVYIAGK	10	344	352	WcALShhER	7
381	401	iMNGEADAMSLDGGFVYIAGK	3	353	365	LKcDEwSVNSVGK	6
434	445	kSASDLTWDNLK	6	355	365	cDEwSVNSVGK	1
435	445	sASDLTWDNLK	1	366	380	iEcVSAETTEDcIAK	1
457	470	tAGWNIPmGLLYNK	7	381	401	iMNGEADAMSLDGGFVYIAGK	126
476	489	fDEFFSEGCAPGSK	1	415	433	SDNcEDTpEAGYFAVAVVK	8
491	511	dSSLcKlCmGSGLNLcEPNNK	15	434	445	KASDLTWDnLK	7
497	511	lCmGSGLNLcEPNNK	1	435	445	SASDLTWDNLK	16
497	522	lCmGSGLNLcEPNNKEGYYGYTGAFR	6	435	447	SASDLTWDNLkGK	8
512	522	eGYGYTGAFR	2	457	470	TAGWNIPmGLLYNK	35
535	545	hQTVPQNTGGK	2	476	489	FdEFFSEGCAPGSK	26
553	568	nLNEKDYELLCdDGTR	18	497	511	LcmGSGLNLcEPNNK	9
569	581	KPVEEYANchLAR	1	497	522	LcMGSGLNLcEPNNKEGYYGYTGAFR	7
664	677	kcSTSSLLLEAcTFR	12	512	522	EGYYGYTGAFR	5
665	677	cSTSSLLLEAcTFR	1	535	545	hQTVPQNTGGK	1
				546	552	NPDpWAK	3
				553	568	NLNEKDYELLCdDGTR	1
				558	568	DYELLCdGTR	1
				569	581	KPVEEYANchLAR	6
				582	590	APNhAVVTR	7
			240				421

Table 21. *In vitro* modified amino acids by highest frequency, Orbitrap

Modified amino acids by highest frequency																	
Glyc AA	#	Count	Glyc AA	#	Count	Oxi AA	#	Count	Oxi AA	#	Count	Oxi AA	#	Count	Oxi AA	#	Count
Tyr	314	26	Ile	381	2	Met	389	99	Phe	211	6	Asp	416	2	His	349	1
Met	309	25	Leu	497	2	Met	382	94	Trp	344	6	Lys	447	2	His	350	1
Lys	312	24	Asn	504	2	Met	109	90	Phe	476	6	Cys	498	2	Cys	368	1
Tyr	317	17	Glu	512	2	Met	464	43	Asp	477	6	Asn	504	2	Cys	418	1
Lys	664	12	His	535	2	Met	499	35	Asp	261	5	Tyr	515	2	Asp	420	1
Asn	553	10	Lys	557	2	Asp	387	33	Pro	422	5	Pro	549	2	Pro	486	1
Asp	24	9	Tyr	45	1	Asn	383	31	Tyr	426	5	Trp	550	2	Tyr	514	1
Lys	27	8	Asn	75	1	Met	256	24	Phe	427	5	Tyr	574	2	Tyr	517	1
Asp	491	8	Asn	76	1	Asn	110	23	Asn	461	5	Arg	581	2	Phe	521	1
Lys	233	7	Lys	78	1	Met	313	21	Trp	264	4	His	14	1	Arg	522	1
Lys	496	7	Lys	206	1	Asp	392	21	Asp	438	4	Lys	18	1	His	535	1
Lys	103	6	Thr	209	1	Phe	107	15	Asn	584	4	Tyr	68	1	Tyr	559	1
Lys	434	6	Cys	240	1	Asn	443	15	His	585	4	Tyr	71	1	Phe	563	1
Thr	457	6	Ser	255	1	Pro	463	13	Tyr	95	3	Pro	74	1	Asp	565	1
Asn	383	5	Lys	259	1	Trp	441	12	Phe	204	3	Asn	75	1	Lys	569	1
Asn	555	5	Ser	279	1	Trp	8	11	Asp	356	3	Asn	76	1	Pro	570	1
Lys	380	3	Lys	280	1	Lys	445	11	Asn	576	3	Lys	78	1	His	578	1
Lys	42	2	Met	313	1	Lys	259	8	Pro	583	3	Pro	79	1			
Tyr	85	2	Thr	373	1	Phe	395	8	Tyr	85	2	Phe	84	1			
Lys	88	2	Asn	435	1	Trp	128	7	Phe	94	2	Lys	88	1			
His	207	2	Trp	460	1	Asn	129	7	Tyr	96	2	Pro	91	1			
Asp	221	2	Phe	476	1	Tyr	314	7	His	207	2	Tyr	199	1			
Cys	355	2	Asn	509	1	Trp	358	7	Tyr	238	2	Asn	213	1			
Trp	358	2	Asn	510	1	Asp	442	7	Asp	240	2	Asp	221	1			
Asn	361	2	Lys	511	1	Trp	460	7	His	242	2	Lys	239	1			
Lys	365	2	Tyr	559	1	Phe	479	7	Tyr	317	2	Phe	282	1			
Ile	366	2	Lys	569	1	Phe	480	7	Tyr	397	2	Phe	285	1			
Thr	374	2	Cys	665	1	Pro	131	6	Lys	401	2	Lys	291	1			

Table 22. Non-simultaneous modification of *in vitro* glycosylated transferrin

Glyc & Oxi modified AA			
Frequency per 1136 peptides			
	#	Glyc	Oxi
Asn	75	1	1
Asn	76	1	1
Lys	78	1	1
Tyr	85	2	1
Lys	88	2	2
His	207	2	2
Asp	221	2	1
Cys	240	1	2
Lys	259	1	8
Met	313	1	21
Tyr	314	26	7
Tyr	317	17	2
Trp	358	2	7
Asn	383	5	31
Trp	460	1	7
Phe	476	1	6
Asn	504	2	2
His	535	2	1
Tyr	559	1	1
Lys	569	1	1

Table 23. *In vitro* glycated and oxidized amino acids

Modified AA by type			
Glyc AA	%	Oxi AA	%
Lys	36.3	Met	49.3
Tyr	19.6	Asn	11.3
Asn	12.1	Asp	10.4
Met	10.8	Phe	7.8
Asp	7.9	Trp	6.7
Thr	4.2	Tyr	4.3
Cys	1.7	Pro	4.1
His	1.7	Lys	3.5
Ile	1.7	His	1.6
Trp	1.3	Cys	0.5
Glu	0.8	Arg	0.4
Leu	0.8		
Ser	0.8		
Phe	0.4		

Table 24. Amino acid glycation by frequency per native frequency in native transferrin

Amino acid	Glyc count	Native count	Glyc %	Native %	Ratio G/N
Met	26	9	10.8	1.3	8.2
Tyr	47	26	19.6	3.8	5.1
Lys	87	58	36.3	8.5	4.2
Asn	29	34	12.1	5.0	2.4
Asp	19	45	7.9	6.6	1.2
Trp	3	8	1.3	1.2	1.1
Thr	10	30	4.2	4.4	0.9
Ile	4	16	1.7	2.4	0.7
His	4	19	1.7	2.8	0.6
Cys	4	38	1.7	5.6	0.3
Ser	2	41	0.8	6.0	0.1
Glu	2	42	0.8	6.2	0.1
Phe	1	28	0.4	4.1	0.1
Leu	2	59	0.8	8.7	0.1
Ala	–	57	–	8.4	–
Gly	–	50	–	7.4	–
Val	–	44	–	6.5	–
Pro	–	32	–	4.7	–
Arg	–	26	–	3.8	–
Gln	–	17	–	2.5	–
	240	679	100.0	100.0	

Table 25. *In vitro* glycated amino acid type less top 4, frequency per frequency in native transferrin

Amino acid	Glyc count	Native count	Glyc %	Native %	Ratio G/N
Lys	63	58	42.6	8.5	5.0
Asn	29	34	19.6	5.0	3.9
Asp	19	45	12.8	6.6	1.9
Trp	3	8	2.0	1.2	1.7
Thr	10	30	6.8	4.4	1.5
Ile	4	16	2.7	2.4	1.1
His	4	19	2.7	2.8	1.0
Tyr	4	26	2.7	3.8	0.7
Met	1	9	0.7	1.3	0.5
Cys	4	38	2.7	5.6	0.5
Ser	2	41	1.4	6.0	0.2
Glu	2	42	1.4	6.2	0.2
Phe	1	28	0.7	4.1	0.2
Leu	2	59	1.4	8.7	0.2
Ala	–	57	–	8.4	–
Gly	–	50	–	7.4	–
Val	–	44	–	6.5	–
Pro	–	32	–	4.7	–
Arg	–	26	–	3.8	–
Gln	–	17	–	2.5	–
	148	679	100.0	100.0	

Table 26. Frequency of amino acid type occurring -1 position N-terminally, and +1 position C-terminally to glycosylated residue

Less 1		Glycosylated residue															Plus 1		Glycosylated residue															
N-terminally		Cys	Asp	Glu	Phe	His	Ile	Lys	Leu	Met	Asn	Ser	Thr	Trp	Tyr	Tot	%	C-terminally	Cys	Asp	Glu	Phe	His	Ile	Lys	Leu	Met	Asn	Ser	Thr	Trp	Tyr	Tot	%
Lys		2	2	1		2	2	3	1	1	1	2				17	30.4	Glu						1	4			1		1		2	9	16.1
Arg			2		1			3		1		1	1			9	16.1	Ser	1	1			1	2		1			1		1	7	12.5	
Gly								2						1	1	4	7.1	Leu						1		3					2	6	10.7	
Ser								2					1		1	4	7.1	Asp	1			1		2		1						5	8.9	
Glu							1						1	1		3	5.4	Ala						1			1	1				3	5.4	
Leu								1			2					3	5.4	Cys		1				1	1							3	5.4	
Met								1			1				1	3	5.4	Gly			1						1				1	3	5.4	
Asn								1			2					3	5.4	Ile						2					1			3	5.4	
Ala								2								2	3.6	Met						1	1			1				3	5.4	
Pro											2					2	3.6	Asn									2				1	3	5.4	
Val								1			1					2	3.6	Pro							3							3	5.4	
Cys								1								1	1.8	His		1				1								2	3.6	
Asp															1	1	1.8	Lys									1	1				2	3.6	
Phe															1	1	1.8	Gln		1			1									2	3.6	
Thr													1			1	1.8	Thr												1		1	1.8	
Tyr																		Tyr								1						1	1.8	
Ile																		Val																
Gln																		Phe																
His																		Arg																
Total		2	4	1	1	2	2	18	1	2	9	3	4	2	5	56	100	Total	2	4	1	1	2	2	18	1	2	9	3	4	2	5	56	100

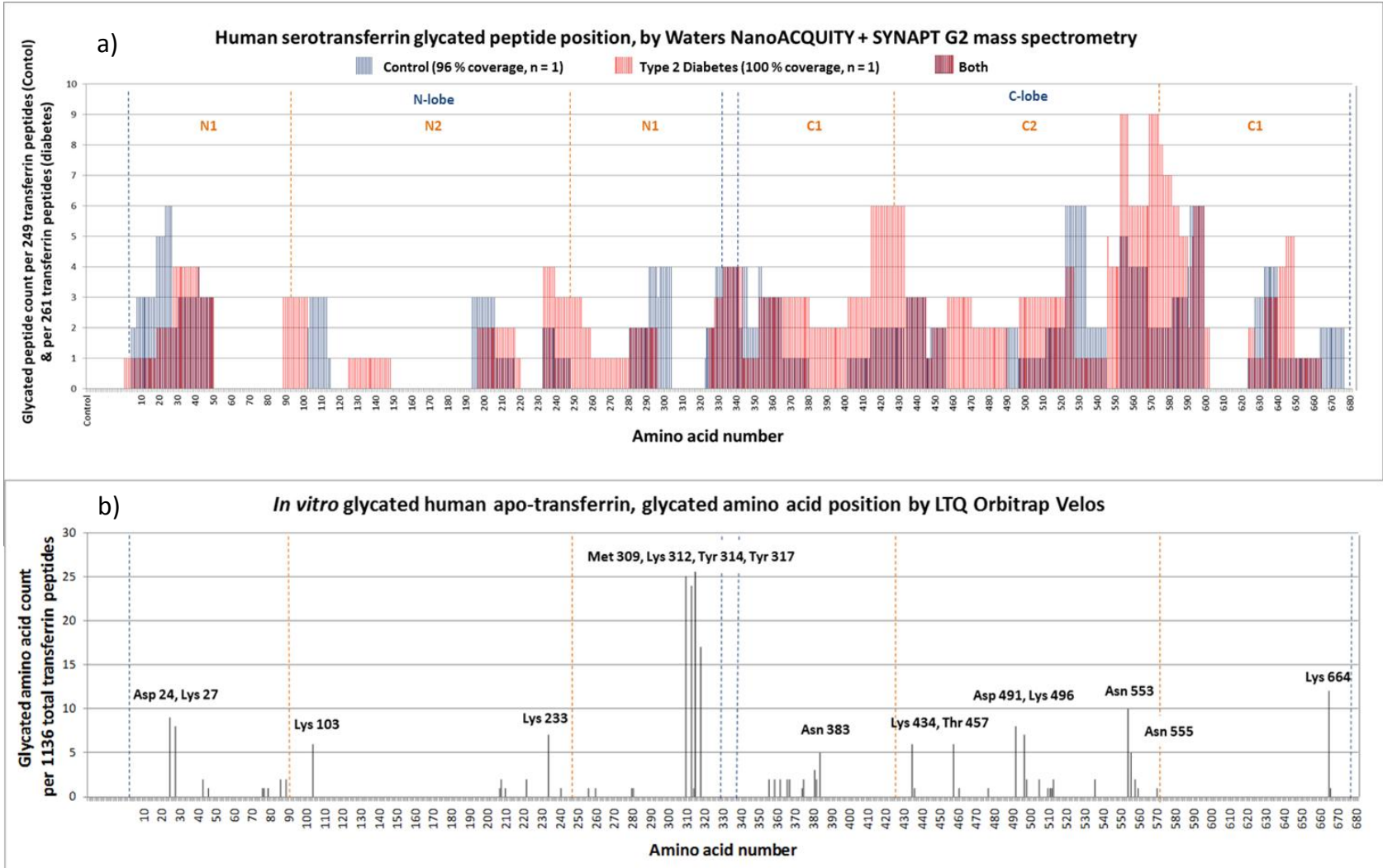


Figure 34. Human serum transferrin glycation by mass spectrometry

- a) Human serum transferrin glycated peptides, by Waters NanoACQUITY mass spectrometry
- b) *In vitro* glycated human serum transferrin, glycated amino acids, by Thermo Orbitrap Velos mass spectrometry.

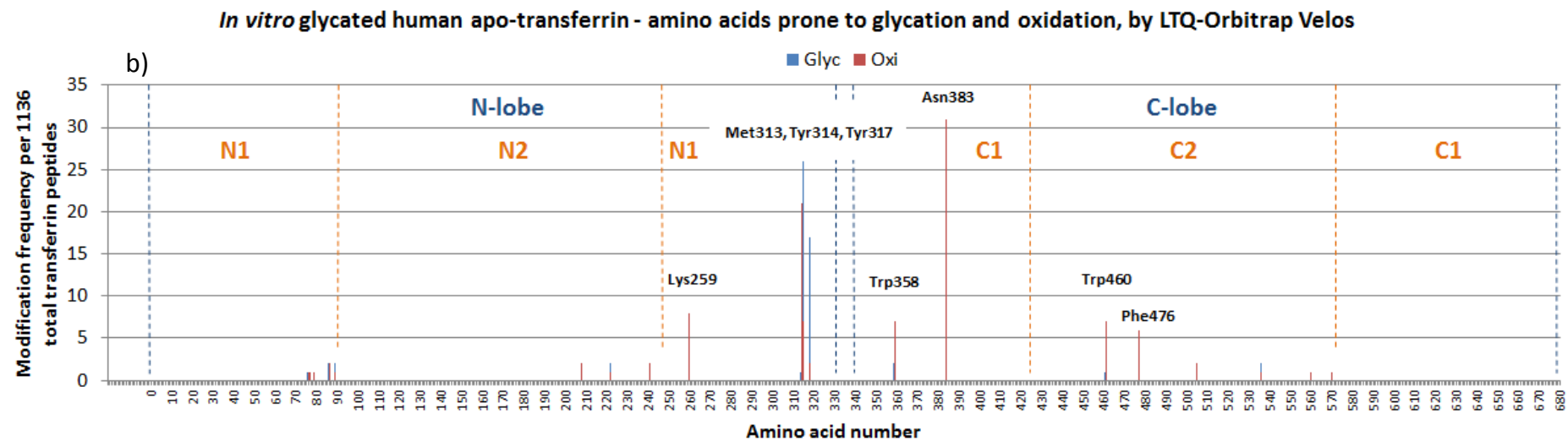
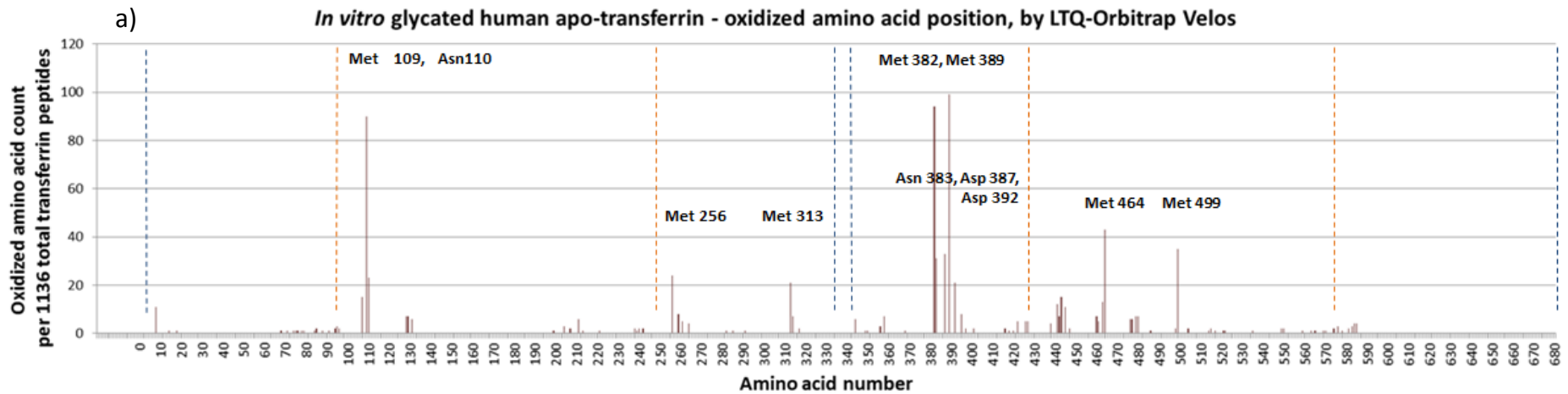


Figure 35. Human serum transferrin, amino acid glycation (a), and oxidation (b), following *in vitro* glycation, by Orbitrap mass spectrometry

3.5 Discussion

The aim of this study was to indicate potential differences between *in vitro* and *in vivo* glycosylated transferrin, in position and extent of glucose modifications which may be relevant to the protein fragmentation seen *in vitro* or other loss of function. Due to the small number of samples, their assessment on different equipment, and the lack of a control sample for the *in vitro* sample, suggestions only can be inferred from these results.

The complete human serotransferrin amino acid sequence was first described by MacGillivray et al (1982), and the sequence used here is that of UniProtKB/Swiss-Prot version 160, a free access, reviewed, compilation of sequence identification data. The human serotransferrin page was last modified in November 2010. The first 19 UniProt amino acids comprise the signal peptide and, in agreement with the majority of authors, have been subtracted from the UniProtKB sequence to give the residue numbers reported here. The three dimensional structure of human serum transferrin is shown in Figure 36, the secondary structure is shown in Figure 37, and the position of amino acids found to be glycosylated following *in vitro* glycosylation, is shown in Table 27, superimposed onto primary and secondary structures, with functional sites indicated.

3.5.1 Glycation and glycosylation

Human serum transferrin is made up of a 679 amino acid sequence, arranged into two structurally similar lobes (designated N and C), with a short linker peptide between them. Within each lobe the protein is sub-divided into two domains, between which lies a binding site for one iron atom (Figure 36).

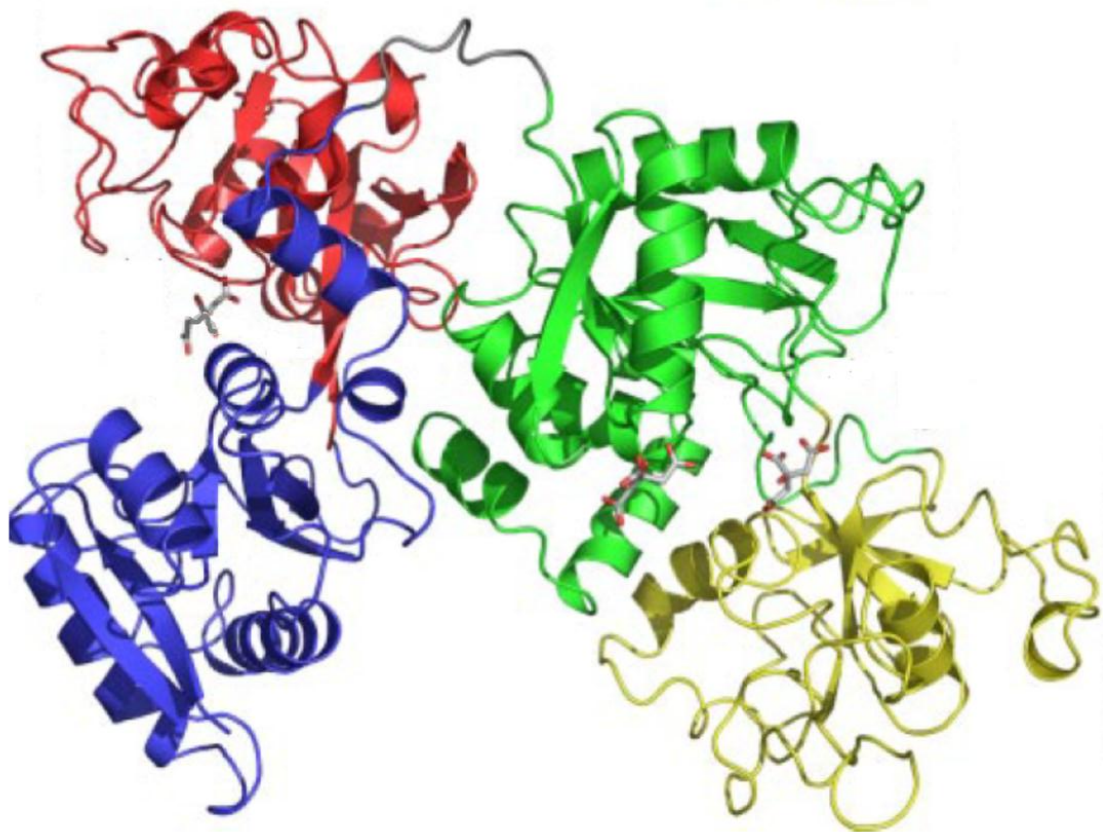


Figure 36. The crystal structure of human apo-transferrin

Showing subdomain N1 in blue, N2 in red, C1 in green, and C2 in yellow. The linker peptide is shown in grey. This figure was originally published in the *Journal of Biological Chemistry*. Wally, J. Halbrooks, P. Vornheim, C. et al. The crystal structure of iron-free human serum transferrin provides insight into inter-lobe communication and receptor binding. *Journal of Biological Chemistry*. 2006; Vol. 281: 24934 – 24944 © the American Society for Biochemistry and Molecular Biology. Reproduced and adapted with permission from the publishers.

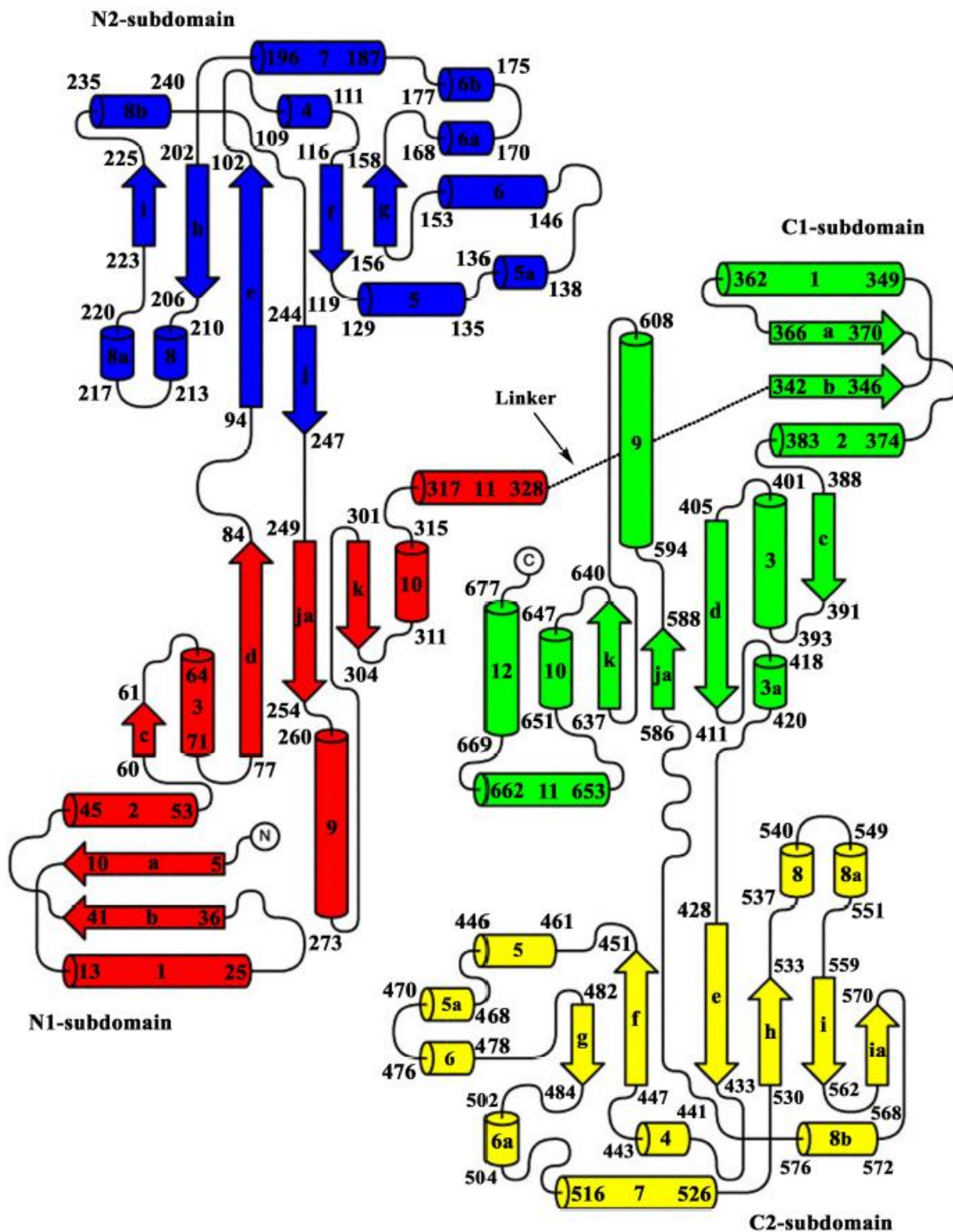


Figure 37. Topology map of the secondary structure of apo-human transferrin
 Showing subdomains, coloured N1 – red, N2 – blue, C1 – green, and C2 – yellow. This figure was originally published in the Journal of Biological Chemistry. Wally, J. Halbrooks, P. Vonrhein, C. et al. The crystal structure of iron-free human serum transferrin provides insight into inter-lobe communication and receptor binding. Journal of Biological Chemistry. 2006; Vol. 281: 24934 – 24944 © the American Society for Biochemistry and Molecular Biology. Reproduced and adapted with permission from the publishers.

The Waters' *in vivo* data showed a clear preference for glycation of the C-lobe over the N-lobe (Table 17), which is also seen in the positioning of the two N-linked carbohydrate glycosylation moieties in human serum transferrin, which both occur within the C-lobe, at Asn413 and Asn611. Around 50% of all proteins in nature are believed to be glycoproteins (Apweiler et al 1999), and have either one or more serine or threonine linked O-glycans, and/or one or more asparagine linked N-glycans. Glycosylation takes place enzymatically, in the Golgi apparatus for O-linked glycans, and in the endoplasmic reticulum for N-linked glycans. O-linked glycans are thought to play a role in antibody recognition, structural rigidity, and as ligands for carbohydrate bonding proteins such as selectins and adhesins (Hounsell et al 1996). N-glycans help to facilitate protein production operations such as folding, sorting and transport, and tagging for enzyme recognition (Helenius & Aebi 2001). N-glycans occur as part of the amino acid sequence N-X-S/T, where X is any residue apart from proline (Apweiler et al 1999). It is interesting that this sequence has been conserved twice within the C-lobe of transferrin, and that the C-lobe is also preferred for non-enzymatic glycation. As, in extracellular proteins, only about two thirds of potential N-glycosylation sites are utilised as such (Apweiler et al 1999), other influences must be involved, which may be relevant to non-enzymatic glycation.

Efforts to determine the factors influencing residue preference within and adjacent to the N-X-S/T sequon, for N-glycans in archaeal protein, have found that simple properties, such as size or charge, did not appear to be explanatory (Igura & Kohda 2010). However, N-glycosylation did occur more frequently within an extended protein conformation, where fully extended β -strands have successive residue side-

chains projecting straight up, and then straight down from the protein back bone in a horizontal plane. This has also been noted in eukaryotic proteins, and in 60% of the 350 glycoprotein chains characterised within the Structural Assessment of Glycosylation Sites (SAGS) database (Petrescu et al 2006). Igura & Kohda (2010) suggest that the extended conformation allows a more efficient scanning by the glycan-catalysing enzyme oligosaccharyltransferase, coupled with the transient interaction of the required sequon with the active site of the enzyme, favouring glycan synthesis.

Work on N-glycan positioning of 21 sites on one particular eubacterial protein (*C. jejuni*, Kowarik et al 2006) has also identified the requirement for aspartic acid or glutamic acid to be present at the -2 position N-terminally, of the glycosylated asparagine. Igura & Kohda (2010) propose that this suggests a turn in the secondary protein structure that brings the hydroxyl group of serine or threonine closer to the asparagine carboxamide group, to increase the nucleophilicity of the asparagine. This occurs post-translationally to the folded protein, and does not involve enzyme scanning, and is not influenced by an extended protein conformation.

To draw relevance to non-enzymatic glycation positioning on transferrin, the requirement for the presence of glutamic acid or aspartic acid is familiar, occurring at 25% of the +1 position residues in the Orbitrap data (Table 26); although the position differs, and the reaction is enzymatic. As the pro-glycosylation effect of an extended, beta, conformation is effected within the endoplasmic reticulum, it is unlikely to have any influence on non-enzymatic glycation, and indeed there were

fewer glycation on beta strands (14) than on helices (17), with 8 on turns (Table 27.).

3.5.2 Glycation frequency by residue

The Orbitrap data shows that the most frequently glycosylated residues *in vitro* are lysine (36.3% of glycosylated total), tyrosine (19.6%), asparagine (12.1%), and methionine (10.8%) (Table 23). When the top 4 residues Met309, Lys312, Tyr314, and Tyr317 are excluded the most frequently glycosylated residues are lysine (42.6%), asparagine (19.6%), aspartic acid (12.8%), and threonine (6.8%) (Table 25). Amino acid glycation frequency per frequency of amino acid occurrence in the native protein finds methionine, tyrosine, lysine, and asparagine the highest, in descending order (Table 24). Excluding the top 4 finds lysine, asparagine, aspartic acid, and tryptophan have the highest ratio (Table 25). Chetyrkin et al (2007) also found tryptophan to be vulnerable to glycation and oxidation within *in vitro* glycosylated lysozyme, and Halliwell & Gutteridge (2007) have highlighted the aromatic ring of tryptophan as particularly susceptible to oxidation by a range of free radicals.

Compared to the top residues predicted by hydrophobicity (arginine, lysine, glutamine, and aspartic acid, Gallet et al 2000), it is surprising that no arginine residues were found to be glycosylated, although they were included in the search during mass spectrometry. Arginine is often cited as being a key glycation residue (Ahmed & Thornalley 2003, Sharma & Santhoshkumar 2009 for example). This might be explained by the observation that arginine occurs less frequently in transferrin compared with lysine, representing 3.8% of residues (the same as

tyrosine), whereas lysine is the second most common (after leucine), representing 8.5% of residues (Table 24). However, tyrosine was frequently glycosylated despite its low occurrence rate, therefore other factors must influence this. Tyrosine, in common with tryptophan, has an aromatic ring, and is also vulnerable to oxidation (Halliwell & Gutteridge 2007) therefore may have a similar susceptibility to glycation.

In some agreement with Priego-Capote (2010), of the 11 transferrin lysine glycation sites they identified (corresponding to Lys 233, 259, 296, 340, 365, 489, 496, 534, 545, 569, and 657), the Orbitrap analysis found lysine glycosylations at: 233, 259, 365, 496, and 569, all of which corresponded to glycosylated peptides in the Waters analysis. Priego-Capote et al only performed a light incubation (30 mM glucose for 24 hours at 37°C), however a heavier degree of lysine glycosylation is seen compared to the 100mM, 14 day incubation used for the Orbitrap study. Priego-Capote et al used 50 µl reconstituted plasma added to 500 µl phosphate buffer, with 30 mM glucose. A very similar protein concentration was used, as total plasma protein is 60-80 g/l, therefore 3.5 mg/50 µl, diluted 3.5 mg/550 µl, = ~ 1.93 mg/ml, compared with 2mg/ml buffer transferrin concentration used prior to Orbitrap analysis. This is suggestive of either: a pro-glycation influence by other plasma components, glycation reversal during the longer incubation, or increase or loss of glycation during processing prior to MS, in one or other (or both) of the samples. The Orbitrap findings may show lysine residues more vulnerable to glycation than others, and at higher glucose concentration (or lower protein concentration) the rest of the lysines identified by Priego-Capote et al may subsequently become glycosylated.

3.5.3 The influence of neighbouring residues

In consideration of the nature of the neighbouring amino acids, the Orbitrap data found, in descending order, glutamic acid, serine, aspartic acid, and leucine to be the top 4 residues occurring +1 position C-terminally of all glycosylated residue types (Table 26). For glycosylated lysine only, glutamic acid was the most common, followed by proline, then serine, aspartic acid and isoleucine equally. This is not in agreement with Zhang et al (2011), who found that the most likely residues to occur +1 position C-terminally of the glycosylated lysine, *in vitro*, are leucine, serine, and glutamic acid. It is in some agreement with Johansen et al (2006), who identified the occurrence of glutamic acid and lysine to be common here. Analysis of the Waters' *in vivo* data found that the amino acid most likely to occur +1 position C-terminally of the trypsin-cleaved lysine following protein digest was aspartic acid, representing 17.8% of residues, followed by serine, lysine, and cysteine. The carboxylic acid group in acidic side-chains is thought to participate in the reaction (Watkins et al 1985). Presumably the combination of a negative charge and a functional hydroxyl group may be destabilising for the area, but the glycosylation occurs on the neighbouring lysine side-chain amine, which has a lower bond energy of $\leq 338.9 \text{ kJ/mol}^{-1}$ (Luo & Kerr 2005), and a positive charge. Serine also has a hydroxyl group, but has a neutral charge, and leucine has two methyl groups, which are relatively unreactive.

The Orbitrap data found one position N-terminally of the glycosylated residue *in vitro*, lysine, to be most frequent (30.4%), followed by arginine, glycine, and serine (Table 26), whereas Zhang et al (2011) identified leucine, glutamic acid, and serine here. Again, the Orbitrap data agrees more with Johansen et al (2006), who found lysine,

valine, and leucine most frequent. There can be seen from the Orbitrap data a general tendency for glycation to favour sites that have a negatively charged residue C-terminally, and a positively charged residue N-terminally, however, other factors are also involved and the influences on glycation are evidently complex.

3.5.4 Comparison between *in vivo* and *in vitro* glycated transferrin

As different protocols and devices were used for the mass spectrometry, only general comparisons can be made between *in vivo* and *in vitro* results. The most striking difference between the two is the heavy glycation *in vitro* of Met309, Lys312, Tyr314, and Tyr317, near to the C-terminal end of the N-lobe, a region which has no glycation *in vivo* (Figure 34). The other, less notable, difference is Cys665 at the carboxyl end of the C-lobe where there appears to be a higher rate of glycation *in vitro*. In general, a broad agreement is seen. Surprisingly, less glycation has occurred overall *in vitro* with 100 mM glucose incubation, suggesting that apart from the glycation position differing, 14 day incubation with approximately 200 mM glucose may provide a degree of glycation similar to that seen *in vivo*. Alternatively, glucose concentration of 100 mM *in vitro* may lead to formation of glycation adducts larger than those measured by MS, and the glycated residues measured may represent only a portion of the total.

Residues Met309, Lys312, Tyr314, and Tyr317 are sited on an α -helix, close to the inter-lobe contact Arg308, which links to Asp376 via a salt-bridge (Table 27, Figure 38). The other inter-lobe contact is between Asp240 and Arg678, and Asp240 was found to have a low level of glycation *in vitro*, and peptide glycation of both cases *in vivo*. As salt-bridges occur as hydrogen bonds between the positively charged

primary amine on the side-chain of lysine or arginine, and the negatively charged carboxylate on the side-chains of glutamic acid or aspartic acid (Dougherty 2006), glycation to this particular aspartic acid may follow the breaking of this bond, which has a weaker bond strength than the adjacent carbonyl double-bond in the side-chain.

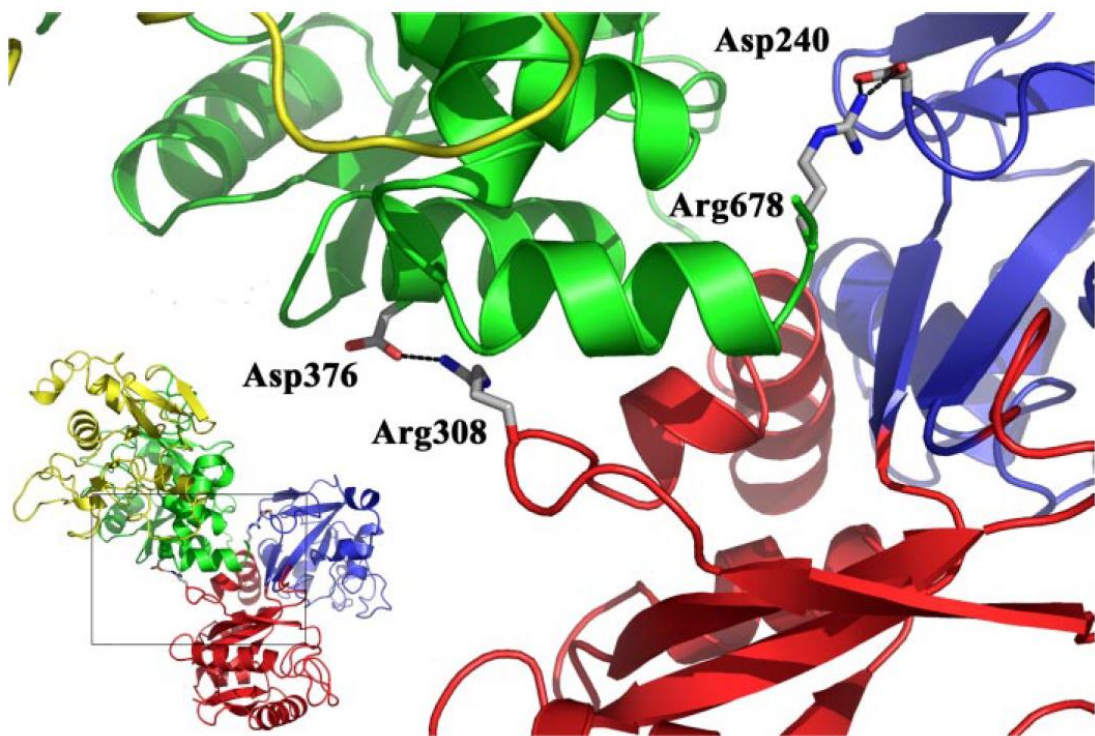


Figure 38. The interface between the two lobes of apo-transferrin

Showing Arg308 in the N1-subdomain to Asp376 in the C1-subdomain, and Asp240 in the N2-subdomain to Arg678 in the C1-subdomain. This figure was originally published in the Journal of Biological Chemistry. Wally, J. Halbrooks, P. Vonrhein, C. et al. The crystal structure of iron-free human serum transferrin provides insight into inter-lobe communication and receptor binding. Journal of Biological Chemistry. 2006; Vol. 281: 24934 – 24944 © the American Society for Biochemistry and Molecular Biology. Reproduced and adapted with permission from the publishers.

To identify reasons why the top 4 (Met309 to Tyr317) residues are heavily glycosylated *in vitro*, but not at all *in vivo*, one difference between the samples is that the *in vitro* protein is entirely in the apo-form, and therefore open in structure, allowing

solvent accessibility to parts of the protein that might otherwise be protected. The difference is not clear-cut however, as transferrin *in vivo* exists in four forms, in the following approximate proportions: apotransferrin (44.5%); monoferric transferrin (44.5%); holotransferrin (11.1%) (in Van Campenhout et al 2004). The N-lobe is preferred for iron-binding by human serotransferrin *in vivo*, in blood taken from the arm (Williams & Moreton 1980). Given the strength of glycation of the top 4 *in vitro*, and the concentration of apotransferrin occurring *in vivo*, it would seem unlikely that none of the *in vivo* peptides would be glycated at these sites, suggesting that structural changes due to iron loading is not the cause. Moreover, these residues lie within the central region of transferrin (Figure 36), not between the two sub-domains which close around and sequester the iron atom, and therefore the iron status of the protein should not affect solvent accessibility of the Met309 to Tyr317 region.

3.5.5 Glycation of functional sites – iron binding

The N- and C-lobes of transferrin open and close via a hinge connecting sub-domains 1 and 2, which closes with a twisting action around the iron atom, and which is co-ordinated by an aspartic acid, two tyrosine residues, and a histidine. The iron is bound by two oxygen atoms from a carbonate anion, which is stabilised by an arginine and a threonine (Lambert et al 2005). Wally et al (2006) demonstrated that that in the apo-form the two lobes are equally open, and therefore differences in glycation position between the open and closed forms might be expected to occur within this iron-binding pocket. In fact, none of the eight iron-binding residues are glycated, despite tyrosine and aspartic acid being

prone to glycation in this experiment. In the C-lobe, one anion-binding residue is glycosylated (Thr457), as is Trp460, which lies adjacent to another proposed anion-binding ligand in that group. Plotting the 3-dimensional structure may identify other glycosylated proteins sited within these clefts. No other glycosylations are seen proximal to binding residues. In the human serum samples glycosylated peptides are seen in the Thr457 region in both subjects, and in the Trp460 region in the diabetes sample, and as no other glycation was seen *in vitro* for 20 residues N-terminally, or 15 residues C-terminally, it may be suggested that glycation to these important residues might be occurring *in vivo*. If that were the case, glycosylation of Thr457 would prevent carbonate anion-binding, and the provision of the two oxygen atoms required for iron-binding within the C-lobe. Failure of iron-binding within the C-lobe may have a greater physiological effect than failure within the N-lobe, as a number of studies have indicated that C-lobe interaction with cell membrane transferrin receptors is stronger than that of the N-lobe, and that, in the monoferric state, N-lobe iron-loaded transferrin may not bind to the transferrin receptor as effectively (Young et al 1984; Zak et al 1994, and Mason et al 1997, in Crichton 2009). Cheng et al (2004) describe C-lobe-receptor binding within the C1 domain only, leaving C2 free to open and release the iron atom once internalised into the cell. In contrast, the N-lobe binds to the transferrin receptor at two sites, requiring the N1 and N2 domains, which is suggested to constrict lobe opening and iron release.

3.5.6 Receptor binding

The human transferrin receptor is an 80 kDa transmembrane bilobed glycoprotein, capable of binding two mono- or diferric-transferrins. The complex is endocytosed, and the iron is released from the transferrin at acidic intracellular pH 5-6, probably by protonation of the carbonate anion and opening of the interlobe cleft. The apo-transferrin remains attached to the receptor and is returned to the cell surface, where the extracellular pH of 7.4 causes the transferrin to disassociate from the receptor, and return to circulation. This is estimated to be repeated more than 100 times within the life of the transferrin (in Crichton 2009).

Consideration of glycation within the transferrin-receptor binding regions finds that two of the residues proposed as such are glycated, Lys365, and Ile366, in the C-lobe (Wally et al 2006). This peptide region is also glycated in both the *in vivo* samples, although alternative candidates for glycation are present. It would be interesting to repeat the Orbitrap procedure with human samples to establish the presence of glycation on proposed receptor binding residues as this would have implications for iron delivery to cells, if Lys365 and Ile366 are proven to be essential for protein docking. To speculate further, iron-loaded transferrin unable to discharge its load (and not lost via the kidney) would ultimately be taken up by hepatocytes, or macrophages, potentially leading to iron deposition in the liver, or within atherosclerotic plaques. However, Dineen et al (1994) found no difference in liver iron in post-mortem examination of 15 non-insulin-dependent diabetes sufferers, compared with 17 age-matched controls.

Two cysteines involved in disulphide bonds were glycated, Cys355 and Cys665. Glycation on these cysteine side-chains may involve breaking the disulphide bonds

within the protein. Both of these are within the C-lobe, Cys355 is bound to Cys368, and Cys665 to Cys474. Cys368 is also a proposed receptor binding residue (Wally et al 2006), but in any case, loss of either bridge would be a significant structural impairment. Both Cys355 and Cys368 lie within glycosylated peptide regions in both *in vivo* samples.

3.5.7 *In vitro* oxidation

Oxidation is a common artefact of mass spectrometry, particularly to methionine, histidine, and tryptophan, and not surprisingly 49.3% of the 823 oxidized residues were methionine (Table 23). Met389 and Met382 alone made-up 23.5% of the oxidations, and were part of a heavily oxidized region between Met382 and Asp392 which included glycosylation at Asn383 (Figure 33). Two of the top 4 glycosylated residues were also subject to non-simultaneous oxidation (Tyr314 and Tyr317), with adjacent Met313 also prone to oxidation. It is a limitation of this study that without an unglycosylated control sample the degree of oxidation due to glycosylation cannot be determined, however, methionine oxidation *in vivo* is repairable by disulphide and methionine sulphoxide (MeSOX) reductases. Methionine and cysteine are particularly vulnerable to oxidation to the sulphur atom *in vivo*, however they are also the only proteins for which oxidation modifications can be repaired (Berlett & Stadtman 1997). Methionine has been suggested to operate as an ROS scavenging system, to protect irreparable proteins, based on the little apparent effect of oxidation on biological function (Levine et al 1996). Therefore, *in vivo*, unless the MeSOX antioxidant system fails or is overwhelmed, methionine (and cysteine) oxidation cannot be expected to contribute to protein damage by oxidation,

irrespective of whether or not the oxidation seen *in vitro* is a consequence of MS. The inclusion of a control sample might have determined whether these high levels of methionine and cysteine oxidation are linked to glycation.

3.5.8 Comparison between Control and Diabetes – *in vivo*

The Waters MS analysis identified 87 glycations in the Control sample, with an average peptide length, for all peptides, of 11.2 amino acids, and 10.6 for glycated peptides. The average length of all peptides for the diabetes sample was 12.7 amino acids, average length for glycated peptides only was 12.9 (data not shown). The diabetes sample peptides may be longer on average than the Controls', because glycated lysine is known to be resistant to trypsin cleavage, and therefore fewer cleavage sites are available.

Many glycated peptides were common to both cases, particularly at the N-terminal end of the N-lobe, and within the C-lobe, corresponding to Asp24 and Lys27, and Asn553 and 555 in the *in vitro* sample. Consequently, these are regions that may be commonly glycated *in vivo* (Figure 33).

It is difficult to quantitatively compare the degree of glycation found in the two human samples with that reported *in vivo* in the literature, due to the differences in methodologies and units of measurement used. Van Campenhout et al (2006) reported μM fructosamine per gram transferrin (Table 15). Austen et al (1987) reported percent glycated (whole) protein (Table 16), finding 5.2% glycated (and therefore presumably 94.8% unglycated) in diabetes. Measuring glycated peptides as a result of MS does not account for how many glycated peptides there may be per protein, never-the-less, the rate of 39.5% of all peptides being glycated for the

Waters diabetes sample, and 34.9% for the control (Table 17), seems to be very high, and accords with the surprising finding of less glycation being present in the *in vitro* sample. The presence of two N-glycans does not explain this, as these modifications are larger than a glycation (in the oligosaccharide structure the first N-acetylglucosamine moiety alone has a molecular weight of 221.21 g/mol, larger than glucose, 180.16 g/mol), and would be identified by MS. In addition, no modification is reported in one of the glycan regions, Asn611. No obvious explanation for the high degree of glycation *in vivo* is apparent, and without being fully involved in the experimental procedure it is difficult to speculate on the cause. Analysis of a number of human samples through an Orbitrap device would provide useful and detailed information about this.

3.6 Conclusion

Due to the small number of samples, their assessment on different equipment, and the lack of a control sample for the *in vitro* sample, suggestions only can be inferred from these results.

Using Orbitrap MS, a surprisingly large range of amino acids were found to be vulnerable to glycation *in vitro*, that has not been seen by other groups who have only searched for lysine glycation. Orbitrap MS should be used to determine whether a similar range is occurring *in vivo*, and if any of the functional sites found to be glycated in this single experiment *in vitro* also occur *in vivo*.

In vitro transferrin glycation experiments such as this one, and those by other groups, may not produce results that are relevant *in vivo*, as 38.3% of all glycations found *in vitro* here were to four residues, all unglycated *in vivo*, which were also

associated with amino acid oxidations. Consequently they provide a likely region for protein fragmentation *in vitro* not seen *in vivo*, and suggest an explanation for the loss of *in vitro* transferrin function seen by this and other groups, which would have no equivalent *in vivo*. The loss of TIBC *in vivo* reported by Van Campenhout et al (2006a) can be explained by renal transferrin loss. Close to half of the oxidation sites observed were to methionine, and therefore potentially MS artefacts, or if not, likely to be repaired by MeSOX *in vivo*. If the later, they also represent modifications seen *in vitro* only, and will contribute to the protein oxidation and fragmentation found in this study and the studies of others.

Chapter 4. Non-transferrin-bound iron in type 2 diabetes

“For the life of the flesh is in the blood.”

Leviticus 17:11

4.1 Aim

To evaluate non-transferrin-bound iron in people with type 2 diabetes and controls, and to assess its association with haemoglobin glycation, lipid peroxidation, antioxidant status, and other iron parameters. To compare NTBI in people with and without diabetes, and with and without cardiovascular and renal disease.

4.2 Background

The potential for iron to be involved in advanced glycation end product development, and the known effects of glycation on some iron binding proteins *in vitro*, have led to the investigation of reactive plasma iron in people with type 2 diabetes by several groups. Very little work has been done specifically on the links between NTBI, diabetes, and CVD, or renal disease, but much of the work done on iron and CVD, and iron and chronic kidney disease (CKD) has relevance. The effects of IV iron supplementation in CKD provide an additional view from an extreme vantage point.

As discussed in Chapters 1 and 2, glycation of iron proteins *in vitro* alters their ability to interact with iron. For transferrin, *in vivo*, the effects of glycation may be far less catastrophic (Chapter 3), and perhaps this is also the case for other glycosylated proteins *in vivo*. Haemoglobin glycation *in vitro* weakens the haem-globin structure (Sen et al 2005), and plasma and sub-endothelial free glycosylated haemoglobin may

represent a form of NTBI in diabetes. NTBI may correlate with HbA_{1c}, as was found to be the case by Shetty et al (2008) ($r = 0.436$, $P < 0.01$), demonstrating this association, however Lee et al (2006) found an inverse correlation between the two ($r = -0.28$, $P = 0.06$).

4.2.1 Cardiovascular disease and non-transferrin-bound iron

Speculation has long been made that iron may be linked to cardiovascular risk in the general population, perhaps contributing to the reduced risk seen in females prior to menopause (Sullivan 1992). Extension of the theory to explain the increased risk in diabetes has quickly been proposed, in the half-light of the serum ferritin-iron status data (reviewed in Chapter 1).

A review of the literature regarding iron status and cardiovascular risk, finds little agreement between studies. A table of relevant studies has been compiled (Table 28), outlining the nature of the study, assessment method for iron status, outcome measures, and findings.

Table 28. Compilation of the literature regarding iron status and CVD risk - key points

Author, year, and study	Iron measures	Outcome measures	Results and Conclusion	
Salonen et al 1992 n=1,931 M, 42 to 60 yrs, 3 yr f-up	sFt, Hb. No control for inflammation	Acute myocardial infarct	sFt > 200 µ/l gave a 2.2 fold risk of acute MI. Hb had no significant effect on risk when ferritin was controlled for	↑
Sempos et al 1992 (NHANES II) n=1,604 M & F, < 16 yr f-up	sFt. No control for inflammation	Mortality from all causes, CVD, CHD, and MI	Significant increased risk for African-American men sFt < 50 µg/l. No other associations found	↑ ↔
Ascherio et al 1994 (HPFS) n=44,933 M, 40 to 75 yrs, 4 yr f-up	Dietary iron intake (total and haem)	Coronary events	No significant association total, non-haem, or haem iron and CHD. Risk for MI increased non-significantly in quintile 5 compared to 1. Highly significant risk for MI in quintile 5 if not taking vitamin E or multi-vitamin supplement	↔ ↑
Corti et al 1997 Review of 11 studies	Range of serum measures and dietary iron intake	Association between iron status or intake, and CVD	Higher iron states do not appear to be strongly associated with increased risk of CVD. The evidence is inconsistent; larger studies find an inverse relationship, with lower iron levels linked to adverse CVD outcomes.	↔
Danesh & Appleby 1999 Meta-analysis of 12 studies, n=7,800 CHD cases	sFt (n=5), TSAT (n=5), TIBC (n=3), serum iron (n=3), dietary iron intake (n=3)	CHD	No good evidence was found for existence of strong associations between iron status and CHD	↔
Sarnak et al 2002 n=14,410 M & F, 6.1 yr f- up	Hb: anaemia Y/N	CVD event	Anaemia was significantly associated with risk for CVD	↑

Menke et al 2009 n=2,662 M & F, (n=219 with PAD)	sFt, TSAT, CRP	OR for peripheral arterial disease (PAD)	Non-significant positive trends were seen between ferritin, and TSAT, and PAD. OR were calculated against tertile 1 however, therefore possible negative influences of lowest tertiles may confound these results	
Zacharski et al 2007 (FeAST) n=1,277 M & F with PAD, 6 yr f-up. Phlebotomy (n=636), control (n=641)	sFt: controls 122.5 ± 87.2 µg/l ± SD; intervention 58.3 ± 31.3 µg/l ± SD.	All-cause mortality; death plus non-fatal MI and stroke	No significant decrease seen with decreased sFt	↔
Ahluwalia et al 2010 n=124 age sex matched pairs, +/-two or more carotid atherosclerotic plaques	sFt, serum iron, sTfR, TSAT, Hb. AGP, hsCRP	OR for two or more plaques	When inflammation was controlled for by CRP, ferritin had a small non-significant influence on OR for plaques. When controlled by AGP, ferritin had a significant influence on OR for plaques (p = 0.03). There were no significant influences shown by the other iron parameters	↔
Menke et al 2011 (NHANES III) n=12,258 M & F, 18yr f-up	sFt, TSAT, CRP	All cause, CVD, or cancer mortality	sFt and TSAT were not associated with risk for all-cause mortality	↔
Casiglia et al 2011 (abstract) n=906 F, 10 yr f-up	Dietary iron intake	Cardio- and cerebrovascular events	Low iron intake independently predicted cerebrovascular and coronary morbidity and mortality. Haem iron intake was not a predictor	↑
Morkdal et al 2011 (abstract) n=28,154 M, 32,644 F, without MI or stroke. 11.4 yr f-up	TSAT, TIBC, serum iron	RR for ischaemic heart disease mortality	RR was higher in quartile 1 for TSAT, and serum iron, compared to quartile 4 for M&F, and for TIBC in F. In M, TIBC quartile 1 was slightly protective compared to quartile 4. Low iron status may be a late sign of IHD pathology	↑

Relevant study				
DePalma et al 2010, (FeAST) (sFt: 25 and 60 µg/l, n=51), control (n=49)	sFt, statins, IL-6, hsCRP	Mortality	Positive correlations are found between sFt and IL-6 (r = 0.185; P = 0.002), and hsCRP (r = .118; P = 0 .04). Statin use reduced ferritin levels independently of phlebotomy, indicating an effect of these agents on iron metabolism	

Key: ↑: support for high iron status and increased CVD risk; ↔ : No support for high iron status and increased CVD risk; ↓ : support for low iron status and increased CVD risk; AGP: α-1 acid glycoprotein; CRP/hsCRP: (high sensitivity) C-reactive protein; F: females; FeAST: Iron and Atherosclerosis Study; Hb: haemoglobin; HPFS: Health Professionals Follow-up Study; M: Males; NHANES II: National Health and Nutrition Examination Survey; OR: odds ratio; PAD: peripheral arterial disease; RR: relative risk; sFt: serum ferritin; TIBC: total iron binding capacity; TSAT: transferrin saturation.

Table 29. Results of a meta-analysis of upper and lower threshold haemoglobin concentrations (with 95% CIs), and excess population risk of mortality attributable to low or high haemoglobin levels, in two patient groups (Shah et al 2011).

Haemoglobin thresholds	Stable angina		Post myocardial infarction	
	Women	Men	Women	Men
Lower threshold Haemoglobin level (g/dl) (95% CI)	11.9 (11.5–13.5)	13.1 (13.0–13.4)	12.8 (12.1–13.5)	13.5 (13.2–13.9)
Relative risk of death below threshold (95% CI)	1.92 (1.44–2.38)	2.03 (1.72–2.40)	1.77 (1.51–2.10)	2.00 (1.76–2.29)
Upper threshold Haemoglobin level (g/dl) (95% CI)	16.3 (14.0–17.5)	17.2 (13.6–18.0)	15.1 (13.9–16.6)	16.4 (15.1–17.8)
Relative risk of death above threshold (95% CI)	2.65 (1.06–10.8)	1.11 (1.00–3.86)	1.81 (1.13–3.53)	1.14 (1.01–1.51)

From Table 28, it can be seen that the only significant association between serum ferritin and CVD risk is evident when inflammation is not controlled for (Salonen et al 1992). Serum ferritin has also been found to not be associated with risk when inflammation is not controlled for (Sempos et al 1992). A review (Corti et al 1997), and a meta-analysis (Danesh & Appleby 1999), found no consistent evidence for an association between iron status and CVD or CHD. Non-significant positive associations are reported when CRP is used to control for inflammation (Menke et al 2009, Ahluwalia et al 2010), but in a larger study no associations are found (Menke et al 2011). Compared with IL-6, CRP does not correlate as closely with sFT (DePalma et al 2010), and therefore the use of CRP to control for inflammation may be inadequate, and the results of studies controlled thus may still include inflammation as a factor in the level of risk seen. No benefit is seen with phlebotomy to lower serum ferritin (Zacharski et al 2007). Three studies find an association between CVD risk and low iron status (Sarnak et al 2002, Casiglia et al 2011, Morkdal et al 2011). No association between higher Hb and CVD risk was found to be evident by Salonen et al (1992), Sarnak et al (2002), or Ahluwalia et al (2010).

Shah et al (2011) (Table 29) conducted a meta-analysis of retrospective cohort studies totalling 20,131 people following diagnosis of stable angina with no previous CHD, and 14,171 people following myocardial infarction survival of seven days, for 3.2 years. They found additional mortality risks below and above a range of haemoglobin threshold levels, suggesting a U-shaped relationship between haemoglobin and CHD risk.

In conclusion, the literature does not clearly support a link between high iron status as measured by a range of iron indices and CVD risk. The evidence is stronger for low iron status and risk for CVD. From the conclusions of the meta-analysis by Shah et al (Table 29), there is an association between low Hb concentration and increased mortality, with evidence that high Hb is also associated with poor prognosis, although in the case of females with stable angina, the 95% confidence interval for relative risk is wide, suggesting some uncertainty in the result. No probability values are reported with the relative risk values.

4.2.2 Non-transferrin-bound iron and cardiovascular disease risk

Considering NTBI and CVD risk, Rajapurkar et al (2011) found a strong detrimental association between catalytic iron and prevalent CVD, in a cross-sectional study of 496 middle-aged people of mixed health. When fully adjusted for confounding variables, the highest third by NTBI had an odds ratio of 3.8 (95% CI, 1.4 to 10.1) for CVD. NTBI was measured by the bleomycin assay (Gutteridge & Xiao-Chang 1981), finding a mean level for the lowest tertile of 0.04 μM , and 3.15 μM for the highest tertile. Disagreeing with this, Van der A et al (2006) found no increased risk of CHD or MI in their highest tertile of NTBI compared with the lowest, in 1132 women over a 4.3 year study. NTBI was measured at baseline using the method of Breuer & Cabantchik (2001), finding a range from -2.06 to 3.51 μM , where negative values are explained as describing the free iron scavenging effect of transferrin.

Of the evidence for iron status in diabetes and link to CVD, Lee et al (2006) instigated their NTBI in type 2 diabetes study as a result of finding that diabetes patients who used supplemental vitamin C had increased CVD mortality. They

hypothesised that NTBI was causing this, and that increased oxidative stress was resulting from chemical reduction of ferric NTBI by ascorbic acid. Their results, and others, are given in Table 30. Sulieman et al (2004) (Appendix 1.ii) measured NTBI (method of Esposito et al 2003) in patients within 24 hours of acute MI, 329 with diabetes, 322 without. Mean NTBI in controls was 0.14 ± 0.23 ($\mu\text{M} \pm \text{SD}$), compared with diabetes, 0.43 ± 0.7 ($\mu\text{M} \pm \text{SD}$). The 30 day mortality rate was 2.5-fold higher in diabetes, and NTBI was associated with increased mortality at 30 days in the diabetes group only. Diabetes patients without NTBI had the same mortality risk as non-diabetes.

Haptoglobin genotype is not reported in Sulieman et al's study, but would be a very interesting addition to these findings, along with HbA_{1c}, as the haptoglobin 2-2 genotype might account for some or part of the increased mortality in diabetes. Glycated plasma haemoglobin may be detrimental via LDL oxidation, possibly from iron-linked redox toxicity from structural damage to haemoglobin (see Chapter 1). Such damage might account for the NTBI being measured in diabetes.

4.2.3 Renal disease and non-transferrin-bound iron

Physiological iron status in advanced renal disease is characterised by anaemia, as measured by transferrin saturation (particularly if receiving erythropoiesis stimulating agents), and the appearance of raised iron stores indicated by ferritin level (Wish 2006). This may be due to inflammation, which is common in CKD (Oberg et al 2004), as hepcidin (which prevents iron release from cells, including across the gut), and ferritin, are acute-phase proteins. Haemodialysis also leads to blood losses from frequent sampling, and residue remaining in dialyzer,

compounded by decreased oral iron intake (Wish 2006). Treatment with oral supplemental iron can lead to NTBI (Hutchinson et al 2004) (Appendix 1.ii), as can intravenous (IV) iron (Kooistra et al 2002, Slotki et al 2005). IV iron in haemodialysis patients leads to oxidative stress (Lim et al 1999), which is improved by supplemental vitamin E (Roob et al 2000). Lim et al do not say if IV iron is given concomitantly to haemodialysis treatment in their study, but simultaneous haemodialysis and IV iron (as commonly performed) might be more toxic than the two received separately. Kooistra et al found NTBI levels of 0.74 ± 0.69 to 3.79 ± 1.41 μM in ten haemodialysis patients undergoing 100 mg iron infusion over 60 minutes. NTBI levels were raised prior to IV infusion, compared to normal, but no controls were included in the study. Prakash et al (2005) found no increased NTBI in CKD patients compared with controls, but NTBI was significantly increased in haemodialysis patients not on intravenous iron therapy compared to CKD and controls. Patients receiving intravenous iron were not included in the study. No associated lipid peroxidation, or protein thiol formation, was found by FOX₂ or 5'5'dithio-bis(2-nitrobenzoic acid) (DTNB) assays, indicating that the NTBI was not redox active. Microhaemolysis was suggested to be a cause of NTBI in haemodialysis, by mechanical damage to red blood cell releasing haem, therefore it is surprising that no associated lipid peroxidation was seen.

Miyata et al (1997) have shown that enhanced AGE formation in haemodialysis patients is not accounted for by elevated glucose levels, but is accelerated by increased oxidative stress, as measured by oxidised ascorbate. This was a linear relationship, not seen in healthy controls, leading Miyata et al to suggest the

presence of an unknown catalyst in its development. This could be suggested to involve iron, either as concomitant IV iron or haem from red blood cell degradation.

In uraemia, serum AGE build-up is observed irrespective of diabetes being present, or haemodialysis. Thornalley et al (2000) investigated serum albumin glycation in 13 renal-function impaired uremic patients, finding renal impairment associated with a low but significant increase in albumin mass, perhaps caused by AGE formation, increased oxidation, or fatty acid binding. As glycated and oxidized albumin binds an increased amount of iron (Silva & Hider 2009, see Chapter 1), NTBI as an albumin-bound species would be a likely scenario in uraemia.

Evidence for iron status and links to CKD include serum ferritin studies. Prakash et al (2005) did not find ferritin to be associated with NTBI in haemodialysis, however, Kalantar-Zadeh et al (2001) did find high serum ferritin to be a reliable predictor of morbidity in haemodialysis patients, though having previously demonstrated that it is likely to reflect inflammation rather than iron overload (Kalantar-Zadeh et al 1999), perhaps the link with morbidity is not surprising.

Replacing red meat with chicken as dietary protein for four weeks can reduce urine albumin excretion by 46% in diabetes patients with microalbuminuria (Gross et al 2002), hinting at haem iron as a possible link. The authors do not include measures of ferritin, haemoglobin, or serum iron, and so it is not known what effect the diets are actually having on iron status. In rats with proteinuria and glomerular sclerosis, investigation of the effect of a low iron diet compared with normal (Remuzzi et al 1991), found that reduction of haematocrit following iron restriction had a reno-

protective effect, significantly lowering urine protein excretion compared with controls. The effect may have been due to lessening of local oxygen radical production, or an increase in blood viscosity and shear stress on glomerular capillary walls. The authors propose that anaemia may be protective in renal disease progression, and that normalising haematocrit may in fact contribute to its progression.

Within the kidney, the presence of redox active iron is proposed to be a factor in the progression of kidney disease (Alfrey et al 1989, Howard et al 1991, Swaminathan et al 2007). Howard et al (1991) found that in patients with diabetes, with varying amounts of proteinuria, transferrin and albumin were leaked through the kidney equally. Urinary iron excretion increased early in the course of renal disease (pre-proteinuria) and became more marked as disease progressed. Iron was present in the urine in excess of transferrin at all stages, leading the authors to suggest that either a) the transferrin was being reabsorbed in the proximal tubule, or b) the iron was occurring by means other than transferrin filtration. In either case, the iron could be redox active and result in tubule-interstitial injury. Human biopsy studies in patients with proteinuria found that protein excretion correlated with iron accumulation in proximal tubular lysosomes, though not with glomerular filtration rate (Nankivell et al 1992).

A mechanism for the involvement of NTBI in progression of renal disease has been proposed by Smith & Thevenod (2009). This model describes iron excretion, via glomerular filtration of transferrin, and re-absorption; with evidence from mouse and rat studies showing that in response to a reduction in dietary iron, divalent

metal transporter-1 (DMT-1) in the proximal tubular membrane increases, and decreases following dietary iron increase. Iron levels reflected these changes, and studies of iron in urine taken from the proximal tubule, and in excreted urine, had demonstrated that 99.3% of glomerular filtered iron was reabsorbed. These examples describe transferrin-iron filtration and reabsorption to varying degrees in the proximal tubule, where it may or may not subsequently cause oxidative damage. Diabetic nephropathy however is characterised by extracellular matrix thickening to tubule *and* to glomerular basement membranes, and so it might appear that diabetic glomerular damage comes about via AGE development (Kanwar et al 2008), without involvement of iron, whereas proximal tubule damage perhaps involve AGE and iron in tandem.

Whilst the dietary intake data is suggestive of a reduction in iron status having a protective effect on protein loss in glomerular filtration, the mechanisms involved are not known. It is not known for example whether the detrimental effect is due to less post-prandial transferrin-iron present in the blood stream, or less stored iron leading over a longer period to less red blood cell haemoglobin, perhaps leading to less plasma haemoglobin. How any form of iron or NTBI species alters glomerular filtration is unknown, despite knowing it can be reabsorbed afterwards.

4.2.4 Non-transferrin-bound iron methodologies

No one standard method is used to measure NTBI, and a comparison study of the commonest of the methods used (Jacobs et al 2005) found significant variations in results within samples. The two methods most commonly used by researchers are the fluorescence-based assay described by Breuer & Cabantchik (2001), and high

performance liquid chromatography (HPLC) assay with nitrilotriacetic acid (NTA) by Singh et al (1990), with modification by Gosriwatana et al (1999). There are a number of problems with measuring NTBI – losing the low molecular weight iron ligands to vacant transferrin iron binding sites, and displacing and chelating iron from loaded transferrin sites. Methods that involve plasma or serum incubation with added ligands risk either of these.

Early methods used antibiotic bleomycin to bind iron, and the subsequent DNA damage by this complex measured by reaction with thiobarbituric acid (Gutteridge & Xiao-Chang 1981). Jacobs et al found that the bleomycin method showed remarkably lower values of mean NTBI than other assays, with poor correlation between values, in their comparison. An alternative biological method, recommended by Halliwell & Gutteridge (2007), but rarely used in practice, involves use of the enzyme aconitase, following mobilisation by apoconalbumin, which was found to provide NTBI levels close to those found by the earlier bleomycin assay. Most recently, research has concentrated on development of fluorescent iron-chelating probes which can scavenge iron from iron-citrate complexes, and have a very slow exchange with apo-transferrin (Ma & Hider 2009). These are useful for monitoring iron-citrate in β -thalassemia patients (Hider et al 2010). Gel filtration followed by inductively coupled mass spectrometry (ICP-MS) has usefully described glycosylated and oxidized albumin bound iron *in vitro* (Silva & Hider 2009), however this method is not suitable for serum as gel filtration cannot resolve albumin from transferrin-bound iron (Hider et al 2010).

Because NTBI is heterogeneous, different iron species may occur in different diseases, and in different tissues (Evans et al 2008), and NTBI present in diabetes may not be fully measured by some assays. In particular, increased iron on glycated and oxidized albumin is not picked up by NTA, and therefore assays employing NTA will under-report NTBI in diabetes (Silva & Hider 2009). Consequently, comparison between the results reported for diabetes is difficult, and the findings of the groups below are diverse. NTA-based assays may not give an accurate picture of NTBI in diabetes. Despite this, Lee et al (2006), and Leoncini et al (2008), find some agreement in levels for established diabetes, Lee et al with NTA, and Leoncini et al without. This suggests that deferoxamine (DFO), used by Leoncini et al, may not displace glycated albumin-iron either. The DFO-iron complex has an association constant of pK_a of 31 (Davis & Porter 2002), while the NTA-ferric iron association constant is 15.9 at 0.1 M KCL (Motekaitis & Martell 1993). The association constant for transferrin-iron binding is between 19 and 20 (Harris 1986).

4.2.5 Methods and findings for non-transferrin-bound iron in diabetes

Four studies of type 2 diabetes have included NTBI measures, using different methodologies (Table 30).

Table 30. Non-transferrin-bound iron reported in diabetes by other groups

Authors (<i>NTBI method</i>)	Controls	Diabetes	Diabetes
Van Campenhout et al (2006b)* (<i>Breuer & Cabantchik 2001</i>)		Type 2 diabetes	Type 1 diabetes
	<i>n</i> = 100	<i>n</i> = 67	<i>n</i> = 39
Age (years)	43.5	62.5	44
HbA _{1c} (%)	5.35	7.3	7.8
Iron (μM)	18	15.5	16
TSat (%)	27	24	26
Hb (g/dl)	No report	No report	No report
NTBI (μM mean)	1.44	0.68	0.91
Lee et al (2006) (<i>Gosriwatana et al 1999</i>)		Newly diagnosed type 2	Type 2, 4-15 years post diagnosis
	<i>n</i> = 47	<i>n</i> = 49	<i>n</i> = 48
Age (years ± SD)	58.3 ± 9.7	59.8 ± 9.7	58.9 ± 9.2
HbA _{1c} (%) ± SD)	No report	No report	8.1 ± 1.7
Iron (μM ± SD)	17.1 ± 7.5	21.5 ± 9.7	18.1 ± 6.0
TSat (%) ± SD)	32.0 ± 15.4	29.4 ± 11.7	33.0 ± 11.2
Hb (g/dl ± SD)	13.1 ± 1.2	13.2 ± 1.6	14.2 ± 1.5 ^a
NTBI (μM mean ± SD)	0.04 ± 0.13	0.24 ± 0.29 ^b	0.62 ± 0.43 ^b
Leoncini et al (2008) (<i>Ferrali et al 1989</i>)		Type 2 diabetes	Type 1 diabetes
	<i>n</i> = 39	<i>n</i> = 51	<i>n</i> = 25
Age (years ± SEM)	46.2 ± 0.8	68.8 ± 1.5	50.6 ± 3.1
HbA _{1c} (%) ± SEM)	5.0 ± 0.2	7.5 ± 0.2 ^c	7.6 ± 0.2 ^c
Iron (μM ± SEM)	No report	No report	No report
TSat (%) ± SEM)	26.6 ± 0.7	26.5 ± 1.6	26.1 ± 1.7
Hb (g/dl ± SEM)	No report	No report	No report
NTBI (μM mean ± SEM)	0.10 ± 0.03 (<i>n</i> = 24)	0.53 ± 0.09 ^d (<i>n</i> = 24)	
Shetty et al (2008) (<i>Nilsson et al 2002</i>)		Good control BG	Poor control BG
	<i>n</i> = 40	<i>n</i> = 53	<i>n</i> = 50
Age (years ± SD)	55 ± 8	50 ± 5	55 ± 10
HbA _{1c} (%) ± SD)	6 ± 1	7 ± 1	11 ± 2 ^e
Iron (μM ± SD)	No report	No report	No report
TSat (%) ± SD)	No report	No report	No report
Hb (g/dl ± SD)	No report	No report	No report
NTBI (μM mean ± SD)	0.5 ± 0.2	0.8 ± 0.5	34.2 ± 27.5 ^e

Key: BG Blood glucose; * Data shown are approx. means of Male and Female results; ^a *p* < 0.01 known diabetes vs newly diagnosed and control; ^b *p* < 0.01 between groups; ^c *p* < 0.001 vs controls; ^d *p* < 0.001 vs controls; ^e *p* < 0.01 vs controls and good BG.

Van Campenhout et al (2006) used the NTBI method developed by Breuer & Cabantchik (2001). This uses fluorescein-apo-transferrin (Fl-aTf) to chelate unbound iron, with readings taken as a ratio of two reagents of varying fluorescence. The serum apotransferrin sites are first blocked with gallium ($\text{Ga}(\text{Cl})_3$), then reaction-available iron is mobilised with oxalate. Serum and reagents are mixed in multiwell plates, with fluorescence determined after 1 hour in a microplate reader. Drawbacks are that not all the apotransferrin sites were filled, leading to some false positives, and that efforts to overcome this led to underestimation of NTBI, and failure to detect it at low levels. The Fl-aTf assay by Breuer & Cabanchik was included in Jacobs et al's (2005) study of different methodologies, and was performed in three different laboratories using identical blockers and scavengers, but differing equipment, reagent preparation, and procedures. Jacobs et al found that of all the methods tested, the Fl-aTf assay, whilst variations in results were seen, had the optimal profile for reproducibility.

Van Campenhout et al (2006) found significantly less NTBI in male controls than males with type 2 diabetes (1.08 ± 2.15 vs 1.37 ± 1.65 μM , mean \pm SD, respectively). Significant differences were seen within each group between males and females. More NTBI was seen in female compared with male controls, and in males compared with females in both diabetes groups. Mean NTBI level for females with type 2 diabetes was -0.02 ± 1.87 μM . A high NTBI level was seen in female controls which did not appear to be due to undiagnosed haemochromatosis as all other iron measures were normal, and which are unexplained. The large spread of values was seen, and negative values were reported, which may have been due to movement of iron onto vacant transferrin sites. All groups had a sizeable

proportion (> 50%) positive for NTBI, with transferrin saturation < 40%. In explanation for their findings, Van Campenhout et al suggest that their assay may not be detecting the various different isoforms of NTBI equally, possibly overlooking differences due to diabetes and gender.

Lee et al (2006) used the HPLC assay of Gosriwatana et al (1999), based on the method of Singh et al (1990). NTA is added to plasma to mobilise and chelate NTBI non-specifically bound to serum proteins, citrate, and low molecular weight ligands. This then undergoes ultrafiltration, and the iron content of the filtrate determined by HPLC, with 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (deferiprone, DHP, CP20, L1), the chromophore of which was read at 450 nm. A drawback of the method of Singh et al is that unsaturated transferrin might compete with low molecular weight iron complexes including the Fe-NTA ligand, leading to artificially low NTBI readings. Singh et al's method was modified by Gosriwatana et al with the addition of sodium-tris-carbonatecobalt (III), to block free transferrin binding sites. Variations on this method use additional chelators such as 3-hydroxyl-1-propyl-2-methyl-pyridin-4-one hydrochloride (CP22) (Kime et al 1996, Buonocore et al 2003), and final NTBI detection by colorimetry or inductively coupled plasma mass spectrometry (ICP-MS), although these appear to be rarely used. Gosriwatana et al's method, with both colorimetry and ICP-MS detection, was included by Jacobs et al and found to have an elevated within-sample variation, suggesting it to be less favourable than the FI-aTf assay, although full comparisons were difficult to make due to the negative values found by some of the methods. Lee et al (2006) filtered through Millipore Microcon YM-30, 30 KDa filters.

Lee et al (2006) found NTBI levels in controls and established type 2 diabetes to be 0.04 ± 0.13 and 0.62 ± 0.43 (μM , mean \pm SD, respectively), and differed significantly between the controls and both newly diagnosed and established diabetes. NTBI was present in 91.7% of patients at 4 to 15 years post-diagnosis, 59.2% of newly diagnosed patients, and 10.6% of controls.

Leoncini et al (2008) used the method of Ferrali et al (1989), whereby plasma samples were reacted with desferrioxamine (DFO), which chelates any available iron forms, producing ferrioxamine. This is ultrafiltrated through 30 kDa filters, and excess DFO removed by silica column chromatography. The remaining DFO-iron complex is determined by HPLC detection at 229 nm. Leoncini et al found NTBI levels of 0.10 ± 0.03 and 0.53 ± 0.09 (μM , mean \pm SEM), in control and diabetes respectively, with significant difference seen between groups.

Shetty et al (2008) used Nillson et al's 2002 bathophenanthroline disulphonate (BPS) assay. BPS is added to plasma samples and left to stand for 15 minutes. The BPS binds to ferrous iron with a binding constant of pK_a 22 (Perrin, in Nillson et al) to produce a measurable colour, and to ferric iron which is measurable after reduction, typically via ascorbate. In Nillson et al's method, samples are then filtered through a "MultiScreen filtration system" with a pore size of $0.45 \mu\text{m}$, through which all individual plasma proteins could be expected to pass. Shetty et al do not specify a pore size, and did not respond to a request for clarification of this point. The sample is read by spectrophotometry at 535 nm, and results found by subtracting the value of an equivalent sample without BPS. Shetty et al found extremely high levels of NTBI in diabetes patients with poor glucose control, $34.2 \pm 27.5 \mu\text{M}$, mean \pm SD. Healthy controls had $0.5 \pm 0.2 \mu\text{M}$, and diabetes patients with

controlled blood glucose to have $0.8 \pm 0.5\mu\text{M}$ (mean \pm SD). Similarly high levels were found by this group in haemodialysis patients, 10 out of 22 of whom had diabetes (the controls had $\sim 3\ \mu\text{M}$) (Prakash et al 2005). These levels seem unfeasibly high, and perhaps the method may include measurement of glycated albumin-iron. However, as there is no agreement on the number of iron atoms that will bind to albumin, and it is not known how many more might bind to glycated albumin (see Chapter 1), it is not possible to say whether these figures are a likely representation of those which might be seen in diabetes. As Shetty et al's reported levels for the newly diagnosed, and control groups, are not unfeasibly high by other reports, it is possible that they might be correct. However, the association constant for BPS is 22, less than the DFO-iron constant of 31, which could not chelate glycated albumin-iron in Leoncini et al's study, and therefore BPS would not be expected to do so either. Another reason for Shetty et al's high NTBI levels may be due to the chelation of iron from transferrin or plasma haemoglobin by BPS, although Nillson et al report in their original description of the assay that this did not occur even after a three-hour incubation.

4.2.6 Summary

Some agreement is seen for levels of NTBI in diabetes, and one study has found a link between NTBI, diabetes, and cardiovascular mortality. In renal disease, NTBI is seen following IV supplementation, and in haemodialysis, but NTBI has not yet been measured in CKD patients prior to dialysis. It is not known what form NTBI takes in diabetes, other than that glycated albumin-bound iron is thought to occur and not be picked-up by methods that use NTA to mobilise NTBI prior to measurement. Plasma and sub-endothelial extracellular glycated haemoglobin may

represent a form of NTBI in diabetes. HbA_{1c} is used here as a marker for total protein glycation, and correlation of NTBI with HbA_{1c} is used to investigate the presence of association between NTBI and protein glycation. Two studies disagree completely on this association, therefore a third investigation is justified. Correlation of NTBI with HbA_{1c} will not demonstrate that extracellular glycated haemoglobin is a source of NTBI, as glycated haemoglobin correlates well with other glycated proteins such as albumin. However, additional correlation, if any, between NTBI and extracellular haemoglobin might suggest this to be the case. If an increased presence of NTBI is found in those with vascular and renal complications, and a source can be suggested, this would be a useful contribution for directing future interventional work.

Our laboratory has developed a method for measuring NTBI based on published techniques, and it has been employed to measure NTBI in a cross-section of people with and without type 2 diabetes. Both groups have a range of cardiovascular and renal function profiles. This will provide valuable additional information on NTBI, iron and antioxidant status, and lipid peroxidation, along with other clinical biochemistry and anthropometry parameters. The measurement of NTBI in CKD patients prior to dialysis is reported for the first time.

4.3 Hypotheses

H₁ NTBI will correlate with HbA_{1c} in type 2 diabetes and controls.

H₂ NTBI will correlate with extracellular (plasma) haemoglobin in type 2 diabetes and controls.

H₃ NTBI will correlate with lipid peroxidation, measured by plasma malondialdehyde, in type 2 diabetes and controls.

H₄ NTBI will correlate with reduced plasma ascorbate, in type 2 diabetes and controls.

H₅ In people with type 2 diabetes levels of NTBI will differ in those with pre-established cardiovascular disease (a history of myocardial infarct, bypass surgery, stent, angina, stroke, transient ischaemic attack), compared to controls with cardiovascular disease.

H₆ In people with type 2 diabetes levels of NTBI will differ in those with chronic kidney disease (CKD) (established by assessment of albuminuria, glomerular filtration rate, plasma creatinine, and blood pressure) compared to controls with CKD.

4.4 Materials and Methods

See Appendix 3.i for laboratory protocols, solutions, and equipment.

4.4.1 Measurement of non-transferrin-bound iron

The NTBI method used is a development of the methods of Singh et al (1990), Kime et al (1996) as described above, and Paffetti et al (2006). Kime et al use NTA mobilisation of loosely-bound iron (100 µl plasma plus 10 µl 0.8 mM NTA pH 7.0, for 20 minutes at room temperature (RT), samples are then diluted in an equal volume of 5 mM 3-morpholinopropanesulfonic acid (MOPS) buffer, left to stand for 10 minutes at RT, then 130 µl sample is placed in a 100 kDa Whatman ultracentrifuge filter and centrifuged at 12,000 x g for 30 minutes and 4°C. The filtrate is placed in a 20 kDa Whatman ultracentrifuge filter and centrifuged at

12,000 x g for 30 minutes and 4°C, filtrates held on ice until HPLC. Iron is detected following reaction with CP22 dissolved within the mobile phase (3 mM CP22 made up in 5 mM MOPS pH 7.0, 800 ml of this added to 200 ml acetonitrile, filtered and degassed. Kime et al found iron contamination in their blanks (mean 1.75 µM), which was traced to the Whatman filters, and consequently sample values were calculated following subtraction of the mean value for contiguous blanks. They report assay sensitivity down to 0.02 µM, recovery following spiked plasma of between 92.0 and 99.2%, intra-batch coefficient of variation of 5.13%, and inter-batch coefficient of variation of 9.97% (but do not give a concentration of iron used for these).

Paffetti et al (2006) use 50 µl of plasma, to which is added 5 ml of 0.8 M NTA, incubated for 30 minutes at RT, then diluted with an equal volume of 5 mM PIPES buffer, then left to stand for 20 minutes at RT. The samples are then filtered using 100 kDa Whatman Vecta-Spin ultracentrifuge filters, at 13,660 x g, 4°C, for 40 minutes. The filtrate is then filtered again through 20 kDa filters under the same conditions. The injected sample reacts with CP20 (Deferiprone, DHP) within the mobile phase (3.5 mM DHP in 5 mM PIPES buffer pH7.0, plus 4% acetonitrile).

Using two filtration steps makes these assays lengthy and expensive, and increases the possibility of contamination. Experimentation by our laboratory with different filters has led to development of a reliable method using a single filtration step. 150 µl of plasma is added to 15 µl of 0.8 M NTA, incubated for 20 minutes at RT, then diluted with 150 µl PBS. Samples are then filtered through 30 kDa Millipore Amicon Ultra filters, at 13,000 x g, 4°C, for 30 minutes. The filtrates are held on ice. Prior to HPLC injection, 150 µl of filtrate received 15 µl 50 mM DHP and incubated for 5

minutes. The mobile phase consists of 3.5 mM DHP in 5 mM PIPES buffer pH7.0, plus 5% acetonitrile.

4.4.2 Non-transferrin-bound iron assay performance – coefficient of variation

The results of the measurement of standards over a 24 month period have been collated to find mean and SD values (Table 31, Figure 39). The standard size most frequently used for finding plasma NTBI are 1, 5, and 10 μM (Figure 40).

Table 31. NTBI assay compilation of standards

Absorbance at 450 nm (AU)			
Fe ³⁺ μM	Mean	SD	n
0.5	0.006		1
1	0.019	0.008	26
2	0.035	0.008	9
5	0.078	0.022	32
10	0.155	0.051	26
20	0.315	0.127	2
30	0.576	0.082	5

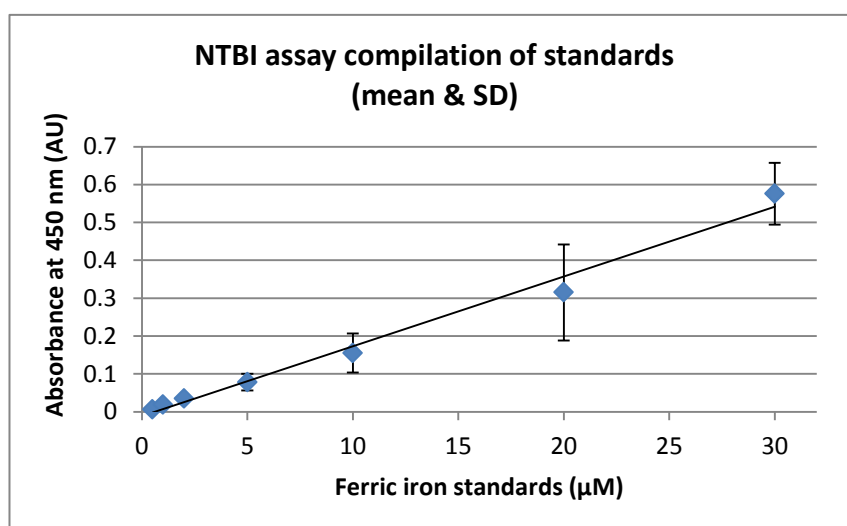


Figure 39. NTBI assay compilation of standards
(Mean and SD). For sample size see Table 31.

Intra-day precision test of four 1 μ M ferric iron standards found a coefficient of variation of 21.9% (mean 0.023, SD 0.005 AU), tested on a single day. Inter-day CV% over 24 months was found to be 43.2% (Table 32). This is high, however it is the result of standards made freshly on the day, and therefore contains pipetting errors. The coefficient of variation given by other groups, for example Paffetti et al (2006), used stored aliquots of a single spiked pooled plasma batch for assay intra- and inter-batch precision.

The system was found to give linear measurements of iron standards down to 500 nM.

Table 32. NTBI assay coefficient of variation

Absorbance at 450 nm (AU)			
Ferric iron standard	Mean	SD	CV
1 μ M (n=26)	0.019	0.008	0.432
5 μ M (n=32)	0.078	0.024	0.309
10 μ M (n=26)	0.155	0.051	0.332

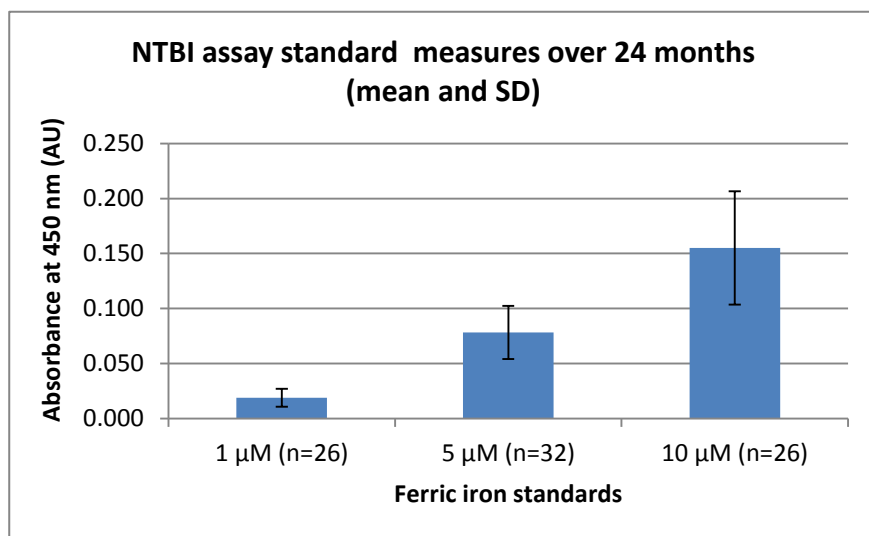


Figure 40. NTBI assay standards over 24 months
(Mean and SD, Table 32.)

4.4.3 Study subjects and design

Subjects were a sub-group of recruits to the SUMMIT (Surrogate Markers for Micro- and Macro-vascular Hard Endpoints for Innovative Diabetes Tools) Study, taking place at The Diabetes and Vascular Medicine Research Centre, University of Exeter. SUMMIT is a pan-European research consortium funded by the Innovative Medicines Initiative (IMI), involving 24 universities, research centres, and pharmaceutical companies, over a five year period. The Exeter cohort comprises 450 participants, made up of type 2 diabetes patients with manifest CVD, type 2 diabetes patients without CVD, and non-diabetic controls. Samples from participants eligible for the NTBI study were included from their baseline visit to the SUMMIT study at Exeter. Sample collection began in April 2011, and ran until the end of the NTBI study period, in April 2012.

4.4.4 Assignment of work

The candidate is responsible for the following work: development of ideas and hypotheses in this thesis, assay of NTBI, total iron, total iron binding capacity, unsaturated iron binding capacity, transferrin saturation, malondialdehyde, reduced and oxidized ascorbate, extracellular (plasma) haemoglobin; compilation and statistical analysis of data. All other work contributing specifically to the results, including participant recruitment and contact, anthropometry, all other clinical biochemistry and measures of physiology, were carried out by the SUMMIT study research team in Exeter and the laboratories at the Royal Devon and Exeter Hospital, Wonford.

4.4.5 Ethical considerations

Ethical permission was granted by the South West regional ethics committee, study number: 10/H0206/67, and the local ethical committees of The Royal Devon and Exeter NHS Foundation Trust, University of Exeter, and Plymouth University.

4.4.6 Statistical considerations

Statistical analyses were carried out by IBM SPSS software, version 19. Parametric and non-parametric methods were employed as required, and are specified within the results. All tests of central tendency used independent samples testing. All tests were 2-tailed, with significance level set at 0.05.

4.4.7 Sample size

For median testing of NTBI between groups (type 2 diabetes versus controls), the numbers used by the four previous NTBI in diabetes investigations have been repeated. As Lee et al (2006), using a similar HPLC assay to our own, found highly significant differences between the groups, these numbers can be expected to be sufficiently powerful for our own analysis.

4.4.8 Inclusion and Exclusion criteria

Inclusion criteria were: older than 18 years; type 2 diabetes patients with manifest CVD or CKD, type 2 diabetes patients without CVD or CKD, non-diabetic controls with and without CVD and CKD, hypertension, dyslipidaemia, angina, proteinuria, rheumatoid and osteoarthritis. Subjects receiving medications including insulin were included and the drugs and dosages recorded. Subjects receiving oral supplemental iron were included as blood samples were taken in the fasted state,

prior to supplement intake. Iron supplementation was recorded. Pre-menopausal females were included and their status noted.

Subjects were excluded on grounds of any malabsorption disease, peritoneal dialysis or haemodialysis, cancer, any known communicable disease.

4.5 Results

The characteristics of the two sample groups, probability values for differences in central tendency, and correlations, are given in Table 33 and Table 34. Significant differences are seen in BMI and body fat % (higher in diabetes), and malondialdehyde (MDA) (lower in diabetes) (Table 33). A highly significant difference is seen for white blood cell count (higher in diabetes, Table 34), and very highly significant differences are seen in HbA1c (higher in diabetes), and total cholesterol and LDL (lower in diabetes) (Table 33).

Table 33. Group characteristics – anthropometry, lipids, and antioxidant status

Group characteristics – anthropometry, lipids, and antioxidant status				
Gender		Female (n)	Male (n)	Total (n)
		Control	7	17
	Diabetes	18	36	54
		Mean	SD	T-test between groups, <i>P</i>
Age (years)	Control	66.5	9.3	0.309
	Diabetes	69.3	11.6	
BMI (kg/m ²)	Control	25.6	9.3	0.028*
	Diabetes	30.5	6.1	
Waist-hip ratio	Control	0.89	0.21	0.155
	Diabetes	1.01	0.35	
Body fat (%)	Control	28.0	8.0	0.016*
	Diabetes	33.6	9.2	
HbA _{1c} (mmol/mol)	Control	40.0	4.4	<0.001***
	Diabetes	55.6	10.8	
Systolic blood pressure (mmHg)	Control	134.8	17.8	0.337
	Diabetes	129.1	24.1	
Diastolic blood pressure (mmHg)	Control	75.8	7.0	0.299
	Diabetes	73.2	14.1	
Total cholesterol (mM)	Control	5.3	1.0	<0.001***
	Diabetes	4.1	1.0	
Triglycerides (mM)	Control	1.5	0.9	0.722
	Diabetes	1.5	0.9	
HDL (mM)	Control	1.6	0.5	0.313
	Diabetes	1.2	0.3	
LDL (mM)	Control	3.1	0.9	<0.001***
	Diabetes	2.0	1.0	
Total/HDL ratio	Control	3.7	1.1	0.676
	Diabetes	3.9	3.0	
Ascorbate ¹ (Reduced) (μM)	Control	33.41	2.94 to 67.62	0.285
	Diabetes	21.53	0.00 to 59.96	
Ascorbate (Oxidized) (μM)	Control	3.47	2.48	0.490
	Diabetes	4.08	5.05	
Urate (μM)	Control	349.7	90.0	0.573
	Diabetes	337.8	78.8	
Malondialdehyde (μM)	Control	2.48	0.82	0.041*
	Diabetes	2.00	1.18	

Key: **P* < 0.05; ***P* < 0.01; *** *P* < 0.001; ¹ non-normal distribution, median & range

Table 34. Group characteristics – iron, and blood cell indices

Group characteristics – iron, and blood cell indices				
		No NTBI (n)	NTBI (n)	Total (n)
		Non-transferrin-bound iron present	Control	5 (20.8%)
	Diabetes	20 (37.0%)	34 (63.0%)	54
	Total	25	53	78
		Median	Range Minimum	Range Maximum
Non-transferrin-bound iron (μM) (non-normal distribution)	Control	0.34	0.00	1.08
	Diabetes	0.21	0.00	1.34
Independent samples median test				$P = 0.326$
		Mean	SD	T-test between groups, P
Total iron (μM)	Control	19.0	6.56	0.557
	Diabetes	17.9	7.56	
Total iron binding capacity ($\mu\text{M}/\text{ml}$)	Control	50.9	13.9	0.377
	Diabetes	53.9	12.1	
Transferrin saturation (%)	Control	39.9	16.7	0.262
	Diabetes	35.2	17.0	
Unsaturated iron binding capacity ($\mu\text{M}/\text{ml}$)	Control	32.0	15.8	0.304
	Diabetes	36.0	15.2	
Plasma haemoglobin ¹ (g/l)	Control	0.26	0.05 to 4.00	0.092
	Diabetes	0.38	0.00 to 1.90	
Whole blood haemoglobin (g/dl)	Control	13.7	0.98	0.104
	Diabetes	13.3	1.33	
Red blood cell count ($\times 10^{12}/\text{l}$)	Control	4.50	0.35	0.574
	Diabetes	4.46	0.32	
Haematocrit (%)	Control	0.40	0.02	0.157
	Diabetes	0.39	0.03	
Mean corpuscular volume ($\times 10^{14} \text{ l}/\text{cell}$)	Control	87.9	3.29	0.236
	Diabetes	86.7	5.41	
Mean corp. haemoglobin ($\times 10^{11} \text{ g}/\text{cell}$)	Control	30.5	1.30	0.098
	Diabetes	29.8	2.35	
Red blood cell distribution width (%)	Control	13.2	0.61	0.152
	Diabetes	13.5	1.11	
White blood cell count ($\times 10^9/\text{l}$)	Control	5.62	1.27	0.007**
	Diabetes	6.57	1.39	
Platelets ($\times 10^9/\text{l}$)	Control	223.5	40.6	0.322
	Diabetes	236.5	56.2	

Key: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ¹ non-normal distribution, median & range

4.5.1 Non-transferrin-bound iron

Within all cases, 25 of the 78 participants had no detectable NTBI. Frequency distributions for all cases are shown in Table 34, and Figure 41. The data has a non-normal, right-skewed distribution and therefore non-parametric methods are used for testing central tendency and associations with other variables.

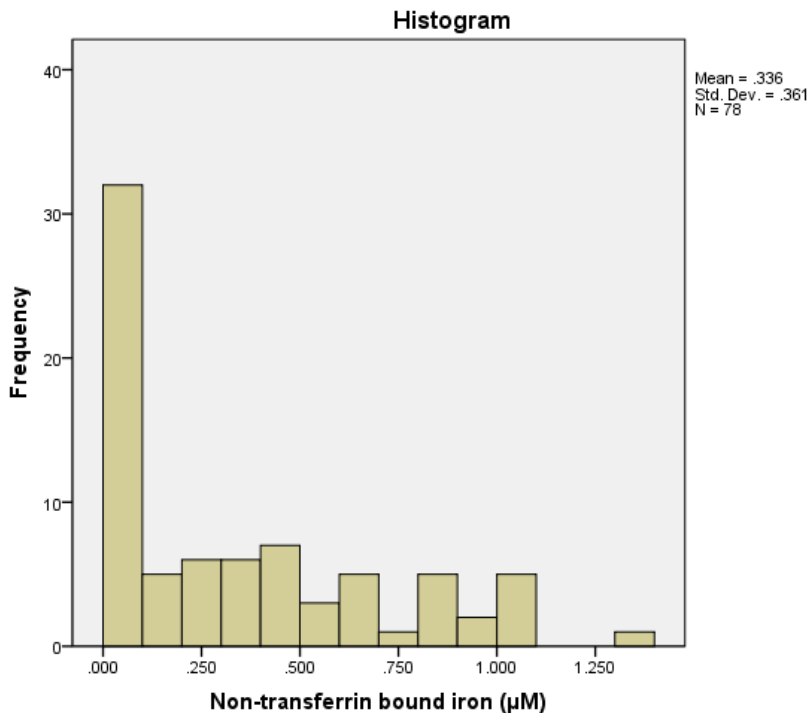


Figure 41. Non-transferrin-bound iron distribution of values
All cases (n = 78).

NTBI is seen to correlate positively with LDL ($P = 0.005$) (Figure 42), malondialdehyde ($P < 0.001$) (Figure 53), total plasma iron ($P < 0.001$) (Figure 43), transferrin saturation ($P < 0.001$) (Figure 44), haemoglobin ($P = 0.001$) (Figure 45), haematocrit ($P = 0.002$) (Figure 46), mean corpuscular haemoglobin ($P = 0.004$) (Figure 47), and red cell distribution width ($P = 0.044$) (Figure 48).

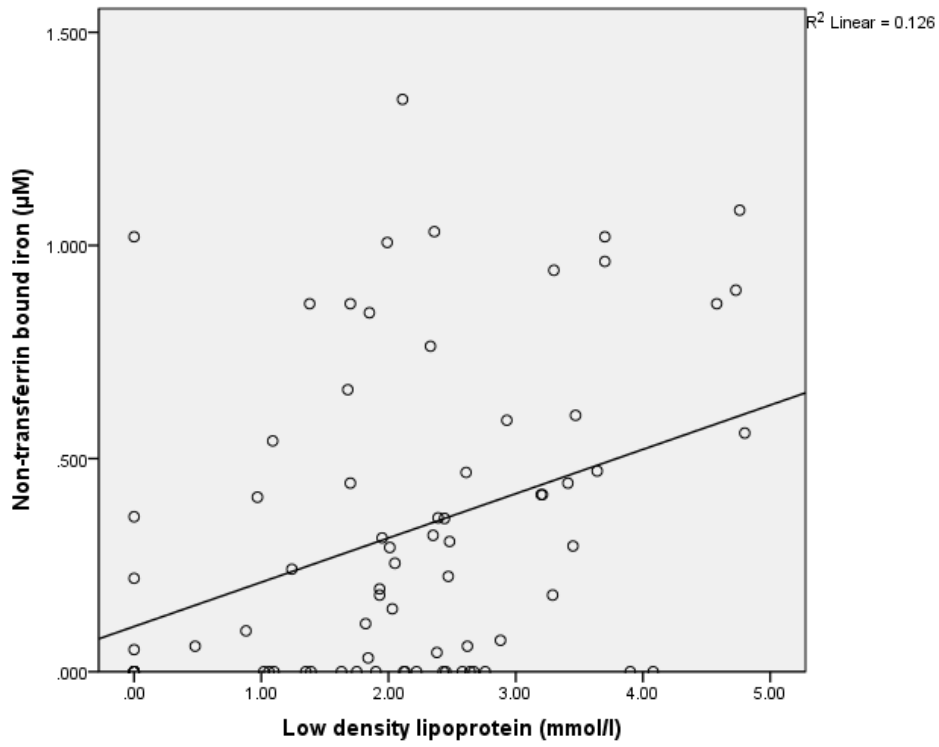


Figure 42. Correlation between low density lipoprotein and NTBI
 All cases ($n = 78$, $r = 0.324$, $P = 0.005$).

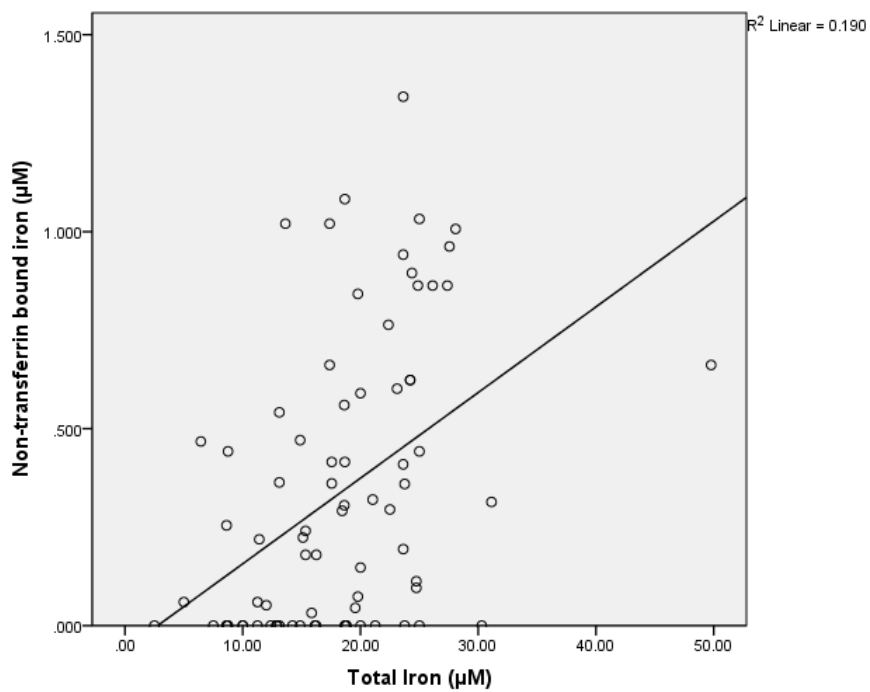


Figure 43. Correlation between total iron and NTBI
 All cases ($n = 78$, $r = 0.460$, $P = 0.000$).

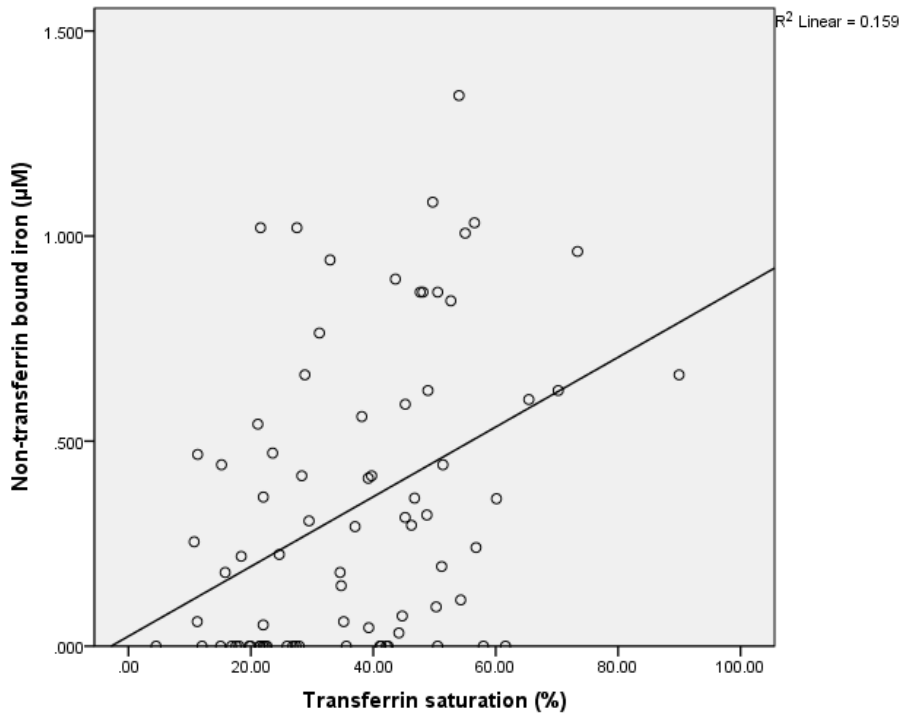


Figure 44. Correlation between transferrin saturation and NTBI
 All cases ($n = 78$, $r = 0.407$, $P = 0.000$).

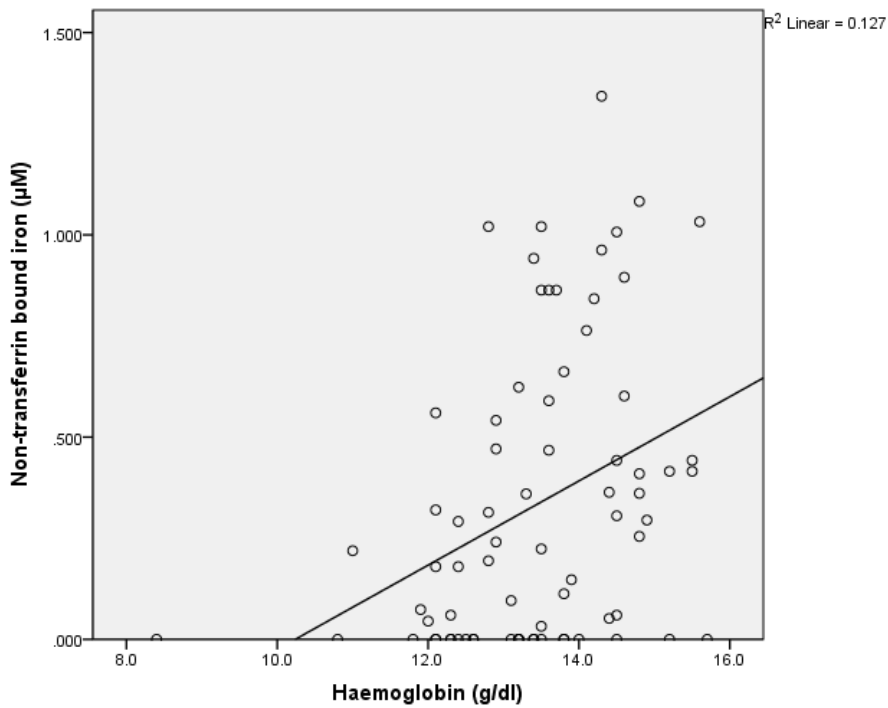


Figure 45. Correlation between haemoglobin and NTBI
 All cases ($n = 75$, $r = 0.386$, $P = 0.001$).

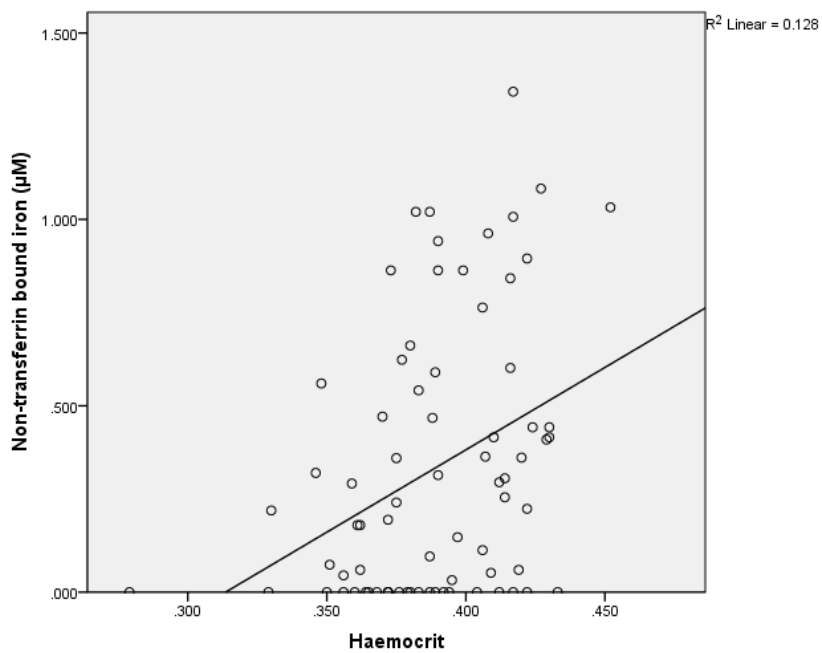


Figure 46. Correlation between haematocrit and NTBI
 All cases ($n = 75$, $r = 0.355$, $P = 0.002$).

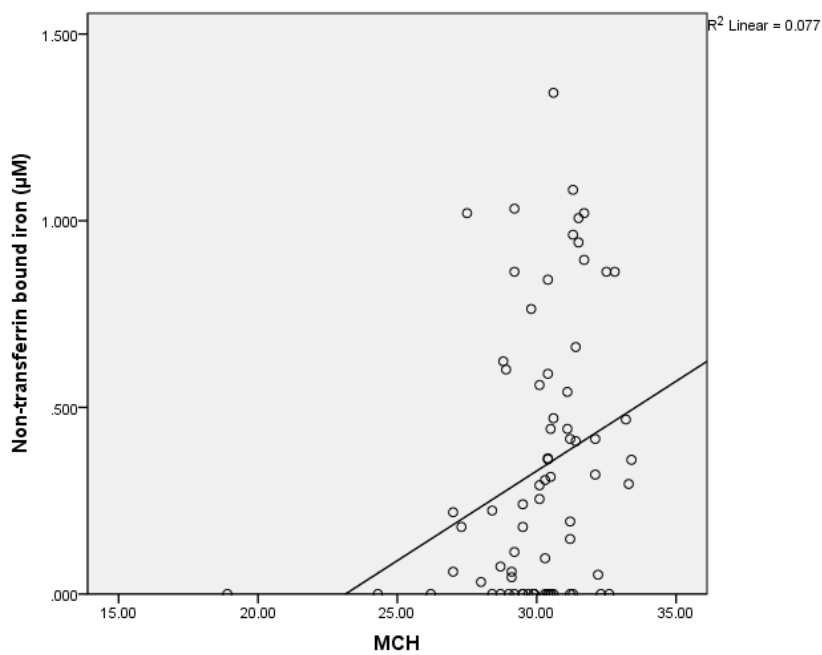


Figure 47. Correlation between mean corpuscular haemoglobin and NTBI
 All cases ($n = 75$, $r = 0.326$, $P = 0.004$).

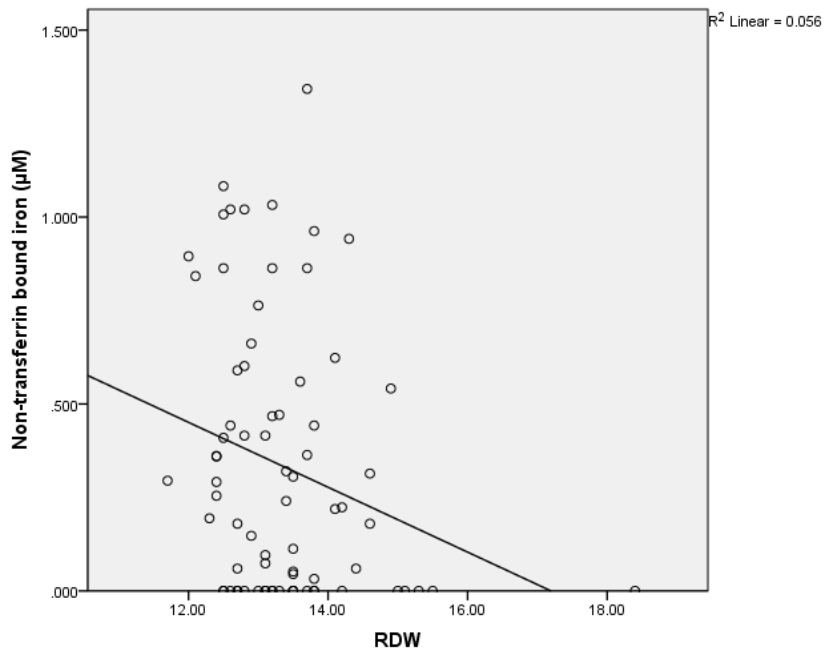


Figure 48. Correlation between red cell distribution width and NTBI
 All cases (n = 75, $r = -0.233$, $P = 0.044$).

4.5.2 Hypothesis testing

4.5.2.1 H_1 NTBI will correlate with HbA_{1c} in type 2 diabetes and controls.

The null hypothesis is retained. No correlation is seen between NTBI and HbA_{1c} ($r = -0.167$; $P = 0.155$) (Figure 49), with results suggestive of a slight negative trend.

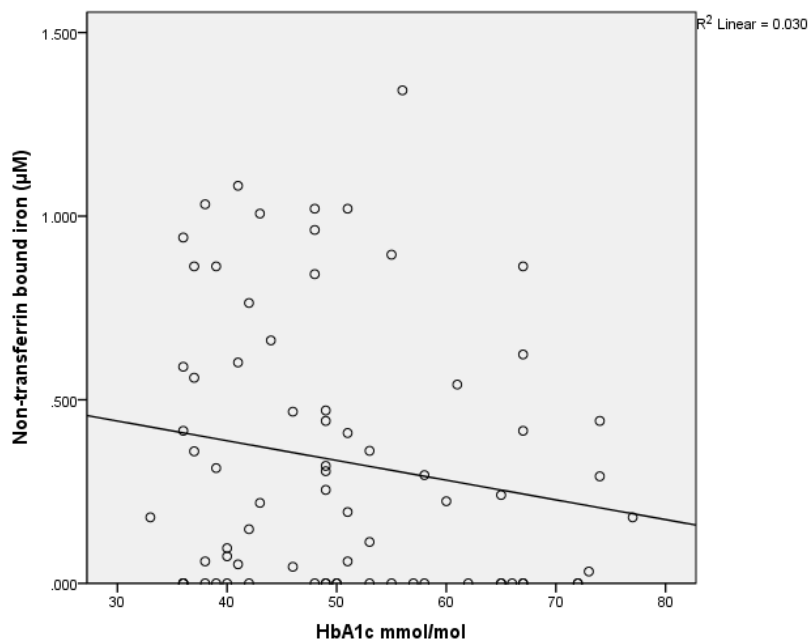


Figure 49. The relationship between HbA_{1c} and Non-transferrin-bound iron
 All cases (n = 78, r = -0.167; P = 0.155).

No significant difference is seen in median NTBI in the control group compared to type2 diabetes (P = 0.326), (Figure 50).

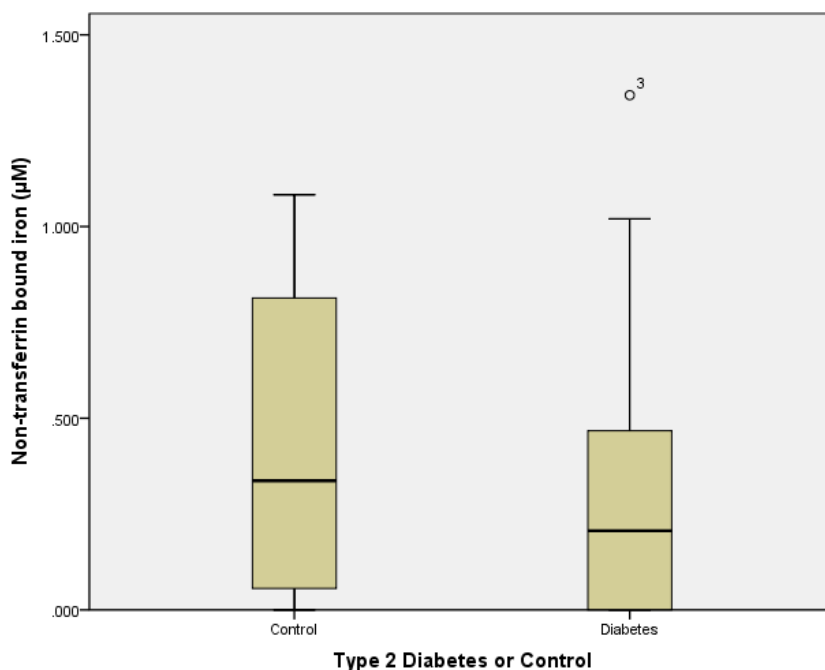


Figure 50. Non-transferrin-bound iron in Controls and Type 2 Diabetes
 Controls (n = 24), Type 2 Diabetes (n = 54, P = 0.326 for difference between medians).

4.5.2.2 H_2 NTBI will correlate with free plasma haemoglobin in type 2 diabetes and controls.

The null hypothesis is retained. No correlation is seen between NTBI and extracellular haemoglobin ($r = -0.056$; $P = 0.640$), (Figure 51).

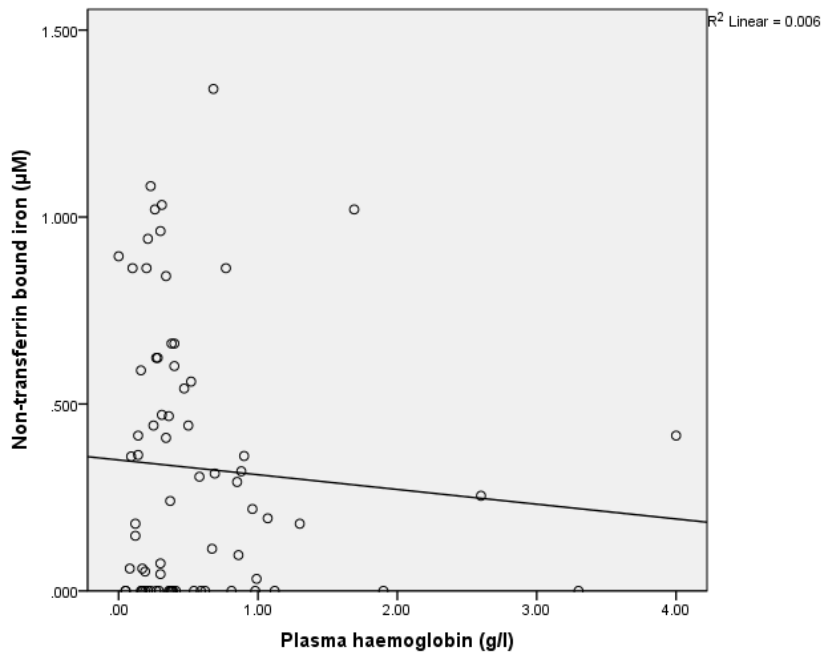


Figure 51. Extracellular haemoglobin vs Non-transferrin-bound iron
All cases ($n = 73$, $r = -0.056$; $P = 0.640$).

No significant difference is seen between medians for Controls or Type 2 diabetes ($P = 0.092$) (Figure 52).

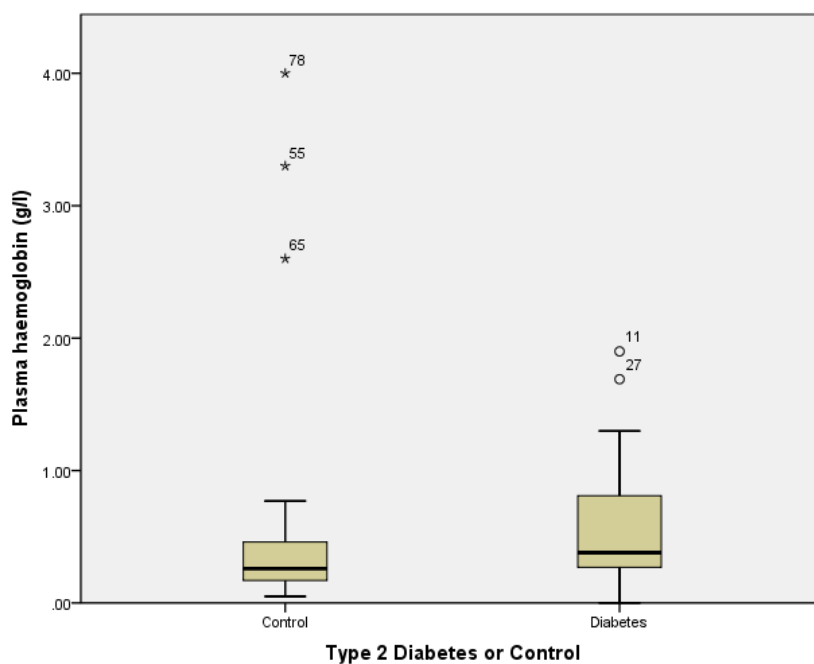


Figure 52. Extracellular plasma haemoglobin in Controls and Type2 Diabetes
 Controls (n = 23), Type 2 Diabetes (n = 50, $P = 0.092$ for difference between medians).

4.5.2.3 H_3 NTBI will correlate with lipid peroxidation, measured by plasma malondialdehyde, in type 2 diabetes and controls.

The null hypothesis is rejected. With all cases pooled, NTBI positively correlates very highly significantly with MDA (0.440, $P < 0.001$) (Figure 53). Within controls, NTBI correlates very highly significantly with MDA ($r = 0.707$, $P < 0.001$) (Figure 54). Within type 2 diabetes, a significant correlation is seen ($r = 0.311$, $P < 0.022$) (Figure 55).

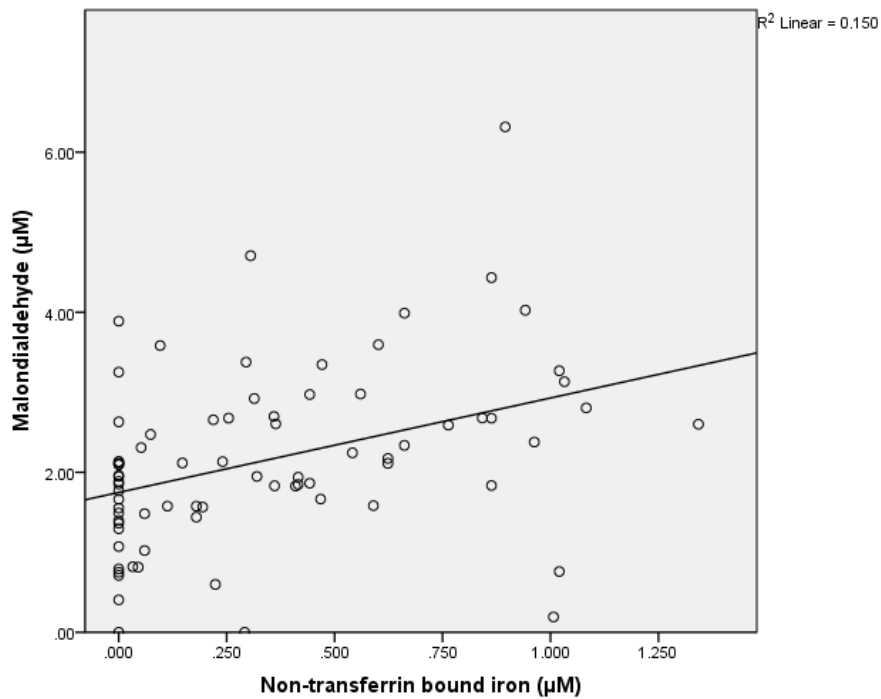


Figure 53. The relationship between Non-transferrin-bound iron and Malondialdehyde

All cases (n = 78, r = 0.440, P < 0.001).

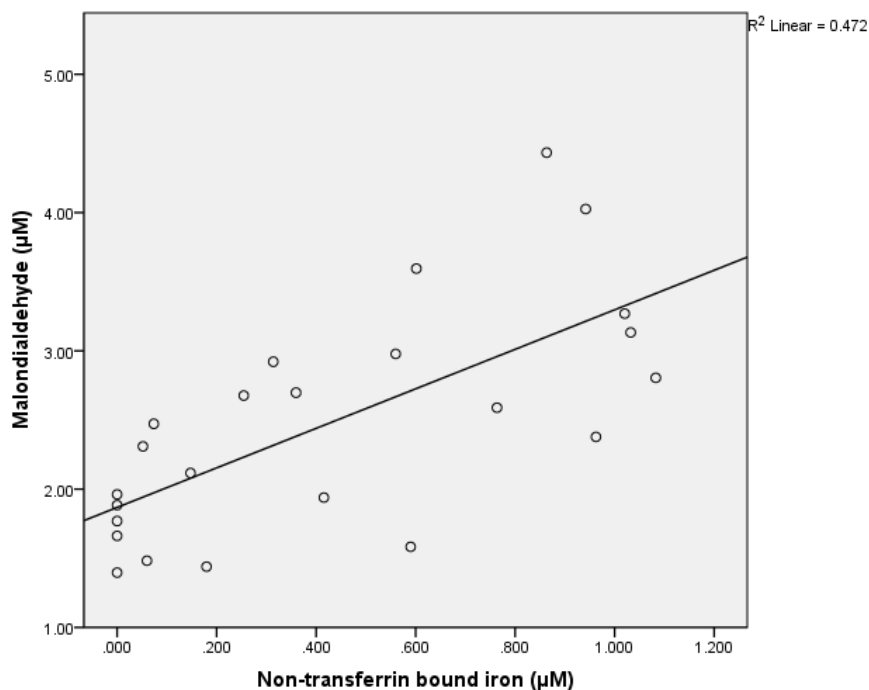


Figure 54. The relationship between Non-transferrin-bound iron and Malondialdehyde, Controls

Controls (n = 24, r = 0.707, P < 0.001).

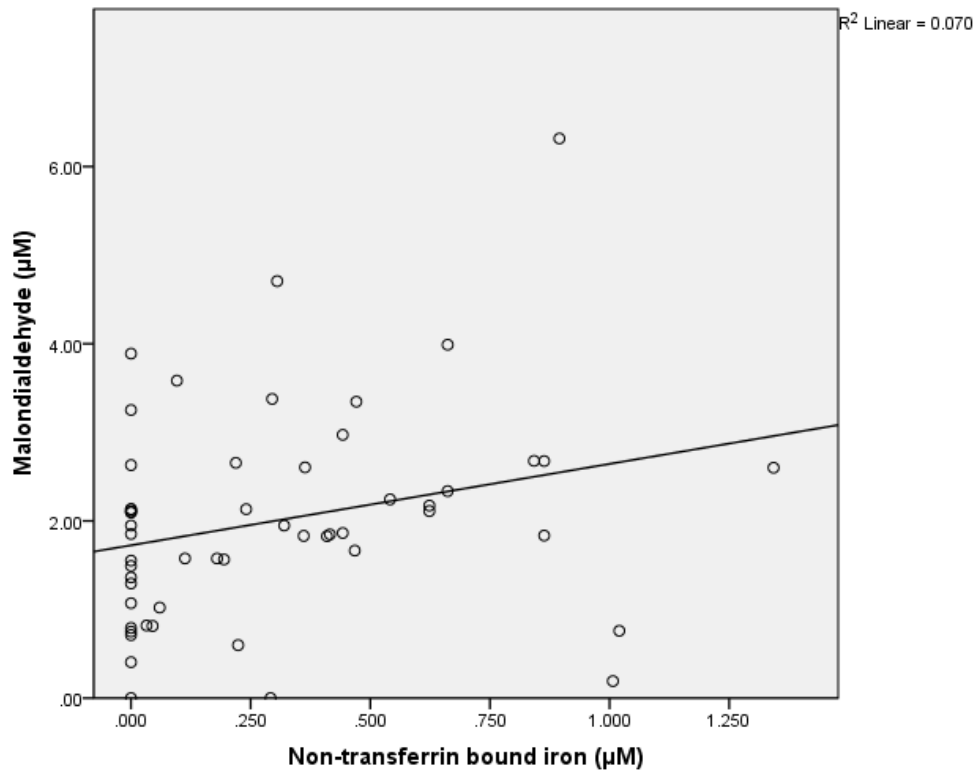


Figure 55. The relationship between Non-transferrin-bound iron and Malondialdehyde, Type 2 diabetes

Type 2 Diabetes, (n = 54, r = 0.311, P < 0.022).

Significantly less malondialdehyde is seen in the type 2 diabetes group compared with controls (P = 0.041) (Figure 56).

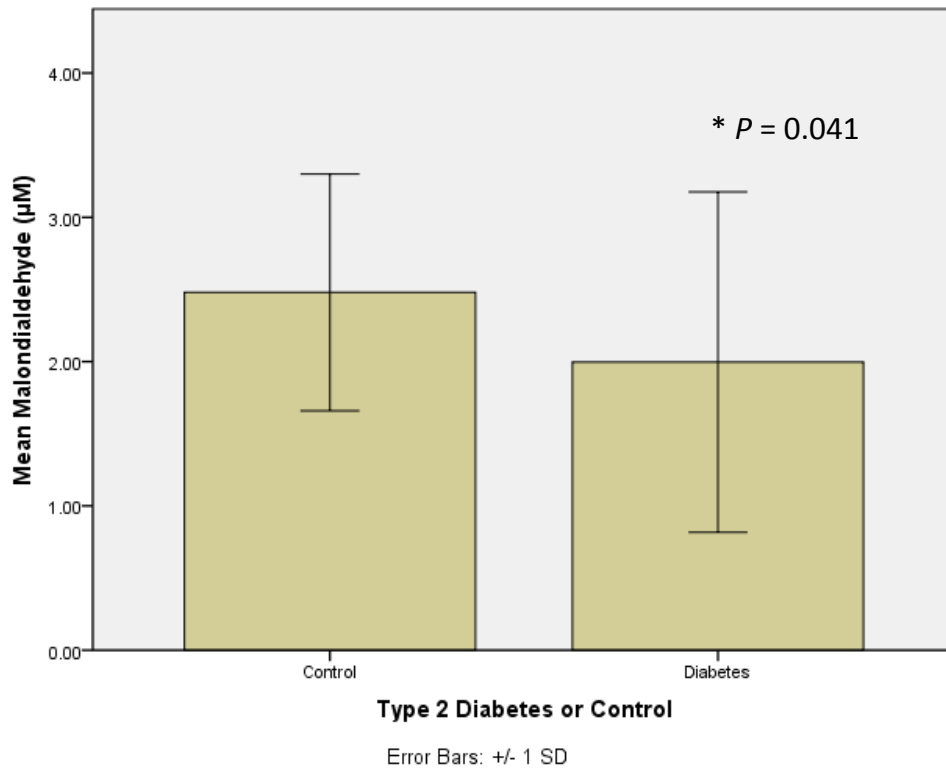


Figure 56. Malondialdehyde in Controls and Type 2 Diabetes

Controls (n = 24), Type 2 diabetes (n = 54) (mean and SD, $P = 0.041$ for difference between means).

4.5.2.4 H_4 NTBI will correlate with chemically reduced plasma ascorbate, in type 2 diabetes and controls.

The null hypothesis is retained. No significant trend is seen between NTBI and reduced ascorbate, by non-parametric correlation, all cases (n = 78, $r = 0.168$, $P = 0.149$) (Figure 57).

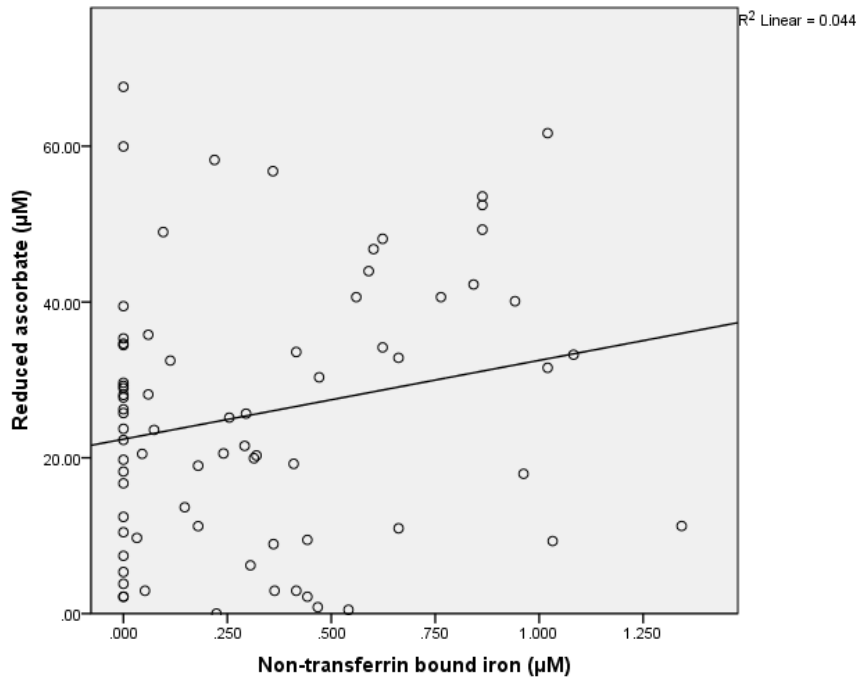


Figure 57. Correlation between NTBI and reduced ascorbate
All cases ($n = 75$, $r = 0.168$, $P = 0.149$).

No significant difference is seen between groups for median values ($P = 0.285$) (Figure 58).

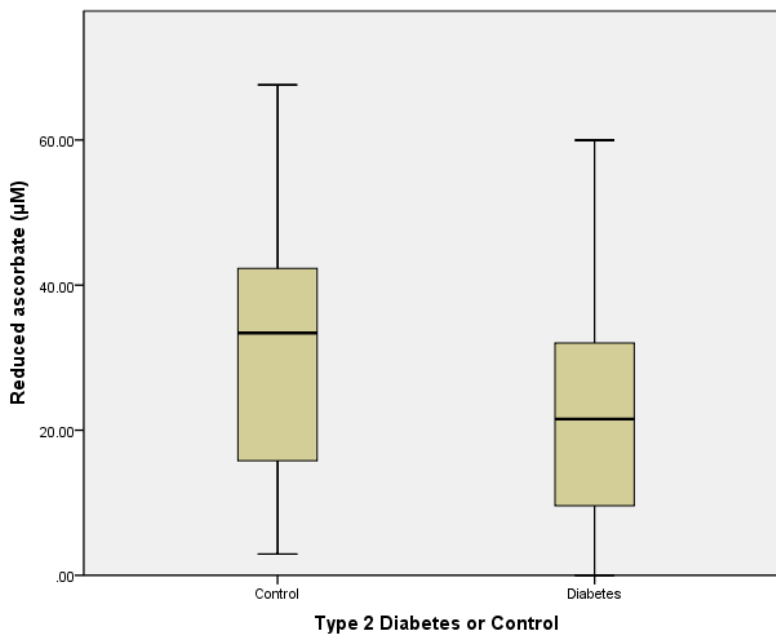


Figure 58. Chemically reduced ascorbate in Controls and Type 2 Diabetes
Controls ($n = 24$) and Type 2 diabetes ($n = 51$, $P = 0.285$ for difference between medians).

4.5.2.5 H_5 In people with type 2 diabetes levels of NTBI will differ in those with pre-established cardiovascular disease (a history of myocardial infarct, bypass surgery, stent, angina, stroke, transient ischaemic attack), compared to controls with cardiovascular disease.

The null hypothesis is retained. No significant difference is found between medians for NTBI across groups, by independent samples median test ($P = 0.379$) (Table 35, Figure 59).

Table 35. NTBI in type 2 diabetes and Controls with co-morbidities

NTBI in type 2 diabetes and Controls with co-morbidities				
	N	NTBI Median (μM)	Range	P
Control	24	0.34	0.00 to 1.08	0.326
Diabetes	54	0.21	0.00 to 1.34	
Control + Cardiovascular disease	8	0.46	0.00 to 1.08	0.426
Diabetes + Cardiovascular disease	15	0.29	0.00 to 1.34	
Control + Chronic kidney disease	18	0.20	0.00 to 1.03	0.272
Diabetes + Chronic kidney disease	38	0.21	0.00 to 1.02	

Table 36. Group characteristics and NTBI – cardiovascular disease

Group characteristics – Cardiovascular disease			
Cardiovascular disease (History myocardial infarct, bypass surgery, stent, angina, stroke, transient ischaemic attack)	No CVD (n)	CVD (n)	Total (n)
Control	15 (65.2%)	8 (34.8%)	23
Diabetes	38 (71.7%)	15 (28.3%)	53
Total	53	23	76
NTBI (μM , median & range)	No CVD	CVD	P
	0.19 (0.00 to 1.02)	0.31 (0.00 to 1.34)	0.212

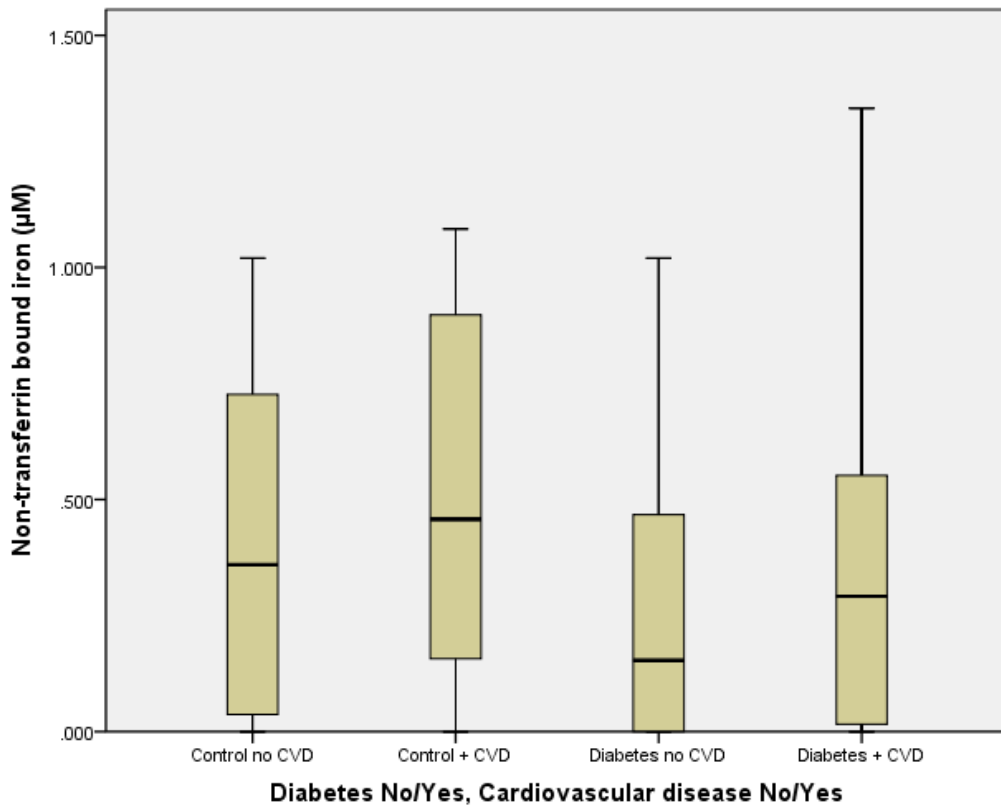


Figure 59. Non-transferrin-bound iron in Controls and Type 2 Diabetes, without and with CVD

Controls without and with CVD (n = 15; n = 8), Type 2 diabetes without and with CVD (n = 38; n = 15).

When all cases are pooled no significant difference is found in NTBI between those without and with pre-established vascular disease ($P = 0.212$ for independent samples median test) (Table 36). A trend towards more NTBI is seen in the CVD group (median 0.19 for subjects without CVD, compared to 0.31 μM in CVD, $P = 0.212$ (Figure 60). Comparing this result with others, Rajapurkar et al (2011) detected less NTBI generally, though found a significant median increase in CVD compared to no CVD in a cohort with mixed morbidities (0.10 μM compared to 0.21 μM in CVD) using the bleomycin detectable iron assay.

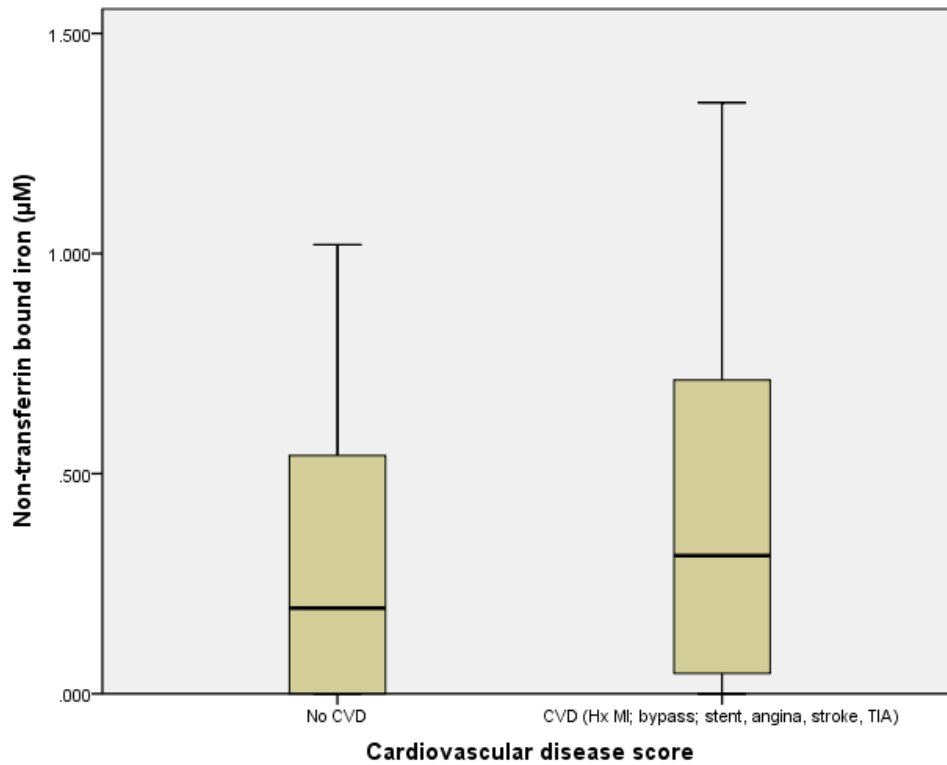


Figure 60. Non-transferrin-bound iron in all cases, without and with CVD
 All cases, without CVD (n = 53), with CVD (n = 23, $P = 0.212$ for difference between medians).

4.5.2.6 H_6 In people with type 2 diabetes levels of NTBI will differ in those with chronic kidney disease (CKD) (established by assessment of albuminuria, glomerular filtration rate, plasma creatinine, and blood pressure) compared to controls with CKD.

The null hypothesis is retained. No significant difference was seen for medians of NTBI across groups, by independent median test ($P = 0.283$) (Table 37, Figure 61).

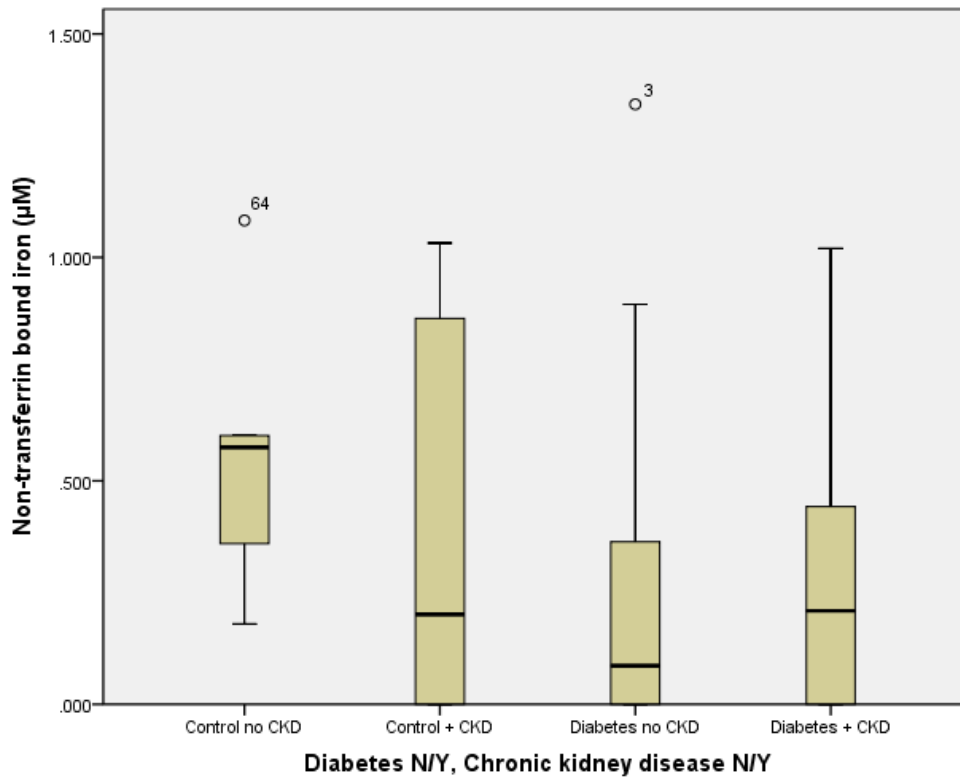


Figure 61. Non-transferrin-bound iron in Controls and Type 2 Diabetes, without and with CKD

Controls, without and with CKD (n = 6 and n = 18), Type 2 diabetes without and with CKD (n = 11 and n = 38).

When controls and type2 diabetes cases are pooled, no significant difference is seen in median NTBI across stages of CKD ($P = 0.406$) (Figure 62), or between those without or those with CKD ($P = 1.00$) (Figure 63).

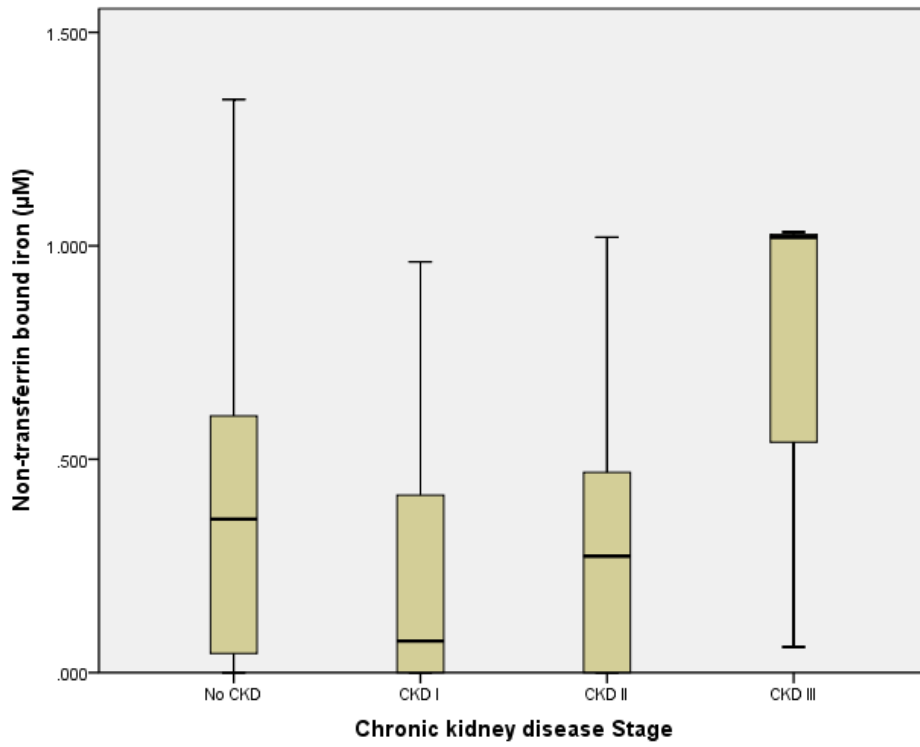


Figure 62. Non-transferrin-bound iron in CKD

All cases. No CKD (n = 17), CKD stage I (n = 25), CKD stage II (n = 28), CKD stage III (n = 3).

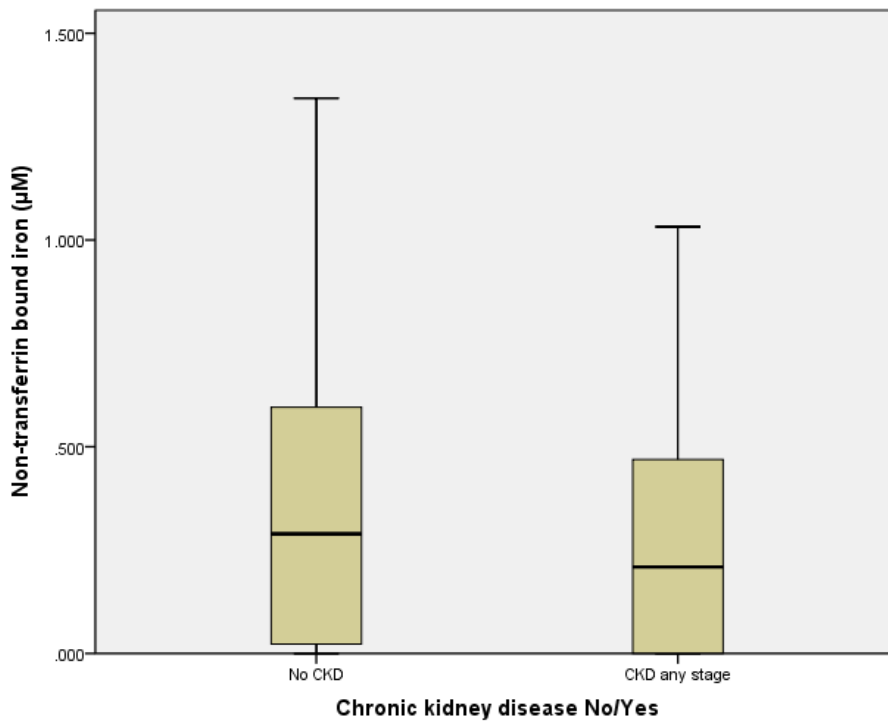


Figure 63. Non-transferrin-bound iron in participants without or with CKD

All cases, without CKD (n = 17), with CKD (n = 56) ($P = 1.00$ for difference in medians).

Table 37. Group characteristics – renal profile, urea and electrolytes

Group characteristics – renal profile, urea and electrolytes					
	Random albuminuria				
		No (n)	Yes (n)	Total (n)	
Random albuminuria	Control	22 (91.7%)	2 (8.3%)	24	
(> 20 mg/l)	Diabetes	48 (88.9%)	6 (11.1%)	54	
No/Yes	Total	70	8	78	
CKD (see Appendix 1.i for classification criteria. No cases of stages IV or V were found)	Chronic kidney disease stage (n)				
		No CKD	CKD I	CKD II	CKD III
	Control	6	5	10	3
	Diabetes	11	20	18	0
NTBI (μM , median) (Range)		0.36 (0.0 to 1.34)	0.07 (0.0 to 0.96)	0.27 (0.0 to 1.02)	1.02 (0.06 to 1.03)
	Median test across groups, $P = 0.283$				
Non-normal distribution		Median	Range	Median test between groups, P	
Random urine creatinine (mmol/l)	Control	5.70	3.0 to 14.5		
	Diabetes	5.6	0.0 to 20.8	0.984	
Random urine albumin (mg/l)	Control	0.00	0.0 to 76.2		
	Diabetes	2.10	0.0 to 213.7	0.240	
Albumin/creatinine ratio	Control	0.00	0.0 to 7.4		
	Diabetes	0.25	0.0 to 32.9	0.384	
Albumin excretion rate ($\mu\text{g}/\text{min}$)	Control	0.00	0.0 to 57.0		
	Diabetes	1.70	0.0 to 591.7	0.500	
Normal distribution		Mean	SD	T-test between groups, P	
Plasma creatinine (μM)	Control	84.6	18.3		
	Diabetes	78.4	16.0	0.139	
Urea (mM)	Control	5.69	2.09		
	Diabetes	5.57	1.83	0.815	
Sodium (mM)	Control	140.8	2.17		
	Diabetes	141.2	2.82	0.521	
Potassium (mM)	Control	4.37	0.34		
	Diabetes	4.35	0.28	0.768	

The investigation of NTBI in pre-dialysis renal disease, with and without diabetes, and all cases pooled, shows no median difference between No CKD, and CKD (Figure 61, Figure 63). When CKD is divided by stage (Figure 62) a trend towards increasing NTBI with renal deterioration is seen, although the small group sample size in stage 3 ($n = 3$) makes this finding insecure. Further work would be useful to increase this sample size, and include cases from CKD stages 4 and 5 (prior to dialysis) to give a fuller picture over the range of disease. No relationships are seen

when linear renal indices were correlation tested against NTBI or haemoglobin (Table 38). Overall, insufficient evidence is found here to support a link between NTBI and pre-dialysis renal disease.

Table 38. Association between NTBI and case characteristics, by non-parametric correlation (Spearman's rho)

Association between NTBI and case characteristics, by Spearman's rho (all cases included unless specified)		
	<i>r</i>	<i>P</i>
Age (years)	0.034	0.769
BMI (kg/m ²)	0.042	0.725
HbA _{1c} (mmol/mol)	-0.167	0.155
Control	0.130	0.544
Diabetes	-0.120	0.408
Systolic BP (mm Hg)	0.142	0.239
Diastolic BP (mm Hg)	0.096	0.425
Total cholesterol (mM)	0.169	0.147
Control	0.165	0.440
Diabetes	0.072	0.618
LDL (mM)	0.324**	0.005
Control	0.481*	0.017
Diabetes	0.187	0.189
Total/HDL ratio	0.290*	0.012
Urate (μM)	0.200	0.095
Ascorbate (Reduced) (μM)	0.168	0.149
Control	0.274	0.195
Diabetes	0.025	0.863
Ascorbate (Oxidized) (μM)	0.202	0.082
Control	0.281	0.183
Diabetes	0.191	0.179
Malondialdehyde (μM)	0.440***	0.000
Control	0.707***	0.000
Diabetes	0.311*	0.022
Plasma haemoglobin	-0.056	0.640
Control	-0.224	0.304
Diabetes	-0.111	0.443
Total iron (μM)	0.460***	0.000
Control	0.451*	0.027
Diabetes	0.437***	0.001
Transferrin saturation (%)	0.407***	0.000
Control	0.307	0.144
Diabetes	0.414**	0.002
Total iron binding capacity (μM/ml)	-0.020	0.863
Unsaturated iron binding capacity (μM/ml)	-0.256*	0.023

Whole blood haemoglobin (g/dl)	Control	0.386***	0.001
	Diabetes	0.376	0.077
		0.395**	0.004
Red blood cell count ($\times 10^{12}/l$)		0.183	0.116
Haematocrit (%)		0.355**	0.002
Mean corpuscular volume ($\times 10^{14} l/cell$)		0.219	0.059
Mean corpuscular haemoglobin ($\times 10^{11} g/cell$)		0.326**	0.004
Red blood cell distribution width (%)		-0.233*	0.044
White blood cell count ($\times 10^9/l$)		-0.106	0.364
Platelets ($\times 10^9/l$)		-0.010	0.930
Random urine creatinine (mmol/l)		0.125	0.287
Random urine albumin (mg/l)		0.079	0.500
Albumin/creatinine ratio		0.063	0.589
Albumin excretion rate ($\mu g/min$)		0.079	0.499

Key: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Table 39. Additional correlation testing by Pearson correlation and Spearman's rho

Additional correlation testing by Pearson correlation unless specified (np)			
		<i>r</i>	<i>P</i>
LDL vs Haemoglobin	All cases	0.335**	0.004
	Controls	-0.104	0.635
	Diabetes	0.446***	0.001
LDL vs Malondialdehyde	All cases	0.362***	0.001
	Controls	0.334	0.111
	Diabetes	0.311*	0.026
LDL vs HbA _{1c} (mmol/mol)	All cases	-0.176	0.135
	Controls	-0.100	0.643
	Diabetes	0.161	0.269
Haemoglobin vs Plasma haemoglobin	All cases	0.044	0.718
	Controls	0.253	0.255
	Diabetes	-0.165	0.262
Haemoglobin vs MDA	Controls	0.084	0.703
	Diabetes	0.207	0.141
MDA vs haematocrit	All cases	0.172	0.139
Reduced ascorbate vs oxi ascorb (μM) (np)	All cases	0.467***	0.000
LDL vs Reduced ascorbate (np)	All cases	0.102	0.395
MDA vs Reduced ascorbate (np)	All cases	0.1783	0.116
Extracellular haemoglobin vs MDA	All cases	-0.110	0.355

Extracellular haemoglobin vs LDL	All cases	-0.117	0.337
Haemoglobin vs LDL	HbA _{1c} Tertile 1 (n = 24)	-0.058	0.789
Haemoglobin vs LDL	HbA _{1c} Tertile 2 (n = 24)	0.495*	0.014
Haemoglobin vs LDL	HbA _{1c} Tertile 3 (n = 24)	0.538**	0.007
Haematocrit vs LDL	HbA _{1c} Tertile 1 (n = 24)	-0.144	0.503
Haematocrit vs LDL	HbA _{1c} Tertile 2 (n = 24)	0.424*	0.039
Haematocrit vs LDL	HbA _{1c} Tertile 3 (n = 24)	0.543**	0.006
HbA _{1c} vs NTBI (np)	HbA _{1c} Tertile 1 (n = 25)	0.018	0.933
HbA _{1c} vs NTBI (np)	HbA _{1c} Tertile 2 (n = 24)	-0.274	0.195
HbA _{1c} vs NTBI (np)	HbA _{1c} Tertile 3 (n = 25)	-0.036	0.866
LDL vs NTBI (np)	No statins (n = 27) Statins (n = 46)	0.545** 0.134	0.003 0.373
Haemoglobin vs NTBI (np)	No statins (n = 27) Statins (n = 46)	0.373 0.408**	0.056 0.005
Haemoglobin vs LDL	No statins (n = 27) Statins (n = 46)	0.472* 0.389**	0.015 0.008
LDL vs MDA	No statins (n = 27) Statins (n = 46)	0.514** 0.130	0.006 0.388
HbA _{1c} vs NTBI (np)	No statins (n = 27) Statins (n = 46)	-0.131 -0.245	0.523 0.101
HbA _{1c} vs NTBI (np) Controls	No statins (n = 14) Statins (n = 9)	0.263 -0.312	0.263 0.413
HbA _{1c} vs NTBI (np) Diabetes	No statins (n = 12) Statins (n = 37)	-0.093 -0.168	0.775 0.320
NTBI vs MDA (np)	No statins (n = 27) Statins (n = 46)	697*** 0.301*	0.000 0.040
Haemoglobin vs MDA	No statins (n = 27) Statins (n = 46)	0.290 0.240	0.142 0.107
NTBI vs Haemoglobin (np)	No statins (n = 27) Statins (n = 46)	0.373 0.408**	0.056 0.005
HbA _{1c} vs Reduced ascorbate (np)	All cases	-0.374***	0.001
HbA _{1c} vs Reduced ascorbate (np)	Controls (n = 24)	-0.041	0.850
HbA _{1c} vs Reduced ascorbate (np)	Diabetes (n = 47)	-0.303*	0.038
HbA _{1c} vs Reduced ascorbate (np)	No statins	-0.405*	0.045
HbA _{1c} vs Reduced ascorbate (np)	Statins	-0.314*	0.038

Key: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; np: non-parametric correlation

4.5.3 The effect of nitrilotriacetic acid on haemoglobin

The SUMMIT NTBI measurements were found to correlate with whole blood haemoglobin, and small quantities of extracellular, plasma, haemoglobin were consistently seen. In order to assess the ability of nitrilotriacetic acid (NTA) to bind iron associated with haemoglobin, potentially forming a species measured as NTBI, a series of tests were undertaken.

To assess the total amount of iron in haemoglobin, bovine haemoglobin (2.5 mg/ml, n = 2) was first measured for total iron by the method of Bothwell (1971), and protein concentration by the method of Bradford (1976), finding a mean value of 24.2 $\mu\text{M}/\text{mg}$. Following protein precipitation, the residual plug had a strong brown colour, indicating that iron may have remained on the protein. It is not clear if this measure was successful, and therefore recovered iron per cent of total has not been calculated for the results below.

4.5.3.1 The effect of nitrilotriacetic acid on haemoglobin, Experiment 1.

To assess the effect of 0.8 M NTA on bovine haemoglobin, concentrations (2 mg and 5 mg) of lyophilised bovine haemoglobin (Sigma lot # 010K7618, no purity given) were dissolved in PBS (1 ml). Aliquots of these (150 μl) were made, and taken through the usual NTBI assay (n = 2), receiving 15 μl of 0.8 M NTA, or PBS for controls.

Experiment 1. Results

	Apparent iron (μM , mean of 2)	
	No NTA	With NTA
2 mg/ml	14.6	24.5
5 mg/ml	25.3	59.3

Table 40. Apparent iron following 0.8 M NTA and NTBI assay

Using 0.8 M NTA with two concentrations of lyophilized haemoglobin, finds that in both cases NTA is associated with increased iron being measured when haemoglobin is separated from the solution (Table 40). The greater concentration of haemoglobin results in increased iron (Figure 64).

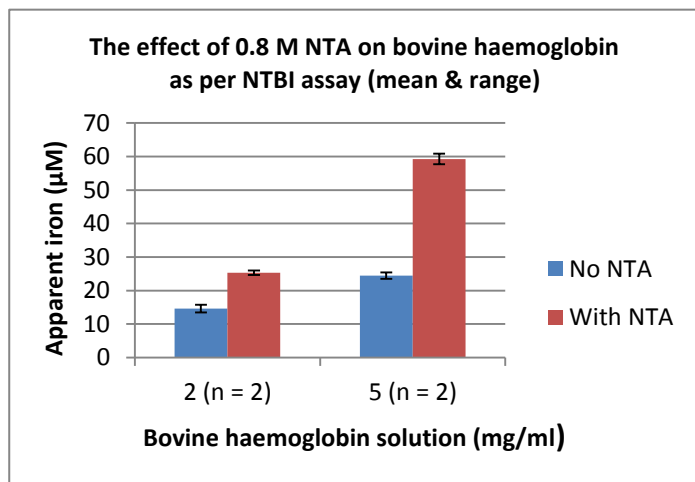


Figure 64. Apparent iron following NTBI assay with and without 0.8 M NTA

NTA leads to more iron being measurable from haemoglobin during the NTBI assay procedure. This may be due to NTA binding loosely-bound haemoglobin-iron; or

binding free iron in solution, perhaps present as a consequence of being prepared from lyophilised protein.

4.5.3.2 *The effect of nitrilotriacetic acid on haemoglobin, Experiment 2.*

To assess the amount of iron removed from haemoglobin by varying concentrations of NTA, single aliquots (500 μ l) of bovine haemoglobin (2 mg/ml) received varying concentrations of NTA (0.05 M to 0.8 M, 50 μ l), followed by 20 minute incubation and the remainder of the usual NTBI procedure.

Experiment 2. Results

Added NTA (M)	Apparent iron (μ M)
0.05	10.9
0.1	10.2
0.2	12.0
0.4	13.7
0.8	14.1

Table 41. Apparent iron (μ M) following varying concentration of NTA, and NTBI assay

Using single aliquots of a constant concentration of dissolved lyophilized haemoglobin, and increasing concentrations of NTA (Table 41), a trend in increased iron is seen, in a dose-dependent manner (Figure 65).

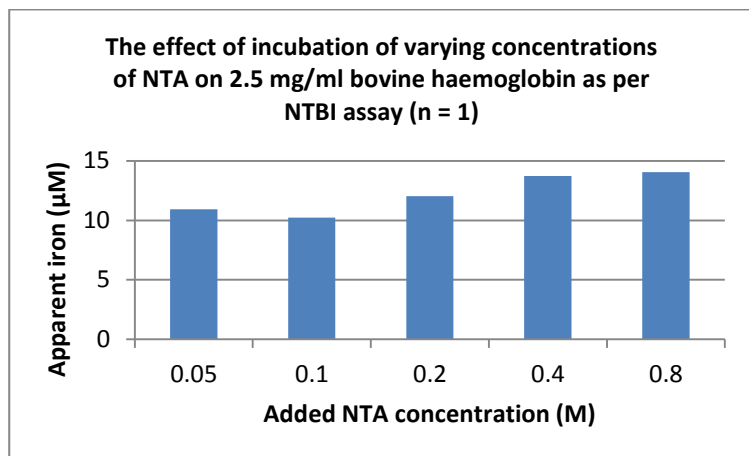


Figure 65. Apparent iron following varying concentrations of NTA
Following assay for non-transferrin-bound iron.

During the NTBI procedure, incubation with NTA results in increases in measurable iron in a generally dose-dependent manner.

4.5.3.3 The effect of nitrilotriacetic acid on haemoglobin, Experiment 3.

To assess the effect of incubation time of bovine haemoglobin (2.5 mg/ml) with 0.8 M NTA, the usual NTBI procedure was performed with and without the 20-minute incubation at room temperature (n = 2).

Experiment 3. Results

	Apparent iron (µM, mean of 2)
No incubation	12.7
20 minute incubation	15.6

Table 42. Apparent iron (µM) following addition of 0.8 M NTA, without and with the 20-minute incubation at room temperature.

Repeating the NTBI assay on further equal aliquots of dissolved lyophilized haemoglobin, but omitting the usual 20-minute incubation at room temperature (Table 42) finds an increase in iron following incubation (Figure 66).

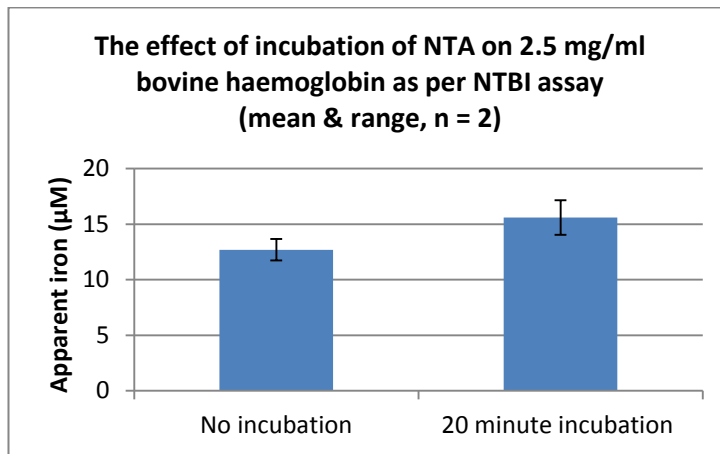


Figure 66. Apparent iron following normal NTBI procedure with and without 20 minute incubation
Following 0.8 M NTA administration.

4.5.3.4 The effect of nitrilotriacetic acid on haemoglobin – conclusion

The studies here give a strong indication that NTA is able to sequester iron associated with lyophilised haemoglobin dissolved in PBS, in a dose-, and time-dependent manner. It is not known if this is due to the nature of haemoglobin in this form, or if the same effect is seen with NTA and extracellular haemoglobin in plasma samples.

4.6 Discussion

The aim of this study has been to evaluate the presence of non-transferrin-bound iron in people with type 2 diabetes and controls, and to assess its association with haemoglobin glycation, lipid peroxidation, antioxidant status, and other iron parameters. Further to these, the comparison of NTBI in people with and without diabetes, and with and without cardiovascular and renal disease has also been undertaken. It can be seen from these results that no positive association is found between NTBI and HbA_{1c} ($r = -0.167$; $P = 0.155$) and there is no significant difference between controls and type 2 diabetes groups for NTBI ($P = 0.326$), as

measured by this assay. The occurrence of NTBI in direct association with protein glycation is not supported here.

The range of levels of NTBI over all cases bears some similarity to that of Lee et al (2006), and Leoncini et al (2008), however the increase in diabetes reported by both groups is not seen, suggesting that the source of NTBI measured by this assay may be different. No correlation is seen between NTBI and extracellular haemoglobin ($r = -0.056$; $P = 0.640$) despite the strong positive association between NTBI and whole blood haemoglobin ($r = 0.386$, $P = 0.001$), suggesting no contribution from this potential source. Neither is an association found between extracellular and whole blood haemoglobin ($r = 0.044$, $P = 0.718$), although it is not clear from the literature whether or not these may be expected to correlate. For the diabetes group here, the correlation hints at being inverse ($r = -0.165$, $P = 0.262$) although the trend is weak, and the probability is poor. A very highly significant correlation is seen between NTBI and MDA ($r = 0.440$, $P < 0.001$), which might be expected given the potential for iron-induced lipid peroxidation.

Across the iron indices results a non-significant trend is seen for a lower iron status in the type 2 diabetes group. These include total iron, transferrin saturation, haemoglobin, red blood cell count, haematocrit, and the NTBI result (Table 34). Readings for ferritin and CRP would be interesting to have in addition, nevertheless the results from this relatively small study indicate a trend towards lower levels of iron by various markers. The highly significantly raised white blood cell count suggests this is probably due to inflammation. Other groups do not agree on iron status in diabetes (Table 30). Lee et al (2006) found non-significantly higher level of total iron and transferrin saturation, and highly significantly more haemoglobin

(and ferritin); Van Campenhout et al (2006) found non-significantly less total iron and transferrin saturation. No clear picture is evident, suggesting perhaps little importance in the overall scheme, although it would be interesting to have haemoglobin values from Van Campenhout et al (2006), Leoncini et al (2008), and Shetty et al (2008).

NTBI significantly positively correlates with total iron ($r = 0.460$, $P = < 0.001$), and transferrin saturation ($r = 0.407$, $P = < 0.001$) in this study (Table 38), in agreement with Lee et al (2006). No such correlations are found by Van Campenhout et al (2006), or reported by the others. As NTBI is normally found to correlate with transferrin saturation (Jacobs et al 2005) this may be due to shortcomings in Van Campenhout et al's NTBI assay. Interestingly, the positive correlation reported here between NTBI and haemoglobin, strengthening with diabetes (Table 38), is in agreement with Lee et al (2006) ($r = -0.11$, $P = 0.07$; $r = 0.22$, $P = 0.14$; $r = 0.37$, $P = < 0.01$; for Controls, Newly diagnosed, and Known diabetes in Lee et al's study). This agreement in correlation is in spite of a disagreement in mean level between controls and diabetes, and although it is difficult to draw firm conclusions it appears that the strengthening correlation between NTBI and haemoglobin in diabetes is independent of haemoglobin level.

To suggest a source of the NTBI measured here, a scenario is proposed whereby MDA (a dicarbonyl compound) is partially the result of erythrocyte lipid membrane peroxidation, leading to cell haemolysis and increased plasma MDA, with further cell membrane and endothelial damage from extracellular haemoglobin, and haem. A number of observations underlie this theory. Firstly, the unexpected correlations of NTBI with haematocrit ($r = 0.355$, $P = 0.002$) and haemoglobin ($r = 0.386$, $P =$

0.001), and the perplexing correlations between NTBI and LDL ($r = 0.324$, $P = 0.005$), and haemoglobin and LDL ($r = 0.335$, $P = 0.004$). Secondly, it is shown here that NTA, at the concentration used in the NTBI assay, can bind iron associated with haemoglobin. Thirdly, there is a growing body of evidence linking altered cholesterol content and composition profile of erythrocyte membranes in atherosclerosis, which is predictive of acute coronary events (Tziakas et al 2007, Shearer et al 2009).

The data showing the ability of NTA to scavenge iron from lyophilised haemoglobin in solution shows a dose, and time-dependent effect. Whether the same occurs with haemoglobin in plasma samples is not known, however Kolb et al (2009) found that NTA concentrations of 10 to 100 mM could influence NTBI measurements in serum following 20 minute incubation and ultrafiltration, in a dose-dependent manner. Kolb et al do not speculate on the source of iron being sequestered by NTA in their unspiked samples, but extracellular haemoglobin might be a donor, based on the results of the small *in vitro* study here.

In acute coronary syndrome, the most vulnerable plaques have a significantly higher level of unesterified cholesterol than is seen earlier in the time-course of CVD, when macrophage in atherosclerotic lesions have more accumulated esterified cholesterol (Tabas 2000). The source of unesterified cholesterol in later-stage plaques is likely to be erythrocyte membranes, which have more unesterified cholesterol than any other cell (Arbustini 2007), and occur alongside increased iron accumulation in the necrotic core of advanced coronary atheroma (Kolodgie et al 2003). The cholesterol content of erythrocyte membranes is acquired via rate-limited exchange between lipoproteins and erythrocytes (Gottlieb 1980), thought

to be independent of circulating cholesterol level, although the mechanism involved is not known (Tziakas et al 2007). Both the total lipid content, and the fatty acid profile of erythrocyte membranes are predictive of acute coronary syndrome (Tziakas et al 2007, Shearer et al 2009), with protective fatty acids being essential n-3 docosapentaenoic acid, and n-6 linoleic acid. Treatment with statins in acute coronary syndrome leads to a significant reduction in erythrocyte cholesterol (Tziakas et al 2009). Zhang et al (2011) have shown that erythrocyte membrane cholesterol is significantly higher in acute coronary syndrome than stable angina pectoris, correlating positively with plasma lipoprotein(a), and negatively with apolipoprotein A-1 (the major apolipoprotein in HDL).

Given the scenario outlined above, with erythrocyte membrane damage and release of haemoglobin, extracellular haemoglobin might be expected to correlate with NTBI if NTBI is a product of this source, but this is not seen. Extracellular haemoglobin level may be thought of as a consequence of rate of haemolysis, and rate of haptoglobin clearance, however the saturating effect of haptoglobin (400 molar excess, Asleh & Levy 2005) should ensure none is normally present. If NTBI is occurring from this source then either haptoglobin capacity must be overwhelmed, the haptoglobin-haemoglobin bond altered, or NTA is able to scavenge iron from haptoglobin-bound haemoglobin. As haptoglobin-haemoglobin binding is normally irreversible, with an association constant greater than 10^{15} mol/l (Handin et al 2003) the last option seems unlikely. Levels of haemoglobin between 0.07 to 0.15 g/dl can normally be bound by haptoglobin (Langlois & Delanghe 1996), and haemoglobin levels in episodes of severe haemolytic disease can exceed 1.0 g/dl (Rother et al 2005). Plasma haemoglobin reported here ranges from 0.0 to 0.4 g/dl,

therefore the haptoglobin capacity may be exhausted. Considering an alteration in binding functionality, it is interesting to note that Jia et al (2007) have described haemoglobin oxidation by H_2O_2 affecting two particularly vulnerable cysteine residues in the β -globin chain, causing irreversible change to cysteic acid. Loss of haem pocket structure occurs, with cross-linking between α -globin chains, which is associated with reduced haptoglobin binding.

A further source of NTBI could be haem, from haemoglobin degradation, perhaps by the effect of glycation weakening the haem-globin linkage (Sen et al 2005), as described in Chapter 1. This would also explain the lack of correlation between NTBI and plasma haemoglobin, as Drabkin's reagent in the haemoglobin assay specifically measures haemoglobin following its oxidation to ferric methaemoglobin. Plasma haem is highly pro-oxidant if not complexed by haemopexin or albumin, and as smaller than haemoglobin will be more invasive of the sub-endothelium. Haem is hydrophobic, and can be rapidly taken up by cell membranes and LDL (Balla et al 2003). Both haemoglobin and haem are known to be potent extracellular oxidants, producing nitric oxide depletion, vascular dysfunction including hypertension, and renal damage from glomerular filtration (Kato 2009). Extracellular haemoglobin and haem perform all the roles that NTBI is implicated for in CVD, and are plausible NTBI sources in diabetes.

A significant decrease in MDA is seen in the diabetes group compared with controls ($P = 0.041$), and a very highly significant decrease in LDL ($P < 0.001$). This probably reflects the more aggressive use of statins for cholesterol lowering, used by 71.7% of the diabetes group compared with 39.1% of controls (Table 43). Significant

differences in LDL are seen across groups for controls and diabetes, and without and with statins (Figure 67).

Table 43. Group characteristics and NTBI – use of statins

Group characteristics – use of statins			
Use of statins	No Statins (n)	Statins (n)	Total (n)
Control	14 (60.9%)	9 (39.1%)	23
Diabetes	15 (28.3%)	38 (71.7%)	53
Total	29	47	76
NTBI (μM , median & range)	No statins	Statins	<i>P</i>
	0.32 (0.00 to 1.02)	0.22 (0.00 to 1.34)	0.479
LDL (mM mean \pm SD)	2.73 \pm 1.42	1.75 \pm 0.94	0.001**
Malondialdehyde (μM mean \pm SD)	2.48 \pm 1.16	1.94 \pm 1.04	0.039*
Reduced ascorbate (μM) ¹	29.1 (2.2 to 67.6)	21.5 (0.0 to 60.0)	0.291

Key: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ¹ median and range

Whilst NTBI and LDL correlate across all cases (Figure 42), no significant difference in NTBI is seen across groups when controls with and without statins, and diabetes with and without statins are compared separately ($P = 0.513$) (Figure 68).

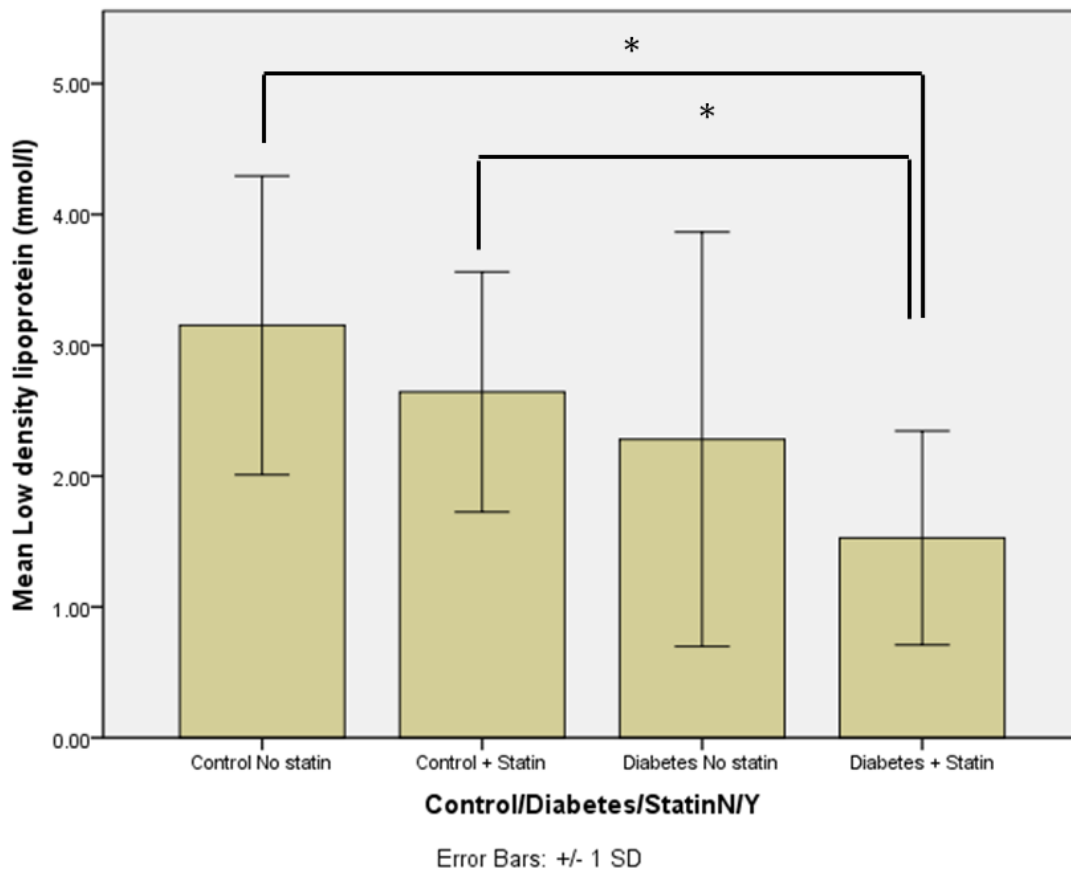


Figure 67. Low density lipoprotein in Controls and Type 2 Diabetes, without and with statins medication

Controls without and with statins (n = 14, and n = 9), Type 2 diabetes without and with statins medication (n = 15, and n = 38) ($P < 0.001$ between Control No statin and Diabetes + Statin; $P = 0.037$ between Control + Statin and Diabetes + Statin).

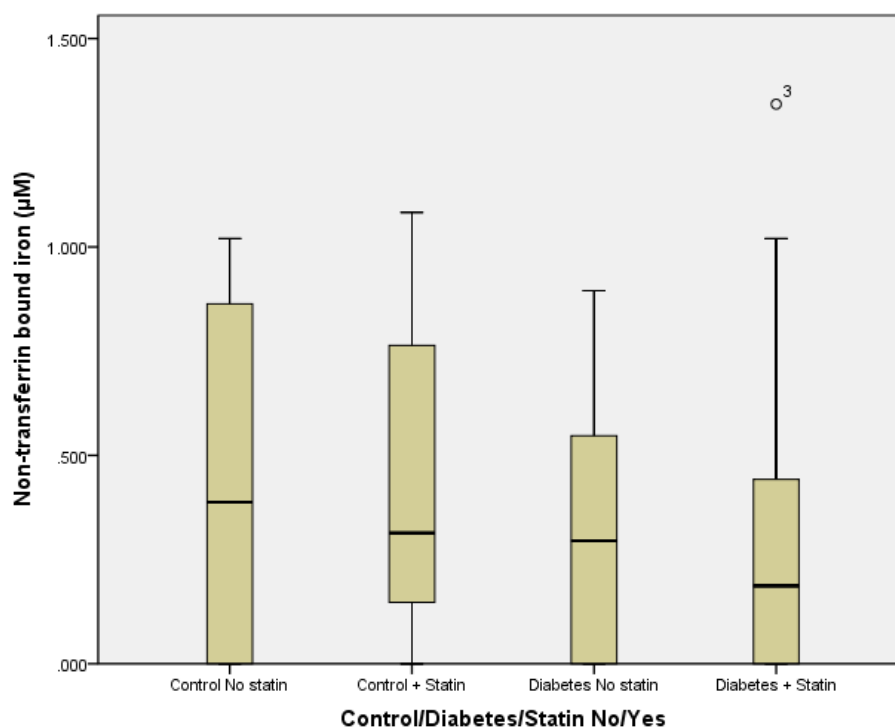


Figure 68. NTBI in Controls and Type 2 Diabetes without and with statins medication

Controls without and with statins (n = 14, and n = 9), Type 2 diabetes without and with statins (n = 15, and n = 38)

To explore further the correlation between haemoglobin and LDL, significant only in diabetes, and negatively associated in controls, the data was stratified by HbA_{1c} into tertiles and further correlations performed. The relationship strengthens significantly with HbA_{1c} (Table 44, Figure 69, Figure 70, Figure 71).

Table 44. Correlation tests between whole blood haemoglobin and LDL by HbA_{1c} tertile

Tertile HbA _{1c} (mmol/l)	Correlation between haemoglobin and LDL by HbA _{1c} tertile					
	1 (< 42.0) n = 24		2 (42.0 to 53.0) n = 24		3 (> 53.0) n = 24	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Haemoglobin vs LDL	-0.058	0.789	0.495*	0.014	0.538**	0.007

Key: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

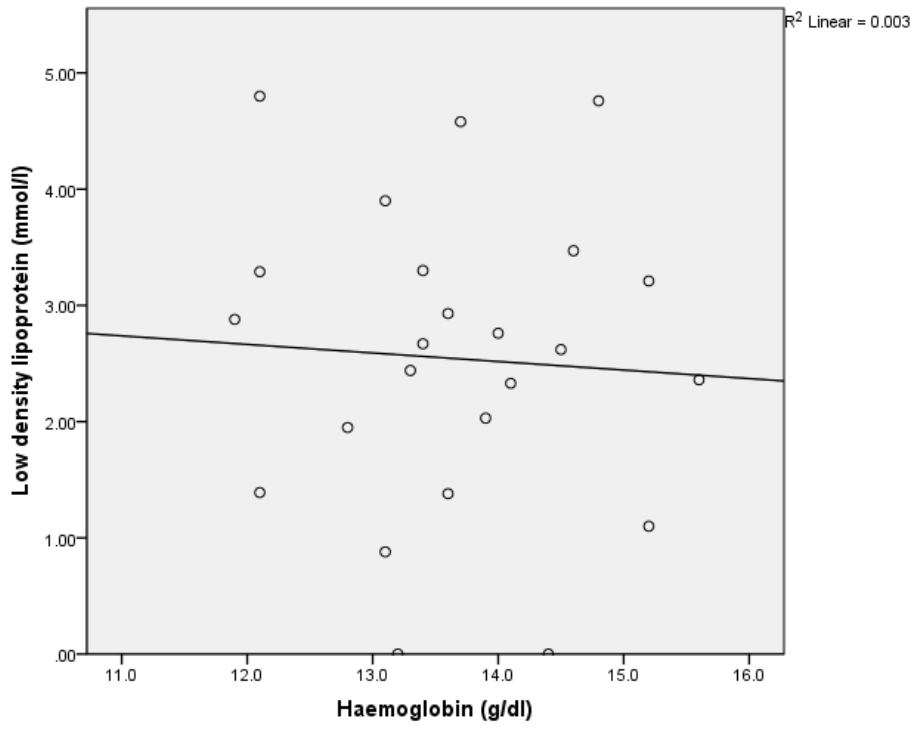


Figure 69. Haemoglobin vs LDL stratified by HbA_{1c}, Tertile 1
 (n = 24, r = -0.058, P = 0.789)

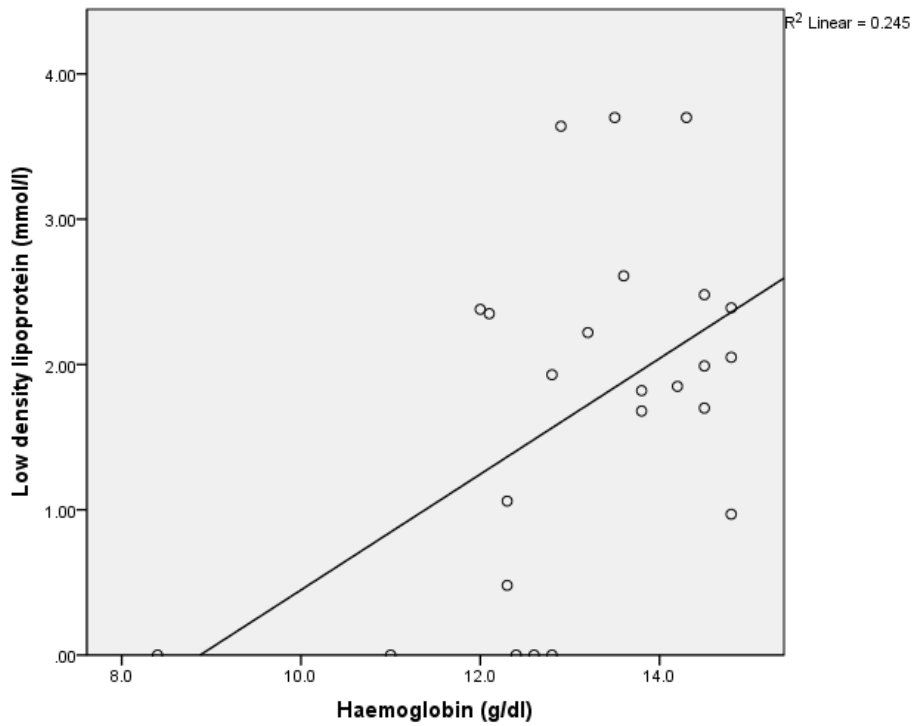


Figure 70. Haemoglobin vs LDL stratified by HbA_{1c}, Tertile 2
 (n = 24, r = 0.495*, P = 0.014)

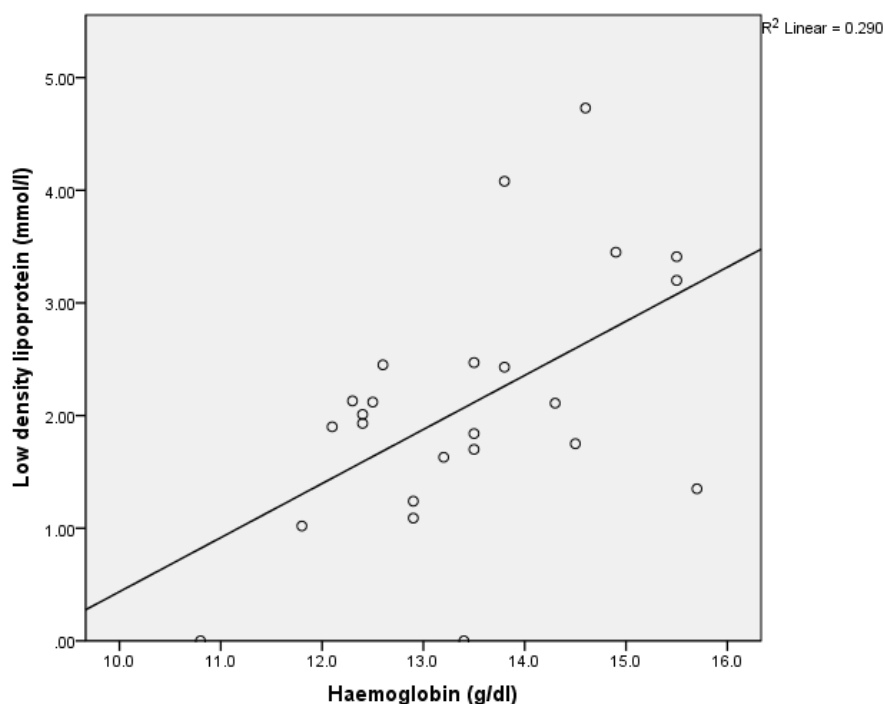


Figure 71. Haemoglobin vs LDL stratified by HbA_{1c}, Tertile 3
(n = 24, r = 0.538**, P = 0.007)

Increased HbA_{1c} clearly associates with increased positive association between whole blood haemoglobin and LDL. If haemoglobin/haematocrit-correlated plasma LDL results from erythrocyte haemolysis and lipid release followed by hepatic LDL synthesis, then it is increased by some factor relating to glycaemia. Erythrocyte membrane alterations in diabetes have long been identified, including increased glycation across the range of membrane proteins (Miller et al 1980), with 952 different erythrocyte membrane proteins found to be glycated, by mass spectrometry (Zhang et al 2011). Increased membrane lipid peroxidation occurs in diabetic erythrocytes, significantly correlating with HbA_{1c} (Jain et al 1989), decreased deformability and unesterified membrane cholesterol (Garnier et al 1990); and membrane spectrin glycation and oxidation (Schwartz et al 1991). Erythrocyte fragility increases with HbA_{1c} in type 2 diabetic patients (Kung et al

2009), and similarly, erythrocyte fragility increases in patients with type 2 diabetes (Lippi et al 2012). A scenario of lipid and protein glycation, oxidation, and ROS-driven oxidative damage would fit this picture, and the significant correlation seen between MDA and LDL in diabetes ($r = 0.311$, $P = 0.026$) may support this.

Further support for this scenario of lipid and protein glycation, oxidation, and ROS-driven oxidative damage may be provided by the results of correlations between HbA_{1c} and reduced ascorbate in this study. With all cases pooled, a highly significant negative correlation is seen ($r = -0.374$, $P = 0.001$), however no correlation is seen in controls ($r = -0.041$, $P = 0.850$). In the diabetes group the negative correlation is significant ($r = -0.303$, $P = 0.038$) (Table 39, Figure 72, Figure 73, Figure 74).

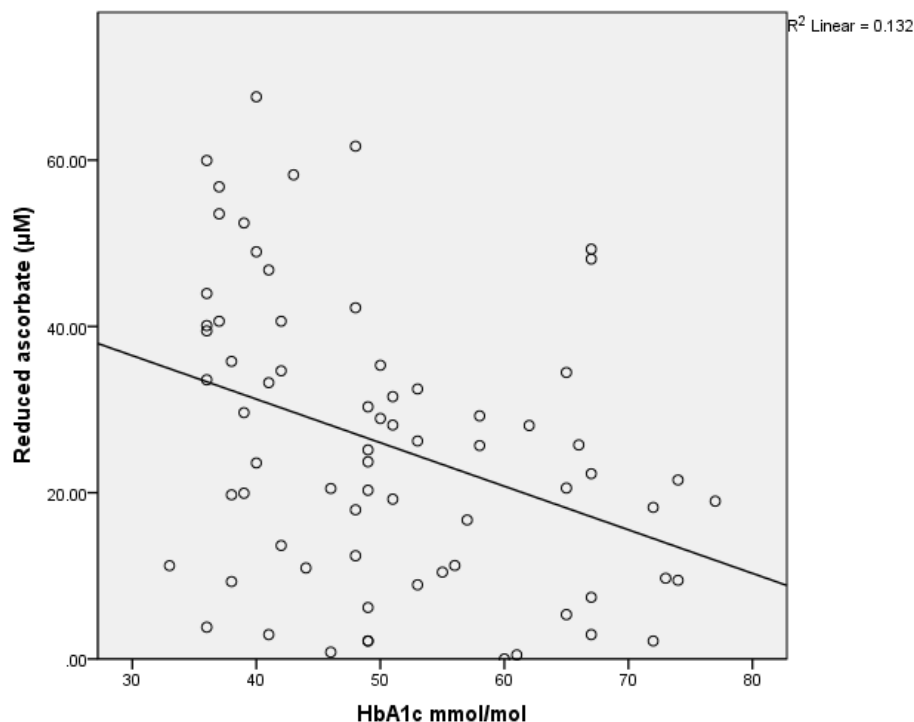


Figure 72. HbA_{1c} vs ascorbate, all cases
($n = 78$, $r = -0.374^{***}$, $p = 0.001$)

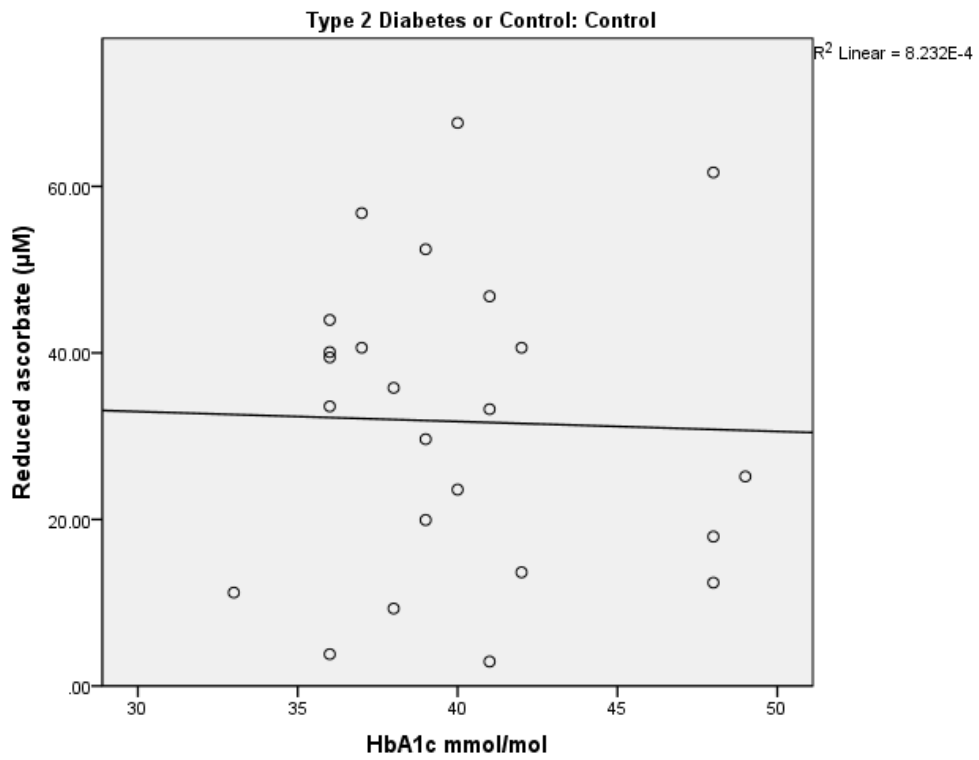


Figure 73. HbA_{1c} vs ascorbate, Controls
 (n = 24, r = -0.041, P = 0.850)

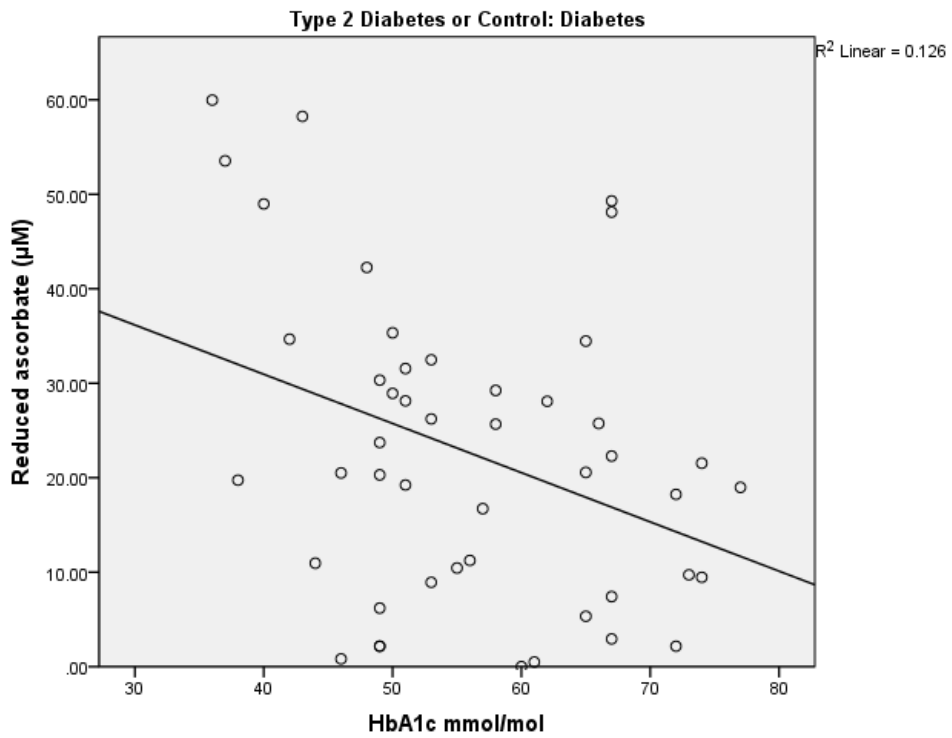


Figure 74. HbA_{1c} vs ascorbate, Type 2 Diabetes
 (n = 47, r = -0.303*, P = 0.038)

The theory of glucose-linked erythrocyte membrane damage leading to plasma haemoglobin and haem suggests that the relationship between HbA_{1c} and NTBI should strengthen with diabetes. This is not the case however, and the trend becomes negative in diabetes (Controls, $r = 0.130$, $P = 0.544$; Diabetes, $r = -0.120$, $P = 0.408$, Table 38). Likewise it seems surprising that NTBI is found here to be significantly correlated with LDL in controls ($r = 0.481$, $P = 0.017$), but not in diabetes ($r = 0.187$, $P = 0.189$), and not correlated with total cholesterol ($r = 0.169$, $P = 0.147$) (Table 38). Table 45, below, describes the mean and median levels of key parameters and their interaction by correlation. It can be seen that the mean level of LDL in controls is significantly higher than diabetes, probably due to the increased use of statins in the diabetes group. According to the theory, a proportion of plasma cholesterol will be derived from haemolysis, this proportion increasing with glycation in diabetes. Due to inflammatory anaemia, iron status and subsequently haemoglobin and haematocrit are lessened in diabetes, resulting in less free haemoglobin and haem (measured as NTBI) despite an increased rate of red cell lysis. Inflammation would also cause up-regulation of haptoglobin and hemopexin as acute phase proteins, reducing plasma haemoglobin and haem. Statins would up-regulate cholesterol take-up by the liver. The combination of inflammation and statins therapy results in no correlation between LDL and NTBI being seen. In controls, the strong correlation between LDL and NTBI would be due to higher levels of iron generally, greater haematocrit, more plasma cholesterol without statins to lessen cholesterol resulting from “normal” haemolysis, with a concomitant increase in NTBI due to lower levels of haptoglobin and hemopexin.

Table 45. Profile of selected results, showing mean and median values, and different associations between controls and diabetes, and without and with statins

Profile of selected measures						
	NTBI (μM) Median	Hb (g/dl) Mean	MDA (μM) Mean	NTBI (μM) Median	LDL (mM) Mean	MDA (μM) Mean
Correlation in Controls (r)	0.376	0.084	0.707***	0.481*	0.334	
Controls	0.34	13.7	2.48	0.34	3.1	2.48
Diabetes	0.21	13.3	2.00*	0.21	2.0*	2.00*
Correlation in Diabetes (r)	0.395*	0.207	0.311*	0.187	0.311*	
No statins	0.32	13.2	2.48	0.32	2.7	2.48
Statins	0.22	13.5	1.94*	0.22	1.7**	1.94*
Correlation, No statins (r)	0.373	0.290	0.697**	0.545**	0.514**	
Correlation, Statins (r)	0.408**	0.240	0.301*	0.134	0.130	

Key: NTBI, non-transferrin-bound iron; Hb, whole blood haemoglobin; MDA, malondialdehyde; LDL, low density lipoprotein; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Controls, $n = 24$; Type2 diabetes, $n = 53$; No statins, $n = 27$; Statins, $n = 46$.

See Table 38 and Table 39 for ranges and SD.

Splitting the cases by use of statins therapy finds a non-significant difference in NTBI between groups (median 0.32 without statins compared to 0.22 μM with statins, $P = 0.479$; Table 43, and Table 45, above). In exploration of a possible protective effect on NTBI by statins, correlations were performed on the two groups (Table 39, and Table 45), finding a highly significant positive correlation between LDL and NTBI without statins ($n = 27$, $r = 0.545$, $P = 0.003$), which is lost with statins therapy ($n = 46$, $r = 0.134$, $P = 0.373$). The positive correlation between haemoglobin and NTBI remains constant, becoming significant in the statins group due to the larger group size. The significantly lower LDL with statins ($P = 0.001$) is reflected in a significantly lower MDA level ($P = 0.039$), and a non-significant trend

for less ascorbate ($P = 0.291$) (Table 43). Correlation tests between NTBI and HbA_{1c} with and without statins and diabetes, find no significance (Table 39). Further work needs to be done to understand the interaction between LDL and NTBI, which is disrupted by statins.

The scenario of glycation, oxidation, and haemolysis implies an influence on plasma LDL level by haemolysis and hepatic LDL synthesis. However, when a rough calculation is performed to show the relative quantities of cholesterol potentially deriving from haemolysis, the figure is quite low. Total unesterified erythrocyte cholesterol per gram haemoglobin is 3.63 ± 0.21 mg/g (Farquhar 1962, in Beutler et al 2001), therefore if 10% of erythrocytes are lysed in the blood stream (Garby & Noyes 1959), plasma haemoglobin may equal 10% of all haemoglobin, i.e. ~ 1.4 g/dl, then ~ 5.1 mg/dl cholesterol will also be present, with a molecular weight of 385.65 g/mol, contributing ~ 132.0 μ M to a typical plasma cholesterol level of ~ 5.0 mM.

From the rough calculation above lysed erythrocyte cholesterol may not appear to contribute significantly to overall cholesterol levels, unless haemolysis is greatly increased, and therefore further explanations may be needed to explain the correlation between haemoglobin and LDL in diabetes. Not all the mechanisms by which statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) operate are understood. Essentially, statins lower plasma LDL by increasing clearance from the circulation, and reducing the production of apoB-containing lipoproteins by the liver. This latter is altered in diabetes by the effect of insulin resistance; and additionally, insulin deficiency leads to reduced LDL receptors and altered LDL composition (for review see Ginsberg 2006). Other actions of statins

are known, such as inhibition of isoprenoids, which are intermediates of cholesterol synthesis, and required for subsequent signalling pathways and GTP-binding protein function; also statins may induce peroxisome proliferator-activated receptors (PPARs), which would have an anti-inflammatory effect (Zhou & Liao 2009). Endothelial function is also improved by statins independently of LDL level, possibly by up-regulation of endothelium nitric oxide synthase (eNOS) and therefore NO (Hamilton et al 2010). All statins have at least one hydroxyl group (as does cholesterol) and it is interesting to speculate on a possible iron-binding capacity contributing to its anti-inflammatory effects and other pleiotropic benefits.

Interestingly, haemoglobin-bound cholesterol has been identified in erythrocytes in healthy people (Nikolic et al 2003), within both the cell membrane and the cell itself. Binding stoichiometry is two molecules each of cholesterol and phospholipid per haemoglobin molecule. In haemolysate, haemoglobin-lipid adduct ranged from 0.2% to 55.0% of total haemoglobin ($n = 36$), and correlated positively with plasma HDL ($r = 0.57$, $P < 0.01$). Lower levels of haemoglobin-lipid adduct are found in cell membranes. The authors speculate that erythrocytes may have a defensive role removing free cholesterol from circulation; however, in a diabetic and haemolytic scenario this grouping might be a potent source of ROS following glycation of the haemoglobin.

In summary, the results for NTBI, LDL, haemoglobin, haematocrit, and MDA, found by this study may be explained by Figure 75, below.

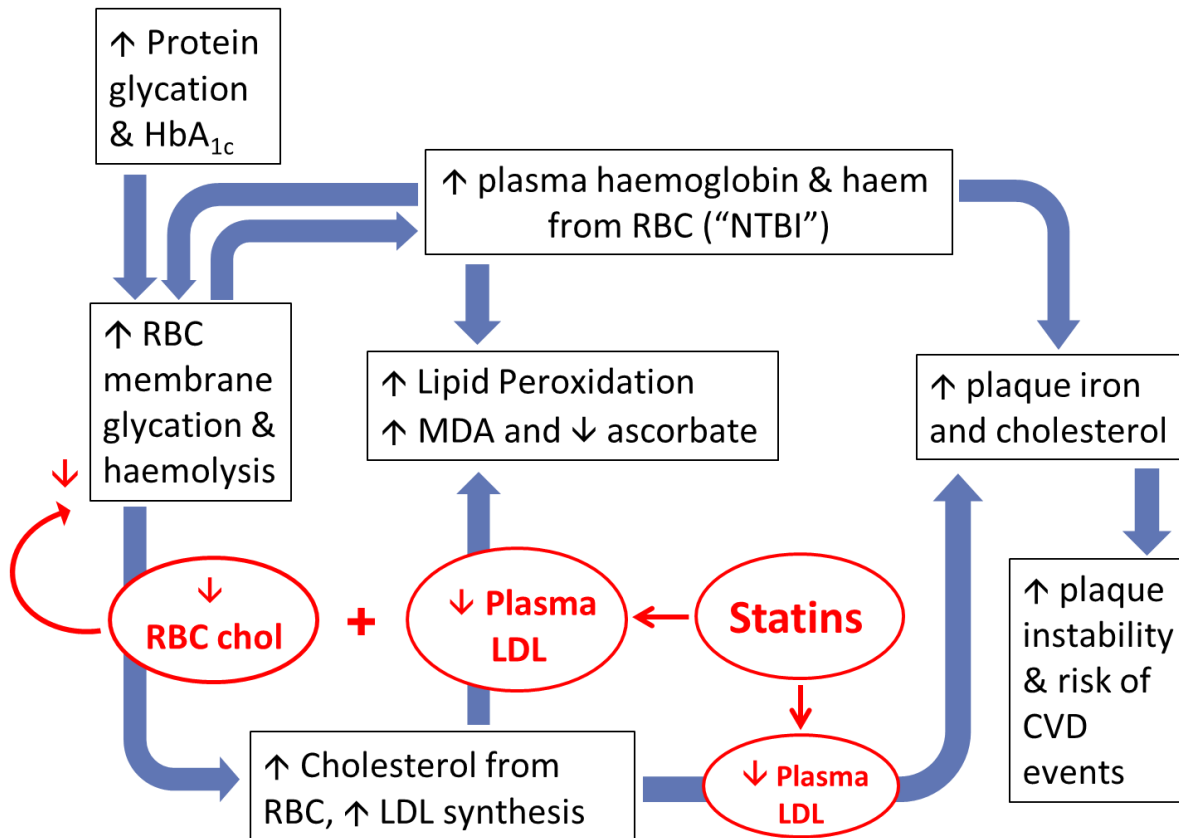


Figure 75. The proposed role of protein glycation in plasma NTBI and LDL, and the protective effects of statins

NTBI results from erythrocyte membrane glycation, oxidation, and subsequent haemolysis (purple pathways), with increased plasma MDA and decreased ascorbate indicating lipid peroxidation and oxidative damage. Plasma haemoglobin from haemolysis oxidizes to methaemoglobin, and degrades to haem, becoming vulnerable to incorporation in atherosclerotic plaques, where, alongside unesterified cholesterol, it can promote hydroxyl radicals and further oxidative damage. The use of statins lowers plasma LDL (red pathway), reducing the cholesterol available for erythrocyte membrane take up, and availability for lipid peroxidation, and thereby decreasing haemolysis and the appearance of NTBI.

The scheme described by Figure 75 shows the plasma iron and plasma cholesterol products of haemolysis as occurring independently, however they might also be bound together, as found in haemolysate by Nikolic et al 2003).

4.6.1 Limitations

There are several limitations to this study. Our assay includes NTA mobilisation, and therefore may not measure glycated albumin-bound iron, as found by Silva & Hider (2009). Our results may therefore underestimate total NTBI levels in people with diabetes. In addition, given the mechanisms discussed in Chapter 1, haptoglobin genotype will influence CVD risk in diabetes. This would be a valuable measure, but due to financial limitations this has not been possible. The measurement of serum ferritin is planned, but these results are not included in the thesis due to timing.

The study is also limited by lack of numbers for the measurement of NTBI in renal disease. Measurement of urine iron would be an interesting contribution to the renal parameters.

The use of statins is associated with decreased NTBI here. Angiotensin-converting-enzyme (ACE) inhibitors and angiotensin II type I receptor blockers (ARB) were also compared, but found to have no effect on NTBI when tested (results not shown). Other medications taken by participants have not been considered however, and these may have an effect on results.

4.6.2 Future work

A theory to explain the correlations between NTBI and LDL; NTBI and haemoglobin; and LDL and haemoglobin in diabetes is suggested, but work needs to be carried out to test the theory and define the processes involved. In addition, the various statins compounds, and cholesterol, could be tested for iron binding function. Mass spectrometry has previously been used to examine erythrocyte membrane proteins for post-translational modifications by Zhang et al (2011) (see Chapter 3), who

found that erythrocyte proteins were more extensively glycosylated than plasma proteins in people with diabetes, in all 952 different membrane, and 853 cytosol proteins. Spectrin alpha and beta chain were consistently up-regulated in diabetes amongst others – perhaps in response to glycation and oxidation-related losses? Increased lipid peroxidation significantly correlates with HbA_{1c} in diabetes (Jain et al 1989), and erythrocyte fragility increases in type 2 diabetes, with HbA_{1c} (Lippi et al 2012, Kung et al 2009), but further laboratory studies could be carried out on erythrocytes to link membrane protein glycation, lipid peroxidation, and the appearance of haemoglobin and haem.

In addition, it has been shown that the formation of advanced glycation end-products (AGE) on the surface of diabetic erythrocytes bind to specific AGE receptors on endothelial cells (Wautier et al 1994), and therefore haemolysis *in situ* should also be considered.

4.7 Conclusions

In people with type 2 diabetes and controls, with and without CVD and CKD, no association between NTBI and HbA_{1c} is seen. Utilizing HbA_{1c} as a marker of protein glycation indicates that no correlation is found between NTBI and protein glycation. Unexpected correlations between NTBI and LDL, and LDL and haemoglobin with increasing protein glycation, are reported for the first time. NTBI is suggested to be iron sourced from haemoglobin or haem, from erythrocyte haemolysis prior to sample collection, possibly facilitated by nitrilotriacetic acid scavenging during laboratory assay. Reduction of LDL by statins therapy is associated with less NTBI in the diabetes group. NTBI is measured in people with pre-dialysis CKD stages I to III for the first time, finding no significant difference in NTBI level between groups, or

correlation with markers of renal function. No association between NTBI and kidney function at this stage of disease is proven.

Epilogue

5.1 Prevention and intervention of non-transferrin-bound iron

On the assumption that NTBI as measured here will be a contributing factor to iron build-up in atherosclerotic plaques, and potentially damaging to the sub-endothelium prior to atherogenesis, efforts should be made to prevent or lessen it. The positive relationship described between LDL and NTBI, despite the lack of understanding of its nature, suggests that reducing high plasma LDL by statins is beneficial. The significant reduction in MDA in the statins group indicates that lipid peroxidation is occurring, correlating highly significantly with LDL without statins ($P = 0.006$), but not with statins ($P = 0.388$, Table 39), and therefore measures to protect against lipid peroxidation can be recommended, primarily by maintaining a good daily intake of dietary vitamin E.

As individual requirements for vitamin E depend on dietary intake of polyunsaturated fatty acids (PUFA), no specific dietary reference values are published. However, minimum levels are set at > 4 mg/day for males (5.9 IU), and > 3 mg/day for females (4.5 IU) (DH 1991), with safe upper levels set at 540 mg α -tocopherol equivalents/day (805.9 IU) (EVM 2003). Most dietary vitamin E is consumed as fats (spreads, margarines and oils), fried snacks, cereal foods, and meat and meat products, with the most concentrated vitamin E levels being in seed and corn oils (Henderson et al 2003). As these are largely n-6 fatty acids, and the n-6:n-3 fatty acid ratio is important for anti-inflammatory and cardio-protective effects (Appendix 1.v., Calder 2006, Kinsella et al 1990), oily fish should be included in the diet, with intakes of at least one 140 g portion/week recommended for the

general population (Mozaffarian et al 2008). For those with a prior cardiovascular event, intakes should be between two and four portions of oily fish per week, giving > 7 g of n-3 fatty acids (Kris-Etherton et al 2002). People with diabetes should include oily fish in their diet at least twice a week, in line with their raised risk for cardiovascular events. In addition to their known anti-arrhythmic and anti-inflammatory properties, n-3 fatty acids are important structural components of cell membranes, and able to lower plasma cholesterol levels (Connor 2000).

For people with diabetes, dietary iron intakes should follow normal recommendations unless iron status is outside the normal range (Section 1.4.3, and Appendix 1.iii). In general, a diet high in wholegrains, pulses, vegetables, fruit, low fat dairy and oily fish should be followed, limiting intakes of saturated and trans fatty acids (NCCCC 2008).

5.2 Overview of work and conclusions

This thesis has undertaken to explore the roles that iron might play in the development and pathologies of type 2 diabetes. From a review and discussion of the literature, in the development of type 2 diabetes a proportion of people with the metabolic syndrome, and non-alcoholic fatty liver disease, will have insulin resistance-associated hepatic iron overload, and, with the metabolic syndrome, will have an increased risk for diabetes. Whilst the build up of iron in the liver is thought to influence insulin resistance, it is not known by what mechanism this occurs. Hepatic-iron build up may take place in those with a predisposing genotype, occurring alongside the progressively increasing risk for diabetes with obesity, inflammation, dyslipidemia, and hypertension.

The literature for increased physiological iron status, as measured by serum ferritin, and soluble transferrin receptor to ferritin ratio, finds a positive association with risk for type 2 diabetes, which is not reduced by regular blood donation. Studies that assess physiological iron status and risk for diabetes by using serum ferritin alone, and which control for inflammation with CRP, may be inadequately controlling for inflammation.

The literature for dietary iron intake and risk for type 2 diabetes suggests that processed red meat is associated with risk (Aune et al 2009, Micha et al 2010), with high intakes of red meat possibly also attached to a small increased risk. There may be a role for iron in the development of advanced lipoxidation products (ALE) during processing, and a mechanism is described that might account for a link between ALE and pancreatic insulin secretion (Peppas et al 2002, Zhao et al 2009). Laboratory-based experiments should be undertaken to determine the involvement of iron in ALE development of different foods, with consideration of the lipid composition of the foods.

In the development of diabetes-related pathologies, the effects of high plasma glucose levels are suggested by numerous authors to lead to functional damage to glycosylated proteins. Glycation of iron-related proteins, and haptoglobin genotypes, are discussed. Further research to determine risk of diabetes complications other than CVD by haptoglobin genotype, is needed.

A sequence of *in vitro* experiments was carried out to test the functional capacity of glycosylated transferrin. These show that *in vitro*, glycosylated transferrin undergoes fragmentation, explaining the loss of function reported by earlier groups. Working

with the University of Bristol Proteomics Facility, *in vitro* glycated transferrin was examined by Thermo Orbitrap mass spectrometry (MS) to chart glycated amino acids in addition to lysine for the first time. A substantial number and range of residues were found to be subject to glycation, including Lys365 and Ile366, residues involved with transferrin receptor binding. Comparison with *in vivo* glycated transferrin MS by Waters suggests that many of the *in vitro* glycation sites are not glycated *in vivo*, and that many of the others involve methionine, therefore are potential MS artefacts, or likely to be repaired by methionine sulphoxide reductases *in vivo*. Further work by Orbitrap mass spectrometry of transferrin samples from people with and without diabetes should be undertaken to establish the extent and positioning of glycation *in vivo*.

In collaboration with the Diabetes and Vascular Medicine Research Group, University of Exeter, a study has been made of a cohort of people with type 2 diabetes, and controls, with and without cardiovascular disease (CVD) and chronic kidney disease (CKD). No association between non-transferrin-bound iron (NTBI) and HbA_{1c} is seen, and therefore, utilizing HbA_{1c} as a marker of protein glycation generally, no direct correlation with protein glycation. Unexpected correlations between NTBI and LDL, and LDL and haemoglobin with increasing protein glycation, are reported for the first time. NTBI is suggested to be iron sourced from haemoglobin or haem, from erythrocyte haemolysis prior to sample collection, possibly facilitated by nitrilotriacetic acid scavenging during laboratory assay. Erythrocyte membrane fragility may be increased by membrane protein glycation, and proximal lipoxidation. Reduction of LDL by statins therapy is associated with less NTBI in the diabetes group. NTBI is measured in people with pre-dialysis CKD

stages I to III for the first time, finding no significant difference in NTBI level between groups, or correlation with markers of renal function. No association between NTBI and kidney function at this stage of disease is proven.

Further work should be undertaken to define the link between NTBI, haematocrit, and erythrocyte and plasma cholesterol. Statins, and cholesterol, should be investigated for iron binding function, and the role of cholesterol-bound-haemoglobin or haem considered in the development of insulin resistance-associated hepatic iron overload.

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Appendices

Appendix 1.i. Classification of stages in chronic kidney disease

Table 1. NICE Guideline for classification of stages in chronic kidney disease

	Stage I	Stage II	Stages III a & III b	Stage IV	Stage V
Albuminuria (mg/24 h)	<20	20-300	≥300 (up to 15 g/day)	≥300 (up to 15 g/day)	≥300 (Can fall)
Glomerular filtration rate (ml/min/1.73 m ²)	Normal/increased ≥90	Slight decrease 60-89	Moderate decrease a) 45-59 b) 30-44	Severe decrease 15-29	Established renal failure <15
Serum creatinine (µmol/l)	Normal 60-100	Normal 60-100	High normal 80-120	High 120-400	Very high >400
Blood pressure (mm Hg)	Normal 90/60 to 120/80*	Slightly increased 120/80 to 140/90*	Increased >140/90*	Increased >140/90*	Increased >140/90*
Clinical signs	None	None	Anaemia ± oedema Increased blood pressure May be none	Anaemia ± oedema Increased blood pressure May be none	Anaemia ± oedema Increased blood pressure Uraemic symptoms

* General ranges, Blood Pressure Association 2008

Table 2. Diagnostic categories for microalbuminuria, macroalbuminuria, and proteinuria (adapted from Watkins 2003, and NICE 2008)

<p><i>Microalbuminuria</i></p> <ul style="list-style-type: none"> ▪ Urinary albumin excretion rate 30-300 mg/24 h ▪ Albumin creatinine ratio >2.5 mg/mmol/l (men) >3.5 mg/mmol/l (women) ▪ Urinary albumin concentration >20 mg/l 	<p><i>Macroalbuminuria</i></p> <ul style="list-style-type: none"> ▪ Urinary albumin excretion rate >300 mg/24 h <p><i>Proteinuria</i></p> <ul style="list-style-type: none"> ▪ Urinary protein excretion >500 mg/24 h ▪ Albumin creatinine ratio >30 mg/mmol ▪ Urinary albumin concentration >200 mg/l
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Appendix 1.ii. Non-transferrin-bound iron in various conditions

Table 1. Occurrence of non-transferrin-bound iron (NTBI) in various conditions					
Patient group	Study population	Transferrin saturation % mean (range)	NTBI ($\mu\text{mol/L}$) mean (range) or SD	NTBI measurement method used	References
Healthy controls	256 healthy male and female non-blood donors, aged 61.3 ± 5.7 yrs	Range of means 30 to 34%	2.51 ± 0.80	Breuer & Cabantchik (2001) Fluorescence based one step assay	Engberink et al (2008)
	17 healthy controls	25% (17 to 43%)	-0.30 (-1.50 to 0.60)	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	De Valk et al (2000)
	63 healthy controls, aged 51 ± 12 yrs	$35 \pm 11\%$	0.154 ± 0.328	Breuer & Cabantchik (2001) Fluorescence based one step assay	Le Lan et al (2005)
	47 healthy controls	$32 \pm 15.4\%$	0.04 ± 0.13 . NTBI detected in 10.6% of sample	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	Lee et al (2006)
	20 healthy controls, aged 34 ± 7 yrs	Unknown	4.35 ± 0.85 S.E.M. (Fe ²⁺ 23%)	Nilsson et al (2002) BPS spectrophotometry	Prakash et al (2005)
	30 healthy controls, aged 24.5 ± 9.0 yrs	$38.1 \pm 11.5\%$	-1.48 ± 0.48	Singh et al (1990) ultrafiltration. Modified by adding AlCl ₃ to prevent NTBI shuttling	Walter et al (2008)
	17 healthy controls, aged 23 to 40 yrs	29% (19 to 41%)	-0.87 (-1.27 to -0.75)	Singh et al (1990), Porter et al (1996) NTA HPLC	Cighetti et al (2002)
Acute myocardial infarct	322 patients within 24 hr of AMI	Unknown	0.14 ± 0.23	Esposito et al (2003) DFO fluorescence	Suliman et al (2004)
Ageing	41 healthy individuals	Unknown	Positive correlation labile cellular iron with ageing	Flow cytometry	Doulias et al (2008)

African iron overload	25 African patients with iron overload, and 170 relatives & neighbours	Unknown	1.47 ± 1.75 (undetectable to 7.9)	Al-Refaie et al 1992 NTA HPLC	Mcnamara et al (1999)
Alcohol abusers	a) 29 active drinkers with cirrhosis, aged 55 ± 11 yrs	a) 67 ± 27%	a) 2.22 ± 2.49	Singh et al (1990), Porter et al (1996) NTA HPLC	De Feo et al 2001
	b) 14 active drinkers without cirrhosis, aged 55 ± 9 yrs	b) 46 ± 22%	b) 0.59 ± 1.64		
Alcoholic cirrhosis	33 patients, with known absence of other causes of liver damage, aged 52 ± 10yrs	56 ± 34%	0.385 ± 0.560	Breuer & Cabantchik (2001) Fluorescence based one step assay	Le Lan et al (2005)
β-Thalassemia & blood transfusion	66 thalassemia patients receiving 2 different iron chelation treatments	a) 89.1 ± 19.8%, b) 95.3 ± 11.0%	a) 3.19 ± 2.21, b) 4.81 ± 1.94	Singh et al (1990) ultrafiltration. Modified by adding AlCl ₃ to prevent NTBI shuttling	Walter et al (2008)
	a) 21 thalassaemia major	a) 82% median (58 to 110%)	a) 2.57 (0.08 to 6.73)	Singh et al (1990), Porter et al (1996) NTA HPLC	Cighetti et al 2002
	b) 13 thalassaemia intermedia	b) 65% median (29 to 88%)	b) 1.28 (-0.45 to 4.08)		
Blood donation	n=443 healthy male and female blood donors, aged 59.1± 5.4 yrs	30 ± 12%	2.33 ± 0.94	Breuer & Cabantchik (2001) Fluorescence based one step assay	Engberink et al (2008)
Chemo-therapy	40 bone marrow transplant patients, female to male ratio 1:11, aged 18 – 64 yrs. 18 received chemotherapy, 22 received radiochemotherapy	98% (75 to 100%)	4.0 (1.90 – 6.90) day 0	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	Durken et al (1997)
Chronic renal failure without dialysis or iron supplementation	24 patients aged 43 ± 14 yrs	Unknown	15.93 ± 2.81 S.E.M. (Fe ²⁺ 22.5%)	Nilsson et al (2002) BPS spectrophotometry	Prakash et al (2005)
Coronary heart disease risk	11,471 females aged 49-70 yrs were followed for ~4.3 yrs.	Range of means 18.2 to 28.7% at baseline	Range -2.06 to 3.51. An inverse relationship was found between increased NTBI & CHD risk	Breuer & Cabantchik (2001) Fluorescence based one step assay	Van Der A et al (2006)

Type 2 diabetes	49 newly diagnosed patients	29.4 ± 11.7	0.24 ± 0.29. NTBI detected in 59.2% of sample	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	Lee et al (2006)
	48 patients with >5 yrs duration diabetes	33 ± 11.2	0.62 ± 0.43. NTBI detected in 91.7% of sample		
Type 1 & type 2 diabetes	Type 1 diabetes n=25, Type 2 diabetes n=51	T1 DM 26.1 ± 1.7%, T2 DM 26.5 ± 1.6%	0.53 ± 0.09, compared with 0.10 ± 0.03 controls	DFO-FE complex, with ultrafiltration, HPLC	Leoncini et al (2008)
Diabetes & acute myocardial infarction	329 diabetes patients within 24 hr of AMI	Unknown	0.43 ± 0.7	Esposito et al (2003) DFO fluorescence	Suliman et al (2004)
End stage renal disease receiving dialysis without iron supplementation or rHuEPO	22 patients aged 46 ± 14 yrs	Unknown	42.41 ± 20.72 S.E.M. (Fe ²⁺ 19.8%)	Nilsson et al (2002) BPS assay	Prakash et al (2005)
Dysmetabolic hepatosiderosis	26 patients with high serum ferritin and insulin resistance syndrome, aged 55 ± 11 yrs	47 ± 14%	0.381 ± 0.381	Breuer & Cabantchik (2001) Fluorescence based one step assay	Le Lan et al (2005)
Haemochromatosis: homozygous - treated	27 patients, mostly receiving maintenance treatment	27 to 85%, mean 57%	1.79 (-0.25 to 3.90)	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	De Valk et al (2000)
	14 male patients receiving maintenance treatment, aged 56 ± 11 yrs	40 ± 14%	0.221 ± 0.559		
Haemochromatosis: homozygous - untreated	23 male patients, with excess iron, pre-treatment, aged 44 ± 10 yrs	87 ± 16%	0.761 ± 0.504	Breuer & Cabantchik (2001) Fluorescence based one step assay	Le Lan et al (2005)
Haemochromatosis: heterozygous	46 subjects, selected for low and high NTBI, aged 43 ± 15 yrs	Range of means 19.6 to 51.0%	Median 2.75 M, 2.30 F (0.44 to 6.21)	Breuer & Cabantchik (2001) Fluorescence based one step assay	Van Tits et al (2006)

	22 symptom free individuals	14 to 40%, mean 27%	0.51 (-0.75 to 2.75)	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	De Valk et al (2000)
Myeloablative treatment for stem cell transplant	6 patients with haematological malignancy	91% (87 - 94%)	Range 0.8 to 1.0 for all 6 patients	Evans & Halliwell (1994), bleomycin assay	Sahlstedt et al (2002)
Neonates	384 neonates, 195 found to have NTBI >1.16 $\mu\text{mol/L}$		Range 1.16 to 15.2	Kime et al (1996) HPLC, chelation by disodium nitryloacetic acid & CP22	Buonocore et al (2003)
Premature neonates	21 infants receiving blood transfusion, median gestational age 27 wks.	Pre BT median 51.4%, Post BT 54.1%	Pre BT range 0.0 to 4.5 (median 0), post BT 0.0 to 20.7 (median 2.1)	Gutteridge et al (1981) bleomycin assay	Hirano et al (2001)
Range of diseases	195 chemotherapy, haemochromatosis and other unspecified patients	Unknown	Range of findings 0 to 2 μM	Colorimetric method: Zhang et al (1995); HPLC: Singh et al (1990)	Kolb et al (2009)

Table 2. The occurrence of non-transferrin-bound iron following iron supplementation (examples)

Patient group	Study population	Transferrin saturation % mean (range)	NTBI ($\mu\text{mol/L}$) mean (range) or SD	NTBI measurement method used	References
Oral iron	7 otherwise healthy anaemic women received 200mg FeSO ₄ (65mg elemental iron) with food (a), without food (b)	a) ~73% 3hr post iron b) ~58% 3hr post iron	a) 4.6 \pm 0.5 SE, b) 3.9 \pm 1.1 SE	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	Hutchinson et al (2004)
Oral iron	35 unfasted pregnant females received 100mg ferrous calcium citrate	Unknown	0.074 (SD 0.23) 1 hr post administration	Breuer & Cabantchik (2001) Fluorescence based one step assay	Gurumurthy et al (2008)
Intravenous iron & healthy subjects	20 healthy volunteers aged 18 - 38 yrs, received 100mg ferrous saccharate (Venofer)	40 \pm 18%	3.3 \pm 2.2, 4 hrs post iron	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	Rooyackers et al (2002)
Intravenous iron & pregnancy	22 pregnant females received 100/5ml Venofer	Unknown	Mean 0.81 (SD 0.95) 1 hr post administration	Breuer & Cabantchik (2001) Fluorescence based one step assay	Gurumurthy et al (2008)

Intravenous iron & immune apheresis	6 patients with autoimmune diseases, aged 42 ± 7.8yrs, received 50mg iron (III) sucrose (Venofer)	45.2 ± 14.4%, 30 minutes post iron	0.0 in 5 patients, 0.16 in 1 patient, within 30 minutes post iron	Gutteridge et al (1981) bleomycin assay	Sengoelge et al (2004)
	6 patients with autoimmune diseases, aged 42 ± 7.8yrs, received 100mg iron (III) sucrose (Venofer)	66.8 ± 25.7%, 5 minutes post iron	Range 0.1 to 0.5 within 30 minutes post iron		
Intravenous iron, haemodialysis, and peritoneal dialysis	71 HD and CAPD patients aged 18-90 yrs, receiving either 100mg iron sucrose or sodium ferric gluconate	29.5±14.4%	Redox active NTBI detected in 19.7% of patients, mean 2.2±1.75	Breuer & Cabantchik (2001) Fluorescence based one step assay	Esposito et al (2002)
	10 HD & rHuEPO treatment patients received 100mg iron saccharate over 60 minutes (a) and 6 minutes (b)	Unknown	a) 3.17 ± 1.18 1hr post iron, b) 2.42 ± 1.13 1hr post iron	Singh et al (1990), Gosriwatana et al (1999), NTA/cobalt HPLC	Kooistra et al (2002)

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Appendix 1.iii. Dietary iron fact sheet



Dietary Iron

Are you getting the right amount?



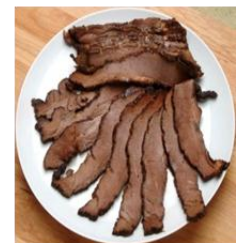
Iron is important for making red blood cells to carry oxygen around the body. Some people have too little iron, other people can have too much

How much do you need?

Women 19-50 years: 14.8 mg/day

Women 50+ years: 8.7 mg/day

Men 19+ years: 8.7 mg/day



You can help absorb the iron in food by having fruit or fruit juice with your meal



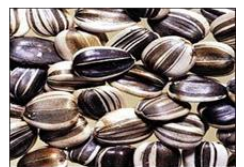
Drinking tea and coffee at mealtimes stops some of the iron being absorbed



Lean, unprocessed red meat is a good source of iron



Aim for less than 70g a day (cooked weight) of red and processed meat, the size of one small beef-burger (SACN 2010)



How much iron is in different foods?

Food	Serving	Iron (mg)
Fortified instant oat breakfast cereal	1 bowl (30g)	3.6
Fortified breakfast cereal	1 bowl (30g)	2.4
Fortified nutri-bar (check wrapper)	1 bar (45g)	2.4
Lean roast beef	2 thick slices (90g)	2.3
Stewed beef mince	3 tablespoons (80g)	2.2
Tofu	2 x 4 x 8 cm (60g)	2.1
Pitta bread	1 small (74g)	2.0
Vege-sausages	2 sausages (60g)	1.9
Baked beans	3 table spoons (120g)	1.7
Sardines	half a tin (58g)	1.7
Anchovies	half a small tin (41.5g)	1.7
Curly kale, boiled	1 serving (85g)	1.7
Dried figs	2 figs (40g)	1.7
Turkey meat, dark	average serving (120g)	1.7
Soya bean burger	1 burger (56g)	1.5
Sunflower seeds, cashews	1 handful (25g)	1.5
Lamb, lean roast leg	2 slices (80g)	1.4
Peas, boiled	average serving (85g)	1.4
Dried apricots	4 apricots (32g)	1.3
Spring greens	average serving (85g)	1.2
Lentils, boiled	1 tablespoon (35g)	1.2
Irish stout	1 pint (568 ml)	1.1
Wholemeal bread	1 slice (36g)	1
Boiled egg	1 egg (50g)	1

Iron data: McCance & Widdowson (2002). Fact sheet: desley.white@plymouth.ac.uk Registered Dietitian [DIFS 17/3/12]

Appendix 2.i. Tables of results

Table 46. Protein in filtrate, % of amount pre-filtration, Batches A to D

Batch	Run	Glucose incubation (mM)				
		Fresh	0	500	750	1000
A	1	1.41	4.38	3.60	3.80	11.20
	2	0.00	0.00	5.62	3.75	6.54
	3	0.68	0.00	1.23	7.34	12.91
B	1	0.00	0.00	3.66	11.32	3.28
	2	0.00	0.00	0.00	1.80	3.68
	3	0.00	0.00	1.05	1.25	1.80
C	1	0.00	0.00	0.00	1.09	0.00
	2	0.00	0.00	3.07	0.00	3.17
	3	0.00	0.00	3.62	0.00	0.00
D	1	0.00	0.00	0.00	0.00	0.00
	2	0.00	0.00	0.00	0.00	0.00
	3	0.00	0.00	0.00	0.00	0.00
	Mean	0.17	0.36	1.82	2.53	3.55
	SD	0.44	1.26	1.98	3.56	4.49
	<i>P</i>		0.626 <i>P</i> vs Fresh	0.043* <i>P</i> vs 0	0.060 <i>P</i> vs 0	0.027* <i>P</i> vs 0

SD: Standard deviation

Table 47. Protein in retentate, % of amount pre-filtration, Batches A to D

Batch	Run	Glucose incubation (mM)				
		Fresh	0	500	750	1000
A	1	90.08	89.13	85.01	81.28	71.17
	2	81.17	75.09	91.41	70.26	89.99
	3	98.20	115.60	84.11	110.24	87.79
B	1	107.43	97.17	75.73	68.98	62.72
	2	107.36	92.63	76.14	71.64	74.85
	3	97.22	96.86	86.04	59.52	75.96
C	1	94.58	125.00	75.15	63.86	74.36
	2	90.49	91.51	67.36	77.79	73.52
	3	86.96	93.14	106.71	60.93	80.03
D	1	82.36	80.94	84.72	81.04	83.78
	2	98.35	96.21	93.06	75.68	74.71
	3	94.92	84.59	90.29	83.17	83.00
	Mean	94.09	94.82	84.64	75.37	77.66
	SD	8.40	13.80	10.40	13.60	7.60
	<i>P</i>		0.877 <i>P</i> vs Fresh	0.053 <i>P</i> vs 0	0.002** <i>P</i> vs 0	0.001** <i>P</i> vs 0

SD: Standard deviation

Table 48. Iron in filtrate, Batches A to D (μM)

Batch	Run	Glucose incubation (mM)				
		Fresh	0	500	750	1000
A	1	26.5	36.2	34.4	28.3	32.1
	2	41.0	37.1	43.2	48.1	47.4
	3	30.3	38.4	32.4	29.4	30.2
B	1	34.3	38.4	43.2	41.7	43.9
	2	34.7	37.9	47.0	47.2	48.6
	3	36.5	38.4	50.9	43.9	49.8
C	1	39.5	47.6	51.2	55.5	46.8
	2	42.8	38.3	48.0	51.6	46.2
	3	41.8	42.3	51.8	42.5	55.2
D	1	50.9	54.4	48.8	46.1	43.7
	2	33.7	33.5	55.8	40.4	46.0
	3	33.1	43.3	46.7	42.5	56.5
	Mean	37.1	40.5	46.1	43.1	45.5
	SD	6.5	5.7	6.9	7.9	7.8
	<i>P</i>		0.189 <i>P</i> vs Fresh	0.041* <i>P</i> vs 0	0.362 <i>P</i> vs 0	0.084 <i>P</i> vs 0

SD: Standard deviation

Table 49. Iron in retentate, Batches A to D (μM)

Batch	Run	Glucose incubation (mM)				
		Fresh	0	500	750	1000
A	1	24.37	16.54	15.34	16.78	14.13
	2	19.07	17.63	10.88	8.83	9.92
	3	15.81	20.31	12.06	11.69	13.31
B	1	25.72	21.54	12.68	13.95	11.42
	2	36.10	21.54	10.53	10.66	11.42
	3	23.19	22.56	10.03	11.16	10.66
C	1	20.08	15.45	10.33	8.33	9.83
	2	18.33	19.20	12.70	10.20	9.95
	3	17.45	16.58	8.58	13.45	8.20
D	1	8.79	6.53	3.27	5.07	8.90
	2	23.32	17.35	17.01	11.49	7.44
	3	10.48	11.27	5.18	5.97	4.51
	Mean	20.23	17.21	10.71	10.63	9.97
	SD	7.24	4.60	3.84	3.31	2.59
	<i>P</i>		0.2359 <i>P</i> vs Fresh	0.0011** <i>P</i> vs 0	0.0006*** <i>P</i> vs 0	0.0002*** <i>P</i> vs 0

SD: Standard deviation

Appendix 3.i. Materials and methods

Materials purchasing

1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (DHP), nitrilotriacetic acid (NTA), butylated hydroxytoluene, and all other laboratory reagents were purchased from Sigma-Aldrich. Amicon Ultra filter units 30 kDa were purchased from Merck-Millipore.

Equipment

See section 2.3.1 for full laboratory equipment list.

UV/Vis HPLC

Dionex UVD170S detector.

Hichrom C18 polyether ether ketone (PEEK) lined column, 100 mm x 4.6 mm, 5 µM particle size, with a PEEK-lined 2 µm inline filter.

Gynkotec pump. Rheodyne injector port and sample loops, all PEEK-lined.

Chromeleon software.

Fluorescence HPLC

Dionex Ultimate 3000 detector and pump

Hichrom C18, 100 mm x 4.6 mm, 5 µM particle size column, with a 2 µm inline filter.

Electrochemical HPLC

BAS LCD 40 electrochemical detector

Milton Roy Constametric pump

150 mm x 5.0 mm Hichrom C18 column

Protocols

Measurement of non-transferrin-bound iron in plasma

SOLUTIONS

PHOSPHATE BUFFERED SALINE [PBS] pH 7.4 (0.154M NaCl)

[137 mM NaCl; 2.7 mM KCl; 8 mM Na₂HPO₄; 1.46 mM KH₂PO₄]

Using a 250 ml volumetric flask weight out the following:

NaCl [MW 58.44] 2.0 g

KCl [MW 74.56] 0.05 g

Na₂HPO₄·12H₂O [MW 358.14] 0.716 g

KH₂PO₄ [MW 136.09] 0.05 g

Dissolve in about 200 ml H₂O. Take pH to 7.4. Make up to 250 ml with H₂O.

Store in refrigerator.

NITRILOTRIACETIC ACID [NTA] (0.8M)

Weigh out 4.702 g of nitrilotriacetic acid disodium salt [MW 235.1] and place in 25 ml flask.

Add about 20 ml H₂O. When dissolved, take pH to 7.0 with 100 mM NaOH. Make up to 25 ml with H₂O. Store in refrigerator

3-HYDROXY-1,2-DIMETHYL-4(1H)-PYRIDONE [DHP]. IRON CHROMOPHORE

[MW 139.15] Weigh out 0.014 g, add 2.0 ml of PBS. This makes a 50 mM solution.

STOCK FERRIC ION STANDARDS

Stock solution 1 mM FeCl₃. [FeCl₃·6H₂O MW 270.3]

Weigh out 0.027 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and make up to 100 ml with HPLC grade H_2O .
Store in small volumes at -80°C .
Prepare standards for calibration, ranging from 1 to 10 μM

MOBILE PHASE

5 mM PIPES buffer pH 7.0 containing 3.5 mM DHP and 5% acetonitrile

1. Weigh out 0.866 g of PIPES disodium salt and place in a 500 ml flask
2. Add 0.244 g of DHP.
3. Make up to 500 ml with HPLC grade water
4. Add 30 ml of acetonitrile
5. Take to pH 7.0 with 1.0 M HCl
6. Filter and degas

PROCEDURE

- [1] Take 150 μl of plasma and add 15 μl 0.8M NTA solution. Incubate at room temperature for 20 minutes.
- [2] Add 150 μl of phosphate buffered saline and mix well.
- [3] Place mixture in 30kDa Millipore amicon ultra filters
- [4] Centrifuge at 13,000 x g for 30 minutes at 4°C .
- [5] Remove ultrafiltrate, measure volume, to every 100 μl add 10 μl of 50 mM DHP.
- [6] Incubate for 5 minutes at RT and then inject into the HPLC system.

UV/Vis HPLC set-up

Pump pressure 1 ml/min. Peaks measured between 3.5 and 5.0 minutes, at 450 nm.

Measurement of ascorbate and urate

Loosely based on method of Sato et al (2010) and Mitton & Trevithick (1994)

SOLUTIONS

Standard Ascorbate & Urate

Ascorbate: 0.018 g/100 ml [0.009 g/50 ml] in H_2O gives a 1.0 mM stock solution. Dilute in PBS to standards 10 μM to 50 μM .

Urate: 0.019 g/100 ml [0.0095 g/50 ml] in H_2O gives a 1.0 mM stock solution.

Dilute in PBS to standards 10 μM to 50 μM .

10% Metaphosphoric acid [MPA] containing 2mM EDTA

Make up a solution of 2 mM EDTA in H_2O . e.g 0.186 g EDTA disodium salt made up in 250 ml H_2O . Use this to make up the MPA solution.

5 g of MPA diluted with 50 ml of H_2O containing 2 mM EDTA.

5% MPA containing 1 mM EDTA

A 1:2 dilution of the above in H_2O

Tris (2-carboxyethyl)phosphine hydrochloride [TCEP] 350 mM in 5% MPA containing 1 mM EDTA (Reducing agent).

0.100 g made up in 1.0 ml of 5% MPA in 1 mM EDTA

MOBILE PHASE

50 mM phosphate buffer pH 2.8 containing 540 μM EDTA and 2% MeOH

3.9 g NaH_2PO_4

0.1 g EDTA

Make up to 400 ml, add 10 ml MeOH and make up to 500 ml with MilliQ H₂O

pH to 2.8 with 1.0 M HCl

Filter and degas

PROCEDURE

- [1] Take 100 µl of plasma and add to 100 µl of 10% MPA containing 2.0 mM EDTA. Mix well and centrifuge at 20,000 x g at 4°C for 10 minutes.
- [2] Store ascorbate extract at -80°C until measured.
- [3] Take 2 x 45 µl aliquots of supernatant.
- [4] To one add 10 µl of 5% MPA containing 1 mM EDTA. Add a further 100 µl of 5% MPA containing 1 mM EDTA. Mix well. This is for measurement of reduced ascorbate. From this sample remove 50 µl and add to a tube containing 450 µl 5% MPA containing 1 mM EDTA. Mix well. This is for measurement of urate.
- [5] To the other sample add 10 µl 350 mM TCEP in 5% MPA containing 1 mM EDTA. Incubate for 20 minutes at room temperature. This is to reduce the oxidised dehydroascorbate back to ascorbate. This measures total ascorbate

Suggested procedure: Inject the reduced sample, and the sample for urate while the total ascorbate sample is incubating. Then inject the total sample.

Standards: Prepare standards as follows:

1. Take 100 µl of standard and add to 100 µl of 10% MPA containing 2 mM EDTA. Mix well and centrifuge at 20,000 x g for 10 minutes at 4°C.
2. Take a 45 µl aliquot and add 10 µl of 350 mM TCEP in 5% MPA containing 1 mM EDTA.
3. Incubate for 20 minutes at room temperature.
4. Inject into HPLC system

Electro-chemical HPLC set-up

Pump: 1 ml/min; Volts: + 0.6; Range: 0.5 µA; Filter: 0.10 Hz

Measurement of malondialdehyde (MDA)

Based on the method of Agarwal & Chase (2002).

SOLUTIONS

Butylated hydroxytoluene (BHT) 0.05% in 95% ethanol

Weigh out 0.0025 g add to 4.750 ml ethanol plus 250 µl dH₂O

Phosphoric acid (0.44 M)

Take 1.0 ml concentrated orthophosphoric acid and makeup to 100 ml with dH₂O

Thiobarbituric acid (TBA) (42 mM)

To 0.303 g of TBA make up to 50 ml with dH₂O. Heat at 50 to 55°C until dissolved

Stock solution of MDA

Weigh out 0.00313 g of MDA and make up to 10 ml with dH₂O. Dilute with PBS to make a range of standards from 500 nM to 10 µM

Mobile phase

Potassium dihydrogen phosphate (KH₂PO₄) (50 mM)

Weigh out 3.403 g of KH_2PO_4 dissolve in HPLC grade H_2O to make 500 ml. Mix 80% 50 mM KH_2PO_4 (500 ml) and 20% HPLC grade MeOH (125 ml). Adjust pH to 6.8 using 5 M sodium hydroxide. Filter and degas.

PROCEDURE

In 2 ml centrifuge tubes with screw caps put 50 μl plasma, standard or blank. Add 50 μl of BHT solution then add 400 μl of phosphoric acid and 100 μl of TBA solution.

1. Mix well on a vortex mixer. Heat for 1 hour at 100°C in a dry block
2. Remove and place on ice to cool
3. Add 300 μl of n-1-butanol. Mix on a vortex mixer for 15 seconds per sample, repeat for a further 15 seconds
4. Centrifuge for 5 minutes at 13,000 x g to separate the aqueous and butanol phases
5. Carefully remove 200 μl of the butanol extract. Inject directly into HPLC system
6. Use methanol to wash system between each sample.

Fluorescence HPLC SET-UP

Pump: 1ml/min; Fluorescence HPLC Wavelengths - Excitation: 515 nm, Emission: 553 nm; Sensitivity: 4

Total iron (Bothwell et al 1971), and Total iron binding capacity (Ramsay 1957), see Chapter 2.

References

Agarwal, R. & Chase, S. (2002) Rapid, fluorimetric-liquid chromatographic determination of malondialdehyde in biological samples. **Journal of Chromatography B** 775: 121-126

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Mitton, K. Trevithick, J. (1994) High performance liquid chromatography-electrochemical detection of antioxidants in vertebrate lens: glutathione, tocopherol and ascorbate. **Methods in Enzymology** 233: 523-39

Ramsay, W. (1957) The determination of the total iron-binding capacity of serum **Clinica Chimica Acta** 2 (3): 221 - 226

Courses, conferences, and presentations

Within the PhD period of study the following courses and conferences have been attended:

December 2008, British Dietetics Association Research Symposium for Dietitian's New to Research, poster presentation

February 2009, Combating Obesity: Strategies for Prevention and Intervention 3: an Erasmus intensive two week programme, Austria, oral presentation

June 2009, Postgraduate Research Day, Plymouth University, Faculty of Health, oral presentation

June 2009, UK Genetic Haemochromatosis AGM, Royal Free Hospital, London

December 2009, First International Conference on Metal Chelation in Biology and Medicine, Bath

June 2010, South West Dietitian's Research and Clinical Dietetics Study Day, Plymouth University, oral presentation

June 2010, Postgraduate Research Day, Plymouth University, Faculty of Health, oral presentation

June 2010, UK Genetic Haemochromatosis AGM, Royal Free Hospital, London, oral presentation

July 2010, Division of Biosciences seminar, Brunel University, Middlesex, oral presentation

July 2010, School of Health Professions Research Group meeting, oral presentation

September 2010, The European Iron Club Annual Meeting, Building bridges with bio-iron from bench to bedside, Nijmegen, The Netherlands, poster presentation

November 2010, South Devon Health Care Foundation Trust, Riviera Research Day, Torbay, poster presentation

March 2011, Cambridge University and ABCAM conference, Iron related diseases: A look to the future, Cambridge, poster presentation

April 2011, Centre for Research in Translational Biomedicine Annual Research Day, Plymouth University, poster presentation

June 2011, UK Genetic Haemochromatosis AGM, Royal Free Hospital, London

June 2011, Postgraduate Research Day, Plymouth University, Faculty of Health, oral presentation

June 2011, Postgraduate Society Conference, Plymouth University, oral presentation

September 2011, European Federation of Associations of Patients with Haemochromatosis, annual meeting, Brussels, Belgium

September 2011, The European Iron Club for Professionals in Biomedical Inorganic Iron, Annual Meeting, Louvain-la-Neuve, Belgium, poster presentation

October 2011, Genetic Haemochromatosis Continuous Professional Development study day, organised by the UK Genetic Haemochromatosis Society, Royal Free Hospital, London

Publications

White & Collard (2010) Iron and disease in diabetes: Protein glycation and the pathologies of advanced diabetes. Building bridges with bio-iron: From bench to bedside. American Journal of Hematology, Volume 85, Issue 12, page E18-E19 [Abstract]

tive transcripts were analyzed by real-time PCR. The effect of treatment with hemin was evaluated by luciferase assay in K562 cells using constructs carrying the different promoter sequences specific for FPN1 alternative transcripts. FPN1 protein from total cellular extracts and in cytosolic and membrane fractions were analysed by Western blot. Possible regulative elements located in genomic DNA upstream of the FPN1 transcription start site were searched for by bioinformatic analysis.

Results: We obtained the following results: a) modulation of FPN1 protein was hemin concentration dependent; b) we observed the FPN1 induction as soon as thirty minutes after treatment; c) FPN1 protein progressively decreased in presence of an heme synthesis inhibitor; d) finally we observed an early induction of non-IRE FPN1 alternative transcripts with an apparent sequential and specific activation and mutual exclusion between the IRE and non-IRE transcripts especially in the early times of exposition; e) only the non-IRE FPN1 transcript is translationally regulated by heme; f) sequence analysis of the non-IRE FPN1 gene promoter revealed the presence of a putative Maf recognition element (MARE).

Conclusions. In conclusion our results showed that in erythroid cells FPN1 gene was regulated by heme levels through non-IRE FPN1 alternative transcript induction. This regulation is of particular interest with regard to disorders such as thalassemias where the ineffective erythropoiesis and hemolysis cause severe anaemia.

040

RELATIONSHIPS BETWEEN HLA-TYPE, T-LYMPHOCYTE SUBSETS, MHC-I LINKED HAPLOTYPES AND THE EXPRESSION OF IRON OVERLOAD IN C282Y HOMOZYGOUS INDIVIDUALS FOUND BY POPULATION SCREENING

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Background: A common feature of C282Y homozygotes identified through population screening is the relatively low iron load as determined by their serum ferritin levels (Scand. J. Gastroenterol. 2001; 36, 1108). The proportion of C282Y homozygotes that will progress to life-threatening complications of the disease is unknown, and it is unclear which patients need closest follow up. Thus, the finding of additional, modifying factors that could help predict the course of the disease may lead to a more targeted treatment. Previous work on T-lymphocyte subsets (J. Hepatol. 1998; 28, 1) has indicated that abnormally high CD4+/CD8+ ratios due to low peripheral blood CD8+ counts are associated with more severe forms of iron overload and with faster re-entry of iron after treatment. Recently, two conserved haplotypes defined by the SNP markers PGBD1, ZNF193 and ZNF165 were found associated with the clinical expression of hemochromatosis

(BMC Med. Gen. 2008; 9, 97). The major haplotype, designated A-A-T, was characterized by low CD8+ T-lymphocyte numbers and severe iron overload, whereas the minor haplotype, designated G-G-G, was associated with high CD8+ numbers and lower iron stores. Furthermore, HLA-type has previously been reported to modify the degree of iron accumulation (Eur. J. Haematol. 2007; 79, 429; *ibid* 2009; 84, 145). We have examined these factors in C282Y homozygotes found through a hemochromatosis screening program.

Methods: A total of 529 screening-detected individuals were invited to participate in the study, and 333 subjects, of which 182 (103 men, 79 women) were C282Y homozygous, responded positively. HLA-type and T-lymphocyte subsets (CD4+, CD8+, total T-lymphocytes) was determined in all C282Y homozygous individuals, whereas the three SNPs PGBD1 (rs1997660), ZNF193 (rs7206) and ZNF165 (rs203878) have been determined in 161 individuals (89 men, 72 women). The newly obtained data were compared with data obtained in the original screening study.

Results: C282Y homozygous men, but not women, carrying two HLA-A3 alleles had significantly increased iron levels, as represented by serum ferritin at the time of diagnosis, compared to those carrying one or no A3 allele. The major SNP haplotype (A-A-T) showed a tendency (not statistically significant) of higher ferritin levels compared to the minor haplotype (G-G-G). In addition, the A-A-T haplotype was associated with HLA-type A3,B7 or A3,B14, whereas the minor haplotype (G-G-G) was found predominantly in HLA-A1,B8 individuals. The frequencies of the minor alleles were PGBD1-G 16.5%, ZNF193-G 23.0% and ZNF165-G 22.7%, yielding homozygote frequencies of 2.7%, 5.3% and 5.1%, respectively, assuming Hardy-Weinberg equilibrium.

041

IRON AND DISEASE IN DIABETES: PROTEIN GLYCATION AND THE PATHOLOGIES OF ADVANCED DIABETES

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Disordered iron homeostasis has been associated with risk for type 2 diabetes, although the specific mechanisms involved are not clear. Haemochromatosis – a genetic iron overload condition – commonly results in diabetes, and is suggested to be the result of oxidative stress to β -cells following pancreatic iron deposition. In established diabetes, studies into the effects of iron depletion have found improvements in insulin secretion and sensitivity, coronary artery responses, and endothelial dysfunction. Significant improvements in kidney function have been demonstrated in microalbuminuric type 2 diabetes patients, following reduction of dietary iron. High plasma glucose levels, as seen in diabetes, cause increase in rate of protein glycation reactions. This can be the result of either the Amadori reaction between acyclic monosaccharides and protein amine groups, such as measured on haemoglobin HbA1c, or by autoxidation of cyclic monosaccharides, which may be catalysed by trace amounts of transition metals. Continuing reactions, also vulnerable to catalysis by iron, and contributed to by dicarbonyl products of glucose and lipid metabolism, lead to the formation of irreversible advanced glycation end products (AGE), and their degraded species. Over time, AGE in the extracellular matrix develop cross-links, causing thickening and stiffening of tissue. Pro-inflamma-

tory responses further contribute to oxidative stress and subsequent irreversible damage in a range of tissues such as blood vessels, kidney basement membrane, and the heart, developing into the endothelial and vascular complications associated with advanced diabetes. Evidence is accumulating that glycation of a number of iron-binding and antioxidant proteins impairs their function, and therefore the potential exists for a vicious circle whereby redox available iron may contribute to glycoxidation damage and further reduction in iron-binding antioxidant capacity. Whilst glucose toxicity clearly drives AGE development, iron may act to amplify effects and consolidate damage. Chelatable loosely-bound plasma iron, iron; oxidant/antioxidant status; protein glycation; nephropathy; cardiopathy; and retinopathy will be assessed in diabetes; impaired glucose tolerance; and healthy controls. Associations between iron and the pathologies of advanced diabetes will be explored in this study.

042

EXPRESSION OF IRON TRANSPORT MOLECULES IN DUODENAL MUCOSA OF PATIENTS WITH CHRONIC LIVER DISEASE

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Iron and its uptake and transport within the body are very closely related to several serious diseases resulting without treatment in very damaging sequels. Therefore, in the present study we examined the gene expression of duodenal iron transport molecules and hepcidin in patients with hemochromatosis (treated and untreated and with different genotypes which represent risk for I. type hemochromatosis), in patients with alcoholic liver disease and iron deficiency anemia. Gene expressions of DMT1, ferroportin, Dcytb, hephaestin, HFE and transferrin receptor 1 were measured in duodenal biopsies using real-time PCR and Western blot analysis. Hpcidin levels were measured employed ELISA assessment. Major mutations in HFE gene responsible for I. type hemochromatosis, i.e. C282Y, H63D and S65C, were analyzed by PCR-RFLP method. On mRNA level, all analyzed molecules were not significantly different in hemochromatotic patients with different genotypes or in untreated hemochromatotic patients compared with controls. DMT1, ferroportin, and transferrin receptor 1 levels were significantly increased in post-phlebotomized hemochromatotic patients and also in patients with alcoholic liver disease and normal iron indices relative to controls. All tested molecules were significantly increased in patients with iron deficiency anemia compared with controls. Protein expression of analyzed molecules was not significantly different in any of the examined groups when compared with controls. Spearman rank correlations showed that DMT1 and Ferroportin, Dcytb and hephaestin mRNA and ferroportin and Dcytb proteins are positively correlated to each other regardless of the underlying disease. Serum ferritin

was negatively correlated to Dcytb, hephaestin, DMT1, ferroportin, and transferrin receptor 1 mRNA but not to proteins in studied groups of patients. Serum hepcidin was significantly decreased in patients with alcoholic liver disease when compared to controls. The decrease was observed in patients with iron deficiency anemia as well, but this was not statistically significant. Our data show that untreated hemochromatotic patients do not have increased iron transport molecule mRNAs. Post-phlebotomized hemochromatotic patients had increased DMT1 and ferroportin levels in response to increased erythropoiesis after phlebotomy. Observed positive correlation among Dcytb, hephaestin, DMT1, ferroportin, and transferrin receptor 1 mRNA indicates the presence of coordinated regulation of these genes. On the other hand, the changes on mRNA level in patients with iron deficiency anemia are probably a part of physiological compensation of iron deficiency in organism.

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043

IS ACTIVATION OF HAEM OXYGENASE – 1 INVOLVED IN IRON ACCUMULATION IN ALCOHOLIC LIVER DISEASE?

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Introduction: The progression of reversible alcoholic steatohepatitis to irreversible cirrhosis of the liver is often associated with accumulation of iron. However, the mechanisms by which iron accumulates in the liver in chronic alcoholism are not clearly known. Alcohol ingestion is known to induce a state of oxidative stress in the liver secondary to up-regulation of cytochrome P450 2E1 (CYP2E1). Oxidative stress is known to activate haem oxygenase 1 (HO-1). We hypothesized that alcohol-induced oxidative stress may lead to induction of HO-1 leading to increased haem degradation and subsequent release of ferrous iron (Fe²⁺), contributing to iron overload in this condition. This study was designed to test the above hypothesis.

Methodology: Male Swiss albino mice (weighing 28–30g) were pair-fed with commercially available Lieber DeCarli alcohol and control diets for 1, 2, 4 and 8 weeks. Alcohol-fed mice received 20% of total calories in the form of alcohol. Daily alcohol consumption by alcohol-fed mice and weights of mice were monitored. They were sacrificed at the end of the study periods and the liver was retrieved. Microsomes isolated from liver tissue were used for western blots for CYP2E1 and HO-1. The activities of CYP2E1 and HO-1 were estimated by spectrophotometric assays. Levels of serum and liver iron are currently being measured. Data from alcohol-fed mice and pair-fed controls were compared.

Results and discussion: All alcohol-fed mice consumed between 12 to 15 grams of alcohol per kg per day. There were no significant differences in weight gain/loss between the alcohol-fed mice and their pair-fed controls. CYP2E1 enzyme activity was found to be significantly increased in alcohol-fed mice at 1, 2, 4 and 8 weeks, with associated significant increases in protein levels of the enzyme at 4 and 8 weeks. No significant induction of HO-1 (either in protein content or enzyme activity) was seen following 1, 2, and 4 weeks of alcohol feeding. Mild induction was seen at 8 weeks; however this increase was not statistically significant. Results on