2015

Relationships between iron, oxidative stress, glycated proteins and the development of atherosclerosis in Type 2 diabetes

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http://hdl.handle.net/10026.1/3222

http://dx.doi.org/10.24382/4159

Plymouth University

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Relationships between iron, oxidative stress, glycated proteins and the development of atherosclerosis in Type 2 diabetes

Husam O.Dakhil Aljwaid

A thesis submitted to the Plymouth University in fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences
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United Kingdom
This project was funded by the Ministry of Higher Education and Scientific Research
Baghdad, Iraq
2015
Abstract

Hyperglycaemia stimulates a variety of biochemical abnormalities. The area of particular interest in this study is the influence of non-enzymatic glycation of proteins on iron homeostasis, and particularly on non-transferrin-bound iron (NTBI) and its possible relation to atherogenesis in both Type 2 diabetic and obese non diabetic subjects. The link between non-enzymatic glycation of proteins and iron homeostasis, and development of macrovascular disease may be mechanistically different in Type 2 diabetic and obese non diabetic subjects due to a difference in the protein glycation pattern.

Because the following in vivo study required storage of samples for up to two years to complete the processing of all the samples, a storage study was carried out using different anticoagulants and addition of reduced glutathione (GSH) to samples to study the effects of storage, thawing and freezing of the samples on the level of malondialdehyde (MDA), a biomarker of lipid peroxidation. This storage study showed that EDTA attenuated the action of lipid oxidation compared with lithium heparin (LiH). A combination of GSH with either EDTA or LiH added more protection from lipid peroxidation in the first week of storage, but due to the thawing and freezing of the sample the action of GSH diminished through its autooxidation, meaning that addition of GSH to samples in the following in vivo study would be useless.

An in vivo study was carried out on iron-related parameters in three subject groups: control (non-diabetic, non-obese), Type 2 diabetic and obese non diabetic. Glycated haemoglobin (HbA1c) was strongly correlated with NTBI in the diabetic group. Also the level of NTBI was significantly increased in Type 2 diabetic subjects compared with other groups while the level of total iron was significantly decreased. The study showed a strong positive correlation between NTBI and a biomarker of endothelium dysfunction (E-selectin) in all groups studied. Although it is not possible from the current data to know if there is a causal relationship between these two parameters, it remains a possibility that iron released from its binding sites could initiate oxidative damage to the endothelial cells and begin the process of atherogenesis. Positive correlation at the 90% confidence level between NTBI and a biomarker of inflammation, high sensitivity C-reactive protein, is another indicator in this study of a link between increases in NTBI, inflammation, endothelium dysfunction and atherosclerosis. This study also showed for first time that NTBI is present in higher levels in the plasma of obese subjects compared to controls despite the obese subjects having significantly lower total iron.

An in vitro study found that glycation of transferrin half saturated with iron increased with increasing glucose concentration, leading to decreased capacity of transferrin to hold iron and increased release of free iron. Also co-incubation of transferrin half saturated with iron with low density lipoprotein (LDL) and glucose showed oxidation of LDL (measured as MDA). This may be explained by the effect of glycation, leading to release of free iron, which catalyses oxidation of LDL. In addition, glycation of LDL may enhance the oxidation of LDL catalysed by iron.

Both studies indicate that the glycation of proteins has a major impact on iron homeostasis leading to release of non-enzymatic glycation and contributing to one of the most common complications of Type 2 diabetes, atherosclerosis.
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<tr>
<td>50% ITf</td>
<td>Transferrin half saturated with iron</td>
</tr>
<tr>
<td>2-DE</td>
<td>Two-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>2hPG</td>
<td>Two hours plasma glucose</td>
</tr>
<tr>
<td>ACR</td>
<td>Albumin: creatinine ratio</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetic Association</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>apoTf</td>
<td>Apotransferrin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DCYTB</td>
<td>Duodenal cytochrome b</td>
</tr>
<tr>
<td>DG</td>
<td>Deoxyglucose</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent metal transporter 1</td>
</tr>
<tr>
<td>DPN</td>
<td>Diabetic peripheral neuropathy</td>
</tr>
<tr>
<td>DPN</td>
<td>Distal peripheral neuropathy</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FPG</td>
<td>Fasting plasma glucose</td>
</tr>
<tr>
<td>FPN</td>
<td>Ferroportin</td>
</tr>
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</table>
Fe1C-Tf  Fe$^{3+}$ ion bound to the C-terminal lobe
Fe1N-Tf  Fe$^{3+}$ ion bound to the N-terminal lobe
FRAP    Ferric reducing ability of plasma
$g$     Gravitational acceleration at the Earth’s surface
GA      Glycated albumin
GDM     Gestational diabetes mellitus
GPx     Glutathione peroxidase
GR      Glutathione reductase
GSH     Reduced glutathione
Hb      Haemoglobin
HbA$_{1c}$ Glycated haemoglobin
HH      Hereditary haemochromatosis
HFE     Haemochromatosis protein
HO$^•$  Hydroxyl radical
holoTf  Holotransferrin
HPLC    High performance liquid chromatography
HSA     Human serum albumin
hs-CRP  High sensitivity C-reactive protein
IDDM    Insulin dependent diabetes mellitus
IDF     International Diabetes Federation
IEF     Isoelectric focusing
IIDM    Insulin independent diabetes mellitus
IL-6    Interleukin 6
LDL     Low density lipoprotein
LIP     Labile iron pool
MDA     Malondialdehyde
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA</td>
<td>Metaphosphoric acid</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non-transferrin-bound iron</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SF</td>
<td>Serum ferritin</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>sTfR</td>
<td>Soluble transferrin receptor</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR1</td>
<td>Transferrin receptor 1</td>
</tr>
<tr>
<td>TfR2</td>
<td>Transferrin receptor 2</td>
</tr>
<tr>
<td>TfRs</td>
<td>Transferrin receptors</td>
</tr>
<tr>
<td>TIBC</td>
<td>Total iron binding capacity</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TPTZ</td>
<td>Tripyridyltriazine</td>
</tr>
<tr>
<td>TSAT</td>
<td>Transferrin saturation measurement</td>
</tr>
<tr>
<td>UIBC</td>
<td>Unsaturated iron binding capacity</td>
</tr>
<tr>
<td>UKPDS</td>
<td>UK Prospective Diabetes Study</td>
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XVI
<table>
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<tr>
<th>USD</th>
<th>U.S. dollars</th>
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<td>WHO</td>
<td>World Health Organisation</td>
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Acknowledgements

In the name of Allah, the Most Gracious, the Most Merciful. Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. First of all, I wish to express my sincere appreciation and deep sense of gratitude to my supervisors Dr John Moody and Dr Keith Collard for their scientific guidance, encouragement and supports through this work. I would like to express my deep gratitude and appreciation to Professor Jonathan Pinkney (Department of Diabetes and Endocrinology, Plymouth Hospitals) for his help to encourage the diabetes patients to participate in my study, and also I would like to express my deep gratitude and appreciation to Professor Paul Winyard and Dr Charles Affourtit for their advice to complete my thesis.

I also extend heartfelt thanks to my sponsor "The Ministry of Higher Education and Scientific Research, Baghdad, Iraq" for financing the scholarship that has enabled me to complete this work. Thanks to the members of the School of Biological Sciences and technical staff who have helped me at various times throughout this study: Professor Waleed Al-Murrani, Dr Michele Kiernan, Dr Desley White, Mrs Liz Preston (Senior Technician) and Mrs Natalie Sweet.

Thank all my colleagues those gave me attention and time during this study Kasim Askar, Khwam Hussein, Ali Abid, I apologize if I have missed anyone to involve in this acknowledgment for their assistance in some parts of my work. Last but not least, it is a pleasure to thank my family, my parents, brothers, sisters, wife and children for their endless love, prayers and encouragement and gave me the incorporeal support during the preparation of this work.

Husam
Dedication

This thesis is dedicated to my parents, brothers, sisters, wife, sons and daughters
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.

This study was financed by the Ministry of Higher Education and Scientific Research of Iraq. During this study blood was taken from volunteers after obtaining appropriate ethics committee approval.

Word count of main body of thesis: 38,174

Signature:

Date: 2015
Literature review
1.1 Diabetes mellitus

Humans around the world face many health threats. One of the most significant threats is diabetes mellitus (DM), often simply referred to as diabetes. Although it has been centuries since DM was first recognised, it is still not fully understood and managed. The global prevalence and incidence of diabetes can lead to a variety of disabling, life-threatening and expensive complications (Yang et al., 2012). Despite the efforts to control the growing number of diabetes patients, the burden of this disease, as well as from its complications, is set to increase globally (Wild et al., 2004).

Of the world’s adult population, the estimated prevalence of all types of diabetes was 366 million in 2011, and it has been predicted that the number of people with diabetes will have risen to 552 million by 2030 according to new figures released by the International Diabetes Federation (IDF) (Whiting et al., 2011). The IDF estimated that in 2011 the five countries with the largest numbers of people with diabetes were China, India, the United States of America, Russia and Brazil. In 2011 about 2.9 million people had been diagnosed with diabetes in the UK; it is estimated that five million people will have diabetes in the UK by 2025 (Diabetes UK, 2012). Furthermore, it is estimated that there are around 850,000 people in the UK who have diabetes but are either unaware, or have no confirmed diagnosis.

In addition to genetic factors there is a broad range of factors that have been implicated in this dramatic increase in the number of diabetes patients in recent decades. These factors are (a) overall growth in population; (b) increased life expectancy resulting in a higher level of aged population more
prone to diabetes; (c) increasing obesity; (d) unhealthy diets; and (e) sedentary lifestyles. Interventions such as dietary modification and physical inactivity play a significant role in controlling diabetes (Shaw et al., 2010). The high prevalence of diabetes has economic impact on countries due to direct costs for treatment and care, and also indirect costs relating to the time taken off work and reduced productivity of people with diabetes (Ryan, 2009, Shaw et al., 2010). In economic terms, it was estimated that diabetes accounted for 12% of health expenditure in 2010 (WHO, 2013). The global health expenditure on diabetes is expected to total at least $376 bn in 2010 and $490 bn in 2030. The expenditure varies by region, age group, gender and country’s income level (Zhang et al., 2010a). In the UK, diabetes cost approximately £23.7 bn in 2010/2011 (Hex et al., 2012).

1.2 Diagnosis of diabetes

Diabetes is characterized by temporarily or permanently elevated blood glucose that results from a defect in insulin production or insulin action (or both) (ADA, 2011), associated with chronic hyperglycaemia and disorders in carbohydrate, fat, protein and electrolyte metabolism (ADA, 2011). The common diabetic symptoms are polyuria (the urge to urinate frequently), polydipsia (the desire to drink large amounts of water to quench constant thirst), polyphagia (the frequent urge to eat), weight loss over a short period of time, dehydration, vomiting, mental state changes and weakness (ADA, 2011, Kitabchi et al., 2006).

In the absence of symptoms above, the following criteria should be applied to confirm the presence of diabetes: fasting plasma glucose (FBG)
≥ 7.0 mmol l⁻¹ or whole blood ≥ 6.1 mmol l⁻¹, and the two hours plasma glucose test (2hPG) ≥ 11.1 mmol l⁻¹. The latter test requires the use of a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water given orally two hours before testing plasma glucose. These criteria should be confirmed by repeating the testing on a different day. If the fasting or random values are not diagnostic, the 2 h value post glucose load should be used.

Glycated haemoglobin (HbA₁c), which reflects an average plasma glucose level over the previous 8 to 12 weeks, has also been used as a diagnostic test for diabetes (Nathan et al., 2007). Diabetes may be diagnosed on any of the criteria shown in Table 1.1 (John, 2012). A repeat confirmatory test is required in most cases.

<table>
<thead>
<tr>
<th></th>
<th>Diabetes</th>
<th>Impaired or 'pre-diabetes'</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA₁c</td>
<td>≥ 48 mmol mol⁻¹</td>
<td>42-47 mmol mol⁻¹</td>
<td>≤ 41 mmol mol⁻¹</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>≥ 7.0 mmol l⁻¹</td>
<td>6.1-6.9 mmol l⁻¹</td>
<td>≤ 6.0 mmol l⁻¹</td>
</tr>
<tr>
<td>2-h glucose</td>
<td>11.1 mmol l⁻¹</td>
<td>7.8-11.1 mmol l⁻¹</td>
<td>≤ 7.7 mmol l⁻¹</td>
</tr>
<tr>
<td>Random glucose</td>
<td>11.1 mmol l⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3 Classification of diabetes mellitus

The classification of glucose metabolism disorders is principally derived from aetiology, and includes staging of pathophysiology based on the degree of deficiency of insulin action. These disorders are classified into four groups: (i) Type 1 diabetes mellitus (T1DM); (ii) Type 2 diabetes mellitus (T2DM); (iii) diabetes mellitus due to other specific mechanisms or diseases; and (iv) gestational diabetes mellitus (Alberti and Zimmet, 1998).

1.3.1 Type 1 diabetes mellitus

Previously this type was called insulin-dependent diabetes mellitus (IDDM) and T2DM was called non-insulin-dependent diabetes mellitus (NIDDM). This nomenclature is not currently used because it is confusing and has frequently resulted in patients being classified based on treatment rather than on pathogenesis (Kuzuya et al., 2002).

The incidence and prevalence of T1DM is increasing worldwide (Soltesz et al., 2007). The disease occurs mostly in individuals under the age of 30 years and is therefore also termed juvenile-onset diabetes, even though it can occur at any age. It accounts for about 10% of all cases of diabetes. The particular manifestation of T1DM is characterized by a loss of pancreatic β-cell function and an absolute insulin deficiency (Harrison et al., 1996). Autoimmunity is the predominant effector mechanism in T1DM resulting in damage to the β-cells in the Islets of Langerhans and insulin deficiency (van Belle et al., 2011). Since insulin is the primary anabolic hormone that regulates blood glucose levels, Type 1 diabetics require a continuous supply
of insulin for survival (ADA, 2011, Standl et al., 2006). Until the present time, known solutions to prevent T1DM have not been discovered.

One of the main complications suffered by Type 1 diabetic patients is diabetic ketoacidosis, which is uncommon in other types of diabetes. The aetiology of T1DM is complex; environmental factors coupled with a genetic predisposition for diabetes trigger an autoimmune response that results in destruction of the pancreatic β-cells over several years (Williams and Pickup, 2004).

1.3.2 Type 2 diabetes mellitus
This form is the most common form of diabetes. It has reached epidemic proportions in both developed and developing countries, and is now recognized as a major global health problem. More than 90% of diabetic patients have T2DM. The prevalence of T2DM has been rising worldwide. Among adults (20-79 y of age), an increase in prevalence from 6.4% (285 million) in 2010 to 7.7% (439 million) in 2030 has been estimated (Shaw et al., 2010). This type of diabetes frequently goes undiagnosed for many years because the hyperglycaemia develops gradually and is often not severe enough for the individual to notice any of the classic symptoms of diabetes. T2DM occurs when insulin secretion is inadequate to meet the increased demand posed by insulin resistance (Druet et al., 2006). The state of insulin resistance is characterised by a reduced ability of insulin to suppress hepatic glucose production and to promote peripheral glucose disposal. T2DM occurs when this condition is accompanied by a failure of the pancreatic β-cells to secrete enough insulin to overcome the degree of insulin resistance.
The specific reasons for the development of these abnormalities are not yet known. Genome-wide association studies have identified over 50 new genetic loci for T2DM (Pasquale et al., 2013). Offspring of diabetic people who share similar environments have more chance of developing diabetes (Doria et al., 2008). Both environment and lifestyle are involved in the development of T2DM, in addition to genetic factors (Qi et al., 2008).

Overall obesity and abdominal adiposity, are by far the most important risk factors for the development T2DM (Wang et al., 2005). Obesity continues to increase in children and adolescents (Goran et al., 2003), and rising levels of obesity are leading to a rapid increase in the prevalence of T2DM. Recent studies have provided evidence that release of non-esterified fatty acids from adipose tissue is chronically elevated (Heilbronn et al., 2004), which can mediate many adverse metabolic effects, most notably insulin resistance (Karpe et al., 2011). Adipose tissue also produces a variety of biologically active molecules (adipocytokines) (Matsuzawa et al., 1999). Hotamisligil et al. (1993) reported that tumour necrosis factor-α (TNF-α) overproduced in adipose tissue in obesity contributes to the development of insulin resistance. Other biologically active molecules called leptins (specific hormones contributing to the regulation of energy expenditure and food intake) also affect insulin sensitivity and may participate in the development of hypertension (Shimomura et al., 1999, Masuzaki et al., 1999).
1.3.2.1 Risk factors for T2DM

1.3.2.1.1 Age

The risk of developing T2DM increases with age (Wild et al., 2004, Klein et al., 2004). Those aged ≥ 45 y are more prone to T2DM in developing countries, while in developed countries the largest number is found in those aged ≥ 65 (Whiting et al., 2011). Previously, T2DM was predominantly a disease of middle-aged and older people. In recent decades, the age of onset has decreased and T2DM has been reported in adolescents and children worldwide (Alberti et al., 2004).

1.3.2.1.2 Excess weight

Obesity is the most critical risk factor for T2DM (Kahn et al., 2006). Increase in body mass index (BMI) is associated with escalation of the incidence of diabetes (Mokdad et al., 2003, Klein et al., 2004). Recent evidence from some studies has suggested that obesity and visceral fat accumulation are closely related to the incidence of T2DM (Nagaya et al., 2005). Diabetes UK state that those with a normal BMI but a large waist – defined as 80 cm or more for women and 94 cm or more for men – are most at risk from developing T2DM.

1.3.2.1.3 Genetic factors (family history of diabetes)

Although promoting factors for the pathogenesis of T2DM are increasingly sedentary lifestyles and overeating, genetic elements are also involved. Family history, by itself, is the most useful predictor of disease when multiple family members are affected. A positive family history confers a 2.4 fold increased risk for T2DM, and about 15-25% of first-degree relatives of
patients with T2DM develop impaired glucose tolerance or diabetes (Pierce et al., 1995). Both family history and obesity are strong risk factors for T2DM (Hu et al., 2009). A family history of diabetes provides significant improvements in the detection of additional cases of undiagnosed diabetes (Yang et al., 2010).

1.3.2.1.4 Physical inactivity

The main culprit for the worldwide epidemic of obesity and T2DM is sedentary lifestyles (Hu et al., 2003, Temelkova-Kurktchiev and Stefanov, 2012). Regular physical activity raises HDL cholesterol; lowers LDL levels; leads to lower blood pressure; reduces insulin resistance; and favorably influences cardiovascular function (Health, 2001). Evidence from prospective studies points to physical inactivity as an independent risk factor for T2DM (Gill and Cooper, 2008). Long periods of inactivity lead to insulin resistance in humans (Smorawiński et al., 2000). Increased physical activity is associated with a better glucose and insulin profile, and enhanced β-cell function (Chen et al., 2013). Recent studies demonstrate that enhanced physical activity may delay or prevent the transition from impaired glucose tolerance to T2MD (Tuomilehto et al., 2001). Even people with T2DM have reduced risk of cardiovascular disease when they have regular physical activity (Kirk et al., 2004).

1.3.2.1.5 Ethnicity

Epidemiological studies have shown that T2D has a global distribution, and its prevalence varies from country to country, and in different ethnic groups in the same country. African Americans are more likely to develop diabetes
than white Americans (Egede and Dagogo-Jack, 2005). Also, Asian Indians who immigrated to Western countries are found to be at risk for the development of T2DM due to the metabolic impact of a westernized diet or reasons based on tissue resistance to insulin (Lee et al., 2011). Other risk factors include the history of gestational diabetes, impaired fasting glucose or impaired glucose tolerance, hypertension, dyslipidaemia, polycystic ovary syndrome and history of vascular disease (Klein et al., 2004).

1.3.3. Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is a temporary condition which occurs during pregnancy (usually during the second and third trimester) (Park et al., 2001). In other women, it may be found during the first trimester of pregnancy, and in these women, the condition most likely existed before the pregnancy. The incidence of GDM has been increasing over the last 20 years (Ferrara, 2007). GDM is defined as glucose intolerance that occurs only during the pregnancy in some pregnant women. GDM-related risk factors have not been completely identified yet. However, family history of T2DM in first-degree relatives, older age, obesity, hypertension, elevated blood platelet count, and increased haemoglobin (Hb) and ferritin levels have been identified as potential risk factors for GDM (Teh et al., 2011, Casanueva and Viteri, 2003). GDM is associated with both insulin resistance and impaired insulin secretion (Ryan et al., 1985, Damm, 1998). The mechanisms behind insulin resistance induced by pregnancy are still not fully understood; it occurs because the body produces insufficient insulin to meet the extra needs of pregnancy (ADA, 2011). Knopp et al. (1981) reported that insulin levels in pregnant rats are reduced due to increased
removal of insulin by the placenta. There are also different hormonal and metabolic changes during the second half of pregnancy which facilitate insulin resistance. One is the high level of progesterone in plasma during the second part of pregnancy (Wada et al., 2010). Oxidative stress is implicated in the development of insulin resistance during pregnancy (Patil et al., 2007).

1.4 Complications of diabetes

Chronic hyperglycaemia clustering with other risk factors such as arterial hypertension and dyslipidaemia, as well as genetic factors, can affect diverse parts of the body and, over time, can lead to serious complications. Even with tight glucose control, improved treatment of hypertension and hyperlipidaemia, diabetic complications occur frequently with time (Gallego et al., 2007). The major types of diabetes complications are microvascular and macrovascular. As a consequence of its microvascular pathology, diabetes affects the renal system (nephropathy), causes eye damage (retinopathy) and causes nerve damage (neuropathy). The macrovascular complications of diabetes are associated with effects on the large arteries that supply the heart, brain and lower limbs. As a result, patients with diabetes have a much higher risk of myocardial infarction, stroke (Janghorbani et al., 2007) and limb amputation (Singh et al., 2005). Also there are many other health problems associated with the presence of diabetes such as hip fracture; infertility and negative pregnancy outcome; psychological problems; and cancer (Zhu et al., 2013).
1.4.1 Vascular disease

Vascular abnormalities begin early in diabetic patients. High glucose concentrations alter vascular function, leading to endothelial dysfunction due to imbalance between nitric oxide (NO) bioavailability and accumulation of reactive oxygen species (ROS) (Creager et al., 2003). NO is the most important endothelium-derived relaxing factor (EDRF) and is derived from L-arginine via the action of endothelial nitric oxide synthase (Figure 1.1).

![Figure 1.1 nitric oxide synthesis (Torreilles, 2001)](image)

NO induces vasodilatory effects by activation of soluble guanylate cyclase via an interaction with the ferrohaem centre of the enzyme resulting in the generation of increased cyclic guanidine monophosphate (cGMP) levels. This in turn leads to activation of cGMP-dependent protein kinase which catalyses the phosphorylation of specific muscle proteins, and causes smooth muscle relaxation and subsequent dilation of vasculature.
Hyperglycaemia-induced generation of superoxide anion radical (O$_2$$^•_-$) via different mechanisms (Figure 1.3) causes inactivation of NO through the formation of peroxynitrite (ONOO$^-$), a powerful oxidant which easily crosses phospholipid membranes and induces substrate nitration (Creager et al., 2003). Nitrosylation has been shown to inhibit antioxidant enzymes and endothelial NO synthase activity (Hink et al., 2001).

![Figure 1.2 peroxynitrite formation and tyrosine nitration](image)

1.4.2 Diabetic retinopathy

Diabetic retinopathy is one of the most common causes of blindness (Forbes and Cooper, 2013). It is highly prevalent and is positively associated with the duration of diabetes (Lutale et al., 2013). The retina is a thin layer of nervous tissue in the back of the eye from which focussed images are carried to the brain via the optic nerve. Diabetic retinopathy is
characterized by changes in the retinal blood vessels. Constriction and dilation of these vessels can cause obstruction of normal blood flow, haemorrhage, and leakage of fluid into rear of the eye causing vision problems.

1.4.3 Peripheral neuropathies

Peripheral neuropathy and specifically diabetic peripheral neuropathy (DPN) is one of the most severe complications of diabetes, occurring in 30-50% of all diabetic patients (Deshpande et al., 2008). Both the age of the patient and the duration of diabetes are independent risk factors for DPN (Adler et al., 1997). DPN is associated with loss of protective limb mechanical sensations, traumatic ulceration injures and therefore amputations (Boulton et al., 2005).

1.4.4 Autonomic neuropathy

Autonomic neuropathy affects the nerves that control involuntary activities of the body. It is among the least recognized and understood complications of diabetes, despite its significant negative impact on survival and quality of life in people with diabetes (Vinik and Ziegler, 2007). The action of many organs such as the stomach, intestine, bladder, penis and sometimes the heart can be affected. The clinical manifestations include orthostatic hypotension, exercise intolerance, gastroparesis, diarrhea, constipation and urinary incontinence (Watkins, 2003).
1.4.5 Diabetic nephropathy

Diabetic kidney disease, also called diabetic nephropathy, is a complication that occurs in some people with diabetes. It can progress to kidney failure in some cases. Approximately 40% of people with diabetes will develop nephropathy. A number of risk factors for the progression of nephropathy have been identified including poor glycaemic control, hypertension, smoking, genetic susceptibility, age, race and obesity. The diabetes control and complications trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that improved glycaemic control reduces the risk of diabetic nephropathy in both T1DM and T2DM patients (Turner et al., 1993, Turner et al., 1998).

The first sign of renal problems in patients with diabetes is microalbuminuria. This is defined by the ratio of urinary albumin:creatinine (ACR). In men microalbuminuria is defined as a ratio greater than 2.5 mg mmol\(^{-1}\) and in women greater than 3.5 mg mmol\(^{-1}\) (Burden and Tomson, 2005). This affects over 20% of Type 1 and Type 2 diabetic patients 10-15 years after the onset of diabetes and subsequently may evolve to macroalbuminuria or proteinuria (ACR > 30 mg mmol\(^{-1}\)) (Remuzzi et al., 2002). Once macroalbuminuria is present, the glomerular filtration rate declines at an average rate of 10-12 ml min\(^{-1}\) y\(^{-1}\) in untreated patients (Remuzzi et al., 2002).

Several factors may take charge in diabetic nephropathy, but the main factors are the persistence of high levels of blood glucose due to poor blood sugar control and the decline of insulin signaling due to insulin resistance or
insulin depletion. The precise mechanisms which are responsible for diabetic nephropathy are still being determined. Hyperglycaemia initiates, sustains and promotes the progression of diabetic nephropathy pathology. Extracellular and intracellular proteins are often exposed to glycation reactions. Outside the cell the major toxicity is from accelerated formation of advanced glycation end products (AGEs) (described in detail later) such as imidazole, carboxy-methyl-lysine (CML), carboxy-ethyl-lysine (CEL), glyoxal-lysine dimer (GOLD) and methyl-glyoxal-lysine dimer (MOLD). These are derived from glycation reactions of proteins with glucose or its metabolites, and result in production of stable, covalent modifications of proteins. These modifications may include cross links within or between proteins. AGE-modified proteins increase in several compartments of diabetic kidneys (Tanji et al., 2000). Collagen and other long-lived extracellular matrix proteins are particularly prone to accumulate AGE crosslinks (Susic et al., 2004); these crosslinks prevent degradation of the extracellular matrix and accumulation of the extracellular matrix leads to thickening of basement membranes (Lubec and Pollak, 1980).

Circulating levels of AGE-modified proteins also increase in diabetics (Penfold et al., 2010). These soluble AGE-modified proteins are ligands for a receptor known as the advanced glycation end product receptor (RAGE); this receptor is increased in several cells in diabetic kidneys including podocytes (Tanji et al., 2000). This increase leads to promotion of the inflammation process (Ramasamy et al., 2011). Binding to the RAGE receptor also increases intracellular production of reactive oxygen species (ROS) leads to activate of NADPH oxidase, which promotes intracellular
damage (Goldin et al., 2006). Inside the cell hyperglycaemia causes cell damage or death through several mechanisms such as the aldose reductase pathway, the intracellular AGE and hexosamine pathways, the PKC pathway and the hexosamine pathway (Giacco and Brownlee, 2010). The abnormal signaling of insulin may act to disrupt important paracrine signaling between podocytes and endothelial cells required for normal maintenance of the filtration membrane (Welsh et al., 2010).

Other factors such as high blood pressure, high cholesterol and smoking increase the leakage of albumin across the glomerular podocyte filtration barrier. In addition, the raised blood glucose level may cause some proteins in the glomeruli to cross-link. These cross-linked proteins can trigger a localised scarring process. This scarring process in the glomeruli is called glomerulosclerosis. It usually takes several years for glomerulosclerosis to develop and it only happens in some people with diabetes.

As the condition becomes worse, scarred tissue (glomerulosclerosis) gradually affects smooth muscle cells surrounding the glomerular capillaries (diffuse mesangial sclerosis). Then there is progressively greater mesangial expansion. As a result the kidneys become less capable of blood filtration. This gradual ‘failing’ of the kidneys may gradually progress to what is known as end-stage kidney failure.

1.5 Iron and Type 2 diabetes

The body requires two types of nutrients, micronutrients and macronutrients. Both types are essential for the proper functioning of cells in the body and
are vital for good health. The impacts of macronutrients on human physiology have been studied extensively. Many of these studies have been carried out into the relationships between macronutrients, especially lipids, carbohydrates and protein, and the aetiology of T2DM (Schulze and Hu, 2005), but the certain role of micronutrients in general and iron in particular in the aetiology of T2DM is not well established (Fernández-Real et al., 2002a). Nevertheless, plausible evidence has emerged recently to explain the positive association between excess iron stores and risk of T2DM (Rajpathak et al., 2009, Smotra et al., 2007), although the underlying mechanisms still remain to be fully determined.

The involvement of iron in the risk of T2DM has generated substantial interest over the past 15 years, initially sparked by an association with increased serum ferritin and the observation that diabetes frequently develops in those individuals with iron-overload diseases such as haemochromatosis and recurrent transfusions in diseases like thalassaemia (Sanctis et al., 2013). Also, a large body of epidemiological evidence suggests that an increase in dietary iron (as haem, mainly from meat and meat products) is associated with an increased risk of diabetes (Shah and Fonseca, 2011). It is recognized that iron influences glucose metabolism, even in the absence of significant iron overload (Fernández-Real et al., 2002a).

Firstly, iron is a redox-active transition metal and this property makes it a first-line pro-oxidant that promotes the generating of hydroxyl radicals (HO•) via the Haber-Weiss reaction.
$\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2$

$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-$ (Fenton reaction)

The net reaction is:

$\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2$ (Haber-Weiss reaction)

Hydroxyl radicals may attack pancreatic β-cells through increased oxidative stress, and thus result in impaired insulin synthesis and excretion (Evans et al., 2002, Cooksey et al., 2010). Also there are lower levels of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets than in other tissues; thus pancreatic Islets are particularly susceptible to oxidative damage (Tiedge et al., 1997).

Iron reduces hepatic extraction and metabolism of insulin, leading to peripheral hyperinsulinaemia, and may increase oxidative stress, which inhibits the internalization and action of insulin (Tuomainen et al., 1997). Insulin also influences iron metabolism by stimulating ferritin synthesis and facilitating iron uptake by the cell through the translocation of transferrin receptors from the intracellular compartment to the cell surface (Davis et al., 1986, Jehn et al., 2004).

Genetic iron-overload disease, haemochromatosis, where serum iron levels may exceed 10× the normal values compared to healthy individuals, is a risk factor for T2DM (Moczulski et al., 2001). Interestingly, the pathophysiology of diabetes in haemochromatosis is thought to be due primarily to defects in the early insulin response to glucose.

Increased iron stores are associated with the development of glucose intolerance and increased risk of T2DM (Fernández-Real et al., 2002a,
Barbieri et al., 2001, Fernández-Real et al., 1998, Ford and Cogswell, 1999, Jiang et al., 2004). In contrast, serum ferritin is a biomarker for iron stores which increase in response to inflammatory stress (De Domenico et al., 2008), so increased ferritin in diabetes could simply reflect the inflammatory component of that disease (De Domenico et al., 2008). Nevertheless, phlebotomy improves insulin sensitivity (Fernández-Real et al., 2002b), supporting the argument that iron may play a causal role in diabetes.

In the general population, higher dietary intake of haem is positively associated with risk for T2DM (Rajpathak et al., 2006, Bao et al., 2012). Dietary total iron, non-haem iron, or supplemental iron intakes are not significantly associated with T2DM risk (Vlassara et al., 2002). In contrast, patients with T2DM and high levels of serum ferritin who undergo frequent phlebotomy have been shown to have decreased levels of glycated proteins (e.g. HbA\textsubscript{1c}) and improved insulin sensitivity (Silva and Hider, 2009). Other iron status parameters have been shown to be altered among people with T2DM. For example, circulating non-transferrin-bound iron (discussed in detail on section 1.6.13) levels were found to be higher in people with T1DM compared to healthy controls (Lee et al., 2006).

Increased oxidative stress is widely accepted as contributing towards the development and progression of diabetes, and its complications (Baynes and Thorpe, 1999, Houstis et al., 2006). Acceleration in the process of atherosclerosis is very common in diabetes, leading to increased prevalence of coronary artery disease and stroke. There are probably multiple mechanisms for the initiation and progression of atherosclerotic
lesions. While the typical cardiovascular risks, such as hypertension, hyperlipidaemia and obesity, are known to be more widespread in diabetic patients, recent studies have provided evidence that hyperglycaemia plays a vital role in diabetic complications (Nathan et al., 2003, Stratton et al., 2000, Cerami et al., 1985). Hyperglycaemia stimulates a variety of biochemical abnormalities (Figure 1.3), for instance activation of the polyol pathway; enhanced non-enzymatic glycation with formation of advanced glycation end-products (AGEs); increased hexosamine pathway flux and changed protein kinase C (PKC) activities, which have been implicated in the pathogenesis of diabetic complications. Recent evidence suggests that they are also concerned in the pathogenesis of diabetic atherosclerosis (Massi-Benedetti and Orsini Federici, 1999, Cerami et al., 1985, Bucala and Vlassara, 1995, Berg et al., 1997, Renier et al., 2003, Ruef et al., 2000).

In contrast to the findings in diabetes, there is evidence that obese non-diabetic subjects have low serum iron levels (Chambers et al., 2006, Freixenet et al., 2009, Tussing-Humphreys et al., 2009b), and this does not appear to be due in all cases to dietary iron deficiency (Menzie et al., 2008). The likely mechanism of the hypoferraemia of obesity is the chronic inflammatory state which accompanies obesity (Yanoff et al., 2007, McClung and Karl, 2009). However, other studies in particular groups of subjects suggest that the cause might be true body iron deficiency rather than a response to inflammation due to poor dietary iron Intake or increased iron requirements (Tussing-Humphreys et al., 2009a). Thus the cause is currently unclear.
Figure 1.3 mechanisms by which hyperglycaemia may cause oxidative stress
Over production of superoxide reduces glucose utilization via glycolysis due to inhibition of the glycolytic enzyme GAPDH. This results in increased flux of dihydroxyacetone phosphate (DHAP) to the protein kinase C pathway and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Also excess superoxide leads to increased glucose flux through the polyl pathway, increased sorbitol and decreased NADPH levels, hence leading to the depletion of GSH. The oxidation of sorbitol to fructose is catalysed by the enzyme sorbitol dehydrogenase, with NAD$^+$ reduced to NADH; fructose-3-phosphate (F3P) is produced by enzymatic phosphorylation of fructose. F3P is an unstable compound, and its spontaneous decomposition leads to formation of 3-deoxyglucosone (3-DG), an AGE precursor. Modified from Brownlee (2001).
1.6 Iron

1.6.1 Functions of iron in the human body

Nearly all living organisms require iron in order to survive. Iron is a vital element for most biological processes such as growth and metabolism (Prakash, 2007). In humans and all aerobic organisms, it is an essential mineral for good health. It has several important functions in the body. The capability of iron to readily donate or accept electrons makes it essential for important cellular redox processes. However, this redox reactivity can also be deleterious if uncontrolled. It is involved in numerous enzymatic electron transfer reactions (Chua et al., 2007). Mitochondrial function is dependent on iron in several ways. Iron is a metal cofactor for haemoproteins involved in the mitochondrial electron transport chain, the ‘engine’ of cellular energy production. Both haem-containing enzymes (cytochromes) and iron-containing non-haem enzymes (NADH dehydrogenase and succinate dehydrogenase) play an essential role in the generation of ATP by oxidative phosphorylation of ADP via the citric acid cycle. Iron is an integral part of the protein compounds such as Hb and myoglobin, where it is the binding site for oxygen. Thus, Hb and myoglobin perform the important function of oxygen transport in human body (Beard, 2001). Iron is vital for brain development, neurogenesis, as well as normal brain function (Crichton et al., 2011). In the central nervous system (CNS), iron is essential for multiple functions, including gene expression, DNA synthesis, neurotransmission, myelination, oxygen transport, storage and activation, mitochondrial electron transport, numerous important metabolic processes and as a cofactor for several key enzymes of neurotransmitter biosynthesis, such as dopamine.
(DA) and noradrenaline. It also plays an important role in cellular immune response (McKie et al., 2001, Muñoz et al., 2009). It is essential for many enzymes including catalase. Table 1.2 summarizes iron-containing proteins and their biological functions.

Table 1.2 Iron containing proteins and their biological function

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
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<tbody>
<tr>
<td>Haemoglobin</td>
<td>Oxygen transport</td>
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<td>Myoglobin</td>
<td>Oxygen storage and transport</td>
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<td>Ribonucleotide reductase</td>
<td>Deoxyribonucleotide synthesis</td>
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<td>Aconitase</td>
<td>Citric acid cycle</td>
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<td>Transferrin</td>
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<td>Lactoferrin</td>
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<td>Ferritin</td>
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<td>Cytochromes</td>
<td>Electron transport/ATP synthesis</td>
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<td>Monooxygenases</td>
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<td>Haemopexin</td>
<td>Haem delivery</td>
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<td>Nitric oxide synthases</td>
<td>Synthesis of nitric oxide</td>
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1.6.2 Iron distribution

Human adults contain about 2-4 g of iron (Andrews, 2005, Nathan et al., 2007), but this amount greatly depends on gender, age, nutrition and health. Thus iron levels are usually lower in women due to their smaller size, a reduced liver mass, and iron loss through menstruation (Andrews, 1999a). More than 50% of the body's iron in normal individuals is found in the Hb of blood erythrocytes and their precursors. Nearly 25% of the iron is maintained as storage iron (ferritin and hemosiderin) (Cheng and Li, 2007) mainly in the liver. The remainder exists in myoglobin, reticuloendothelial macrophages and haem enzymes.

In humans, about 20-25 mg of iron is the daily requirement for adults to produce new erythrocytes (Figure 1.4), most of which comes from recycling of iron already in the body. Daily absorption of approximately 1-2 mg is needed to replace the normal iron losses (around 1-2 mg per day) through the stools (0.6 mg d⁻¹), urine (0.15-0.2 mg d⁻¹) and skin (0.2-0.25 mg d⁻¹) (Green et al., 1968). The tissues and cells that generate major iron flows into the blood plasma include the duodenal enterocytes involved in dietary iron absorption; hepatocytes that are the main site of iron storage; macrophages that recycle iron from senescent red blood cells; and the placental syncytiotrophoblasts involved in iron transfer from mother to fetus during pregnancy (Abdul-Ghani et al., 2006, Andrews, 2005, Nathan et al., 2007).

The human excretory system has no active mechanism to excrete iron through the kidney or liver; iron is lost from the body only when cells are
lost, particularly epithelial cells from the gastrointestinal tract, epidermal cells of the skin, and red blood cells in menstruating women (Nathan et al., 2007). As a result, iron homeostasis must be tightly regulated and the balance is achieved by control of absorption rather than by control of excretion (Sun et al., 2008).

Figure 1.4 Distribution of total body iron among tissues. From Hentze et al. (2004).
1.6.3 Iron absorption

Normally, mammals obtain iron exclusively from the diet (Ganz and Nemeth, 2006). The daily diet contains two forms of iron which are typically classified as either haem or non-haem iron (Rajpathak et al., 2009). Both two types are absorbed by distinct pathways and in different amounts. Non-haem iron is found in a wide variety of plant and animal foods (Rajpathak et al., 2009), in the intestinal tract many complexes containing non-haem iron are formed, which can decrease or increase absorption. Foodstuffs contain phytates (high-fibre diets), calcium, phenolic compounds (coffee, tea) and phosphates which tend to form insoluble complexes with iron and therefore limit its absorption. The absorption of non-haem iron can be diminished by co-administration of tetracycline, proton pump inhibitors and antacid medication. In addition, *Helicobacter pylori* infection produces gastric atrophy, which can lead to profound iron-deficiency anaemia (Marignani et al., 1997), whereas ascorbate and compounds from meats can increase absorption (Rajpathak et al., 2009, Hurrell, 2002).

Dietary haem iron is derived from the proteolytic breakdown of Hb and myoglobin found in meat products in the intestinal lumen (Dunn et al., 2007). Haem iron is absorbed more efficiently than non-haem iron (Anderson et al., 2005, Majuri and Gräsbeck, 1987). Also haem iron absorption is little affected by dietary iron chelators or enhancers (Rajpathak et al., 2009). Most iron absorption takes place in the duodenum and proximal jejunum (Johnson et al., 2012). Non-haem iron is primarily present in the ferric form (Fe$^{3+}$). Duodenal cytochrome b (Dcytb), a ferric reductase, which is expressed in the duodenal brush border, plays an important role in
dietary iron absorption by reducing ferric to ferrous iron (Fe$^{2+}$) (Figure 1.5), which is then taken up by the protein involved in ferrous iron absorption, divalent metal transporter 1 (DMT1) (Donovan et al., 2006, Anderson et al., 2005). Haem iron seems to be transported intact by haem carrier protein 1 from the gut lumen into the enterocytes (Krishnamurthy et al., 2007), but the process is less well understood (Anderson et al., 2005). Once internalised inside the enterocyte it is likely that most dietary haem is metabolised by haem oxygenase to release Fe$^{2+}$; part of the iron is oxidized back to the ferric state and bound to the ferritin for storage (Andrews and Schmidt, 2007).

Most of the iron is transferred across the basolateral membrane of the enterocyte surface and oxidised to Fe$^{3+}$ via hephaestin before being bound by plasma transferrin. The major molecule that carries iron across the membrane is ferroportin (FPN; also known as SLC40A1, IREG1 and MTP1) (Ganz and Nemeth, 2006). As the sole known iron exporter, FPN is highly expressed in duodenal enterocytes, hepatocytes, macrophages and placental cells (Ganz and Nemeth, 2006).

The critical role of FPN in intestinal iron absorption and macrophage iron release was confirmed in FPN knockout mice (Donovan et al., 2005). Similar to DMT1, FPN likely conducts ferrous (Fe$^{2+}$) ions. However, Fe$^{2+}$ must be oxidized to Fe$^{3+}$ before it can enter the circulation and load onto plasma transferrin. Thus another ferroxidase protein, hephaestin in enterocytes or ceruloplasmin in macrophages, is also required for cellular iron export (Ganz, 2005, Chen et al., 2006). Hephaestin is a membrane-bound multicopper
ferroxidase expressed mainly in the mammalian small intestine. The ferroxidase activity of hephaestin is thought to play an important role during iron export from intestinal enterocytes and the subsequent iron loading of the blood protein transferrin, which delivers iron to the tissues (Chen et al., 2003).

Figure 1.5 Systemic iron transport
Dietary ferric iron (Fe³⁺) is converted to ferrous iron (Fe²⁺) by ferric reductase in the duodenum proximal jejunum, then DMT1 transports Fe²⁺ into enterocytes. Intact haem from the diet is also imported into enterocytes by haem carrier protein 1 and then converted into ferrous iron by HO1. Ferrous iron is then exported into the bloodstream by FPN. Most of the iron is transferred across the basolateral membrane of the enterocyte surface and oxidised to Fe³⁺ via hephaestin and ceruloplasmin before being bound by plasma transferrin. Iron export by FPN can also be inhibited by the peptide hepcidin. In the bloodstream, ferric iron binds to Tf, which enables import into target cells by binding to TfR. Once bound to the cell membrane, Tf changes shape and releases the iron. It then returns to the portal circulation to bind more iron. DMT1: Divalent metal transporter 1. FPN: Ferroportin. Hp: Hphaestin. Ce: Ceruloplasmin. Tf: Transferrin. TfR: Transferrin receptor. From Rines and Ardehali (2012).
1.6.4 Iron transport
Iron released into the circulation binds to transferrin (Tf) and is transported to sites of use and storage. Tf is an abundant protein mainly found in blood plasma and lymph (2.5 mg ml⁻¹) (Crichton et al., 2008). It is the primary iron transporter in the plasma (Anderson et al., 2007, Atanasiu et al., 2007). Tf is a β globulin and is produced in the liver. Each molecule of Tf can bind and transport two molecules of iron in the Fe³⁺ state. In the circulation, Tf carries nearly all serum iron while small amounts of iron may be loosely associated with other proteins including albumin (Andrews and Schmidt, 2007). Tf saturation rapidly responds to local circumstances and is usually used as an indicator of body iron status. Under normal circumstances, approximately one third of the Tf is bound with iron (Beutler, 2006, Donovan et al., 2006). In mice, the Tf saturation is much higher, ranging from 60% to 80% (Andrews and Schmidt, 2007). The majority of transferrin-bound iron uptake occurs in the bone marrow, where erythroid precursors incorporate the iron into the haem moiety during synthesis of Hb (Donohue et al., 1958). but also other organs uptake iron if the bone marrow is damaged or excessive amounts of iron are already stored in the bone marrow (Andrews, 2005). Once bound to the cell membrane, Tf changes shape and releases the iron. It then returns to the portal circulation to bind more iron.

1.6.5 Iron uptake in the cells
Precursor erythroid cells, hepatocytes, macrophages, placental cells and most other cells can take up the transferrin-bound iron from the circulation using transferrin receptors (TfR) (Donovan et al., 2006, Ganz and Nemeth, 2006). Erythroid bone marrow is the largest consumer of iron. Normally,
around two thirds of the body iron is found in developing erythroid precursors and mature red blood cells (Donovan et al., 2006). Erythroid precursors express cell-surface TfR that take up Fe-Tf by receptor-mediated endocytosis (Abdul-Ghani et al., 2006).

The affinity of the TfR for Tf depends on two parameters, pH and Tf iron saturation. The affinity is highest for fully saturated Tf. However, when the pH decreases to the pH of the endosomes, pH 5.5, the TfR affinity for apotransferrin increases, whereas the affinity for saturated Tf decreases (Huebers et al., 1983, Welch, 1992). Although TfRs are widely expressed, most cells apparently can use non-TfR mechanisms to assimilate iron. However, the utilization of iron by muscle cells and other cell types such as neurons is far less well understood (Andrews and Schmidt, 2007). Once iron leaves the endosome (Figure 1.6), it moves to the mitochondria for incorporation into protoporphyrin IX via the action of ferrochelatase to form haem.
Figure 1.6 Iron uptake in cells
Transferrin receptor 1 on the cell surface binds to transferrin-bound iron from the circulation (holotransferrin). The resulting complex is internalized and acidified through the action of a proton pump to the pH of the endosomes, 5.5. Iron is subsequently released from transferrin and transported out of endosomes via the divalent metal transporter DMT1. Once iron leaves the endosome, it moves to the mitochondria for incorporation into protoporphyrin IX via the action of ferrochelatase to form haem. Apotransferrin and transferrin receptor 1 both returned to the cell surface, where they dissociate at neutral pH, and both participate in another round cycle of iron uptake. From Qian et al. (2002).
1.6.6 Transferrin receptors

The transferrin receptor (TfR) is an essential glycoprotein involved in iron uptake and the regulation of cell growth. This glycoprotein has a molecular weight of approximately 180 kDa. The human transferrin receptor has homodimeric structure composed of two subunits linked by two disulphide bridges. Each monomer can be divided into an extracellular region, an intermediate transmembrane region and an intracellular part. The intracellular part is necessary for normal endocytosis of the receptor (Rothenberger et al., 1987, McGraw and Maxfield, 1990). All cells express a TfR on their surface; the quantity of receptor molecules reflects the cellular iron requirement. There are two different TfRs, TfR1 and TfR2. TfR1-mediated endocytosis is the usual pathway of iron uptake by body cells. TfR2 participates in low-affinity binding of Tf, supporting growth in a few cell types, but the true role of TfR2 is unknown (Halliwell and Gutteridge, 2007, Gkouvatsos et al., 2012).

1.6.7 Iron storage

Two cell types are important for systemic storage of iron (Ganz, 2005, Ganz and Nemeth, 2006), macrophages and hepatocytes. Macrophages can recover iron from senescent erythrocytes. Hepatocytes can acquire both Tf-bound and non-Tf-bound iron from the plasma, and also have a large capacity for iron storage. Inside the cells, iron storage occurs primarily in ferritin which can frequently be found within lysosomes (Beutler, 2007). Ferritin, like Hb, is a major iron storage protein. The iron complex with ferritin is water soluble and nontoxic yet the iron is in a bioavailable form. Ferritin consists of 24 subunits, each subunit containing up to 4500 hydrated and polymerized ferric iron ions within a protein shell with a hollow interior of
approximately 9 nm diameter. In vertebrates the molecules of ferritin are composed of two types of subunit, L and H, while in plants and bacteria there is only the H type (Briat et al., 2010, Welch et al., 2002).

The protein shell surrounding the iron core is penetrated by six channels through which ferrous iron enters to interact with a ferroxidase at the centre of the molecule (Harrison and Arosio, 1996). Iron is able to exit after it has been reduced. This iron depot is readily accessible for Hb synthesis. Ferritin binds iron in a catalytically inactive manner; accordingly, oxidative reactions cannot be promoted by iron bound to ferritin. Because the main iron pool of most cells is located within the ferritin core, it is important to know under what circumstances this pool may become a source of free iron capable of causing oxidative damage (Rudeck et al., 2000). Ferritin iron can be removed by strong iron chelators but this occurs extremely slowly (Chasteen, 1997, Linert and Jameson, 2000), due to their ability to reduce tightly bound Fe$^{3+}$ to the more mobile iron Fe$^{2+}$ form. Direct chelation of Fe$^{3+}$ may also mobilize iron from ferritin with or without reduction to Fe$^{2+}$. In the absence of chelators, the reduced core of ferritin iron appears to remain within the protein shell (Hynes and Ó Coincceanainn, 2002).

Another iron-storage complex, haemosiderin, also deposits iron in the Fe$^{3+}$ state but in a water-insoluble form (Chen et al., 2006). Haemosiderin is most commonly found in macrophages and is especially abundant in situations following haemorrhage (Ganz, 2005). Deposition of iron in haemosiderin can cause organ damage and has been found to be associated with several diseases (Chen et al., 2006).
Ferritin is often elevated by infections, inflammation, chemotherapy, radiation and liver injury (Nielsen et al., 2000). Small amounts of ferritin are normally found in the circulating plasma within the range 15-300 µg l$^{-1}$. Serum ferritin levels increase as a result of iron overload, aging, infection, inflammation, liver disease, juvenile rheumatoid arthritis, leukaemia and Hodgkin’s disease, and they decrease as a result of iron deficiency (Forouhi et al., 2007, Muñoz et al., 2011).

1.6.8 Iron recycling

Due to lack of a regulated mechanism for iron excretion, iron absorption is controlled. Each day less than 0.1% of the total body iron (1-2 mg) enters the circulation through intestinal absorption (Andrews and Schmidt, 2007), and most circulating iron comes from senescent erythrocytes recycled by macrophages. Tissue macrophages recognize old and damaged erythrocytes (particularly in the spleen), and then remove them from circulation (Donovan et al., 2006). Within the macrophages the erythrocyte membranes are broken and the Hb is degraded. Haem oxygenase catalyzes the release of iron from haem. The amount of iron daily recycled by macrophages approximates the amount needed for erythropoiesis (about 20 mg). Some iron remains in macrophages while another portion is exported to blood plasma (Ganz, 2005). FPN is critical for macrophage iron export and is involved in the regulation of the ratio between stored and released iron (Ganz, 2005, Donovan et al., 2005).
1.6.9 Iron homeostasis

The content of iron in the human body is regulated by a complex mechanism for maintaining homeostasis. Iron homeostasis is achieved by regulating the major iron flows into the plasma; absorption of dietary iron by duodenal enterocytes; release of stored iron from hepatocytes; and release of iron from macrophages recycling senescent red blood cells (Ganz and Nemeth, 2006). The stimuli known to modulate the iron homeostatic mechanism are erythroid iron needs, hypoxia, iron deficiency, iron overload and inflammation (Andrews, 2005). For a long time, it was believed that there are two regulators (iron store regulator and erythroid regulator) responsible for iron homeostasis although the identities of these were not known (Finch, 1994). Since the beginning of this century, the discovery of hepcidin (Krause et al., 2000, Park et al., 2001, Pigeon et al., 2001) and the ferroportin receptor (McKie et al., 2000) has allowed a fuller appreciation of the regulation of iron homeostasis.

1.6.10 Hepcidin

Hepcidin is small cysteine-rich cationic peptide hormone responsible for controlling the amount of circulating iron. It circulates in the serum in free form or in combination with β-2 microglobulin and is eliminated mainly with the urine (Atanasiu et al., 2007). Hepcidin is mainly produced by hepatocytes (Shayeghi et al., 2005, Hentze et al., 2010). It is also synthesised by other cells and organs such as monocytes, macrophages, heart, kidney, brain and adipose tissue. Hepcidin acts as a key regulator by binding to the iron transporter ferroportin resulting in internalization and lysosomal degradation of ferroportin, and preventing egress of iron from cells into plasma (Ganz,
Most types of hereditary haemochromatosis result from the mutations that target hepcidin or ferroportin themselves or the pathways that control hepcidin expression. This emphasizes that hepcidin and ferroportin are main players in regulation the systemic levels of iron (Brissot et al., 2011).

The process of regulation of plasma iron by hepcidin takes place in three ways, by regulation of the absorption of dietary iron from the intestine (Laftah et al., 2004); by the release of recycled iron by macrophages (Knutson et al., 2005); and by the movement of stored iron from hepatocytes. Decrease in hepcidin concentration may be the main cause of hereditary haemochromatosis, and elevated levels of hepcidin may be responsible for effects observed in patients with anaemia of inflammation and chronic disease (Ganz and Nemeth, 2012).

The expression of hepcidin is increased in response to increased serum iron, iron overload and inflammatory signals such as interleukin-6 (IL-6) and lipopolysaccharide (LPS), as well as by leptin (Chung et al., 2007), and is diminished in response to increased erythroid production, hypoxia and iron deficiency (Pigeon et al., 2001, Nicolas et al., 2002), all of which are known to affect iron homeostasis. Moreover, all forms of genetic haemochromatosis are found to be associated with decreased hepcidin production or activity (Bridle et al., 2003, Papanikolaou et al., 2003, Roetto et al., 2002). Hepcidin has been shown to be the linker between inflammation and anaemia (Ganz, 2003).
1.6.11 Iron loading disorders

Under normal circumstances the iron concentration in plasma and extracellular fluid remains in a relatively narrow range despite fluctuating iron supply and demand. Any factor that leads to alterations in iron requirements has the potential to influence iron entry into the plasma. Disorders of iron deficiency and iron overload occur when iron balance is disrupted. Andrews (2005) listed four situations that lead to measurable changes in intestinal iron absorption and tissue iron distribution: abnormal iron availability (iron overload or deficiency), accelerated erythropoiesis, hypoxia and inflammation. Under iron overload or inflammatory conditions, iron absorption and plasma availability will be decreased, while they are increased in response to iron deficiency, accelerated erythropoiesis and hypoxia (Anderson et al., 2007).

1.6.12 Hereditary haemochromatosis

This is a genetic phenomenon resulting from uncontrolled iron absorption, that can lead to iron overload, which causes tissue damage (Allen et al., 2008). The most common type of primary iron overload is HFE-related haemochromatosis (Pietrangelo, 2002). Patients with homozygous haemochromatosis have a mutation within the HFE gene on chromosome 6 consisting of a substitution of cysteine to tyrosine at position 282 (Cys282Tyr) (Andrews, 1999a). Haemochromatosis that is not associated with mutations in HFE is termed non-HFE haemochromatosis. This results from mutations in the genes encoding TfR2, hepcidin and haemojuvelin. The pattern of iron deposition is similar to that seen in HFE-related disease (Bomford, 2002). Other primary types of haemochromatosis are FPN-related haemo-
chromatosis, which shows a difference in its histological appearance. The untreated condition can result in pathologies such as hepatic cirrhosis, hepatocellular carcinoma, diabetes, changes in skin pigment, endocrine failure, heart disease and arthritis (Edwards and Kushner, 1993, O'Neil and Powell, 2005). This hereditary disease can be avoided by preventing accumulation of iron in the sites of storage in two ways, by frequent donation of blood or by therapeutic venesection (Falize et al., 2006).

1.6.13 Non-transferrin-bound iron

In normal physiological conditions Tf carries iron safely from sites of supply to all needy cells which express the TfR (Hider et al., 2010). The form of iron found in plasma unbound to Tf or other proteins, and composed of complexes of iron-citrate and phosphates or iron-ligands bound to proteins, is called non-transferrin-bound iron (NTBI); this form appears when Tf is saturated with iron due to high absorption of iron or frequent blood transfusion.

Body fluids and tissues which contain NTBI are prone to oxidative stress due to ability of NTBI to catalyse the Fenton reaction and hence its potential for catalysing the formation of free radicals (Gutteridge et al., 1985), thereby inducing cellular toxicity (Anderson, 2007).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^* \\
\]  
\text{(Fenton reaction)}

There is normally little or no NTBI present in the serum of healthy individuals (Breuer et al., 2000b). NTBI is typically present in concentrations up to 10 μM (Porter et al., 1996) and its existence correlates with high levels of transferrin
saturation (Jacobs et al., 2005a). NTBI appears in patients that are very severely iron overloaded such as those with haemochromatosis, who have 100% transferrin saturation (Lee and Jacobs, 2004) and other diseases like haemoglobinopathy, as well as during chemotherapy and after cardiac operation (von Bonsdorff et al., 2002) and in people with liver cirrhosis due to alcohol addiction (Feo et al., 2006). However, the existence of NTBI has also been shown in patients with partially saturated transferrin (Gutteridge et al., 1985, Lee et al., 2006).

Although the existence of NTBI was discovered over 30 years ago its precise definition and chemical nature still needs to be clarified. The form and nature of serum NTBI is not fully understood (Espósito et al., 2002, Hider, 2002). According to Grootveld et al. (1989), a significant portion of NTBI in haemochromatosis is in the form of citrate and, possibly, acetate complexes. The ability of iron to form large iron-polymers under physiological conditions could lead to different types of iron-citrate complexes, varying in size from monomeric to large oligomer complexes with even 17-19 iron ions (Hider, 2002). Albumin could also act as an iron binding ligand due to the presence of large number of negatively charged carboxylate sites (He and Carter, 1992). The mechanism of uptake of NTBI does not require a receptor, which may cause iron accumulation in the cells (Kaplan et al., 1991). Excess NTBI in haemochromatosis may directly initiate the formation of free radicals and enhanced lipid peroxidation which destroys membrane structure resulting in increased oxidative stress and cellular damage (Gutteridge et al., 1985) (Figure 1.7). Peroxidation of LDL is an important step in the process of atherogenesis and is catalyzed by iron (De Valk and Marx, 1999).
Inside the cell the equivalent of NTBI is called the labile iron pool (LIP), which is bound mainly to low molecular weight compounds. The labile iron pool is catalytically active and capable of initiating free radical reactions. The expansion of the LIP and simultaneously increased NTBI may trigger cell toxicity. Generation of LIP leads to unregulated iron uptake and subsequent intracellular storage either within ferritin molecules or as haemosiderin. The adverse effects of iron overload can arise from the elevation of NTBI and LIP in plasma, and might also cause organ damage mediated by the accumulation of tissue iron in target organs. The equilibrium between the LIP and iron locked in the ferritin shell is critical to maintain the normal function of
cellular iron enzymes. Imbalance in this equilibrium results in the uncontrolled loading of organs, such as the liver, heart and endocrine glands causing cell damage such as liver, endocrine glands and heart (Lee and Jacobs, 2004, Malcovati, 2007).

NTBI is capable of participating in oxidative injury, and plays a pathogenic role in diabetes and its complications such as microangiopathy and atherosclerosis (Figure 1.8). Glycation reduces the Tf concentration in diabetes due to elevated urinary Tf excretion rates (Narita et al., 2006) and increased glycation may reduce iron binding to transferrin, which may result in an increase in NTBI.

Figure 1.8 Role of NTBI in diabetes complications
1.6.14 Methods for measuring NTBI

Several assays have been developed to detect NTBI in body fluids. In all these methods different laboratory techniques have been used such as chromatography, fluorimetry, atomic absorption spectroscopy and spectrophotometry. However, none of them is still widely accepted or considered to be a gold standard (Jacobs et al., 2005b); because of the heterogeneous structure of NTBI different iron species may occur in different diseases, and in different tissues (Evans et al., 2008). An additional problem in some cases is the high complexity of methodology, requiring sophisticated specialized instruments and accessories, as well as debatable reliability (Patel and Kushwaha, 2013).

The oldest method, based on indirect determination of NTBI with the help of the antibiotic bleomycin, was first suggested by Gutteridge et al. (1981). This method was based on ability of the bleomycin iron complex to catalyse the generation of extremely reactive hydroxyl radicals (OH\(^\cdot\)) which lead to DNA damage. Only iron in the ferrous form can catalyze such a reaction, and so to facilitate the reaction all the ferric ions are converted to the ferrous form by using a reducing agent such as ascorbate. The degradation of DNA present in the reaction mixture is measured in the form of malondialdehyde. This method has many problems, important ones being the difficulty in controlling the pH of the medium and the variable purity of commercial bleomycin (Graham et al., 1979). Another important aspect is that the ascorbate which is used as reducing agent may not convert all the ferric iron to ferrous iron and so can lead to false low results. The procedure also includes a heating step for production of the end point coloured complex [(thiobarbituric acid) 2-
malondialdehyde], which adds to the complexity of the assay (Burkitt et al., 2001). Another drawback of this method is the indirect way in which NTBI is detected. Jacobs et al. (2005b) found that the bleomycin method showed significantly lower values for mean NTBI than other assays, with poor correlation between values obtained by this method compared to the others.

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 and Hider, 2009). Another method uses fluorescein-apotransferrin (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 (GaCl₃), then reaction-available iron is mobilised with oxalate. Serum and reagents are mixed in multiwell plates, with fluorescence determined after 1 h in a microplate reader (Breuer and Cabantchik, 2001). Drawbacks are that not all the apotransferrin sites are filled, leading to some false positives, and that efforts to overcome this leads to underestimation of NTBI, and failure to detect it at low levels. The Fl-aTf assay was included in the study of Jacobs et al. (2005a) on 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.

Among these different systems for measuring NTBI, the HPLC method which is used in this study is one of the most convenient methods that was developed initially and has been widely used. Consequently because many of the previous methods are complex or inaccurate or require a large quantity of sample often making them unsuitable for clinical use (Paffetti et al., 2006), HPLC has been considered to be a ‘gold standard method’ for NTBI measurement (Sasaki et al., 2011). The assay shows excellent linearity, reproducibility and compatibility. In the method used here NTBI is measured using a slight modification of the HPLC methods of Kime et al. (1996a) and Paffetti et al. (2006). These methods have two steps. In the first step iron is chelated using a chelating agent such as EDTA, oxalate or NTA. Chelating iron by NTA is better than other chelators because it exhibits minimum iron mobilisation from Tf (Singh et al., 1990). NTA concentration is also found to play a critical role in this assay; with 80 mM NTA more than 5-fold higher NTBI values are found compared to using 10 mM NTA (Kolb et al., 2009). This chelated fraction of iron is separated from plasma protein using 30 kDa Amicon ultrafilters (Millipore) (Jacobs et al., 2005b). The method also uses a specialized HPLC instrument, which is free of stainless steel, to reduce contamination from iron, and 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (DHP) is used as the chromogen for detection of the iron.
1.7 Protein glycation

In general, non-enzymatic oxidation and glycation of proteins, lipids and nucleic acids produces heterogeneous compounds called Advanced Glycation End products (AGEs) (Thorpe and Baynes, 2003). The glycation reaction is a process that involves the non-enzymatic reaction between any reducing sugars (sugars such as glucose, fructose, lactose and maltose that contain aldehyde or ketone groups that are oxidised to carboxylic acids are classified as reducing sugars) and peptides or proteins such as Hb, albumin, collagen, transferrin, insulin, erythrocyte membrane proteins, lens crystallins, enzymes and immunoglobulins. The rate of glycation of any protein or peptide is dependent on both the ambient glucose concentration and the duration of exposure (the biological half-life) of the protein, and the number of available free amine groups. Glycation often leads to alterations in the conformation and/or function of proteins (Olufemi et al., 1987). For example, the glycation of erythrocyte Cu-Zn superoxide dismutase leads to a significant reduction in enzyme activity (Arai et al., 1987). This reaction was first recognized by a French chemist Louis-Camille Maillard in 1911, who noticed a characteristic yellowing that took place when a solution containing glycine was incubated with D-glucose. It was later determined that this characteristic colouring was due to complex reactions that eventually led to the formation different classes of heterogeneous compounds.

1.7.1 Maillard reaction and AGE synthesis in humans

Maillard’s reactions have been extensively studied in food chemistry; however, it was only in the ‘70s that they were also shown to occur in vivo, due to the high concentration of D-glucose, which is found in human plasma.
(Koch et al., 2010). The Maillard reaction starts as a reaction between the carbonyl group of a reducing sugar such as glucose and an amine group on proteins, lipids or nucleic acids (Figure 1.9) leading to the production of an unstable compound known as a Schiff base (Lapolla et al., 2005, Lapolla et al., 2006).

![Figure 1.9 The Maillard reaction](image)

The predominant amine groups involved in glycation are therefore lysine residues and the N-terminus of a given polypeptide. This step is reversible and usually takes a few hours to occur (Figure 1.9). Over weeks the Schiff base turns into a more stable compound called the ‘Amadori’ product through various molecular rearrangements (Ahmed and Thornalley, 2003). Over
months and years, the Amadori products undergo further structural changes through a series of reactions such as oxidation, dehydration and degradation (Figure 1.10) to finally yield highly stable AGE compounds (Brownlee, 1995, Taguchi et al., 2000). Under physiological conditions, non-enzymatic glycation can be detected in the process of ageing, but the reactions are considerably faster and more extensive in pathophysiological conditions such as diabetes mellitus that are associated with a persistently elevated concentration of glucose (Peppa et al., 2003, Wendt et al., 2003).

Figure 1.10 Chemical structure and formation of AGEs in the Maillard reaction
1.8 Oxidative stress in diabetes

Studies concerning oxidative stress parameters have increased because oxidative stress has been implicated in the etiopathogenesis of many diseases including cancer, atherosclerosis and other cardiovascular diseases, diabetes, rheumatoid arthritis, Alzheimer's disease and other neurological disorders, and pulmonary diseases (Yorek, 2003, Marlatt et al., 2008). Oxidative stress has been defined as when the balance of formation of oxidants exceeds the ability of antioxidant systems to remove or scavenge them (Bonnefoy et al., 2002). Under this definition oxidants are electron acceptors that damage proteins, DNA and lipids whereas antioxidants are electron donors that are insufficient to neutralize them.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two main classes of oxidants produced continuously in all cells. Each class contains two types of oxidant, free radical and non-free radical species. Free radicals are defined as “any species capable of independent existence that contains one or more unpaired electrons” (Halliwell & Gutteridge 2007). Transition metal ions are also free radicals by this definition, and may donate or accept electrons depending on their electronic state. Table 1.3 lists some ROS and RNS.
### Table 1.3 Radical and non-radical reactive oxygen species

<table>
<thead>
<tr>
<th>Radical</th>
<th>non-radical</th>
</tr>
</thead>
<tbody>
<tr>
<td>superoxide, $O_2^-$</td>
<td>hydrogen peroxide, $H_2O_2$</td>
</tr>
<tr>
<td>hydroxyl, $OH^-$</td>
<td>hypochlorous acid$^a$, HOCl</td>
</tr>
<tr>
<td>peroxyl, $RO_2^-$</td>
<td>ozone, $O_3$</td>
</tr>
<tr>
<td>alkoxy, $RO^-$</td>
<td>singlet oxygen, $^1O_2$</td>
</tr>
<tr>
<td>hydroperoxyl, $HO_2^-$</td>
<td>peroxynitrite$^b$, ONOO$^-$</td>
</tr>
<tr>
<td>nitric oxide, $NO^-$</td>
<td></td>
</tr>
<tr>
<td>nitryl, $NO_3^-$</td>
<td></td>
</tr>
<tr>
<td>hydrogen atoms, $H^+$</td>
<td></td>
</tr>
<tr>
<td>chlorine atoms, $Cl^-$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ can also be called a reactive chlorinating species, $^b$ can also be called reactive nitrogen species.

The production of oxidant species is inevitable as a by-product of metabolic pathways such as aerobic metabolism and oxidative phosphorylation in mitochondria (Stadtman and Berlett, 1998, Aruoma et al., 1989). Low concentrations of these species are important for cell proliferation, but high concentrations are also used for defense against invading pathogens and in regulation of the activation of several signaling pathways (Apel and Hirt, 2004, Stadtman and Berlett, 1998). For example, NO is essential for the regulation of vascular tone while superoxide is necessary for proper immune function (Oury et al., 1996), but unneutralized ROS and RNS can cause oxidative damage to lipids, proteins and nucleic acids, thus leading to aberrant molecular activities (Avery, 2011).
Accumulating evidence indicates that overproduction of ROS is associated with insulin resistance and obesity, and may be a major risk factor in the onset and progression of diabetes (Maritim et al., 2003), as well as to its later complications (Wolff, 1993). However, the link between oxidative stress and the development and progression of T2DM and its complications is still not fully understood. It has also emerged from various studies that diabetes and the associated hyperglycaemia increase oxidative stress by different mechanisms and elevated oxidative stress plays an important role in the pathogenesis of vascular dysfunction and subsequent diabetic complications including the diabetic retinopathy (Fiorentino et al., 2013, Giugliano et al., 1996).

The management of iron absorption and distribution in the organism, and inside cells must be tightly regulated in order to avoid the deleterious consequences of free iron-induced oxidative stress. Iron becomes a harmful element in cells due to its ability to participate in the generation of ROS (Kell, 2009). ROS are formed in the course of normal metabolism through leakage of electrons from the electron transport chain and by activities of oxidoreductase enzymes such as NADPH oxidase, myeloperoxidase, xanthine oxidase, glucose oxidase, cytochromes P450 and cyclooxygenases (Andrews, 1999a). Under normal conditions, up to 1% of the mitochondrial electron flow leads primarily to the formation of $\mathrm{O}_2^{-}$·. Interference with electron transport can dramatically increase $\mathrm{O}_2^{-}$· production, which is rapidly converted within the cell to $\mathrm{H}_2\mathrm{O}_2$ and $\mathrm{O}_2$ via superoxide dismutase (SOD).

$$2\mathrm{O}_2^{-} + 2\mathrm{H}^+ \xrightarrow{\text{SOD}} \mathrm{H}_2\mathrm{O}_2 + \mathrm{O}_2$$
H₂O₂ can react with the reduced transition metal Fe²⁺ via the Fenton reaction (Figure 1.11) to produce the highly reactive hydroxyl radical. Alternatively, H₂O₂ may be converted into water catalysed by the enzymes catalase and glutathione peroxidase (Stadtman and Berlett, 1997). The modification of proteins is initiated mainly by the reactions with OH•, however, the oxidation process is determined by the availability of O₂ and O₂⁻.

![Figure 1.11 Schematic representation of the sequence of events leading to the Fenton reaction. From Bogdanova and Nikinmaa (2001)](image)

Cells are protected against oxidative stress by an interacting network of antioxidants. These include antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase as well as low molecular weight scavengers such as vitamins C and E, and reduced glutathione (GSH). Under normal conditions there is a well-managed balance between formation and neutralization of ROS, so there is little or no modification of biomolecules.
1.9 Obesity

Obesity is a chronic disease in which abnormal or excessive body fat has accumulated in adipose tissue to the extent that health may be adversely affected and poor health increases mortality (WHO, 2013). Globally, the prevalence of obesity has doubled in the last two decades, leading to obesity being considered as a global epidemic. In 2008, more than 1.6 billion adults over 20 years old were overweight. Of these, over 200 million men and nearly 300 million women were obese meaning that more than 1 in 10 of the world’s population was obese (De Onis et al., 2010). The majority of the world’s adult population will be either overweight or obese by 2030 (Kelly et al., 2008).

In most large-scale studies, obesity is estimated from body mass index, BMI, a measurement obtained by dividing a person's weight in kilograms by the square of the person's height in metres. BMI tends to overestimate body fat in people who are very muscular and underestimate body fat in people who are highly sedentary. People who are very muscular, particularly men, may have a BMI higher than the normal range of 20 to 25, and still appear to have a normal weight and be healthy. In addition the BMI does not show where the body fat is located. New research shows that there are better ways to work out the level of obesity by waist circumference and waist-to-hip ratio (Snijder et al., 2006), although waist circumference and waist/hip ratio have been used as measures of central obesity (where visceral adipose tissue is stored), and BMI has been used as a measure of general obesity (Molarius and Seidell, 1998).
Obesity, especially of visceral fat, causes or exacerbates large numbers of diseases, including hypertension (Matsumura et al., 2001), dyslipidaemia, insulin resistance, T2DM (Kishida et al., 2012, Greenberg and Obin, 2006), metabolic syndrome, coronary heart disease (Paeratakul et al., 2002), stroke, non-alcoholic fatty liver disease, osteoarthritis, sleep apnea (Young et al., 1993, Peppard et al., 2013), and several forms of cancer.

Many factors contribute to causing obesity. In addition to genetic factors, the main factors relate to increased consumption of high energy density foods and a sedentary lifestyle that leads to a positive energy balance with subsequent accumulation of fat. Other factors which have effect on the energy balance include increased meal size, drinking sweet drinks and frequent consumption of carbohydrate. Obesity is linked with increased risk of developing insulin resistance and T2DM. Excess adipose tissue in obese individuals leads to release of increased amounts of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines and other factors which are involved in the development of insulin resistance. When insulin resistance and dysfunction of the β-cells occur together, the subsequent fall in insulin secretion results in a failure to control blood glucose levels leading to T2DM (Kahn et al., 2006). Many genes interact with the environment leading to obesity and in some also to diabetes.
1.9.1 Iron and obesity

A large number of studies have revealed a link between being overweight or obese and having poor iron status (Yanoff et al., 2007, Nead et al., 2004), but the mechanisms that underlie this are still poorly clarified (Tussing-Humphreys et al., 2009a). A first, unexpected, study found a significantly lower mean serum iron concentration in obese compared with non-obese adolescents (Wenzel et al., 1962).

Obesity is regarded as a pro-inflammatory condition characterised by the presence of chronic, low grade systemic inflammation (Ausk and Ioannou, 2008). Adipose tissue is known as an active endocrine organ and releases a number of cytokines and adipokines (Rosen and Spiegelman, 2006), which may in turn influence iron metabolism. Secretion of IL-6 and leptin from adipose tissue triggers hepcidin production. Increased hepcidin production leads to down regulation of iron absorption by the gut (McClung and Karl, 2009), associated with lower iron levels, transferrin saturation and higher serum transferrin receptor levels (Tussing-Humphreys et al., 2009a). This mechanism was reinforced by the observation that bariatric surgery improved iron status in obese patients, as it reduced the adipose mass and inflammation in the patients (Anty et al., 2008). There is a possible mechanism (Figure 1.12) that connects iron and obesity: the initial step is possibly related to an increased iron excess and hepatic iron loading followed by increased visceral adiposity, inflammation and hepcidin up-regulation, leading to iron deficiency (Ruivard et al., 2009).
1.9.2 Obesity and oxidative stress

Oxidative stress is a manifestation of obesity (Keaney et al., 2003). Several mechanisms contribute to the formation of free radicals during obesity, which leads to oxidative stress.

1.9.2.1 Increased fatty acid oxidation

Free fatty acids (FFAs) are oxidised inside the mitochondria after peroxisomal breakdown of very long chain fatty acids through \( \beta \) -oxidation, and this oxidation results in the overproduction of ROS which causes oxidative stress. The excessive production of ROS could also be due to preexisting conditions such as increased leptin and decreased activity of antioxidant enzymes that allow for overproduction of ROS (Inoguchi et al.,...
Oxidative stress can cause changes in mitochondrial DNA, which may result in abnormal changes in the structure of mitochondria and reduced ATP production.

1.9.2.2 Antioxidant depletion

Low molecular mass antioxidant sources can be depleted due to the increase of ROS production. Also the expression of antioxidant enzymes such as catalase, SOD and GPx is decreased in the adipose tissue of obese individuals which may lead to decrease in the level of these enzymes and also the activity of the enzymes may be decrease through the detrimental effects of ROS (Furukawa et al., 2004). Depletion of antioxidants leads to many health problems in obesity (Ozata et al., 2002).

1.9.2.3 Over consumption of oxygen

Total body oxygen consumption increases as weight increases (Gibson, 2000). The consequence of increasing oxygen consumption is increased production of ROS by the mitochondrial respiration process due to the loss of electrons from the electron transport chain, resulting in the formation of the superoxide radical (Amirkhizi et al., 2010, Khan et al., 2006).

1.9.2.4 Adipose tissue

Adipocytes produce various biologically active substances, such as tumour necrosis factor $\alpha$ (TNF-$\alpha$) (Hotamisligil et al., 1993), IL-6 (Fried et al., 1998), monocyte chemoattractant protein 1 (MCP-1) (Sartipy and Loskutoff, 2003) and leptin (Friedman and Halaas, 1998). Thus, obesity is considered a state of chronic inflammation. These cytokines are potent stimulators for the
production of reactive oxygen and nitrogen species by macrophages and monocytes; therefore, a rise in the concentration of cytokines could be responsible for increased oxidative stress.

1.9.2.5 Diet

A diet with high calorific value is likely to lead to the formation of ROS due to altered oxygen metabolism. Stored fat in the body is susceptible to oxidation reactions and the production of ROS. If the production of these ROS exceeds the antioxidant capacity of the cell, oxidative stress resulting in lipid peroxidation, which could contribute to the development of atherosclerosis (Khan et al., 2006).

1.10 Endothelium dysfunction

Circulating blood from the heart to the smallest capillaries is separated from vascular smooth muscle by a simple monolayer of cells called the endothelium; this layer acts as an endocrine organ, producing a variety of bioactive substances that play a crucial role in the maintenance of vascular homeostasis and endothelial integrity (Hirase and Node, 2012), as well as in the maintenance of blood fluidity. To fulfil this range of functions the endothelium produces or releases several vasoactive agents including the vasodilators, NO, prostacyclin and endothelium-derived hyperpolarising factors (EDHFs), and the vasoconstrictors angiotensin II and endothelin-1 (Cooke, 2000). Under physiological conditions, the endothelium acts as an inhibitory regulator of vascular contraction, leukocyte adhesion, vascular smooth muscle cell growth and platelet aggregation (Moncada et al., 1991). However, a growing list of conditions, including hypercholesterolaemia,
systemic hypertension, smoking, diabetes, congestive heart failure, pulmonary hypertension, estrogen deficiency, hyperhomocysteinaemia, and the aging process itself, have been associated with impaired function of the endothelium. Diabetes mellitus in humans is associated with impaired endothelium-dependent relaxation (De Vriese et al., 2009).

1.11 Atherosclerosis

Atherosclerosis is an chronic inflammatory disease resulting in plaque formation in the arterial vessel wall and is generally a disease of the large arteries that leads to myocardial infarction, stroke and lower limb ischaemia (Ross, 1986). The plaque may start already as a fatty streak during the fetal period and continues to develop throughout childhood, adolescence and adulthood (Napoli et al., 1999, McGill et al., 2000). In the early stages, atherosclerosis remains asymptomatic for decades, but in the progressive stage symptoms arise which depend on the affected vascular site (Murray and Lopez, 1997). If the extremities are targeted by these plaques the patients suffer from claudication. If these plaques present in the coronary artery then angina pectoris or myocardial infarction can occur. Transient ischaemia attack or stroke takes place if the carotid arteries are affected (Davies, 1996, Rothwell et al., 2000).

Inflammation and hyperlipidaemia play important roles in initiating and advancing atherosclerosis (Epstein and Ross, 1999, Libby et al., 2002). Inflammation is boosted by many factors including cigarette smoking, insulin resistance, diabetes and hypertension, particularly that mediated by renin angiotensin aldosterone, which is associated with atherosclerosis (Libby and
Aikawa, 2002). Many studies have mentioned the fundamental role of cholesterol in the atherosclerosis process (Steinberg, 2004, Steinberg, 2005, Steinberg, 2006). The first step in atherosclerosis process starts in the endothelial cells (EC). The activation of EC results in the increase expression of adhesion molecules such as P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the EC surface. Circulating leucocytes migrate to the intimal layer, where differentiation of migrated monocytes into macrophages occurs. Macrophages have a great capacity to take up exogenous material, such as oxidatively modified low density lipoproteins (oxLDL). OxLDL is taken up into macrophage lysosomes via receptor-mediated endocytosis, but poorly degraded, resulting in foam-cell formation and generation of fatty streaks. Further recruitment of inflammatory cells and proliferation of smooth muscle cells lead to the development of an atherosclerotic plaque (Epstein and Ross, 1999, Libby et al., 2002).

Smooth muscle cells form a fibrous cap covering the atherosclerotic plaque. Thinning of the fibrous cap has been shown to be caused by decreased collagen production as a result of decreased smooth muscle cell content, or degradation by matrix metalloproteinases excreted by macrophages (Galvis et al., 1994). In end-stage atherosclerosis the fibrous cap overlying the atherosclerotic plaque can rupture (Finn et al., 2010). As a result, the content of these unstable plaques is passed to blood stream resulting in thrombus formation. Very small particles can embolise and end up in smaller vessels of a target organ’s tissue, leading to local occlusion and ischaemia. Problems with vision can occur if these particles end up in the retinal artery. Transient
ischaemic attack or stroke can occur if these small particles are stuck in a small blood vessel (artery) in the brain.

Poor glycaemic control, low grade inflammation and low HDL-cholesterol (HDL-c), are all established promoters of atherosclerosis, and have been reported to correlate with each other in patients with T2DM (Gatti et al., 2009). There is compelling evidence that iron plays a significant role in the development of atherosclerotic plaques (Sullivan, 2009, Sullivan, 2007). The major findings in support of this are those that have demonstrated that reducing iron bioavailability through chelation therapy (Duffy et al., 2001), dietary restriction (Lee et al., 2003) or by frequent blood donation (Zheng et al., 2005), reduces the size of the atherosclerotic lesions in animal studies (Minqin et al., 2005) and improves vascular function in humans (Duffy et al. 2001, Zheng et al. 2005). Obesity is a major risk factor for vascular disease due to changes in adipokine secretions from the enlarged adipose tissue mass (Hajer et al., 2008, Gustafson, 2010).

**1.11.1 Atherosclerotic risk factors**

There are number risk factors that accelerate atherosclerotic process (Table 1.4). Although the American Heart Association (AHA) Conference statement in 1999 suggested a classification which divides risk factors into three classes, the method for classification of these factors has not been agreed around the world. The three categories are: major independent risk factors, predisposing and conditional risk factors (Grundy et al., 1999).
Table 1.4 Classification of risk factors for atherosclerosis as presented by the AHA

<table>
<thead>
<tr>
<th>Major independent</th>
<th>Predisposing</th>
<th>Conditional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>Obesity*</td>
<td>Elevated serum triglycerides</td>
</tr>
<tr>
<td>Elevated serum total and</td>
<td>Physical inactivity*</td>
<td>Small LDL particles</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low serum HDL cholesterol</td>
<td>Abdominal obesity</td>
<td>Elevated serum homocysteine</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>Family history of premature CHD</td>
<td>Elevated serum lipoprotein(a)</td>
</tr>
<tr>
<td>Advancing age</td>
<td>Ethnic characteristics</td>
<td>Prothrombotic factors</td>
</tr>
<tr>
<td>Elevated blood pressure</td>
<td>Psychosocial factors</td>
<td>Inflammatory markers</td>
</tr>
</tbody>
</table>

* These risk factors are designated major risk factors by the AHA.

1.11.2 Iron and atherosclerosis

The link between iron and atherosclerosis has been proposed by Siperstein et al. (1953), and this suggestion was built on the information which was gathered from clinical observations and experimental studies in the early 1950s. In 1981, Sullivan spotted that people with high serum ferritin concentrations have a risk of myocardial infarction and therefore he suggested the hypothesis, which is called the “iron hypothesis”. He also observed that chronic iron depletion by blood donation has a protective effect against ischaemic heart disease (Sullivan, 1981). Before this hypothesis oestrogen was considered to be a possible explanation for difference in risk of vascular disease between sexes, while Sullivan argued that iron is more likely culprit than oestrogen (Sullivan, 1989, Sullivan, 1992, Sullivan, 2001, Sullivan, 2003, Sullivan, 2005, Sullivan, 2007). Inflammation plays a major
role in the atherosclerosis process. Increasing levels of hepcidin due to the inflammation process lead to an increase in the deposition of iron in macrophages which are critical cells in the formation and fate of atherosclerotic plaques. Increased deposition of iron is an important contributor to promoting the generation of reactive oxygen species, and could therefore promote lipid peroxidation and accelerated development of atherosclerosis plaque instability (Ramakrishna \textit{et al.}, 2003).

Iron induces the production of ROS locally which impairs vascular function, decreasing the bioavailability of NO, impairing vasorelaxation, and promoting platelet adhesion and aggregation (Russo \textit{et al.}, 2002). Zheng \textit{et al.} (2005) provided evidence in support of the Sullivan hypothesis when they found that high frequency blood donors have decreased levels of serum ferritin (a marker of body iron store); decreased levels of serum 3-nitrotyrosine (a marker of oxidative stress); and greater flow-mediated dilation in the brachial artery (a marker of vascular function). An interesting study noted that iron chelation with deferoxamine improved endothelial function in patients with coronary artery disease (Duffy \textit{et al.}, 2001).

Evidence from experimental studies has shown that decreased vascular iron content following dietary iron restriction in Apo E-deficient mice leads to lower matrix degradation capacity and increased plaque stability, and significantly attenuates the progression of atherosclerotic lesions in these animals (Lee \textit{et al.}, 1999, Lee \textit{et al.}, 2003).
1.11.3 Oxidative stress and atherosclerosis

Several lines of evidence support the potential role of oxidative stress in accelerating the atherosclerosis process (Nishikawa et al., 2000, Baynes and Thorpe, 1999, Baynes, 1991). Studies in experimental animals have demonstrated that increased production of ROS from the layers of arteries are common risk factors for atherosclerosis (Cai and Harrison, 2000).

All conditions and diseases such as diabetes, hypertension, nitrate intolerance, smoking and aging which increase the production of ROS via several mechanisms have the ability to initiate several processes which contribute to atherosclerosis, by stimulating several processes which lead to atherosclerosis. In the diabetic condition ROS are produced by different pathways and are involved in development of atherosclerosis in different aspects such as activation of glycation reactions and increased electron flux on the mitochondrial electron transport chain. Increased ROS are involved in the development of atherosclerosis in various aspects. First, endothelial dysfunction is an early key event in atherosclerosis (Cai and Harrison, 2000). It has been thought that ROS are involved in the progression of endothelial cell dysfunction, which is accompanied by inactivation of endothelial nitric oxide synthase (eNOS) and decrease of NO levels (Madamanchi et al., 2005). Second, ROS also induce expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), which facilitates inflammatory cell recruitment and lipid deposition in the intimal layer. The subsequent ingestion of excess oxidized LDL particles by macrophages and monocytes leads to release of various inflammatory cytokines and growth factors. Finally, proliferation of vascular
smooth muscle cells (VSMCs) is a key step in the development of atherosclerosis.

Obesity is characterized by an increase in ROS production and endothelial dysfunction, which leads to a reduction in the bioavailability of vasodilators, particularly NO. Several mediators and mechanisms are involved in the decrease in NO including leptin and homocysteine (Guzik et al., 2006). Elevated leptin and homocysteine concentrations in obese individuals with and without hypertension induce oxidative stress, which leads to a free-radical-induced decrease of NO (Konukoglu et al., 2006).

Lipid peroxidation, which is often developed during oxidative stress, leads to production of the end products of lipid peroxidation, reactive aldehydes. The most relevant in current biomedicine are malondialdehyde (MDA) and 4-hydroxynonenal (HNE). These aldehydes are able to 'simulate' complex oxidative stress and its harmful consequences, hence it is commonly accepted that these molecules are considered as ‘second toxic messengers’ of oxygen free radicals. Metal ions like Cu\(^{2+}\) or Fe\(^{2+}\) are frequently used to initiate LDL oxidation \textit{in vitro}, since the resulting oxyLDL by metal ions has similar biological activation to that oxidized \textit{in vivo} (Esterbauer \textit{et al.}, 1992).

\textbf{1.12 Novelty of project}

Most of the recent studies in this area (the relationship between iron oxidative stress, protein glycation and development of atherosclerosis in T2DM) have provided conflicting findings with regard to both Type 2 diabetic and non-diabetic obese subjects. There is a number of reasons that account for these
discrepancies (see below), and these are addressed here by using more appropriate methodology to measure the factors involved. There are several studies that question the validity of traditional methods of measuring iron status in subjects with obesity and/or diabetes (Swaminathan et al., 2007, Jiang et al., 2004). For example, ferritin is often considered a marker of body iron stores but it is an acute-phase reactive protein that may be influenced by coincidental infections and, more importantly, in this setting, by the presence of low-grade inflammation associated with obesity (Yanoff et al., 2007, Ausk and Ioannou, 2008, McClung and Karl, 2009). Moreover, ferritin itself does not participate directly in the oxidant reactions related to iron. Critical to iron’s importance in biological processes is its ability to cycle reversibly between its ferrous and ferric oxidation states. This precise property, which is essential for its functions, also makes it dangerous, because free iron can catalyse the formation of free radicals that can damage the cell. Thus, from a pathophysiological standpoint, it is important to measure iron pools that consist of chemical forms that can participate in redox cycling. This pool is referred to as labile iron, and extracellularly as non-transferrin bound iron (NTBI). A recent editorial concluded that progress into the relationship between iron, oxidative stress and the causes and consequences of diabetes will not occur unless the involvement of the physiological pool (NTBI) is fully investigated (Shah and Fonseca, 2011). The aim of this study was to initiate these essential investigations into the role of NTBI in these conditions.

There is no ‘gold standard’ for measuring NTBI. An improved method for doing this has been developed in our laboratory. NTBI is measured using a slight modification of the high performance liquid chromatography (HPLC)
methods of (Kime et al., 1996b, Paffetti et al., 2006). Recent studies have revealed the involvement of iron and oxidative stress in the development of atherosclerosis (Sullivan, 2007, Sullivan, 2009). Both obesity and diabetes are high risks for developing atherosclerosis. Iron overload is commonly reported in T2DM, and this fits with the current view. In contrast, obesity is commonly reported as being an iron deficient state (Tussing-Humphreys et al., 2009a, Pinhas-Hamiel et al., 2003). This does not fit with the view proposed above. However, no studies have investigated NTBI in obesity. It is theoretically possible to have low iron status (as indicated by classical measures) yet still have high circulating NTBI. This current study was aimed to address this dilemma.

In addition to the points raised above, this study related changes in iron status as measured by circulating soluble transferrin receptors (sTfR) (which are unaffected by inflammation) (Yanoff et al., 2007), total serum iron, iron binding capacity, to NTBI and atherogenesis as indicated by the biomarkers E-selectin and high sensitivity C-reactive protein (hs-CRP). A number of studies have validated the value of hs-CRP and hence it has been used extensively as a biomarker of atherosclerosis and cardiovascular risk (Ridker, 2007, Ridker and Silvertown, 2008, Calabrò et al., 2009).

Most recent studies have found that obese subjects tend to have higher Hb and ferritin concentrations, and lower transferrin saturation compared to the non-obese subjects. The higher ferritin and lower transferrin saturation is consistent with the mechanism of obesity related inflammation (Yanoff et al., 2007). However, assessment of other biomarkers such as sTfR (which is
elevated in iron deficiency) in concert with clearly defined obesity population are required to clarify the causal relationship between obesity and disturbed iron metabolism.

1.13 Conclusions
Iron is a vital element in many biological processes and the body has an efficient system to maintain iron homeostasis. However, elevated body iron levels may be associated with the development of several diseases including T2DM, and contributes to the progression of its complications, such as atherosclerosis, due to ability of iron to catalyse the production of ROS through Fenton chemistry. Excess iron is known to be one of the major contributing causes for oxidation of LDL, which plays a major role in atherosclerosis plaque formation. In obesity, iron distribution is altered both at the cellular and tissue levels with adipose tissue playing a predominant role in this change. On other hand, high levels of circulating glucose (which occur in diabetes) leads to the deposition of glucose onto proteins (protein glycation) which interferes with their normal function. 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. This process may further enhance iron availability because some of these proteins function to store and transport iron. Increases in the availability of iron may lead to the production of ROS, and these can lead to tissue damage which occurs in diabetes such as damage to the blood vessels. There are many gaps in the
research in the area of iron and T2DM, and its complications, some of which are listed below.

1) The impact of iron on T2DM and its complications has been studied recently but many of these studies deal with total iron and not with the redox active form of iron (NTBI). Furthermore, some previous studies have used newly diagnosed T2DM subjects and have not mentioned the duration of diabetes, which may have a great impact on glycation of proteins, and hence on the release of NTBI.

2) In previous studies, standard tests of iron status have been used, which are affected by inflammation; sTfR is a better indicator of iron status because it is unaffected by inflammation.

3) No previous research has assessed NTBI in obese non-diabetic subjects. Because obese subjects are already iron deficient, it would be interesting to know whether NTBI is present in this group.

4) In addition, studies in which NTBI has been measured have not used the most up-to-date methods for measuring it. Current methods of measuring NTBI have higher sensitivity and this may allow detection of lower concentrations of NTBI.

5) Although there have been previous studies on the effects of glycation on Tf these have used either apoTf or holoTf, neither of which are normally present in plasma. A study in which partially iron loaded Tf is used would be more physiologically relevant than previous studies. In addition, the effects of co-incubation with LDL remain to be studied.
1.3 Hypotheses

Release of NTBI from transferrin and possibly other proteins *in vivo* due to glycation processes may have a great effect on T2DM and its complications due to the induction of oxidative stress by a variety of different mechanisms. This leads to a decrease in antioxidant defence and an increase in oxidative damage in the major organs responsible for glucose homeostasis such as the liver and pancreas.

Obesity is known to be associated with low iron status, suggesting that the absence of diabetes in some cases and not in others may be due to lower levels of NTBI in some obese individuals compared to others.

*In vitro* the incubation of 50% ITf with glucose may have a major effect on the iron-binding capacity of Tf and the release of NTBI, but less so than for the incubation of glucose with holoTf, which is less physiologically relevant, and for which there is likely to be some NTBI already present. In addition, the presence of LDL during the incubation with glucose should enhance the effects of glycation of 50% ITf leading to increased NTBI release.
1.2 Aims and objectives

The purpose of this study was to determine in lean healthy controls, obese non diabetic subjects and subjects with diagnosed Type 2 diabetes the following relationships:

1. the degree of haemoglobin glycation (HbA$_{1c}$), and the plasma level of ‘free’ non transferrin-bound iron [NTBI];

2. the level of NTBI and the degree of oxidative stress as indicated by the plasma levels of malondialdehyde (a marker of lipid peroxidation), and the levels and redox state of the major small molecular weight antioxidants ascorbate, urate and glutathione;

3. the relationship between NTBI, oxidative stress, biomarkers of atherosclerosis and central obesity; and

4. the relationship between the NTBI, oxidative status, and biomarkers of atherosclerosis in advanced diabetes.

In addition to this in vivo study, an in vitro study on transferrin glycation with different concentrations of glucose to confirm the influence of glycation in iron binding to transferrin was carried out, as well as another to show the influence of glycation on oxidation of LDL.
Chapter 2: An *in vivo* study on the relationship between non-transferrin bound iron and other common iron-related, inflammation and atherosclerosis biomarkers in Type 2 diabetes and obesity
2.1 Introduction

In recent years the prevalence of Type 2 diabetes mellitus (T2DM) has increased worldwide (Shaw et al., 2010). Iron overload has been implicated in T2DM and other diseases such as cancer, Alzheimer's disease and cardiovascular disease (Jomova and Valko, 2011). Organs such as the pancreas, liver, muscle and adipose tissue, which are heavily involved in glucose and lipid metabolism are affected by iron overload (Fernández-Real and Manco, 2013). The precise mechanisms by which iron induces T2DM are not clear, but iron overload initiates abnormalities in glucose metabolism, including insulin resistance and hyperinsulinaemia, followed by impaired insulin secretion (Wilson et al., 2003). The ability of iron to participate in the generation of powerful oxidant species such as the hydroxyl radical may be considered as a link between iron overload and risk of T2DM (Halliwell and Gutteridge, 1989). The formation of hydroxyl radicals induces oxidative stress and may contribute to insulin resistance or long-term pancreatic failure (Swaminathan et al., 2007).

Hereditary haemochromatosis (HH), a genetic disorder of iron metabolism, provides further evidence of an association between iron and T2DM due to impaired glucose tolerance which leads to insulin resistance and increased risk of diabetes; about 30% to 60% of patients with HH have diabetes (Hatunic et al., 2010). An increased incidence of diabetes is also seen in hereditary aceruloplasminaemia, a condition where the lack of synthesis of apoceruloplasmin affects the distribution of tissue iron and leads to a progressive accumulation of iron (Miyajima, 2003). Pancreatic cells have the tendency to express the divalent metal transporter (DMT1), and so facilitate
the entry and accumulation of iron in comparison to other cells (Andrews, 1999b). Another study found a strong association between clinically raised ferritin, below the range indicative of clinical haemochromatosis, and development of diabetes (Forouhi et al., 2007). These observations have opened new avenues to study the relationships between iron and many diseases.

Under normal conditions, iron circulates in the plasma bound with three major proteins: transferrin, Hb and ferritin. Transferrin is responsible for the delivery of iron to all cells in the body which need iron to perform cellular functions, such as red blood cells during erythropoiesis (Hentze et al., 2010). In serum, transferrin is usually only 30% saturated so that it has the capacity to bind excess iron and thus help in controlling the build-up of toxic amounts of excess iron. Circulating iron, which is not bound to transferrin, referred to as non-transferrin-bound iron (NTBI), becomes important in iron overload disorders, in which plasma iron is present in excess of transferrin-binding capacity, due to its redox activity through Haber-Weiss and Fenton chemistry (Breuer et al., 2000a, Esposito et al., 2003). When NTBI appears in the plasma, due to iron overload or lack of transferrin (apotransferrinaemia), it is rapidly cleared by the membrane-bound transport system constitutively present on parenchymal cells of organs particularly those of liver, heart, pancreas and the adrenal glands. This system does not require endocytosis for iron transport.
2.1.1 Non-transferrin-bound iron and diseases

The presence of NTBI is associated with pathological conditions; in healthy individuals NTBI is found in very low concentrations, which do not exceed 1 µmol l⁻¹ and are often undetectable by most of the methods available (Anderson, 1999). NTBI is a form of iron most susceptible to redox activity, and plays an important role in acceleration the pathogenicity of diseases. NTBI has found in different iron overload diseases such as haemochromatosis, β-thalassaemia, sickle cell anaemia and myelodysplastic syndrome (Hershko et al., 1978, Walter et al., 2006, Le Lan et al., 2005). NTBI is also present in megaloblastic anaemia (Gafter-Gvili et al., 2004); the end stage of renal disease patients on haemodialysis (Prakash et al., 2005); patients with end stage of renal disease on supplemental iron therapy (Zager et al., 2002); and alcoholic cirrhosis. The presence of NTBI in non-iron overloaded conditions such as myocardial infarction, renal disease, diabetes and liver disease (Lee et al., 2006, Lele et al., 2009), has led investigators to consider NTBI as a potential mediator for complications of these diseases. NTBI appears in serum when transferrin is fully iron saturated (Batey et al., 1980). However, recent work has shown the existence of NTBI in some conditions even when transferrin is not fully saturated (Breuer et al., 2000a, Valk and Marx, 2000).

2.1.2 Non-transferrin-bound iron and Type 2 diabetes

The first study which reported the presence of NTBI in uncontrolled Type 2 diabetic patients was carried out by Lee et al. (2006); this study also found that the existence of NTBI in diabetes patients even when transferrin was not fully saturated due to the persistent high plasma glucose levels causing an
increase in the rate of protein glycation reactions (Armbruster, 1987, Garlick and Mazer, 1983). The initial and end stages of glycation of proteins can significantly impair the function and structure of extracellular proteins such as Hb, albumin and transferrin (Singh et al., 2001). AGEs are known to bind with higher amounts of transitional metals such as iron and extract more iron from stable iron compounds thereby increasing the redox activity of AGEs (Qian et al., 1998), which act as Fenton reagents, and catalyse the production of ROS particularly hydroxyl radicals. This causes increased oxidative stress, which is widely accepted as contributing towards the development and progression of diabetes, and its complications such as atherosclerosis. Uncontrolled T2DM is characterized by autooxidation; this process leads to generation of ROS, which can also release the iron from stable forms such as ferritin and haem, which exacerbates the condition (Chau, 2000) (Figure 2.1).
2.1.3 Non-transferrin-bound iron and oxidative stress

Under normal physiological conditions the amount of redox-active iron is tightly controlled; the transferrin (Tf) molecule is adapted to hold iron safely and prevent its participation in redox reactions that could give rise to ROS (Gutteridge and Halliwell, 2000). NTBI is associated with the generation of hydroxyl radicals and lipid peroxidation which lead to oxidative stress (Gutteridge et al., 1985, Kartikasari et al., 2006), but higher NTBI concentrations do not always correlate with lipid peroxidation (Dani et al., 2004, Driss et al., 2005, Scheiber-Mojdekar et al., 2004). Walter et al. (Walter et al., 2006) studied the relationship between biomarkers of oxidative damage and NTBI in both thalassaemia and sickle cell disease (SCD), and
noticed that they are increased in both of these conditions. NTBI concentration is inversely related to plasma vitamin E concentration in SCD (Marwah et al., 2001) and patients with idiopathic haemochromatosis have a considerable ascorbate deficiency (Young et al., 1994). Bongiorno et al. (2011) observed that thalassaemia patients, who are continuously exposed to iron-induced oxidative injury through repeated blood transfusion, have altered patterns of all serum antioxidants. Published data on serum NTBI levels in obesity and T2DM are scarce. Obesity may increase hepcidin production which in turn lowers circulating iron levels by decreasing intestinal absorption and decreasing release from macrophages (Means Jr, 2004).

2.1.4 Non-transferrin-bound iron and atherosclerosis

The potential relationship between iron and heart disease arose from the observation that the incidence of this disease is much higher in men and postmenopausal women than premenopausal women (Sullivan, 1981). Data obtained from epidemiological and experimental work may strengthen the potential role of iron in atherosclerosis (De Valk and Marx, 1999). Excess body iron is associated with increased cardiovascular disease risk, possibly via NTBI mediated enhancement of inflammation and oxidation of low density lipoprotein (LDL). The relationship between iron and atherosclerosis is complex, and remains contradictory. It is hypothesized that free iron-catalyzed oxidation of LDL which may lead to injury of the arterial wall and hence, initiate atherosclerosis (Chisolm and Steinberg, 2000). Oxidized LDL is pro-inflammatory; it attracts monocytes via induction of chemokines, and up regulated scavenger receptor expression on macrophages. The scavenger receptors subsequently recognize oxidized LDL and mediate its
uptake into the cells. This process leads to lipid accumulation in the vessel wall and fatty streak formation, the beginning of atherosclerosis (Steinberg et al., 1989).

The association between NTBI and increased LDL oxidation has not been observed in vivo in subjects with the C282Y mutation of the haemochromatosis gene (Tits et al., 2006). Also NTBI was not associated with in vitro LDL oxidation in a healthy population study (Derstine et al., 2003). In a cross-sectional haemochromatosis screening study, NTBI showed a relationship with intercellular adhesion molecule-1 (ICAM-1), a critical molecule in recruiting monocytes to the extravascular compartment and initiating atherosclerosis (van Tits et al., 2007). A similar study also found that NTBI was associated with ICAM-1, vascular adhesion molecule-1 (VAM-1) and E-selectin expression, and increased cellular stress (Kartikasari et al., 2006).

2.2 Stability of biomarkers in plasma samples during sample preparation and after storage

In clinical research the stability of biomarkers in serum or plasma samples during sample preparation and after storage is of great importance (Lippi et al., 2011). In clinical studies it is usually essential to store samples because of several potential difficulties such as the requirement to collect samples over a period of time, plus funding and instrument problems. For example, in the present study the samples were stored for up to two years until all the analysis was completed. For this reason, some understanding of the influence of storage on the parameters measured would be useful.
In this study the effect of storage, freezing and thawing of plasma sample on a biomarker of oxidative stress (MDA) was examined using tubes treated with different anticoagulants. Other parameters were either previously examined in our laboratory or the manufacturer’s instructions contained information about the conditions for sample storage.

2.2.1 Background information about MDA

Cells produce reactive oxygen species (ROS) as part of metabolic activity. Over production of these species may result in oxidative stress, and this oxidative stress has been implicated in the etiopathogenesis of a number of diseases and clinical conditions. These include atherosclerosis, cancer and other chronic diseases, and oxidative stress may also play an important role in the aging process (Sastre et al., 2000). Many biological molecules are targets of oxidative damage but lipids and lipoproteins are the most susceptible classes of these biomolecules. Lipid peroxidation occurs when a strong oxidant is generated close to the cell membrane. This, after combination with oxygen through peroxyl radical formation, leads to lipid hydroperoxidation with associated membrane disruption and formation of a great variety of compounds which including highly cytotoxic products such as malondialdehyde (Ceconi et al., 2003). At neutral pH the enolate anion form of malondialdehyde has relatively low chemical reactivity (Esterbauer et al., 1991). Nevertheless, several different adducts are formed between the enolate anion form of MDA and nucleic acid bases such as deoxyadenosine, deoxycytidine and deoxyguanosine (Marnett, 1999). The major adduct is a pyrimidopurinone. Other work has reported that deoxyadenosine is the first endogenous DNA lesion found to be a target of nucleotide excision repair
enzymes, and may be a major endogenous DNA adduct that significantly contributes to cancer (Wang et al., 2004). MDA also reacts in vivo with primary amines and including lysine.

2.2.2 Sources of malondialdehyde and health aspects

MDA is a highly reactive organic compound (dialdehyde of malonic acid) with the formula CH$_2$(CHO)$_2$. It is produced as a by-product of polyunsaturated fatty acid peroxidation (Janero, 1990), and also during prostaglandin and thromboxane synthesis (Shimizu et al., 1981, McMillan et al., 1978). MDA can be found in most biological samples including foodstuffs, serum, plasma, tissues and urine, as a result of lipid peroxidation. It was suggested that the bulk of MDA in human plasma is bound to protein; this would explain the low levels of MDA in plasma as measured under standard assay conditions (Lefèvre et al., 1996). The concentration of MDA in both men and women increases with age, which may be relevant to the increasing prevalence of atherosclerosis with age (Rumley et al., 2004). The level of MDA has been reported to be high in diabetes, hepatitis C infection and HIV seropositive children (Higueras et al., 1994, Jareño et al., 1998, Losada and Alio, 1997). It has also been reported that MDA derived from lipid peroxidation in chondrocytes mediates oxidation of cartilage collagens and leads to alteration of biochemical and biophysical properties of cartilage collagen fibrils, making them prone to degradation and initiating the changes observed in aging and osteoarthritis (Tiku et al., 2003). The determination of MDA-modified LDL was reported as a useful marker for identifying patients susceptible to coronary heart disease (Holvoet et al., 1999). MDA is found in the atherosclerotic plaque deposits promoted by diabetes (Kume et al., 1995).
2.2.3 Effect of storage of plasma on TBARS

Malondialdehyde (MDA) is widely used as oxidative stress biomarker in biomedical research (Carone et al., 1993, Requena et al., 1996). During clinical studies this parameter is estimated mostly in stored blood/plasma samples (Carbonneau et al., 1991). The malondialdehyde concentration in plasma increases linearly with time during storage at 4 °C when the sample is exposed to air, but the storage of samples treated with antioxidants such as EDTA and GSH effectively suppresses this process (Lee, 1980). Another study stated that storage of plasma at -20 °C was suitable for only one week after which the concentration of MDA decreased (Carbonneau et al., 1991).

2.3 Aims of the study

Because few data have been published on serum NTBI levels in T2MD and obesity this study aimed to establish:

1. if higher levels of NTBI are present in patients with Type 2 diabetes and obese subjects compared with a control group;

2. if there a relationship between NTBI and common iron-related biomarkers;

3. if there any correlation between NTBI and inflammatory biomarkers;

4. if there a relationship between NTBI and oxidative stress biomarkers in all groups;

5. if there a relationship between NTBI and biomarkers of atherosclerosis; and

6. if there is any correlation with age across all parameters in this study.
Other objectives were:

1. to examine the effect of storage at -80 °C, and repeated freezing and thawing of samples on the measured level of MDA in human plasma;

2. to compare the effects of two anticoagulants, EDTA and lithium heparin, used during the isolation of plasma, on the measured level of MDA; and

3. to determine the effect of GSH addition on limiting changes in the measured level of MDA.
2.4. Materials and Methods

2.4.1 Materials

Light magnesium carbonate, disodium EDTA, metaphosphoric acid, acetonitrile, 95% ethanol, KCl, KH₂PO₄, NaCl, Na₂HPO₄•12H₂O, FeCl₃•6H₂O, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), phosphoric acid, 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (DHP), nitrilotriacetic acid (NTA), butylated hydroxytoluene, thiobarbituric acid and all other laboratory reagents were obtained from Sigma-Aldrich (Poole, UK). Amicon Ultra filter units (30 kDa cut off) were purchased from Merck-Millipore (Fisher Scientific Loughborough, UK).

2.4.2 Venesection and sample handling

The study was conducted after obtaining appropriate ethics committee approval. Signed consent forms (Appendix 8) were obtained for subjects and controls before the study to confirm their willingness to take part. All subjects were anonymised by numbering them and numbering the blood samples taken from them. Blood samples were obtained from 73 subjects: 28 were Type 2 diabetics aged >40 y, all patients recruited from Derriford Hospital; 17 were obese non diabetic subjects aged >40 y; and 28 healthy subjects aged >40 y were enrolled as controls. Medical history of alcohol consumption, smoking and medications were prime considerations in questionnaire forms (Appendix 9). Anthropometric indices were measured which included BMI, body fat percentage and waist to hip ratio (Appendix 10). Phlebotomy of each subject was conducted using 3 × 4 ml Vacutainers (Becton Dickinson, Plymouth, UK), one with lithium heparin as anticoagulant, one with EDTA as anticoagulant and one plain tube for serum. Blood samples in EDTA and
lithium heparin tubes were centrifuged at 1,000 × g for 5 min to provide plasma; the blood in the plain tubes was centrifuged after 30 min at room temperature, and then plasma and serum were stored at -80 °C prior to biochemical analysis. From the EDTA plasma 400 µl were removed and added to 400 µl of 10% metaphosphoric acid (MPA) containing 2 mM EDTA and then centrifuged at 20,000 × g at 4 °C for 10 min. The supernatant was removed and stored at -80 °C prior to the measurement of ascorbate and urate.

2.4.3 Phlebotomy and experimental design for the effect of glutathione on the level of malondialdehyde in stored human plasma

Blood samples were obtained from six healthy volunteers. Phlebotomy was conducted using 4 ml Vacutainers, one anticoagulated with lithium heparin and another with EDTA. The blood samples were centrifuged at 1,000 × g for 5 min to provide plasma. Each plasma sample was divided into two parts; one part was spiked with 1 mM GSH, and the other without GSH. After this all samples were divided into six × 100 µl aliquots and stored at -80 °C before measurement of MDA levels on different days as described below. At various points during the experiment samples were kept at room temperature until all the plasma had thawed and then they were stored on ice until the analysis was complete (up to 8 h), after which they were stored again at -80 °C.
2.4.4 Determination of serum iron

Different methods have been used to measure the concentration of iron. The method used here was adapted from Bothwell et al. (1971).

2.4.4.1 Principle of method

Ferric iron is dissociated from its carrier protein, transferrin, and other iron binding proteins in an acidic medium and simultaneously reduced to the ferrous form. The ferrous iron forms a complex with the chromogen ferene, 3-(2-pyridyl-5-6-bis-[2-(5-furyl sulfonic acid)]-1,2,4-triazine, a sensitive and low-cost iron indicator (Pieroni et al., 2001), to produce a blue chromophore which absorbs maximally at 593 nm.

2.4.4.2 Method

Protein precipitation solution (400 μl of 0.6 M TCA and 0.4 M thioglycolic acid in 1 M HCl) was added to 400 μl of sample, iron standards (10 to 100 μM made up in PBS) and blanks. These were mixed thoroughly for 1 min, and then heated at 56 °C for 15 min in a water bath. Samples were then centrifuged at 1,000 × g for 5 min, and 500 μl of the resulting supernatant were removed and added to 500 μl of 0.5 mM ferene solution. After incubation for 5 min at room temperature the absorbance was read at 593 nm using a CamSpec M302 UV/vis spectrophotometer (Cambridge, UK).
2.4.5 Determination of total iron-binding capacity (TIBC) of plasma samples

2.4.5.1 Principle of method

A colorimetric method was used to determine the total iron-binding capacity (TIBC) in plasma with LiH anticoagulant or in serum samples. An excess of iron was added to the serum to saturate the transferrin. The unbound iron was then precipitated with basic magnesium carbonate, and after centrifugation the iron in the supernatant was determined. The procedure was adapted from the method of Ramsay (1957).

2.4.5.2 Method

To 350 µl of the serum or heparinised plasma were added 350 µl of iron saturating solution (100 µM ferric chloride dissolved in 5 mM HCl, made up from a 1:10 dilution of a stock ferric chloride solution of 1 mM in 50 mM HCl). This was mixed and incubated for 5 min at room temperature. To this were
added 35 mg of light magnesium carbonate. This was agitated frequently and thoroughly over 30-60 min, and then centrifuged at 1,000 × g for 5 min. The supernatant was removed and re-centrifuged at 1,000 × g for 5 min. The resulting supernatant was removed and analysed for iron as in the measurement of total iron (Section 2.4.4).

2.4.6 Unsaturated iron binding capacity (UIBC)

The UIBC was determined by subtracting the serum iron from the TIBC, i.e.

\[ \text{TIBC} - \text{total serum iron} = \text{UIBC}. \]

2.4.7 Transferrin saturation (TSAT)

Transferrin saturation is given as a percentage. It is the ratio of serum iron to total iron-binding capacity (Beilby et al., 1992). It is a more sensitive and specific indicator of iron status than serum iron alone (Bainton and Finch, 1964).

2.4.8 Non-transferrin-bound iron (NTBI)

NTBI was measured using a slight modification of the HPLC methods of Kime et al. (1996b) and Paffetti et al. (2006). Plasma (300 µl) was mixed with 30 µl of 0.8 mM NTA which removes and complexes all low molecular weight iron, and iron non-specifically bound to serum proteins. The mixture was incubated at room temperature for 20 min, and the placed in 30 kDa Amicon ultrafilters (Millipore) to separate the chelated NTBI from transferrin-bound iron. The filters were centrifuged at 13,000 × g for 30 min at 4 °C. The ultrafiltrate was removed and the volume measured. For every 100 µl, 10 µl of 50 mM 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (DHP) was added. Finally
the sample was incubated for 5 min at room temperature before injection into
the HPLC system (20 μl sample loop). The mobile phase consisted of 5 mM
PIPES buffer, pH 7.0, containing 3.5 mM 3-hydroxy-1-propyl-2-methyl-
pyridon-4-one and 5% acetonitrile. The column was a PEEK-lined 100 mm ×
5 mm C18 column (Hichrom, Reading, UK). All tubing was composed of
PEEK. The mobile phase was pumped at a flow rate of 1 ml min⁻¹ using a
Dionex pump (P580). The absorbance of the iron-chromophore complex was
determined using a Dionex UV/VIS detector (UVD170S) at a wavelength of
450 nm and the chromatography conducted using Chromeleon software
(Thermo Fisher Scientific, Loughborough, UK). The concentration of NTBI
was computed from blanks and standards taken through the whole procedure
along with each batch of samples. This method was validated in our
laboratory (White, 2012) (see Appendix 7). Chromatograms of a standard
and a plasma sample for NTBI are shown in Figure 2.3.
2.4.9 Estimation of total antioxidant activity

Total antioxidant activity was measured using the ‘Ferric-Reducing Ability of Plasma’ (FRAP) assay of Benzie and Strain (1996) with slight modifications.

2.4.9.1 Ferric-Reducing Ability of Plasma (FRAP) assay

This assay depends on the reduction of ferric tripyridyltriazine (Fe$^{3+}$ TPTZ) to ferrous tripyridyltriazine (Fe$^{2+}$ TPTZ) at low pH, producing an intense purple
colour which can be measured at 595 nm. The colour intensity depends on ability of components in the plasma to reduce Fe$^{3+}$ TPTZ to Fe$^{2+}$ TPTZ.

2.4.9.2 Preparation of FRAP reagent

FRAP reagent was prepared by adding 3.4 ml of 300 mM acetic acid to 100 ml of water and then adjusting the pH to 3.6 by adding 2 mM NaOH. The solution was then made up to 250 ml with water, and then 0.081 g of FeCl$_3$ were added, followed by 0.039 g of TPTZ which had been dissolved in dilute acid (10% hydrochloric acid). The FRAP reagent was stored for up to two weeks at 4 °C.

2.4.9.3 Procedure

The samples, standards and FRAP reagent were warmed to 25 °C and then 10 μl of sample or the standard were added to 260 μl of FRAP reagent. Water was used as a blank. The absorbance at 595 nm was measured for 45 min in a plate reader (Versamax, Molecular Devices, Sunnyvale, CA). The measurements were compared to a standard curve prepared using 0-2 mM FeSO$_4$·7H$_2$O.
2.4.10 Determination of total and reduced ascorbate

Total and oxidised ascorbate (DHA) were measured by the method described by Sato et al. (2009). Two (90 µl) aliquots of the 10% metaphosphoric acid (MPA) extract (Section 2.4.2) were removed from the stored extract. One was for the measurement of reduced ascorbate and the other for measurement of total ascorbate. For the measurement of total ascorbate, 10 µl of 350 mM Tris (2-carboxyethyl) phosphine hydrochloride in 5% MPA, containing 1 mM EDTA, were added, and the tubes were capped and the contents mixed well on a vortex mixer. The mixture was incubated for 20 min at room temperature (this is to reduce the oxidised dehydroascorbate back to ascorbate). Finally 200 µl of 5% MPA containing 1 mM EDTA were added to the mixture, which was injected into the HPLC system. The other 90 µl of supernatant was mixed with 10 µl of 5% MPA containing 1 mM of EDTA, and then 200 µl of 5% MPA containing 1 mM of EDTA was added. The mixture was mixed well on a vortex mixer before injection into the HPLC system. The HPLC system consisted of a 150 mm × 4.6 mm ACE 5 AQ C\textsubscript{18} column (Hichrom, Reading, UK), a Milton Roy Constametric pump (Milton Roy, Sunderland, UK) coupled to a BAS LCD40 electrochemical detector (BASi, West Lafayette, IN, USA) set at a voltage of +0.6 V. The mobile phase consisted of 50 mM potassium phosphate buffer containing 0.54 mM EDTA and 2% methanol at pH 2.8. This was pumped at a rate of 1.0 ml min\textsuperscript{-1}.

This method is in accordance with previous stability studies where MPA-stabilized plasma samples had been stored at -70 °C from 2 weeks to over 10 years (Chung et al., 2001). A standard curve for total ascorbate is shown in Figure 2.4.
2.4.11 Thiobarbituric acid reactive substances (TBARS) assay

The TBA assay is one of the most frequently used methods for the measurement of MDA and other products of lipid peroxidation in fatty acids, food products, membrane systems, plasma and tissue samples (Halliwell and Gutteridge 1990a; Draper and Hadley 1990). The general procedure simply involves heating the material under test with TBA under acidic conditions and measuring the absorbance of the resulting pink chromogen, either spectrophotometrically or using High Performance Liquid Chromatography (HPLC). The chromogen, which forms by the condensation of two molecules of TBA and one molecule of MDA, absorbs light at 532 nm and fluoresces at 553 nm.
2.4.11.1 Measurement of malondialdehyde in plasma

MDA is routinely measured by reacting it with thiobarbituric acid (TBA) which, under appropriate conditions, gives a pink coloured product that absorbs light at 532 nm. The method of Agarwal and Chase (2002) was followed. Plasma (50 µl) or standard (up to 2 µM) or blank was mixed with 50 µl of BHT (0.05% in 95% ethanol), 400 µl of phosphoric acid (0.44 M) and 100 µl of TBA (42 mM). The tubes were capped and the contents mixed well on a vortex mixer. All tubes were heated for 1 h at 100 °C in a dry block, and then were removed and placed on ice to cool. Butan-1-ol (300 µl) was added and mixed well using the vortex mixer. The mixture was centrifuged for 5 min at 13,000 × g to separate the aqueous and butanol phases. Two hundred microliters of the butanol extract were removed carefully and placed in tubes ready for injection directly into a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific) comprising an Ultimate 3000 pump and Ultimate 3000 fluorescence detector. Chromatography was conducted using a 100 mm × 4.6 mm Hichrom C_{18} column, protected by a guard column of the same resin and a 2 µm inline filter (Hichrom). Chromatography was controlled using Chromeleon software (version 6.8) (Thermo Fisher Scientific). The mobile phase (50 mM potassium phosphate/methanol 80:20 v/v, pH 6.8) was pumped at a rate of 1.0 ml min\(^{-1}\). MDA was detected using an excitation wavelength of 515 nm and an emission wavelength of 553 nm.

2.4.11.2 Interference with the method

Determination of MDA, which is the most abundant of lipid peroxidation products, is a convenient and sensitive method for quantitative estimation of lipid peroxide concentration in many types of samples including drugs, food
products and human and animal tissues (Lykkesfeldt, 2007). However, although this technique is easy to use (mainly the spectrophotometric assay), its use to assess oxidative stress status in human fluids is problematic for several reasons: (a) aldehydes other than MDA may react with TBA to produce a compound that absorbs light in the same wavelength range as MDA; (b) decomposition of lipid peroxides during the test itself may mask the actual MDA content in the fluid before testing; (c) the presence or absence of metal ions or other undefined radicals may affect the rate of this decomposition, making reliability a problem; and (d) most TBARS, including MDA, in human body fluids are not specific products of lipid peroxidation and may produce false-positive results (Halliwell and Whiteman, 2004). Several compounds including bile pigments, carbohydrates and amino acids have also been found to react with TBA (Esterbauer et al. 1982; Knight et al. 1988) and generate interfering chromogen. Use of HPLC has enabled the separation of these compounds from the true MDA-TBA adduct, thereby increasing the specificity of the assay (Volpi and Tarugi, 1998).

Several precautions were taken in order to minimize interference with this assay. These included collecting blood samples in tubes containing EDTA and LiH, and rejecting any samples showing signs of haemolysis. Since platelets are sources of lipid peroxides, care was taken to avoid the ‘buffy coat’ layer whilst separating the plasma from the red cells. Finally, the purest source of HPLC grade water (Elga-Purelab Q7/15) was sought and used for the preparation of all samples and solutions.
2.4.12 Background information on human E-selectin

E-selectin (Endothelial Leukocyte Adhesion Molecule-1, ELAM-1) is a member of the selectin family of adhesion molecules (Hogg, 1992, Lasky, 1991). Together with LECAM-1 (L-selectin) and GMP-140 (P-selectin), E-selectin mediates the initial interactions of leukocytes and platelets with endothelial cells (Shimizu et al., 1991, von Andrian et al., 1992). E-selectin belongs to the single-chain glycoprotein selectin family which includes E, L and P-selectins that mediate the initial step of leukocyte recruitment in the inflammatory process. E-selectin is a 58.6 kDa protein containing 535 amino acid residues. It is synthesised by endothelial cells in response to cytokines including interleukin-1 beta (IL-1β) and TNF-α, and to bacterial lipopolysaccharide (Bevilacqua et al., 1989, Suárez et al., 2010). E-selectin expression begins after 1-2 h and shows peak expression after 3-4 h, and decay after 16-24 h, in response to activation. E-selectin ligands, expressed on neutrophils, monocytes and a subset of memory T cells are sialylated, fucosylated molecules which bind to the lectin domain of E-selectin. Immunocytochemical techniques have demonstrated the expression of E-selectin on healthy and diseased tissue.

E-selectin mediates the attachment of flowing leukocytes to the blood vessel wall during inflammation by binding to E-selectin ligands on leukocytes (Vestweber and Blanks, 1999). These interactions are labile and permit leukocytes to roll along the vascular endothelium in the direction of blood flow. This initial interaction is followed by a stronger interaction involving ICAM-1 and VCAM-1 that leads eventually to extravasation of the leukocytes through the blood vessel wall into the extracellular matrix tissue. ELISA techniques
have shown that detectable levels of soluble E-selectin are present in the biological fluids of apparently normal individuals. Furthermore, a number of studies have reported that levels of E-selectin may be elevated in subjects with a variety of pathological conditions including kidney inflammation, rheumatoid arthritis, hypertension, dyslipidaemia, diabetes, obesity, and smoking (Roldán et al., 2003, Matsumoto et al., 2002b). Several studies reported that E-selectin levels are elevated in patients with T2DM (Boulbou et al., 2004, Pasini et al., 1995). Up-regulation of endothelial adhesion molecules, including endothelial-leukocyte adhesion molecule (E-selectin), intercellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), play a pivotal role in the earliest phases of atherosclerosis by mediating the binding and subsequent recruitment of monocytes into arterial intima.

2.4.12.1 Principle of measurement of human E-selectin by enzyme-linked immunosorbent assay (ELISA)

The human E-selectin ELISA kit is based on standard sandwich enzyme-linked immunosorbent assay technology. Human E-selectin specific polyclonal antibodies are precoated onto 96-well plates, and the human specific detection monoclonal antibodies are biotinylated. The test samples and biotinylated detection antibodies are added to the wells and then followed by washing with PBS or TBS buffer. Avidin-biotin-horseradish peroxidase (HRP) conjugate is added and unbound conjugate washed away with PBS. The HRP substrate TMB is used to visualize the bound HRP via the peroxidase enzymatic activity. The oxidation of TMB by H$_2$O$_2$ is catalyzed by HRP to produce a blue coloured product that changes into yellow after
adding an acidic stop solution. The absorbance of the yellow product is proportional to the amount of human E-selectin in the sample captured on the plate.

2.4.12.2 Procedure

The assay was carried out according to the manufacturer’s instructions (Biorbyt, UK). Briefly, 100 μl of standard, control, and samples were added per well, and the plate was then covered and incubated for 90 min at 37 °C. The liquid in each well was removed followed by incubation with 100 μl of biotinylated anti-human E-selectin antibody for 60 min at 37 °C. The plate was then washed three times using 0.01 M phosphate-buffered saline (PBS) and each time the buffer was left in the well for 1 min to wash. The liquid was discarded each time and the plate was blotted onto adsorbent material. After that, 100 μl of prepared avidin-biotin-peroxidase complex (ABC) working solution were added into each well and the plate was incubated for 30 min at 37 °C. The plate was washed five times with 0.01 M PBS, the washing solution being left for 1-2 min each time before being discarded. The plate was then blotted onto absorbent material after which 90 μl of TMB were added to each well, which was then incubated for 8-12 min at 37 °C. Finally 100 μl of TMP stop solution were added to each well. The absorbance of the contents of each well was determined within 30 min after adding the stop solution, using a micro plate reader (Versamax, Molecular Devices, Sunnyvale, CA) set to 450 nm. The detection range of the kit was 62.5-4000 pg ml⁻¹. A standard curve for E-selectin is shown in Figure 2.5.
Figure 2.5 ELISA calibration curve for Human E-selectin. The curve is a 4-parameter fit to the data.

2.4.13 C-reactive protein (CRP) structure and function

CRP was first discovered in 1930 in the sera of patients acutely infected with pneumococcal pneumonia, and named after its reactivity with polysaccharide C from Streptococcus pneumonia (Tillett and Francis Jr, 1930). It is a calcium-binding pentameric protein consisting of five identical non-glycosylated 23 kDa polypeptide subunits.

CRP is an acute phase reactant, increasing 1000-fold in response to infection, ischaemia, trauma and burns (Westhuyzen and Healy, 2000). In inflammatory conditions it is produced predominantly in the liver in response to interleukin (IL)-6, which is one of several cytokines released by activated leukocytes and smooth muscle cells in atherosclerotic plaques within hours of an injury or in the presence of inflammation (Li and Fang, 2004). However, adipose tissue is also a significant source of IL-6, which partially explains the
strong correlation between obesity and CRP (Yudkin et al., 2000). CRP may also be produced away from the liver by vascular sources including cells residing in atherosclerotic plaques (Lind, 2003). Its levels remain elevated throughout the acute phase response until normal tissue function and structure are restored. Normal CRP levels are generally defined as being < 10 mg l⁻¹ and healthy individuals generally have < 1 mg l⁻¹ (Armani and Becker, 2005).

The role of CRP is not fully understood, but two functional properties of CRP including the ability to activate the complement system (Volanakis, 1982) through the classical complement pathway, and the ability to modulate the function of phagocytic cells (Li and Fang, 2004) have been demonstrated (Li and Fang, 2004). CRP is also known to bind in a wide variety of substances such as microbial polysaccharides and damaged cell membranes.

2.4.13.1 Storage of samples

CRP is stable during storage both in whole blood for a short period and in serum or plasma for longer times. Samples containing CRP should be stored at 4 °C for short periods of time or at -70 °C for longer periods (Aziz et al., 2003). Comparable results were obtained by Ishikawa et al. (2007) for plasma (heparin and EDTA treated) and serum samples, and levels were unaffected by delays in sample processing and storage temperature. They also found that CRP levels were unaffected by up to seven freeze-thaw cycles (Ishikawa et al., 2007).
2.4.13.2 High sensitivity C-reactive protein (hs-CRP)

CRP has been assayed for many years as a non-specific marker of acute inflammatory diseases, infections, neoplastic diseases, and other conditions where inflammation occurs. It is still assayed in this manner as a marker of inflammation by immunoassay methods that are sensitive to concentrations of 5-20 mg l⁻¹. Atherosclerosis is a subclinical chronic inflammatory condition. Highly sensitive measurements of CRP have been developed to detect this protein at lower levels (0.5-10.0 mg l⁻¹). This assay is referred to as high sensitivity C-reactive protein (hs-CRP), and there are commercially available kits for this purpose that provide similar results in stored, fresh or frozen plasma (Koenig, 2005).

2.4.13.3 High sensitivity C-reactive protein as a biomarker atherosclerosis

CRP is an important inflammatory biomarker and a risk factor for cardiovascular disease. Much evidence indicates that the CRP contributes to the atherosclerosis development process (Epstein and Ross, 1999). An inflammatory process plays a central role in the initiation, progression, and the final steps of this pathology as vulnerable plaques rupture.

2.4.13.4 Procedure

The assay was carried out according to the manufacturer’s instructions (Biorbyt, UK). To each well of a 96 well plate 100 µl of standard or samples were added and incubated at room temperature for 1 h. The contents of the plate were then discarded and the plate was tapped on a clean paper towel to remove residual solution in each well, after which it was washed three
times with washing buffer (300 µl). The plate was then tapped with paper
towel to remove residual wash buffer. This was followed by the addition of
100 µl of detection antibody solution to the plate which was then incubated at
room temperature for 1 h. Again the contents of plate were discarded and it
was washed with washing buffer 4 times, after which it was also tapped with
clean paper towel. Then 100 µl of substrate solution were added and
incubated for 15 min in the dark, after which 100 µl of stop solution were
added and the contents of the plate mixed gently. The absorbance of the
contents of each well was determined immediately after adding the stop
solution, using a Versamax micro plate reader (Molecular Devices,
Sunnyvale, CA) set to 450 nm. A standard curve for CRP is shown in Figure
2.6.

![Figure 2.6 ELISA calibration curve for Human C-RP. The curve is a 4-parameter fit to the data.](image)

Figure 2.6 ELISA calibration curve for Human C-RP. The curve is a 4-parameter fit to the data.
2.4.14 Soluble transferrin receptor

Many parameters have been used to indicate the body iron status in clinical studies such as Hb, serum iron, TIBC, transferrin saturation and serum ferritin. Serum ferritin and serum iron are the most commonly used markers of iron status (Thomas and Thomas, 2002). Unfortunately, some of these parameters are influenced by a number of non-related conditions, e.g. acute phase reactions, which may complicate the clinical interpretation of their results and create an inaccurate picture of the body iron status.

Recently, soluble transferrin receptor concentration has been recognized as a useful indicator of iron status. Soluble transferrin receptor is a soluble protein that is produced by proteolytic cleavage of the membrane-bound transferrin receptor. Both the expression of transferrin receptor on the cell surface and its intracellular concentration are inversely related to intracellular iron concentration (Baynes and Cook, 1996).

Soluble transferrin receptor (sTfR) reflects the number of iron receptors expressed on cell membranes and is raised once tissue iron starts to become limited (Koulaouzidis et al., 2009). Soluble transferrin receptor results from the proteolysis of TfR at a specific site in the extracellular domain, producing monomers that are measurable in plasma and serum (Shih et al., 1990). Major advantages of measuring serum transferrin receptors involve the fact that the assay is not significantly affected by infection or inflammatory processes, and it does not vary with age, gender or pregnancy (Kohgo et al., 1986, Carriaga et al., 1991). However, sTfR levels may be elevated when
there is increased red cell production, turnover, or both, such as in the case of haemolytic anaemia (Kohgo et al., 1987).

2.4.14.1 Principle of test

According to manufacturer’s instructions for the human sTfR ELISA kit (BioVendor, UK), standards, quality controls and samples are incubated in microplate wells pre-coated with monoclonal anti-human sTfR antibody. After 90 min incubation and washing, monoclonal anti-human sTfR antibody conjugated with horseradish peroxidase (HRP) is added to the wells and incubated for 60 min with captured. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and the absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of sTfR. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

2.4.14.2 Procedure

To each well of a 96 well plate 100 µl of diluted standard, quality control, dilution buffer (blank) and sample were added. The plate was incubated at 25-30 °C for 1 h. After that the plate was washed 3 times with 350 µl of washing solution and after the final wash the plate was inverted and tapped strongly against a paper towel. Then 100 µl of conjugate solution were added to each well and the plate was incubated at 25-30 °C for 1 h. Then the wells were washed 3 times with 350 µl of washing buffer and after the final wash
the plate was inverted and tapped strongly against a paper towel. Then 100 µl of substrate solution was added to plate and the plate was incubated for 10 min at room temperature after the plate was covered with aluminium foil to avoid direct sunlight. After that the colour development was stopped by adding 100 µl of stop solution the absorbance was determined by using a Versamax plate reader (Molecular Devices, Sunnyvale, CA) set to 450 nm.

2.4.14.3 Stability of sTfR

Previous experiments (Erhardt et al., 2004) have shown that undiluted serum samples containing sTfR could be thawed and refrozen a few times without any change in analyte concentration. Diluted serum samples also appear to be stable to freeze-thawing. A standard curve for sTfR is shown in Figure 2.7.

![ELISA calibration curve for Human sTfR. The curve is a 4-parameter fit to the data.](image)

Figure 2.7 ELISA calibration curve for Human sTfR. The curve is a 4-parameter fit to the data.
2.5 Statistical analysis

Conventional statistical methods were used to calculate the means, standard deviation (SD) and standard error (SE). The data were analysed using a General Linear Model (GLM) with subject age as a quantitative factor and subject group as a qualitative factor. In all cases there was no significant interaction between subject age and subject group, so the interaction term was omitted from the model. This was followed by Tukey’s test. This allowed differences between the groups to be distinguished from the any relationship between parameters and subject age. Correlation among the different parameters was also investigated. All statistical analysis was carried out using StatGraphics version 16.2.04 (StatPoint Technologies, Inc.) or Minitab (Minitab Ltd, Coventry, UK).
2.6 Results

2.6.1 Effect of glutathione on the level of malondialdehyde in stored human plasma

2.6.1.1 Method development and improvement

Measurement of malondialdehyde (MDA) is an important contribution to the assessment of oxidative stress. Initially quantification of MDA to get precise results without interfering peaks posed difficulties in our laboratory. The original Agarwal and Chase (2002) method used 40:50 (v/v) methanol/buffer as the mobile phase, but with this method an interfering peak was found close to the MDA. However, with 20:80 (v/v) methanol/buffer as the mobile phase a single sharp peak corresponding to MDA was obtained; this mobile phase caused the interfering peak to shift, such that the method now worked properly. Many trials were followed in order to obtain chromatograms with a sharp peak corresponding to MDA (Figure 2.8 A and B). This was eventually achieved following studies in which the ratio of aqueous to organic solvent in the mobile phase was varied, ultimately arriving at the method described above. Figure 2.9 shows a representative chromatogram for a plasma samples obtained using this method.
Figure 2.8 Representative chromatograms showing multiple peaks obtained with the original Agarwal and Chase (2002) method with 40:50 (v/v) methanol/buffer as mobile phase (A). However, with 20:80 (v/v) methanol/buffer as mobile phase a single sharp peak corresponding to MDA was obtained (B). Due to the increase in the polarity of the mobile phase the other peaks took more time to appear. The retention time for MDA with a 2 μM standard was 2.30 min. CVs within and between days were 4-6% and 6-7%, respectively. This finding was obtained by repeating measurements of standard solutions (1,1,3,3-tetraethoxypropane) (n = 5) of MDA (0.2 to 2 μM) within days and between days.
2.6.1.2 Determination of MDA standards

The reagent 1,1,3,3-tetraethoxypropane was used in preparing MDA standards during the MDA assay which yields equivalent amounts of MDA under the conditions of the reaction (Section 2.4.12.1). A linear standard curve was obtained ($R^2 = 0.9995$) (Figure 2.10).
2.6.1.3 Changes in the level of measured MDA with time of storage

Higher levels of MDA were found in plasma stored in tubes treated with LiH compared with those in tubes treated with EDTA. The combination of GSH with EDTA reduced the level of MDA compared with EDTA without GSH, as did the combination of GSH with LiH compared to LiH without GSH but this reduction was less than that seen with the combination of EDTA and GSH. The level of MDA in samples treated with GSH increased over time so that by day 7 the effect of GSH was lost (Figure 2.11 A and B). Using GLM analysis a significant interaction between time and treatment was found ($P < 0.0005$). There were also significant differences between treatments in that there were significantly higher ($P < 0.0005$) levels of MDA in the plasma samples that were treated with EDTA compared with plasma samples that were treated...
with LiH. To investigate the interaction in relation to the effects of GSH pairwise paired t tests were used.

There were significant differences ($P = 0.001$; paired t test) in the levels of MDA at time zero between plasma that was stored in EDTA with and without GSH (Figure 2.11 A), but there were no significant differences ($P = 0.199$) in the levels of MDA in plasma that was stored in EDTA with and without GSH on day 21 (Figure 2.11B). There were significant differences ($P = 0.002$) in the levels of MDA at time zero between plasma that was stored in LiH with and without GSH (Figure 2.12 A), but there was no significant difference ($P = 0.099$) in the levels of MDA in plasma that was stored in LiH with and without GSH on day 21 (Figure 2.12 B). There were no significant differences ($P = 0.075$ and $0.286$, respectively; paired t test) between the levels of MDA at time zero and on day 21 between plasma that was stored in EDTA without GSH and plasma that was stored in LiH without GSH. There were no significant differences ($P = 0.054$ and $0.379$, respectively; paired t test) between the levels of MDA at time zero and on day 21 between plasma that was stored in EDTA with GSH and plasma that was stored in LiH with GSH.
Figure 2.11 Effect of storage at -80 °C on the MDA concentration in human plasma samples. Data shown are means ± SEM. (A) samples stored in EDTA with or without GSH; (B) samples stored in LiH with or without GSH. Effects of time and treatment were analysed using GLM. There was no significant difference between days ($P > 0.05$). However, there was a significant interaction between time and treatment ($P < 0.0005$) and a significant difference between treatments ($P < 0.0005$, $n = 6$).
Figure 2.12 MDA (means ± SEM) in plasma of 6 subjects stored with EDTA or with LiH, with or without GSH (A) at time zero and (B) on day 21. Bars with different letters are significantly different ($P < 0.05$; paired t tests; $n = 6$).
2.6.2 Results of the in vivo study

Venous blood samples (5 ml) were drawn from all subjects. These subjects were categorised into three groups: T2DM, obese non-diabetic and control groups. Although originally intended, the groups were not age matched due to problems with recruitment of subjects for all the groups and the exclusive recruitment criteria used in this study. There were 28 subjects in the T2DM group (10 female and 18 male; 36% and 54%, respectively), aged from 41 to 76 y (mean ± SD, 58 ± 7 y); 28 subjects in the control group (7 female and 21 male; 25% and 75%, respectively), aged from 41 to 64 y (mean ± SD, 52 ± 7 y); and 17 subjects in the obese non-diabetic group (9 female and 8 male; 53% and 47%, respectively), aged from 40 to 74 y (mean ± SD, 50 ± 9 y). See Appendix 3 to find more detail about all the parameters measured in the study.

2.6.2.1 Iron indices in different groups

There were no correlations between age and iron indices except for a positive correlation between TIBC and age (see Section 2.6.2.2 and Appendix 1). For those indices where there was no correlation, the data were analysed using one-way ANOVA. Total serum iron levels were significantly lower ($P = 0.005$) than controls in both the diabetes and obese groups, but there was no significant difference ($P > 0.05$) between the obese and diabetes groups (Figure 2.13). The percentage iron saturation was significantly higher ($P < 0.01$) in the diabetes group than the control and obese groups, but there was no significant difference ($P > 0.05$) between the control and obese groups (Figure 2.14). The NTBI levels were significantly higher ($P < 0.001$) in the diabetes group than in the control and obese groups.
There was a slightly higher level of NTBI in the obese group compared to controls, but this was not significant (Figure 2.15). Serum soluble transferrin receptor levels were significantly lower ($P < 0.05$) in the controls than in the obese and diabetic groups, and the level in the obese group was significantly higher ($P < 0.05$) than that of the diabetic group (Figure 2.16).

![Figure 2.13](image)

**Figure 2.13** Total serum iron (means ± SEM) for control, T2DM and obese groups. There was a significant difference between the control group, and both the diabetes and obese groups ($P < 0.05$; Tukey’s HSD), but there was no significant difference between the diabetes and obese groups. Bars with the same lower case letter are not significantly different (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).
Figure 2.14 % transferrin saturation (means ± SEM) for control T2MD and obese groups. There was a significant difference between the diabetes group, and both the control and obese groups ($P < 0.05$; Tukey’s HSD), but there was no significant difference between the control and obese groups. Bars with the same lower case letter are not significantly different (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).

Figure 2.15 NTBI (means ± SEM) between control, T2MD and obese groups. There was a significant difference between the diabetes group, and both the control and obese groups ($P < 0.05$; Tukey’s HSD), but there was no significant difference between the control and obese groups. Bars with the same lower case letter are not significantly different (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).
There was a significant difference between the control group, and both the diabetes and obese groups ($P < 0.05$; Tukey's HSD), and between diabetes and obese groups ($P < 0.05$; Tukey's HSD). Bars with the same lower case letter are not significantly different (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).

### 2.6.2.2 Correlations between age and iron indices

As noted above there was a positive correlation between TIBC and age for all groups ($P = 0.032$); $R^2 = 0.344$, 0.057 and 0.101 for the T2DM, control and obese groups, respectively (Figure 2.17). GLM analysis of these data showed total iron binding capacity was significantly higher ($P < 0.005$) in the control group than in the diabetes and obese group, but there were no significant differences ($P > 0.05$) between the diabetes and obese groups.
Figure 2.17 Correlations between age and TIBC. 

\( P = 0.032; \) \( R^2 = 0.344, 0.057 \) and 0.101 for the T2DM, control and obese groups, respectively (control, \( n = 28 \); T2DM, \( n = 28 \); and obese, \( n = 17 \)).
2.6.2.3 Correlation between age and all oxidative stress

There were no correlations between age and all oxidative stress indices (see Appendix 2) except for a positive correlation with MDA \( (P = 0.017); \ R^2 = 0.347, 0.131 \) and 0.378 for the T2DM, control and obese groups, respectively (Figure 2.18). GLM analysis of these data showed that MDA levels were significantly lower \( (P < 0.001) \) in controls than in obese and diabetic groups, and that of the diabetic group was significantly higher \( (P < 0.05) \) than that in the obese group.

![Figure 2.18 Correlations between age and MDA](image)

\( P = 0.017, \ R^2 = 0.347, 0.131 \) and 0.378 for the T2DM, control and obese groups, respectively (control, \( n = 28; \ T2DM, n = 28; \) and obese, \( n = 17)\).

2.6.2.4 Oxidative stress indices in different groups

The data for the remaining indices were analysed by one-way ANOVA. Total ascorbate (i.e. ascorbate + DHA) was significantly lower \( (P = 0.0077) \) in the
diabetic group compared to controls and obese subjects, but there was no significant difference between total ascorbate in control and obese subjects \((P > 0.05)\) (Figure 2.19). Ascorbate was significantly lower \((P < 0.05)\) in the diabetic group compared to the control and obese groups but there was no significant difference between the control and obese groups \((P > 0.05)\) (Figure 2.20). DHA was a significantly lower in the control group \((P < 0.01)\) compared to both the diabetic and obese groups, and also significantly lower in the diabetic group compared to the obese group \((P < 0.05)\) (Figure 2.21). Total antioxidant capacity was significantly higher in the control compared to the diabetic group \((P < 0.05)\) but there were no significant differences between control and obese, and obese and diabetic groups \((P > 0.05)\) (Figure 2.22). The percentage of ascorbate in the oxidised form (DHA) was significantly greater than that of the control group in the diabetes and obese groups, but there was no difference between the diabetes and obese groups. (Table 2.1).

<table>
<thead>
<tr>
<th>DHA [µmol l⁻¹] (mean ± SD)</th>
<th>Control</th>
<th>Diabetes</th>
<th>Obese</th>
</tr>
</thead>
</table>

Significant differences between groups are shown as follows: \(^{θ}\), between controls and diabetics; \(^{θ^†}\), between controls and obese; \(^{Δ}\), between diabetics and obese.
Figure 2.19 Total ascorbate (means ± SEM) between control, T2MD and obese groups. There was a significant difference between both control and obese groups and diabetes group \((P < 0.05; \text{Tukey's HSD})\), and there was no significant difference between control and obese groups. Bars with the same lower case letter are not significantly different (control, \(n = 28\); T2DM, \(n = 28\); and obese, \(n = 17\)).

Figure 2.20 Ascorbate (means ± SEM) between control, T2MD and obese groups. There was a significant difference between both control and obese groups and diabetes group \((P < 0.05; \text{Tukey's HSD})\), and there was no significant difference between control and obese groups. Bars with the same lower case letter are not significantly different (control, \(n = 28\); T2DM, \(n = 28\); and obese, \(n = 17\)).
There was a significant difference between the control group and both the diabetes and obese groups ($P < 0.01$), and between diabetes and obese groups ($P < 0.05$; Tukey's HSD). Bars with the same lower case letter are not significantly different (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).

There was a significant difference between the control and diabetes groups ($P < 0.05$; Tukey's HSD), and there was no significant difference between control and obese and obese and diabetes groups. Bars with the same lower case letter are not significantly different (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).
2.6.2.5 E-selectin and high sensitivity C-reactive protein level differences between groups

There were no correlations between age, and either E-selectin or hs-CRP (Appendix 3), and so one-way ANOVA was used for the analysis of the data. E-selectin was significantly lower \( (P < 0.001) \) in the control and obese groups compared to the diabetic group but there was no significant difference \( (P > 0.05) \) between the obese and control groups (Figure 2.23). hs-CRP was significantly lower \( (P < 0.01) \) in the control group compared to the diabetic and obese groups but there was no significant difference between diabetic and obese groups \( (P > 0.05) \) (Figure 2.24).

2.6.2.6 Correlations between NTBI and other iron indices

There were no correlations between NTBI and total serum iron, TIBC and %TS (Appendix 4), but there was a significant correlation between NTBI and soluble transferrin receptor (Figure 2.25).
Figure 2.23 E-selectin (means ± SEM) between control, T2MD and obese groups. There was a significant difference between both control and obese groups and diabetes group ($P < 0.05$; Tukey’s HSD), and there was no significant difference between control and obese groups (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).

Figure 2.24 Plasma hs-CRP levels (means ± SEM) between T2MD, obese and control groups. There was a significant difference between the control group and both the diabetes and obese groups ($P < 0.01$; Tukey’s HSD), but there was no significant difference between the diabetic and obese groups (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).
2.6.2.7 Correlation between NTBI and biomarkers of oxidative stress

There were no correlations between NTBI and MDA, total ascorbate, ascorbate DHA and total antioxidant capacity (Appendix 5).

2.6.2.8 Correlation between NTBI and biomarkers of inflammation

There was a significant correlation at the 90% confidence level between NTBI and a biomarker of inflammation, hs-CRP (Figure 2.26).

2.6.2.9 Correlation between NTBI and biomarkers of atherosclerosis

There was a significant correlation between NTBI and a biomarker of atherosclerosis, E-selectin (Figure 2.27), but there was no correlation between E-selectin and hs-CRP (Appendix 6).
Figure 2.26 Correlations between NTBI and hs-CRP

$P = 0.054$, $R^2 = 0.338$, $0.098$ and $0.256$ for the T2DM, control and obese groups, respectively (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).

Figure 2.27 Correlations between NTBI and E-selectin

$P = 0.001$, $R^2 = 0.469$, $0.017$ and $0.232$ for the diabetes, control and obese groups, respectively (control, $n = 28$; T2DM, $n = 28$; and obese, $n = 17$).
2.6.2.10 Correlation between NTBI and HbA\(_1c\)

There was significant correlation between NTBI and level of HbA\(_1c\) in subjects from the T2DM group (Figure 2.28).

![Correlation between NTBI and HbA\(_1c\)](image)

Figure 2.28 Correlation between NTBI and HbA\(_1c\) \((P < 0.0005; R^2 = 0.742)\) in the T2DM group \((n = 28)\).
2.7 Discussion

2.7.1 Storage of plasma with and without GSH

This study showed a significant difference in the level of MDA on day zero between plasma samples that were stored in EDTA and LiH with and without GSH. MDA levels on day zero were higher in EDTA without GSH compared with those in plasma stored in EDTA with GSH. The explanation of this result may be due to the combination of GSH with EDTA attenuating the oxidation of lipids and lipoproteins in the plasma sample during the time in storage. Addition of GSH should reduce the rate of oxidation and formation of MDA. This appeared to be the case on day zero, but after that the action of GSH gradually diminished with time (Figure 2.4A). Diminution of the action of GSH may explain the non-significant result on day 21 of storage. The results of the present study agree with the results of (Kumar et al., 2012). Kumar et al. stored samples for long periods (708 days) and they found little change in MDA on storage at -80 °C over the first 28 days.

Many studies have shown that addition of antioxidants to the reaction mixture may inhibit metal-catalyzed autoxidation of coexisting polyunsaturated fatty acids (Lepage et al., 1991, Bonnefont et al., 1989, Lee, 1980, Arshad et al., 1991). Hendriks and Assmann (1988) showed that addition of GSH at a final concentration of 0.65 mmol l⁻¹ had no significant influence on serum TBARS concentration. Similar results were obtained for GSH concentrations in the range 0.32-7.80 mmol l⁻¹. Also, they showed that short-time storage of plasma at either 4 °C or -20 °C induces large day to day variations in TBARS results, and that addition of GSH plus EDTA prevented this variation. However, in a study by Hendriks and Assmann (1988) samples were stored
at -80 °C. It is proposed to use untreated plasma and to perform the assay immediately after collection of plasma or to add both glutathione and EDTA before storage. Some studies disagree on the value of the addition of antioxidant; for example, Richard et al. (1992) stated that addition of an antioxidant such as BHT did not change the results for TBARS determination. On the other hand, Richard et al. report quite high values for native TBARS because of their use of an acidic pH during reaction and extraction at low pH leads to an increased yield of MDA.

The present study also showed a significant difference in the level of MDA in day zero between plasma samples was stored in LiH with and without GSH. This was probably due to the action of GSH in reducing lipid peroxidation during storage. However, this action of GSH gradually declined with storage time (Figure 3.4B) such that no significant differences in MDA were seen on day 21. Combination of LiH with GSH was associated with a reduced level of MDA but the combination of EDTA with GSH showed a greater effect. Glutathione is a tripeptide thiol found in virtually all cells. It is one of the most important biological antioxidants. GSH plays an important role in the protection of cells and tissue structures by scavenging free radicals, and participating in several redox and detoxification reactions. It protects cells against the destructive effects of reactive oxygen intermediates (Meister, 1983). GSH is also important for the maintenance of θ-tocopherol and ascorbate in the reduced state (Scholz et al., 1989). Therefore, release of reduced and oxidized glutathione (GSH and GSSG) into the plasma and the ratio of GSH/GSSG are considered a reliable index to evaluate the status of oxidative stress in biological systems. Irreversible cell damage results when
the cell is no longer able to maintain its GSH content (Griffith and Meister, 1978). Glutathione not only protects cell membranes from oxidative damage, but also helps to maintain the sulfhydryl groups of many proteins in the reduced form, a requirement for their normal function (Reed and Fariss, 1984). Alternatively, GSH can be lost by autoxidation which occurs with a half-time of about 5 min in plasma at room temperature (Anderson and Meister, 1980). Only partial inhibition of autoxidation of GSH is achieved by EDTA or potassium cyanide in human plasma. *In vitro*, autoxidation of GSH is less at pH < 6 and may be attributed to the catalytic action of traces of heavy-metal ions such as copper and iron. These actions could account for the transient effect of GSH seen in the present study. It should be noted that in the present study, in order to measure MDA samples were thawed and stored on ice for up to 8 h while analysis was carried; during this time autoxidation of GSH may have occurred.

### 2.7.2 Storage of plasma in EDTA and LiH without GSH

In this work there was no significant difference between the level of MDA in plasma that was stored in EDTA and LiH without GSH on both days zero and 21, but the mean value in EDTA was less than in LiH on both days. Although this was not statistically significant, it is in agreement with the findings of Knight *et al.* (1987) who showed that plasma from liquid EDTA-anticoagulated blood had significantly lower TBARS concentrations than that in serum or plasma from blood treated with LiH, sodium citrate or a “citrate, phosphate, dextrose, adenine” solution. The explanation of this result is that EDTA is known to protect samples against autoxidation; EDTA may chelate free iron in the plasma sample which would limit iron-induced free radical
generation and reduce lipid peroxidation. EDTA also reduced the artefactual formation of MDA during sample processing (Nielsen et al., 1997). However, the formation of Fe$^{3+}$-EDTA may promote oxidative effects, possibly later during storage. The results of this study disagree with those of Lee (1980) and also with those of Carbonneau et al. (1991); this disagreement may be due to the difference in the storage conditions (Lee used 4 °C for storage of samples and Carbonneau et al. used -20 °C and 4 °C).

On the other hand, the combination of EDTA with transition metals such as iron and copper when present in the presence of low concentrations of ascorbate may induce the pro-oxidant activity of ascorbate. Fe$^{3+}$-EDTA can be reduced to Fe$^{2+}$ by ascorbate to generate the ascorbate radical thereby enhancing lipid peroxidation (Buettner and Jurkiewicz, 1996).

### 2.7.3 Iron indices in Type 2 diabetes

In the present study, the concentration of the NTBI and other parameters were determined in the plasma of Type 2 diabetic, obese (non-diabetic) and control subjects. Levels of NTBI in T2DM have been investigated by several groups, using different methods and reporting a range of results. Results from this study indicate that the concentration NTBI was significantly higher in the diabetes group compared with the control group. Our finding is supported by the studies of Sulieman et al. (Sulieman et al., 2004, Lee et al., 2006). However, another study found that NTBI is not present in significant amounts in all cases of T2DM (Van Campenhout et al., 2006b). Lee et al. (2006) found NTBI to be present in 92% of a known diabetic group and 59% of newly diagnosed diabetic group compared with a control group. Sulieman
et al. (2004) found a labile iron pool (LIP) in diabetic patients that was significantly different compared with the control. The non-significant result for some diabetic cases in the study of Van Campenhout et al. (2006b) may be due to the method which was used to measure NTBI and may be due to the unstable nature of NTBI in vivo or differences in the molecular masses within the plasma which are capable of binding different NTBI species. There is no ‘gold standard’ method for the measurement of NTBI (Jacobs et al., 2005a), and variations between studies often occur. The method used in this study was similar to that used by Van Campenhout et al. (2006b), so the results should be reasonably comparable. The reason for the differences between the two studies may therefore reside elsewhere, possibly in the treatments received by the two populations studied, and the extent to which the hyperglycaemia was controlled. For example, on-going studies in our laboratory (unpublished) indicate that the rise in NTBI levels in diabetic patients may be attenuated by statin treatment. Thus the small number of subjects receiving statins (11%) could have influenced the overall levels of NTBI measured in the current study. Without knowing more about these potential interactions there is little strong data to provide a convincing argument for the cause of the differences seen between the two studies. A very interesting relationship between the use of statins and ferritin levels was found recently by Zacharski et al. (2013).

The level of total iron in the diabetic group was lower than in the control group while Lee et al. (2006) found no significant difference between diabetic and control groups, and also between newly diagnosed and control groups. The reason behind the decrease in the level of total iron in T2DM may be the
increased production of hepcidin due to the inflammatory conditions in T2DM, which in turn hinders iron absorption. The reason that hepcidin levels are increased in T2DM may be the increase in ferritin and IL-6 levels; the elevated hepcidin might have adaptive value through down-regulation of iron absorption, and plays an important role in the pathogenesis of T2DM (Jiang et al., 2011).

The reason for the presence of NTBI in T2DM patients may be due to the effects of glycation of proteins such as Hb and transferrin, which is consistent with the strong correlation ($R^2 = 0.741$) between HbA1c and NTBI in T2DM subjects (Figure 4.38). Persistence of hyperglycaemia allows more glycation to occur, not only of Hb, but of other proteins such as transferrin, albumin and immunoglobulins, and this can lead to abnormal consequences for the structure and function of these proteins. The major step in the modification of proteins by glucose is Schiff base formation, followed by Amadori rearrangement (Cohen and Wu, 1994). Hyperglycaemia induces $H_2O_2$ formation by different pathways which promote more iron release from HbA1c than that from non-glycated haemoglobin. This free iron, acting as a Fenton reagent, might catalyse the production of free radicals and lead to degradation of cell constituents (Sen et al., 2005). In addition to haemoglobin glycation, transferrin is also prone to glycation, and glycation of haemoglobin and transferrin induced by the higher glucose levels can impair iron binding and promote even higher levels of free iron in the body (Kar and Chakraborti, 1999, Van Campenhout et al., 2004). Another explanation for the presence of NTBI in T2DM may be related to the decreased lifespan of red blood cells (RBCs) in diabetic patients (Cohen et al., 2004, Engström et al., 2014).
decrease in the lifespan of RBCs in diabetic patients may be due to alteration in their cell membranes (Mazzanti et al., 1992). Increased lipid peroxidised in T2DM interferes with chemical and physical properties of the cell membrane which lead to increased rigidity and decreased fluidity. Erythrocytes are considered as prime targets for oxidative stress due to free radical attack owing to the presence of both high membrane concentrations of polyunsaturated fatty acids (PUFA) and the O2 transport. This may induce alterations in protein and lipid structures and compositions of the RBC membranes, leading to damage to RBCs (Nayak et al., 2008). In addition, the serum transferrin (Tf) concentration is often diminished in diabetes as a consequence of decreased synthesis and/or increased loss (Telci et al., 2000). T2DM is closely correlated with chronic inflammation, with increased levels of circulatory acute response proteins and cytokines in affected subjects. Immune responses and inflammation are suggested to play roles in the development and complications of T2DM (Dandon et al., 2004).

This study also showed a significantly decreased TIBC in the T2DM group compared with the control group. Our results are supported by the study of (Van Campenhout et al., 2006b). In diabetes iron metabolism is disturbed. The reason for the low TIBC in T2MD may be a decrease in the capacity of transferrin to hold iron due to the glycation process which is increased in T2DM patients, or more likely due to Tf being a negative acute phase protein. There was a significant increase in % transferrin saturation in the T2DM group compared with the control group. Our results are supported by those of (Ellervik et al., 2011, Thomas et al., 2004). The reason for the increased the transferrin saturation in T2DM may be due to oxidative damage to the
proteins which hold iron. This study showed that the concentration of sTfR significantly increased in T2DM compared with the control and supported the studies of (Fernández-Real et al., 2007, Van Campenhout et al., 2006a), although other studies showed no difference between T2MD and the control (Hernández et al., 2005, Abou-Shousha et al., 2005). Another study showed sTfR decrease in T2DM (Yamada et al., 2000). The reason for increased sTfR in the current study may be due to hyperinsulinaemia which contributes to the inappropriately high sTfR concentration because insulin is known to cause a rapid and marked stimulation of iron uptake by fat cells, redistributing sTfR from an intracellular membrane compartment to the cell surface (Davis et al., 1986). Also, production of cytokines such as IL-6 and tumour necrosis factor α (TNFα) is elevated in T2DM (Pickup et al., 2000). Blood has the capacity to produce cytokines in diabetes which contributes to the augmented acute-phase response which may cause an increase in transferrin receptors on the cell surface, tissue deposition of iron and insulin resistance (Hirayama et al., 1993, Fernandez-Real et al., 2001).

2.7.4 Oxidative stress in Type 2 diabetes

Previous studies have demonstrated that oxidative stress, induced by elevated concentrations of both glucose and free fatty acids, plays a significant role in causing insulin resistance and β-cell dysfunction (Evans et al., 2002, Kajimoto and Kaneto, 2004). In the present study the concentration of MDA (a marker of lipid peroxidation) was significantly higher compared with the control group. Similar results were reported by (Bhutia et al., 2011, Gallou et al., 1993, Uzel et al., 1987, Aydin et al., 2001, Bikkad et al., 2014). A possible explanation for the increased concentration of MDA in T2DM is
due to increased lipid peroxidation, and the decline in antioxidant defences which may appear early in T2DM. Hyperglycaemia can induce oxidative stress by several different mechanisms such as autooxidation of glucose and the non-enzymatic glycation of proteins to generate superoxide (O$_2^-$) (Baynes, 1991).

In addition, levels of antioxidants decrease in diabetes. For example, ascorbate is an antioxidant vitamin which plays an important role in protecting against free radical induced damage, and a decrease in basal ascorbate levels has been documented in T2DM in contrast to normal subjects (Padayatty et al., 2003, Mullan et al., 2002). This study observed a significant decrease in the level of total ascorbate, and supported the studies of (Som et al., 1981, Sinclair et al., 1994, Bode et al., 1993, Will et al., 1999, Madhikarmi et al., 2013). Several reasons may explain the reduction in ascorbate levels such as decreased intake, increased renal loss or increased utilization in the face of oxidative stress. Ascorbate reacts with free radicals to produce dehydroascorbate. Dehydroascorbate is then reduced to ascorbate via dehydroascorbate reductase, at the expense of GSH present in the blood, back to the functional ascorbate. Reduction of dehydroascorbate may also be non-enzymatic or catalysed by other proteins with dehydroascorbate reductase activity. An increased concentration of dehydroascorbate in T2DM compared with control subjects was found here, supporting the study of (Hisalkar et al., 2012). This result may be explained by the finding of low ascorbate levels in this study and is consistent with a higher turnover rate of ascorbate, with increased oxidation to the oxidised form, dehydroascorbate.
Compared with control subjects, T2DM subjects are subjected to severe oxidative stress identifiable as a depletion of the total antioxidant capacity. In this study the total antioxidant capacity of plasma measured by the FRAP assay was decreased significantly for T2DM compared with control subjects, consistent with the study of (Korkmaz et al., 2013). The reason for the decrease in total antioxidant capacity in T2DM may be to disturbance in the balance in cells between the formation of reactive oxygen species and the protection against the damage they can cause.

2.7.5 Endothelial dysfunction in Type 2 diabetes

Endothelial dysfunction in the current study was assessed using E-selectin as a biomarker. Our study showed significantly increased levels of E-selectin in T2DM compared with control subjects, and is supported by the studies of (Steiner et al., 1994, Ryysy and Yki-Järvinen, 2001, Boulbou et al., 2005). The reason for the increase in E-selectin in T2DM subjects may be insulin resistance, which increases vasoconstriction factors which in turn increases levels of circulating soluble adhesion molecules. Insulin stimulates the vascular endothelium to cause the release of NO and hence vasodilation. Insulin resistant states are extremely useful in explaining the mechanisms underlying the increased macrovascular risk. Inhibition of NO production leads to increased circulating soluble adhesion molecules and increased platelet and leukocyte adhesion (Adams et al., 1997, Ding et al., 2000). The currently observed relationship between NTBI and plasma E-selectin is consistent with the suggestion that NTBI causes an increased risk of atherosclerosis. Adhesion molecules such as E-selectin play a critical role in the monocyte recruitment to the extravascular compartment by mediating
adhesion and transmigration of the cells to the vascular endothelial wall (Adams and Shaw, 1994).

**2.7.6 Inflammation in Type 2 diabetes**

In present study the biomarker of chronic inflammation, hs-CRP, was significantly higher compared with the control, supporting the study of (Tan et al., 2004). The increased level of hs-CRP in T2DM may due to increased oxidative stress. Another reason for increased hs-CRP in T2DM may be that insulin resistance causes changes in the plasma lipid profile, the consequence of which is enhanced cytokine production in adipose tissue. This would lead to increased systemic inflammation with higher levels of inflammatory markers such as C-reactive protein (CRP), tumour necrosis factor-α (TNF-α), and interleukin 6 (IL-6) (Garcia et al., 2010).

**2.7.7 Iron indices in obese non diabetic subjects**

The levels of NTBI in obese non diabetic subjects were increased compared with the control but not significantly. The presence of NTBI in obese non diabetic subjects, even though the level of total serum iron is significantly lower compared with the control, may be due to reduced transferrin concentration. Inflammation is associated with changes in serum levels of the acute-phase proteins that can be used to stage the inflammatory process and be used as biomarkers to evaluate the impact of treatment. Some acute-phase proteins are increased during inflammation, while others, such as albumin, transferrin, and transthyretin, are decreased (Ritchie et al., 1999). The association of iron deficiency with obesity has been confirmed in other populations that include children and adults of both sexes (Cepeda-Lopez et
The mechanism which explains this relationship remains unknown but may be due to an increased iron requirement as the result of larger blood volumes and poor iron absorption, and low iron intake due to restrictive dietary practices aimed at promoting weight loss (O'Connor et al., 2011). Another reason for low iron in obese subjects may be increased hepcidin production. Hepcidin is a pro-inflammatory adipokine and may play an important role in hypoferraemia of inflammation in obesity (Bekri et al., 2006).

Obese non diabetic subjects in this study also had significantly decreased TIBC compared with the control group. Low TIBC in obese subjects may be due to changes in the iron binding sites due to glycation of proteins which hold iron such as transferrin and Hb. Study of the degree of protein glycation in obese non-diabetic subjects showed that there was increased glycation of Hb and decreased glycation of albumin (Koga et al., 2007). The reason for the reduction in the degree of glycation of albumin in obese non-diabetic subjects may be due to the decrease in the concentration of albumin in obese subjects compared with the control due to increase turnover of albumin in obese subjects (Salas-Salvado et al., 2006). The rate of albumin synthesis is decreased and the rate of catabolism is increased as a consequence of the increased the state of inflammation (Temelkova-Kurktschiev et al., 2002). The longer the albumin is present in the plasma, the more likely it is to become glycated. Thus, an enhanced turnover will reduce the half-life of albumin in plasma and reduce the likelihood of glycation.
This study also showed a significant difference in % transferrin saturation between the obese non-diabetic group and the diabetic group, and between the diabetic group and controls. The level of transferrin saturation was, however, not significantly different in obese subjects compared with the controls. The increased transferrin saturation seen in the diabetic group is consistent with the enhanced NTBI also seen in this group. The greater the occupancy of the iron binding sites on the transferrin molecule, the less there are available for binding any circulating non-sequestered iron. Consequently NTBI will increase.

There was also a significant increase in the level sTfR in the obese group compared with the control. sTfR is a truncated form of the membrane-associated transferrin receptor, and is considered to be a sensitive indicator of iron deficiency that is not an acute phase reactant such as ferritin (Mast et al., 1998). The circulating level of sTfR is proportional to the cellular expression of membrane-associated transferrin receptors. Cellular expression of transferrin receptors increases with increased cellular iron needs, such as in iron deficiency. This finding is consistent with that of Yanoff et al. (2007) and supports the consistently reported observation of lower iron status of obese subjects.

2.7.8 Oxidative stress in obese non-diabetic subjects

Obesity may induce systemic oxidative stress such as increased production of free radicals in accumulated fat (Furukawa et al., 2004). Increased markers of oxidative stress have been observed in obese humans (Urakawa et al., 2003). The present study showed that the level of an oxidative stress biomarker, MDA, was significantly higher compared with the control group,
consistent with the findings of (Zwirska-Korczala et al., 2003, Yesilbursa et al., 2005, Prázny et al., 1999). The level of MDA in the T2DM group was significantly higher than in the obese group. Various mechanisms can induce the oxidative stress in T2DM such as increased formation of AGEs (Brownlee et al., 1988); enhanced polyol pathway (Williamson et al., 1993); increased superoxide release from mitochondria (Desco et al., 2002); and activation of NAD(P)H oxidase (Inoguchi et al., 2000). Obese individuals have a micronutrient deficiency compared to normal weight controls of the same age and sex (Kimmons et al., 2006, Kaidar-Person et al., 2008). In the present study ascorbate was significantly lower in the obese group compared with the control. The depletion of ascorbate in obese subjects may be due to role of ascorbate in scavenging of reactive molecular species, protection against protein glycation and prevention of lipid peroxidation (Garcia-Bailo et al., 2011, Calder et al., 2009).

2.7.9 Inflammation in obese non-diabetic subjects

There was a significant difference in hs-CRP between the obese and control groups. Our result is supported by studies of (Fröhlich et al., 2000, Yudkin et al., 1999, Visser et al., 1999). Obese subjects have low-grade of systemic inflammation which may lead to increase the level of hs-CRP. Increased hs-CRP in obesity is probably due to the increased production of inflammatory cytokines by adipose tissue such as IL-6. These cytokines induce acute-phase protein production in the liver (Papanicolaou et al., 1998).
2.7.10 Endothelial dysfunction in obese non-diabetic subjects

The level of E-selectin in the present study was significantly higher in the obese group compared with the control group, consistent with previous observations (Ferri et al., 1999, Pontiroli et al., 2004, Bošanská et al., 2010). The increased concentration of E-selectin in obese subjects may be due to an increased state of inflammation in obese subjects which may lead to the secretion of a variety of factors such as IL-6 which cause endothelial dysfunction and induce release of E-selectin. Obesity may induce endothelial activation or increased shedding of cell surface E-selectin that leads to subsequent increase in soluble E-selectin levels. The high serum concentrations of E-selectin closely correlated with increased total fat volume (Matsumoto et al., 2002a).

2.7.11 Correlations between age and measured parameters

In the present study, we did not find any significant correlation between age and all parameters, except for MDA and TIBC. One of the reasons for a lack of any association in our study may be the small sample size. Many factors may cause a positive correlation between age and level of MDA. Accumulation of free radical mediated oxidative stress increases with age, and thus may overwhelm the natural repair systems in the elderly (Kowald and Kirkwood, 2000). Hyperglycaemia can induce oxidative stress, which increases with age, via several mechanisms including glucose autoxidation, the formation of AGEs, and activation of the polyol pathway. Other circulating factors that are elevated in diabetics such as free fatty acids and leptin also contribute to increased ROS (Jay et al., 2006). Oxygen free radicals react with all biological substances; however, the most susceptible ones are
polyunsaturated fatty acids. Reactions with these cell membrane constituents lead to lipid peroxidation. Membrane phospholipids, specifically esterified polyunsaturated fatty acids, are converted by peroxidation to MDA. The results for MDA are in concordance with previous studies of (Mutlu-Türkoğlu et al., 2003, Mecocci et al., 1999). Mecocci et al. (1999) found age-dependent significant increases in the level of malondialdehyde. Bhatia et al. (2003) found positive correlation between HbA$_{1c}$ and MDA in T2DM. The increase in MDA with age may be linked to a number of factors reflecting increased oxidative damage during aging (Ozcankaya and Delibas, 2002). A correlation of TIBC with age was also found, supported by the study (Mukhopadhyay and Mohanaruban, 2002). The reason for correlation TIBC with age may due to increase may be due to increased inflammation with age. People with advancing age may be developing chronic inflammatory disease which leads to iron deficiency and increased TIBC.

**2.7.12 Correlations between NTBI and measured parameters**

The present study also showed correlations between NTBI and E-selectin, hs-CRP and sTfR. The positive correlation of E-selectin with NTBI in this study is supported by the study of (van Tits et al., 2007). The reason for this result may be because increased NTBI induces oxidative stress, which leads to endothelium dysfunction. Zhang et al. (2010b) found that the use of deferoxamine (DFO) for chelation of iron leads to decreased induction of E-selectin, VCAM-1 and ICAM-1 in endothelial cells. The finding of this study showed that the correlation between hs-CRP and NTBI, supported by the study of Marx et al (2006). The reason behind this our finding may be due to role of NTBI in increasing ROS production through the Fenton reaction.
2.7.13 Correlations between NTBI and HbA\textsubscript{1c} in T2DM

Positive correlation between NTBI and HbA\textsubscript{1c} was found in T2DM group in this study this result was in agreement with the results of Shetty et al. (2008). Shetty \textit{et al.} also found a positive relation between poor glycaemic control and increase in glycation of Hb, which contributes to the increase in free iron pool which is known to increase oxidant generation.

A major purpose of this study was to examine the potential relationship between iron, oxidative stress and vascular dysfunction in both obese non-diabetic subjects and those with T2DM. Both conditions predispose towards vascular disease, but previous studies have indicated that while diabetic subjects may present with symptoms of iron overload, obese subjects tend to be iron deficient (Yanoff \textit{et al.}, 2007). Also, obese subjects are at risk of developing T2DM. In the transition stage from obesity to diabetes there need to be some changes in iron homeostasis. Findings from this study suggest that the presence of protein glycation may be a major factor in the process. With regard to diabetes, the initial step in linking iron dysregulation, atherosclerosis and vascular disease may be the glycation of proteins such as Hb, transferrin, albumin and others. Albumin will non-specifically bind iron, while Hb and transferrin are specific iron-containing proteins. Glycation of the latter two proteins leads to the loss of some of the iron contained within the structure which may lead to oxidative damage to DNA, phospholipid and protein (Sen \textit{et al.}, 2005). The iron released from Hb and transferrin may drive a number of oxidative reactions with the potential to damage indiscriminately a number of tissue types. This study is particularly interested in the relationship between oxidative stress and endothelial function.
Endothelial dysfunction is the first stage in the development of atherosclerosis. Interestingly this study showed a strong positive correlation between NTBI and E-selectin in all groups studied. Although it is not possible to determine a causal relationship between these two parameters, it remains a possibility that iron released from its binding sites could initiate oxidative damage to the endothelial cells and begin the process of atherogenesis. While it is relatively easy to relate this to diabetic patients and obese non-diabetic patients who show both enhanced NTBI relative to controls, it is less easy to relate endothelial dysfunction to NTBI in control subjects. Thus, the question is, does enhanced NTBI drive endothelial dysfunction, or endothelial dysfunction influencing NTBI release? There seems little evidence of the latter, so at present it may be proposed that enhanced NTBI, through the generation of ROS may initiate atherogenesis by damaging endothelial cells. While there was good correlation between NTBI and endothelial dysfunction in the obese group, there was no difference in NTBI or E-selectin between the control and obese group. Thus, the link between obesity and macrovascular disease may be mechanistically different from that proposed for the diabetic patients where the evidence is strong, not from this study, but from others (Kuo et al., 2014).

The one parameter where the obese group stands out as different from the other two groups is in the sTfR data. The high levels of sTfR indicate an overall state of iron deficiency. However, sTfR is mainly a biomarker of overall iron status and not the situation in individual cells in which the number of membrane-associated transferrin receptors available is increased in obese subjects. Thus, intracellular iron in some cells may be increased which may
induce intracellular oxidative damage. The data from this study is not able to
develop this prospect further, or to relate this to endothelial dysfunction in
obese individuals.

The link between obesity and endothelial dysfunction is more likely to be due
to changes in adipokine production in adipose tissue, and the production of
inflammatory cytokines by the inflammatory cells that invade developing
adipose tissue. The strong link between NTBI and E-selectin seen in this
group of patients and in control subjects remains a mystery.
Chapter 3: Effects of glycation of transferrin *in vitro* on iron binding
3.1 Introduction

The majority of proteins are multi-functional. Transferrins are examples of proteins that have many functions (Gomme et al., 2005). The transferrins (Tfs) are a group of non-haem iron-binding glycoproteins that are widely distributed in a variety of fluids in both invertebrates and vertebrates. The main function of transferrins is to act as extracellular proteins which are responsible for transporting iron between sites of absorption, storage and utilization, and to control the level of free iron in physiological fluids by binding this element (Sun et al., 1999). The main proteins of this group are serum transferrins (Tf) the prototype found in blood serum; in other bodily secretions there are lactotransferrin (L Tf), ovotransferrin (OTf) and a fourth class of transferrins, melanotransferrins, which was first identified in human skin cancer cells (Woodbury et al., 1980). Lf is found in secretory fluids such as milk or tears, and also in white blood cells, while OTf is present in egg white. Interestingly, there appears to be no iron transport role for transferrins such as L Tf and OTf. Instead, the major function appears to be to keep the iron concentration low in bodily fluids and to prevent invading bacteria from acquiring iron due to high affinity of transferrins to sequester iron (Brock, 2002). However, some bacteria can avoid the resultant iron-deficient environment created by transferrins by expressing outer membrane proteins that can bind and remove the iron from transferrins. The ability of transferrins to sequester free iron leads to a reduction in infection (Bonsdorff et al., 2006). In addition, apoTf has the effect of reducing the adhesion of Gram-positive and Gram-negative bacteria to the surface of cells (Ardehali et al., 2003).
3.1.1 Serum transferrin structure

Serum transferrin (Tf) is the most important extracellular protein that plays a crucial role in the homeostasis and transport of iron. Tf is a glycoprotein with a molecular weight of ~79 kDa consisting of a single polypeptide chain containing approximately 700 amino acids arranged in two independent metal ion-binding globular domains (Parkkinen et al., 2002). The molecule is stabilized by 19 intra-chain disulphide bonds and protected by two carbohydrate side chains called glycans, with terminal sialic acid residues (De Jong et al., 1990); each glycan has been shown to consist of two sialic acids residues, two galactose, three mannose and four N-acetylglucosamine residues (Gomme et al., 2005). The carbohydrate moiety sites occur in the C-terminal domain and not in the N-terminal domain. The biological function of the glycans in transferrin is not fully understood. However, it has been suggested that they might play a role in Tf solubility and binding to its receptor (Hemmaplardh and Morgan, 1976).

The polypeptide chain of transferrin has a combination of alpha helices and beta sheets to form two structurally similar but functionally different lobes, the N-lobe and C-lobe. Each lobe consists of two domains (NI, residues 1-92 and 247-331; NII, residues 93-246; CI, residues 339-425 and 573-679; CII, residues 426-572). These lobes are held together by a short peptide linker (residues 332-338) and each creates a deep hydrophobic site to hold iron in the ferric state. A common feature of all Tf family members is the large conformational changes that take place upon the binding and release of iron in each lobe. The amino acids residues that bind the ferric ion are the same for both lobes: two tyrosines, one aspartic acid and one histidine. The binding
of iron also needs an anion which is usually carbonate ($\text{CO}_3^{2-}$). The uptake of the metal by Tf is initialized by the binding of the carbonate ion to the apoTf present in blood serum (Rinaldo and Field, 2003), then the metal reaches the metal-binding site of the protein, and the complex is recognized by the transferrin receptor (TfR) and internalized in the cytoplasm by receptor-mediated endocytosis (Dautry-Varsat et al., 1983). There the release of the metal from Tf is influenced by the Tf-TfR interaction (Eckenroth et al., 2011) and modulated by the lower endosomal pH of 5.5, which is significantly lower than the serum pH (7.4). Crystallography shows that conformational changes in the structures of the transferrins depend on iron binding or release. There are two different conformations, an open conformation when they are metal free (Wally et al., 2006), and a closed conformation upon the binding of Fe$^{3+}$ (Guha Thakurta et al., 2003).

Human serum transferrin (hTf) is synthesized in the liver and secreted into the plasma. Because each domain contains an Fe$^{3+}$-binding site, serum transferrin exists as four molecular forms: apotransferrin (Fe0-Tf or apoTf), with no Fe$^{3+}$; one Fe$^{3+}$ ion bound to the C-terminal lobe (Fe1C-Tf); one Fe$^{3+}$ ion bound to the N-terminal lobe (Fe1N-Tf); and both binding sites occupied (Fe2-Tf) (De Jong et al., 1990), but all transferrin-bound iron can be considered physiologically as a single homogeneous pool. Isoelectric focusing is a technique used to separate these isoforms in human blood because each isoform has a different isoelectric point ($\text{pI}$) due to differences in the iron. In addition to differences in iron content there are also different isoforms based on differences in sialic acid content (van Eijk and van Noort, 1992).
3.1.2 Transferrin glycation

Many plasma proteins such as immunoglobulins, albumin and lipoproteins are prone to glycation reactions. These reactions are increased in diabetic patients who have poor glycaemic control compared with normal subjects (Austin et al., 1987). Like other proteins, transferrin may lose its function when glycated and may contribute to diabetic complications such as cardiovascular disease, via a reduction in antioxidant capacity, and the potential occurrence of redox-active plasma iron (Fujimoto et al., 1995, van Eijk and van Noort, 1992). Fujimoto et al. (2002) found that the total level of iron and the iron-binding capacity decreased in the serum of rats when rat serum was incubated with glucose, and the degree of glycation increased with time of incubation.

Many in vitro studies have observed that glycation of transferrin leads to increased oxidative stress by impairing iron-binding antioxidant capacity. Fujimoto et al. (1995) used bovine holotransferrin and apotransferrin (Wako Pure Chemicals); van Campenhout et al. (2004) used human apotransferrin (Sigma); and Goudarzi and Rashidi (2010) used human apotransferrin (Sigma). All these researchers used different glucose concentrations, times of incubation and also different methods for measuring iron, iron-binding capacity and for assessment of glycation. All three groups found positive associations between glucose concentrations, degree of glycation, and reduction in function, with significant differences between glucose concentration and controls, and between different concentrations or increased incubation time.
3.1.3 LDL and atherosclerosis

An increase in plasma levels of triacylglycerol and low density lipoprotein cholesterol (LDL-C), and a decrease in high density lipoprotein cholesterol (HDL-C) have been shown to play a central role and are considered powerful risk factors for atherosclerosis in T2DM (Temelkova-Kurktschiev and Hanefeld, 2004). Elevated levels of LDL, for example, due to genetic abnormalities in the LDL receptor, is a well-known risk factor for atherosclerotic disease and accelerated coronary artery disease (Goldstein and Brown, 1977, Brown and Goldstein, 1986). Determination of the structure of LDL has been a difficult task because of its heterogeneous structure. LDL particles contain a variable and changing number of fatty acid molecules. There is a distribution of LDL particle mass and size (Segrest et al., 2001). LDL particles are sensitive to oxidative damage because of their complex lipid-protein composition. Each LDL particle contains about 700 molecules of phospholipids; 600 of free cholesterol; 1600 of cholesteryl esters; 185 of triglycerides; and 1 molecule of apolipoprotein (apo) B, which in turn is composed of 4536 amino acid residues.

Analysis of data for atherosclerotic lesions has emphasised that the transition metals such as iron and copper are likely to play a central role as triggers of LDL oxidation in vivo (Smith et al., 1992). The concentration of copper is low in the blood system appearing in the form of ceruloplasmin, which is not considered a pro-oxidant (Burkitt, 2001), while, the concentration of iron in blood is extremely high (millimolar) compared with other transition elements. A possible link between iron and atherogenesis has been suggested by the finding that iron chelation blocks oxidation of LDL, whereas iron released
from the protein-bound form causes oxidation of LDL (Abdalla et al., 1992). Oxidative modification of low-density lipoprotein (LDL) plays a causal role in human atherogenesis and the risk of atherosclerosis is increased in patients with diabetes by approximately three-fold (Lyons et al., 1986). One hypothesis is that increased modification of LDL by oxidation and/or glycation may induce endothelial cell injury and accelerate foam cell formation in the arterial intima (Steinberg et al., 1989).

Hyperglycaemia induces lipoprotein modification through the glycation of apolipoproteins. Glycated lipoproteins are more prone to oxidation than native lipoproteins (Sobal et al., 2000). Glycation itself has been demonstrated to be more toxic than oxidation in the case of LDL (Galle et al., 1998). The toxic properties of glycated LDL include the inhibition of NO production in endothelial cells (Posch et al., 1999); alteration of the antithrombotic properties of vascular endothelium (Wen et al., 2002); enhanced expression of adhesion molecules; and also increased platelet aggregation (Takeda et al., 1992).

Glycation and glycoxidation of LDL have been shown to occur under similar conditions of hyperglycaemia. Glycation and oxidation of LDL reduces its affinity for the LDL receptor, leading to reduced hepatic catabolism, increased accumulation of cholesteryl esters in macrophages and altered endothelial function (Steinberg, 1989, Witztum and Steinberg, 1991, Lopes-Virella et al., 1988).
3.2 Aim

The aim of the work described in this chapter was to investigate, *in vitro*, the effects of half saturation of apotransferrin with iron on transferrin and LDL glycation and oxidation, and on iron-binding capacity of transferrin.

3.3 Methods

3.3.1 Transferrin loading with iron

3.3.1.1 Preparation of Fe(NTA)$_2$ solution

Due to the low solubility of Fe$^{3+}$ at pH 7.4, the iron was loaded into transferrin using iron nitrilotriacetic acid, Fe(NTA)$_2$. The solution was prepared by dissolving 22.25 µmol of FeCl$_3$$\cdot$6H$_2$O and 45 µmol of nitrilotriacetic acid in 2 ml of 1 M HCl, adjusting the pH to 4.0 with 1 M NaOH solution and diluting to 10 ml. This solution was freshly prepared (Battin et al., 2009).

3.3.1.2 Preparation of human apotransferrin solution

Human apotransferrin (20 mg, ~0.25 µmol; Sigma-Aldrich) was dissolved in 3 ml of Tris buffer (50 mM Tris, 150 mM NaCl, 20 mM NaHCO$_3$; pH adjusted to 7.4), and the UV/vis spectrum was taken from 250 nm to 750 nm (Figure 3.1); Tris buffer was used as a reagent blank. The concentration of transferrin was calculated by measuring the protein absorbance at 280 nm (Figure 3.1) and using an extinction coefficient of 87.2 mM$^{-1}$ cm$^{-1}$ (Battin et al., 2009).

3.3.1.3 Exposure of 50% loaded transferrin to glucose

Fe(NTA)$_2$ solution (0.4 equivalents per transferrin protein) was added to 800 µl of apotransferrin solution, and, after 5 min incubation, the UV/vis spectrum was scanned. This process was repeated every 5 min until no further changes in the UV/vis spectra were observed (Figure 3.2). Tris buffer was
used as a reagent blank. The percentage of iron loading in the transferrin was estimated by measuring the absorbance at 470 nm, and using an extinction coefficient of 4.86 mM$^{-1}$ cm$^{-1}$ (Ando et al., 2002).

Based this data, the volume of Fe(NTA)$_2$ solution which made the apotransferrin half saturated was known. Aliquots (250 µl) of human apotransferrin solution were freshly prepared as described in Section 3.3.1.2, after which Fe(NTA)$_2$ solution freshly prepared as described in Section 3.3.1.1 was added to make transferrin half saturated with iron. Glucose solutions in Tris buffer were added to this half saturated transferrin to give 1 ml of 0, 250, 500, 750 and 1000 mM glucose. All solutions nominally contained 0.4 µg l$^{-1}$ transferrin. The Tris buffer was prepared with Milli-Q HPLC grade water, and all plastic tubes and tips were autoclaved (at 121 °C for 15 min at 15 PSI). The transferrin solutions were transferred to 2 ml HPLC sample containers (Fisher Scientific, UK), which were then tightly sealed with a small gas phase (~200 µl) at the top. After this the containers were incubated at 37 °C for 14 days. For each container, the absorbance spectrum of the transferrin was taken every day. Selection of containers and their orientation was done based on comparison of the spectrum of a food dye between a normal quartz cuvette and the sealed HPLC containers.

3.3.2 Buffer exchange

The transferrin solutions were removed from the incubator on day 14 and placed on ice, prior to buffer exchange into glucose-free Tris buffer. A Sephadex G-25 column (10 ml) was used for the exchange. This volume of column was found to give a good separation of protein from glucose, with 1 ml fractions. The recovery of protein was tested using bovine serum albumin
An example of buffer exchange and the separation of protein from glucose are shown in Figure 3.7.

### 3.3.3 Measurement of protein

The protein concentration was measured by the Bradford method (Bradford, 1976). As a control a solution of fresh apotransferrin (1 mg ml⁻¹), was made up in Tris buffer.

#### 3.3.3.1 Preparation of Bradford reagent

Bradford stock solution (30 ml) was prepared by mixing 35 mg of Coomassie Blue G-250 (Fisher Scientific, UK Ltd) with 10 ml of 95% ethanol and 10 ml of 88% phosphoric acid. From this stock solution, 6 ml was mixed with 6 ml of 88% phosphoric acid, 3 ml of 95% ethanol and 85 ml of distilled water to produce a working reagent. The working reagent was filtered through Whatman Grade No. 1 filter paper before use. After filtration the solution was stored in a brown bottle at room temperature for several weeks.

#### 3.3.3.2 Procedure

For each sample, blank and control, 100 µl were added to 1 ml of Bradford working solution. After 5 min the absorbance was measured at 595 nm. A calibration curve was established each time a protein assay was performed with apotransferrin dilutions of known concentrations. Using the standard curve (Figure 3.5), the concentration of each sample was determined according to its absorbance.
3.3.4 Measurement of glucose in samples

The glucose concentration was measured by method of Miller (1959), using the colorimetric reagent dinitrosalicylate. The results for glucose standards are shown in Figure 3.4.

3.3.5 Assessment of glycation

The degree of glycation was measured using the 2,4,6-trinitrobenzene sulfonic acid (TNBSA, also known as picrylsulfonic acid) assay, which reacts with NH$_2$ groups at pH 8, to form adducts detectable at 335 nm. TNBSA is a rapid and sensitive assay for the determination of free amine groups (Habeeb, 1966).

To 25 μl of glycated transferrin, L-lysine standards and blanks, the following were added: 25 μl of 4% Na$_2$HCO$_3$ and 25 μl 0.1% TNBSA. The samples were incubated at 37 °C for 2 h and then the following were added: 25 μl of 10% SDS, 12.5 μl of 1 M HCl and 787.5 μl of distilled H$_2$O, to make a final volume of 900 μl. After that, 300 μl of sample, control and standards were transferred to a 96-well plate. The absorbance of the resulting solution was read immediately in plate reader (Versamax, Molecular Devices, Sunnyvale, CA) at 340 nm. Calibration results are shown in Figure 3.8.

3.3.6 Measurement of total iron

The method used was as described in Section 2.4.4.

3.3.7 Measurement of total iron-binding capacity

The method used was as described in Section 2.4.5.
3.3.8 Electrophoresis

3.3.8.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate (SDS) polyacrylamide gels were prepared according to the method of Laemmli (1970) using a 8% separating gel and a 4% stacking gel. The procedure used was as follows.

Plates and combs were cleaned with 70% ethanol, and then the plates were set up on the plate rack according to Bio-Rad instructions, with the bottom edges of the two plates flush to avoid leakage. The plates were locked in the green holder, and the holder placed on the rack. Separating gel mix containing 2.7 ml of solution A (30% acrylamide/bis-acrylamide), 2.5 ml of solution B (1.5 M Tris-chloride, 0.4% SDS, pH 8), 4.8 ml H₂O, 50 μl of 10% ammonium persulfate (APS) and 5 μl of tetramethylethylenediamine (TEMED) was prepared. All these materials were mixed in advance except the TEMED which was added just before pouring the gel into the vertical glass. The main gel mix was poured between the assembled gel plates until the level was just below where the comb sat, after which it was overlaid with 150 μl H₂O. The gel mix was allowed to polymerise for about 30 min, and then the water was removed from the top of the gel.

The stacking gel mixture was prepared using 0.67 ml of solution A, 1.0 ml of solution C (0.5 M Tris-chloride, 0.4% SDS, pH 6.8), 2.3 ml H₂O, 30 μl of 10% APS and 5 μl of TEMED. The stacking gel mix was poured carefully on top of main gel, and the comb inserted, avoiding air bubbles. The gel mix was allowed to polymerise for about 30 min.
Gels were run using a running buffer consisting of Tris base (12 g), glycine (57.6 g), and SDS (4 g) made up to 1 l. The running buffer was added to cover the top of the gel in the Bio-Rad system, before the comb was removed and the wells rinsed out with running buffer using a Pasteur pipette. The tank of the Bio-Rad system was then filled with the desired running buffer volume. The gel was run at 200 V for 45-50 min until the blue tracking dye ran off the end.

Protein concentration for the sample load was assessed as in Section 5.3.3, and then 10 µl containing 50 µg of protein was mixed with 10 µl loading buffer prepared by mixing 1 ml of 0.5 M Tris-chloride buffer, pH 6.8, 0.8 ml glycerol, 16 ml of 10% SDS and 0.32 ml 0.05% bromophenol blue with 4.18 ml distilled water. The samples were heated at 95 °C for 5 minutes before loading them into the gel wells.

Gels were visualised by using Coomassie blue. The dye was prepared by dissolving 0.2% Coomassie Brilliant blue R-250 in 30% methanol and 10% acetic acid. The dye was poured on the gel in a suitable container and left on an orbital shaker (17 rpm) overnight. The gel was then washed with destain solution consisting of 40% methanol and 10% acetic acid for 2 or 3 times until it became clear. Finally the gel was imaged using visible light (Universal Hood 11, Bio-Rad Laboratories, Italy).
3.3.9 LDL incubation with half-saturated transferrin and glucose

3.3.9.1 Preparation of LDL suspension

The LDL used was supplied as a lyophilized powder (Sigma). At first an attempt was made to resuspend the lyophilized LDL in 1 ml of distilled water following the manufacturer’s (erroneous) instructions. However, this was unsuccessful. Eventually a solution containing 150 mM NaCl and 0.01% EDTA, pH 7.4, was used following reference to an internet forum. This resuspended material was homogenised manually using an all-glass Potter homogeniser over a period of about 3 h. This process was carried out under oxygen-free nitrogen gas to avoid LDL oxidation.

3.3.9.2 Procedure

LDL suspension was incubated in the same tubes as were used for the previous experiment Section 3.1.1 with combinations of 50% ITf plus glucose, iron, apoTf and glucose the experimental design shown in Table 3.1.

Table 3.1 LDL incubated with 50% ITf, iron, apoTf and glucose. The concentration of Tf, where present, was 0.44 µM, and the pH was 7.4.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>50% ITf (µl)</th>
<th>LDL (µl)</th>
<th>500 mM glucose in Tris buffer (µl)</th>
<th>Tris buffer (µl)</th>
<th>Fe(NTA)2 (µl) (0.14 µM)</th>
<th>apoTf (µl) (0.4 µg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% ITf + LDL + glucose</td>
<td>250</td>
<td>100</td>
<td>650</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL + iron</td>
<td>100</td>
<td></td>
<td>650</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apoTf + LDL</td>
<td>100</td>
<td></td>
<td>650</td>
<td></td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>LDL</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>LDL + glucose</td>
<td>100</td>
<td></td>
<td>900</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
All tubes were incubated at 37 °C for 14 days. After this the tubes were placed on ice before the LDL was separated by ultracentrifugation using a Beckman Coulter Optima XPN-100 ultracentrifuge (125000 g at 15 °C for 5 h).

3.4 Results

3.4.1 Spectrum of human apotransferrin solution

UV/visible spectra were taken from 250 nm to 750 nm for 1 ml of human apotransferrin solution prepared as described in Section 3.3.1.2. Tris buffer was used as the reagent blank. The concentration of transferrin was calculated by measuring the protein absorbance at 280 nm (Figure 3.1).

![Figure 3.1 UV/visible spectrum of human apotransferrin. A solution of apotransferrin was prepared as described in Section 3.3.1.2. This was diluted 10 fold in Tris buffer. Based on the A280 and an extinction coefficient of 87.2 mM$^{-1}$ cm$^{-1}$ the concentration of transferrin in the stock solution was 136 µM. This was higher than expected (83 µM) based on the nominal mass of protein supplied by Sigma.](image_url)
3.4.2 Apotransferrin titration with iron

UV/visible spectra were taken from 250 nm to 750 nm for human iron transferrin (ITf) freshly prepared as described in Section 3.3.1.3. The spectra are shown in Figure 3.2. The red line represents apotransferrin; the other lines represent different levels of iron saturation of transferrin.

![UV/visible spectra of transferrin during titration with iron.](image)

*Figure 3.2 UV/visible spectra of transferrin during titration with iron. As described in Section 3.3.1.3. Based on A_{470} and an extinction coefficient of 4.86 mM\(^{-1}\) cm\(^{-1}\) for iron-loaded transferrin the concentration of the stock solution was 120 µM, which is in good agreement with the value obtained from the data in Figure 3.1.*

3.4.3 Use of HPLC sample vials as cuvettes

Visible spectra between 400 to 700 nm were taken of a food dye solution in a quartz cuvette (blue line) to compare with the spectra of the same solution in the containers (2 ml HPLC sample vials) which were to be used in the incubation of apotransferrin with different concentrations of glucose. Different orientations of the vials were used in order to find orientations that gave spectra comparable to that obtained in the quartz cuvette. Inappropriate
orientations were labelled to ensure that reliable spectra were obtained (Figure 3.3).

![Graph showing absorbance vs wavelength for different orientations of a food dye solution.](image)

Figure 3.3 Comparison of the visible spectrum of a food dye. The solution in a quartz cuvette (blue line) was compared with those of the same solution in an example of the containers used for incubation of transferrin with glucose, in different orientations (other coloured lines). The orientation represented by the red line shows the most similar spectrum compared with that obtained with a quartz cuvette after used same dye in quartz cuvette.
3.4.4 Glucose standards

The results for glucose standards are shown in Figure 3.4. Glucose was determined using the dinitrosalicylic colorimetric method. The absorbance was measured for each concentration in triplicate for two sets of standards.

![Calibration graph for the determination of glucose (means ± SEM) as described in Section 3.3.4.](image)

3.4.5 Protein standards

The results for protein standards are shown in Figure 3.5. Protein was determined using the Bradford assay. The absorbance was measured for each concentration in triplicate for two sets of standards.
Figure 3.5 Calibration graph for the determination of protein using the Bradford assay (means ± SEM). Bovine serum albumin was used for the standards.

### 3.4.6 Recovery of protein following buffer exchange, and the separation of protein from glucose

The recovery of protein from the 10 ml Sephadex G-25 column (Section 3.3.2) was tested using 1 ml of a 2 mg ml$^{-1}$ bovine serum albumin (BSA) solution. Following loading of the BSA solution, 1 ml aliquots of Tris buffer were added to the column to elute the BSA and 1 ml fractions were collected (Figure 3.6). The protein was recovered in three 1 ml fractions (fractions 3-5). The experiment was repeated using a solution containing BSA and 1 mM glucose. Figure 3.7 shows the recovery of protein following the buffer exchange and separation of protein from glucose. Fractions 3, 4 and 5 were found to be suitable for use in the experiments with transferrin.
Figure 3.6 The recovery of bovine serum albumin (inexpensive compared to Tf) following application of 1 ml of 2 mg ml\(^{-1}\) of protein to a 10 ml Sephadex G-25 column.

Figure 3.7 The separation of bovine serum albumin (red) from glucose (blue), following application of 1 mg ml\(^{-1}\) of protein in Tris buffer containing 1 mM glucose (1 ml) to a 10 ml Sephadex G-25 column.
5.4.7 L-lysine standards

The results for lysine standards are shown in Figure 3.8. L-lysine was determined by detection of free \(-\text{NH}_2\) groups using the TNBSA assay. The absorbance was measured for each concentration in triplicate for two sets of standards.

![Figure 3.8 Calibration graph for the determination of \(-\text{NH}_2\) groups using the TNBSA assay (means ± SEM).](image)

Lysine was used as a source of amine groups. Despite being carried out in Tris buffer the absorbance of the blank (0.0123) was low indicating that Tris does not react with TNBSA.

3.4.8 Exposure of 50% ITf to different concentrations of glucose

50% ITf was exposed to a range of concentrations (0-1000 mM) of glucose as described in Section 3.3.1.3, after which a range of parameters relating to glycation and iron binding were determined.
3.4.8.1 Effect of glucose on the absorbance spectrum 50% ITf

On incubation of 50% ITf with glucose changes in the absorbance spectrum of Tf over time were seen; there was a shift in the 280 nm absorption band towards higher wavelengths (Figure 3.9). Unfortunately a writing error when transferring the spectra for the control (50% ITf without glucose) meant that these were lost. However, the spectrum changes seen in the other samples were not glucose concentration dependent, but may be due to structural changes caused by the relatively high concentration of glucose in the environment of the protein. Because of the large protein spectral changes it was not possible to use to use the 470 nm region to monitor changes in iron binding as originally intended.

![Figure 3.9 Spectra for 50% ITf incubated with 1000 mM glucose on day 1 (red line) and on day 14 (black line). Insert: difference spectrum (between days 14 and 1) shows the shift of the 280 nm peak toward higher wavelength. The absolute spectra were baseline corrected (subtracted average absorbance in the range 700-800 nm) and normalized using A280, i.e. A280 was taken to be equal to one.](image-url)
3.4.8.2 Assessment of glycation

The degree of glycation was assessed by measurement of –NH₂ groups. Figure 3.10 shows the levels of –NH₂ groups after the incubation of 50% ITf without glucose and with different concentrations of glucose for 14 days. The level in 50% ITf incubated with 1000 mM glucose was significantly lower than that for 50% ITf without glucose ($P = 0.02$). Also the level in 50% ITf with 1000 mM glucose was significantly lower than that in 50% ITf with the other concentrations ($P < 0.05$), but there were no significant differences between 50% ITf without glucose and 50% ITf with 250, 500 and 750 mM glucose. There was inverse relationship between concentration of glucose and number of amine groups found after incubation of 50% ITf with different concentrations of glucose (Figure 3.11).

![Glycation of 50% ITf (means ± SEM) incubated with different concentrations of glucose for 14 days. Bars with different letters are significantly different ($P < 0.05$, $n = 3$ replicate measurements).](image)

**Figure 3.10** Glycation of 50% ITf (means ± SEM) incubated with different concentrations of glucose for 14 days. Bars with different letters are significantly different ($P < 0.05$, $n = 3$ replicate measurements).
3.4.8.3 Assessment of transferrin iron-binding capacity

The iron binding capacity of transferrin was measured by the method used previously for the TIBC of plasma (Section 2.4.5). The iron-binding capacity of transferrin was reduced significantly with increasing glucose concentration. There was a significant difference \( (P < 0.01) \) between 50% ITf without glucose and with all the concentrations of glucose. There were also significant differences \( (P < 0.01) \) between 50% ITf with 1000 mM of glucose, and 250 and 500 mM glucose. There were no significant differences \( (P > 0.05) \) between 50% ITf with 750 mM glucose and with 250 or 500 mM glucose (Figure 3.12). There was an inverse relationship between TIBC concentration and the different concentrations of glucose (Figure 3.13).
Figure 3.12 TIBC of 50% ITf (means ± SEM) incubated with different concentrations of glucose for 14 days. Bars with different letters are significantly different. \( n = 3 \) replicate measurements.

Figure 3.13 correlation between TIBC and different concentrations of glucose. Incubated with 50% ITf. \( R^2 = 0.875, P = 0.020, n = 3 \) replicate measurements.
3.4.8.4 Free iron levels

Loss of iron from transferrin increased with increasing concentration of glucose but the increase was not significant ($P > 0.05$) between 50% ITf without glucose and 50% ITf with different concentrations of glucose (Figure 3.14). There was a positive relationship between free iron and different concentrations of glucose (Figure 3.15).

![Figure 3.14 Free iron (means ± SEM) ‘released’ from 50% ITf incubated for 14 days with different concentrations of glucose. Bars with different letters are significant different. n = 3 replicate measurements.](image-url)
Concentrations of glucose (mM) incubated with 50% ITf

![Graph showing the correlation between free iron and different concentrations of glucose incubated with 50% ITf.](image)

Figure 3.15 Correlation between free iron and different concentrations of glucose incubated with 50% ITf. $R^2 = 0.837$, $P = 0.022$, $n = 3$ replicate measurements.

3.4.8.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared according to the method of Laemmli (1970) using an 8% separating gel and a 4% stacking gel as described in Section 3.3.8.1. The gels showed no evidence of fragmentation of the 50% ITf incubated with different concentrations of glucose (Figure 3.16). However, unexpectedly more than one band was found. While it is possible that this is because of impurity arising from the manufacturing process, another possibility is that it relates to differences in the glycoprotein component. It is known that there are isoforms of Tf that differ in the sialic acid component (van Eijk and van Noort, 1992).
3.4.9 Co-exposure of LDL and 50% ITf to glucose

LDL and 50% ITf were incubated with 500 mM glucose as described in Section 3.3.9.2. In addition, as controls, LDL was incubated with apoTf, a low level of free iron, 500 mM glucose and in isolation.

5.4.9.1 Assessment of oxidation of LDL using MDA

There was an increase in LDL oxidation when LDL was co-incubated with 50% ITf and glucose compared to LDL alone, but this was not significant ($P > 0.05$) (Figure 3.17).
Levels of MDA (means ± SEM) from oxidation of LDL. After incubation with 50% ITf plus 500 mM glucose, free iron, apoTf and 500 mM glucose. There were no significant differences in MDA levels ($P > 0.05$). $n = 3$ replicate measurements.

3.4.9.2 Free iron

There was a significantly ($P < 0.01$) higher level of free iron in 50% ITf with 500 mM glucose co-incubated with LDL compared to LDL incubated with iron, apotransferrin or glucose alone (Figure 5.18).
Figure 3.18 Free iron (means ± SEM) ‘released’ from incubation of LDL with 50% ITf plus 500 mM glucose, free iron, apoTf and 500 mM glucose. Bars with different letters are significantly different ($P < 0.01, n = 3$ replicate measurements).

Figure 3.19 TIBC (means ± SEM) after incubation of LDL with 50% ITf plus 500 mM glucose, free iron, apoTf and 500 mM glucose. Bars with different letters are significantly different ($P < 0.01$), nd = not detectable. Note that the LDL was removed by ultracentrifugation before measurement of TIBC of the remaining Tf. $n = 3$ replicate measurements.
Figure 3.20 Glycated LDL (means ± SD) after incubation LDL with 50% ITf plus 500 mM glucose, iron, apoTf and 500 mM glucose.

Bars with different letters are significantly different ($P < 0.01$). Note that these measurements were carried out on LDL separated from the other components by ultracentrifugation. $n = 3$ replicate measurements.
3.5 Discussion

The reactions of glucose and other reducing sugars with proteins have been thoroughly studied *in vitro* and *in vivo*. These reactions change functions, activities and structure of proteins. Many studies have focused on the effects of glycation on protein structure and function in the extracellular proteins, such as collagens (Guilbert *et al.*, 2013), lens crystallins (Katta *et al.*, 2009), Hb (Bose *et al.*, 2013) and albumin (Rondeau and Bourdon, 2011). Glycation of albumin significantly impairs its antioxidant properties (Baraka *et al.*, 2013), and reduces its drug-binding capacity (Baraka *et al.*, 2012). Glycated albumin shows changes in the 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 was in a more hydrophobic environment, and presumably more buried due to the protein glycation (Shaklai *et al.*, 1984).

Most studies have focused on haemoglobin glycation and the use of HbA\textsubscript{1C} as a biomarker of glycaemic control in the diagnosis of diabetes. Other studies use albumin as an alternative to HbA\textsubscript{1C} (Chujo *et al.*, 2006), because it represents 60% of total plasma protein and has a shorter half-life, ranging from 12 to 19 days for glycated albumin (Yatscoff *et al.*, 1984).

Due to the importance of transferrin in iron transport and its acting as antioxidant because of its ability to sequester iron in inactive redox form, Austen *et al.* (1987) started their work on transferrin glycation *in vivo* and demonstrated that transferrin is prone to glycation and that this may affect iron binding to transferrin especially in diabetes.
The present work was aimed at demonstrating the effect on glycation of in vitro incubation of transferrin half saturated with iron (50% ITf) with different concentrations of glucose, and the consequent effects on the iron binding sites and iron release. Previous studies on the effects of glycation on the release of free iron from Tf have been done with 100% ITf (holoTf). With holoTf if there is any release of iron from Tf then there is no remaining iron-binding capacity to take up this free iron, thereby potentially exacerbating the effects of high concentrations of glucose on the release of further iron. The current experimental scenario is more physiologically relevant because the plasma transferrin is usually only about 33% saturated.

The study showed that the glycation of 50% ITf without glucose was significantly lower than 50% ITf incubated with 1000 mM glucose. This study also showed a positive linear relationship between concentration of glucose and degree of glycation. Association between glucose concentration and the degree of glycation in this study is supported by the studies of the (Fujimoto et al., 1995, Van Campenhout et al., 2004, Goodarzi, 2010). Fujimoto et al. (1995) used bovine holoTf and apoTf (10 mg ml\(^{-1}\) protein with 0 or 1 M glucose, in 50 mM phosphate buffer, pH 7.4, at 37 °C for 10 days) and used different methods for assessment the glycation (thiobarbituric acid reaction using 5-hydroxymethyl-2-furfural (HMF) as a standard compound). Fujimoto et al. (1995) found no glycation in zero glucose of both apo and holo bovine transferrin but they found a degree of glycation in apo and holoTf with 1 M glucose. Van Campenhout et al. (2004) used human apoTf (0-33.3 mM or 1000 mM glucose, pH 7.4, at 37 °C for 14 days) and used the fructosamine method (fructosamine measured by reaction of nitroblue tetrazolium with
fructosamine) to assess the glycation and found that glycation of apoTf increased with increasing concentration of glucose. Goodharzi et al. (2010) used human apoTf (0-200 mM glucose, pH 7.4, at room temperature for 10 and 20 days). This study also found that the degree of glycation increased with increased glucose concentration.

In the present study the TIBC in 50% ITf without glucose was significantly higher than that in 50% ITf incubated with all the different concentrations of glucose (15.5% remaining with 1000 mM). This finding was supported by the study of (Van Campenhout et al., 2004). Van Campenhout et al. found that TIBC decreased with increased glucose concentration (81% remaining with 33 mM and 1.5% with 1000 mM glucose). The effect of glycation in lowering transferrin iron-binding capacity might be due to influence the glycation of lysine residues on neighbouring amino acid residues and an overall decrease in positive charge, due to the attachment of sugar residues to the protein amine groups in the glycation process (Handa and Kuroda, 1999), which causes the iron-binding ability of transferrin to be impaired. In this study incubation of glucose with 50% ITf showed less effect on loss of TIBC when compared with the study of Van Campenhout et al. (2004), who used holoTf; this difference in result may arise because of the greater possibility of free iron being present in a preparation of holoTf than in 50% ITf.

In this study denaturing electrophoresis (SDS page) was used to examine the effect of glycation on the fragmentation of 50% ITf in the presence of glucose. There was no evidence of fragmentation of the protein. Fujimoto et al. (1994) used denaturing electrophoresis to further examine Tf samples, finding that substantial fragmentation of the protein could be seen only in the glycated
holoTf. The fragmentation of the protein only appears in previous studies in case of glycated holoTf, which might be due to the high release of iron from the holoTf.

All proteins are glycated when they are exposed to glucose and no exception has been reported (Baynes, 1991). In the present study LDL was also incubated with 50% ITf with 500 mM glucose. In addition, LDL also was incubated with apoTf, iron and with glucose only. Control LDL was prepared in the same way without adding glucose and incubated under identical conditions. Glycation of LDL was estimated in the present study by the number of lysine residues in apolipoprotein B. There was a significant difference between glycation of LDL incubated with 500 mM glucose and LDL which was used as a control. However, unexpectedly there was no evidence of glycation in LDL incubated with 50% ITf and glucose. In addition, LDL incubated with apoTf showed an apparent increase in available lysine residues. Since only a single run of this experiment was possible due to the expense of the reagents, there is a question about the reliability of the results. This is particularly the case given the difficulty in handling the LDL, distributing it between the tubes and especially recovering it after ultracentrifugation.

*In vitro*, glycation of LDL occurs easily by the incubation of LDL with glucose. The part of LDL that is prone to glycation is side chain amine groups of lysine residues in which LDL is rich (Younis *et al*., 2008). The speed of the reaction is dependent on the environment of these residues and not all the residues will be glycated to the same extent. The glycation process also depends in glucose concentration.
Malondialdehyde (MDA) is known to be an end-product of lipid peroxidation formed by oxidation of LDL (Requena et al., 1996). In present study MDA was used as biomarker of LDL oxidation. MDA was increased but there was no significant difference between LDL co-incubated with 50% ITf and LDL incubated with iron, apoTf, glucose and LDL that was used as a control. This result may be explained by the process of glycation of 50% ITf in the presence of LDL changing the binding capacity of transferrin and causing release of free iron, which may cause more oxidation of the LDL. Another possible reason for the increase in the oxidation of LDL may be due to glycation of LDL itself making it more prone to oxidation (Sobal et al., 2000).
Chapter 4 General discussion
4.1 General discussion

Iron is an essential trace element for normal cellular functions, and both iron deficiency and overload have deleterious effects. There is significant evidence which suggests that accumulation of iron in the cells of the body is associated with increased risk of T1DM and T2DM, and this iron has been proposed to be involved in the pathophysiological mechanisms of T2DM (Cooksey et al., 2004, Thomas et al., 2004, Rajpathak et al., 2009, Ellervik et al., 2011, Huang et al., 2011, Montonen et al., 2012). T2DM is more common in hereditary haemochromatosis due to a defect in β-cell function resulting from iron deposition in these cells (Wood et al., 2014), and patients with β-thalassaemia, with high serum ferritin levels, are subject to insulin resistance. Large prospective cohort studies reported that high iron dietary intake, particularly of haem iron derived from meat, is associated with a significant increased risk of T2DM (Li et al., 2008), and serum ferritin levels (a biomarker of body iron stores) are positively associated with diabetes risk (Liu et al., 2009, Fernández-Real et al., 2002a, Zhao et al., 2012). The mechanism(s) which explain(s) this relationship is still not clear but some plausible reasons may be:

1. Increased iron status may increase the risk of T2DM by increasing ROS production via the pro-oxidant properties of free iron and the known relationship between ROS and insulin resistance (Goldstein et al., 2005).

2. Increased iron in the liver may induce insulin resistance by impeding the liver’s capacity for insulin extraction thereby increasing hepatic glucose production. Iron deposits contribute to impaired insulin
metabolism by reducing the insulin extracting capacity leading to hepatic insulin resistance (Bugianesi et al., 2004).

3. Impairment by iron of insulin action and interference with glucose uptake in adipocytes and skeletal muscle (Fernández-Real et al., 2002a, Lepage et al., 1991).

4. Increased muscle iron stores may enhance fatty acid oxidation and therefore could interfere with glucose disposal. Thus increased glucose production and decreased utilization may occur with increasing the level of iron (Rajpathak et al., 2009).

5. Excess of body iron may be also cause iron deposition in pancreatic β-cells resulting in impaired insulin secretion.

High plasma glucose levels, as seen in T2DM, cause an increase in the rate of protein glycation reactions, such as glycation of haemoglobin, transferrin and albumin. This can be the result of the reaction between protein amine groups and glucose or by a reaction catalysed by trace amounts of transition metals, known as glucose autoxidation (Wolff and Dean, 1987). Initial glycation products (Amadori products) are reversible, but further oxidation by ROS and RNS (also vulnerable to catalysis by iron) lead to the formation of AGEs in an irreversible process. AGEs are themselves redox active, and may accept a hydrogen atom from nearby biomolecules, leading to their autoxidation. In the case of low density lipoproteins, this leads to non-recognition by cellular LDL receptors, increasing LDL plasma levels and subsequent foam cell take-up in atherogenesis, increasing cardiovascular risk (Cerami and Ulrich, 2001).
In the human body different species of iron are distributed in the extracellular and intracellular compartments. The potential one in the extracellular compartment is NTBI due to its high tendency to induce ROS which is responsible for catalysing redox reaction in the blood and endothelium (Hider, 2002, Fiorelli et al., 2002, Bonnefont et al., 1989). In addition, NTBI may accumulate in cells due to the mechanism of uptake differing from the strongly regulated mechanism of uptake of transferrin-bound iron (Chua et al., 2004). The accumulation of NTBI may cause damage to different intracellular organelles such as in endothelial cells, macrophages, and vascular smooth muscle cells (Brissot et al., 2012).

The aim of this study was to evaluate the presence of NTBI in T2DM, obese non-diabetic and control subjects, and to examine the association between NTBI with other iron parameters, lipid peroxidation and antioxidant status. Further to these, this study examined the link between NTBI and biomarkers of inflammation and of atherosclerosis in all groups in the study. In addition, an *in vitro* study evaluated the effects of glycation on half saturated transferrin (50% ITf) and also the effects of glycation on oxidation of LDL co-incubated with 50% ITf.

This study showed a marked increase in the level of NTBI in T2DM compared with the control and obese groups. The presence of NTBI in T2DM, which is not an iron-overload condition, may be due to impairment of the binding capacity of the plasma transporter proteins such as Hb and transferrin to hold iron tightly due to structural changes resulting from the glycation process (Kisugi et al., 2007). The increased level of NTBI may be
due to decreased synthesis or increased loss of Tf in diabetes due to renal problem.

All previous studies used high concentrations of glucose and loaded transferrin with iron to high percentages of saturation, which makes these studies not physiologically relevant. In this study, use of 50% ITf with high concentrations of glucose led to low levels of free iron release which suggests that in vivo, where glucose concentrations are much lower, glycation of Tf is likely to have little effect on NTBI release, and that the most part of the NTBI release is from other proteins such as Hb and albumin.

In the case of haemoglobin glycation, structural changes occur that destroy the haem group and release the iron held within due to increased H$_2$O$_2$ production, and its steady-state concentration, resulting in the substantial changes in protein structures which expose the haem moiety and promote its degradation (Cussimanio et al., 2003, Sen et al., 2005, Goodarzi et al., 2014). Normally, Hb damage would be prevented by haptoglobin binding to Hb, but this function is decreased when haemoglobin is glycated (Asleh et al., 2003). The presence of Tf in this study was assessed by measurement of TIBC. This was significantly decreased compared with the control; this loss of iron binding capacity may result from the stereochemical effects induced by the glycation of lysine that prevent the conformational changes (from open to closed transferrin forms) required for metal binding (Andre et al., 2014). The present study showed significant increase in % transferrin saturation, which may be due to process of glycation; it is still not entirely clear why the % transferrin saturation would go up. The first possibility is that damage to the transferrin would limit its capacity to bind further iron, so the % saturation
would appear to increase. The second possibility is that damage to transferrin could lead to it being removed, which would mean that the remaining transferrin became more saturated with iron. The third possibility is due to Tf acting as a negative acute reactive protein. In contrast to the situation with T2DM, obesity is generally associated with depletion of iron stores. This has been suggested to be linked to hepcidin, which plays the role of master regulator of iron metabolism, itself also regulated by iron levels, inflammation and erythropoietic activity (Nicolas et al., 2002). Inflammation, from cytokine release by adipocytes and invading inflammatory cells, may lead to increased hepcidin production, which then down regulates iron absorption by the gut and release of iron from macrophages (McClung and Karl, 2009).

Although there are many studies on iron indices in obese non-diabetic subjects none of these mentions the presence of NTBI in obese subjects. This study found the presence of NTBI in the obese group even though the level of total iron in this group was significantly lower compared with the control and T2MD groups. That NTBI in obese non-diabetic subjects was significantly lower compared with the diabetic group may be explained by obese non-diabetic subjects facing levels of protein glycation intermediate between control and diabetic subjects, as a result of pre-diabetic changes in glucose homeostasis. Previous studies have demonstrated strong, positive associations of BMI with fasting glucose and HbA1c with both obese and diabetic subjects (Eldeirawi and Lipton, 2003).

The occurrence of NTBI in direct association with protein glycation is supported by studies of Lee et al. (2006) and Leoncini et al. (2008). T2DM is
closely correlated with chronic inflammation and increased circulating concentrations of IL-6 and TNF-α were found in T2DM (Pickup et al., 2000). The inflammation process causes increased hepcidin production which inhibits intestinal iron absorption (Arshad et al., 1991), and also inhibits release of iron from the liver and macrophages leading to decreased iron circulation and also increased iron deposition in the cells. In the present study total iron showed a statistically lower concentration in the T2DM group compared to the controls. Also soluble transferrin receptor levels were higher in the T2DM group possibly due to increased deposition of iron as ferritin in cells of T2DM subjects, which may be due to combination age-related accumulation of iron (Killilea et al., 2004) and inflammation which leads to increased cytokine production causing an increase in transferrin receptors on the cell surface (Hirayama et al., 1993).

ROS production in T2MD is an inevitable process due to consequences of metabolic dysfunction (Hider, 2002, Evans and Williams, 1980). Initially antioxidant defence may prevent the detrimental effects of ROS, but later these mechanisms may become exhausted and oxidative damage may develop. Redox-active iron is tightly controlled in human body, but when NTBI is present in the circulation it is more likely to participate in the generation of reactive oxygen species and induce lipid peroxidation (Hider, 2002). In present study there was an increase in plasma MDA in T2DM compared with the control, possibly due to the presence of NTBI which could be initiating Fenton chemistry in vivo, leading to induction of lipid peroxidation, and hence increased MDA. Also increased was the level of DHA which indicates the increase in oxidative stress in T2DM. There are several pieces
of evidence showing that the concentrations of antioxidants were decreased in T2DM. Ascorbate is an antioxidant vitamin which plays an important role in scavenging the damage of free radical and sparing other endogenous antioxidants from consumption. A decrease in overall ascorbate level was documented in T2DM in this study. Antioxidant capacity is an important determinant of tissue injury, especially in patients with increased oxidant stress. This study showed a significant decrease in total antioxidant capacity (as measured using the FRAP assay) in T2DM compared with the control group.

T2DM is characterized by abnormalities in the levels of plasma lipids and lipoproteins, including reduced HDL cholesterol, a predominance of small dense LDL particles, and elevated triglycerides (Krauss, 2004, Siribaddana et al., 1994). In T2DM glycation of lipoprotein fractions might be more important than serum lipid and lipoprotein abnormalities (Yegin and Yegin, 1995). One common change induced by glycation of lipoproteins is that they are rendered more susceptible to oxidation (Sobal et al., 2000). The levels of glycated LDL have been shown to be elevated in DM patients (Lyons et al., 1986). The non-enzymatic glycation of LDL occurs mainly to apolipoprotein B, in which lysine is the major amino acid that undergoes glycation. (Steinbrecher and Witztum, 1984) have reported levels of 2-17% of LDL-lysine to be glycated. Transition metal ions prompt LDL glycation (Imanaga et al., 2000). The present in vitro study showed an increase in glycation of LDL after incubation of LDL with 500 mM glucose; incubation of LDL with 50% ITf showed a lower degree of glycation compared with LDL incubated with 500 mM glucose. The study also showed that the level of MDA was increased in
the case of incubation of LDL with 50% ITf compared with LDL incubation with 500 mM glucose alone. This may be due to the loss of iron from transferrin over time in the incubation, which may in turn increase the oxidation of LDL. Also the level of MDA was increased in the case of incubation LDL with 50% ITf compared with LDL incubation with low level of free iron. This may be due to glycation inducing the oxidation of LDL more than that of native LDL (Sobal et al., 2000).

In the present study the level of the biomarker of atherosclerosis, E-selectin, was correlated with levels of NTBI. NTBI, which is present in T2DM subjects, may induce the oxidation of glycated LDL in the atherosclerotic plaque. NTBI activates the endothelium and increases production of adhesion molecules such as E-selectin which may be involved in recruitment of monocytes. These monocytes convert to macrophages thus enhancing subendothelial LDL retention and macrophage progression to foam cells. It has been previously shown that NTBI levels positively correlate with the expression of adhesion molecules, ICAM-1, VCAM-1 and E-selectin, but not to the inflammatory marker CRP (Kartikasari et al., 2006).

In people with T2DM there was a correlation between NTBI and HbA1c. This maybe indicates that glycation of haemoglobin is a major player in the release NTBI in plasma rather than other proteins such as transferrin, the focus of this study. Regardless of the sources of NTBI, it plays a major role in diabetes complications such as atherosclerosis. NTBI is thought to be easily accessible to many cell types within the plaque, likely accumulating in endothelial cells, macrophages, and vascular smooth muscle cells (Vinchi et al., 2014).
Combinations of haem, Hb and NTBI also play major roles in complications of diabetes such as atherosclerosis. All these species contribute to the oxidation of LDL in plasma and induce the oxidative stress by formation ROS. They also promote endothelial activation, by enhancing adhesion molecule expression and monocyte recruitment (Figure 6.1).

![Diagram](image)

**Figure 4.1 A role for NTBI in atherosclerotic lesions in uncontrolled T2DM.** NTBI from different sources may be introduced to the endothelium directly and activate the endothelium to release different types of adhesion molecules such as E-selectin leading to recruitment of monocytes from the circulation. The resulting macrophages uptake oxidised LDL. Circulating monocytes may also contain NTBI, and its presence enhances the overall process.

The present study needs more strength by studying other factors which may contribute to NTBI release such as protein glycation in RBC membranes. The RBC is unique among cells in that the plasma membrane, its only structural component, accounts for all the diverse biochemical and mechanical characteristics of the cell (An and Mohandas, 2008). The balance, distribution and interplay of the proteins and lipids in the RBC membrane play major
roles in the function of the membrane. The lipid bilayer is composed of equal proportions by weight of cholesterol and phospholipids. The membrane proteins serve numerous functions including transport, cellular interaction and, with the lipid component, the structural integrity of the membrane. Both proteins and lipids are susceptible to glycation and/or oxidative modification. The RBC is therefore particularly susceptible to the consequences of poor regulation of circulating glucose levels. Glycation of RBC membrane proteins may have an effect on the integrity and function of the RBC. Protein glycation may in turn lead to the formation of reactive oxygen species which may damage intracellular and membrane associated macromolecules such as other proteins and lipids (Resmi et al., 2005). A number of in vitro studies have confirmed the damaging effects of glycation on RBC membranes and their components, leading to reduced antioxidant protection, enhanced fragility, reduced deformability and evidence of haemolysis (Resmi et al., 2005, Shin et al., 2008, Suravajjala et al., 2013). Few studies have examined glycation of proteins in the RBC membrane of diabetic patients in vivo. Extensive proteomic analysis of glycation of plasma proteins and proteins of the RBC membrane of diabetic patients demonstrated the presence of a number of glycated proteins in the erythrocyte membrane (Zhang et al., 2008). Furthermore, RBC membrane proteins were more extensively glycated than plasma proteins (Zhang et al., 2011). Glucose concentration as low as 1% can induce non-enzymatic glycation of cell surface proteins in RBC membranes which modifies the visco-elastic properties of the RBCs and decreases their deformability (Riquelme et al., 2005). Another study has provided evidence of glucose-induced changes in lipid and protein
composition and function in RBCs from subjects with T2DM (Starodubtseva et al., 2008). Future studies could focus on the glycation of RBC membrane proteins and lipids, in association with accompanying free radicals, leading to a combination of increased membrane permeability and damage to Hb and haem, leading to the enhanced appearance of both compounds in the plasma along with free iron. These three components then could contribute to the vascular complications of diabetes. It has been suggested that low grade haemolysis in conditions such as diabetes may contribute to the vascular disease associated with the condition (Kato and Taylor, 2010).

An-going study in our laboratory has shown that the level of NTBI is reduced by using statins. Therefore a study of influence of statins on iron haemostasis and RBC membrane integrity would be appropriate in the future, also including other drugs such as anti-diabetics (e.g. metformin) which reduce protein glycation (Rahbar et al., 2000), and maybe reduce release of NTBI and then maybe reduce the consequences of NTBI in the development of atherosclerosis in T2DM.

In addition, an animal model such as the streptozotocin diabetic rat might give more information on the role of iron dysregulation. The following is an outline of a possible future study using this approach. Wistar rats would be randomly divided into five groups, each group containing 30 rats (groups 1, 2, 3, 4 and 5). Group 1 would be injected with iron-dextran at low dose (200 mg/kg) as a control group; group 2 would be injected with iron-dextran at low dose (200 mg/kg) and injected with the diabetes-inducing drug streptozotocin with multiple low doses (60 mg/kg) to induce T2DM; group 3 would be injected with iron-dextran at low dose (200 mg/kg), 400 mg/kg of
deferoxamine to chelate iron (Wu et al., 2004) and also injected with the diabetes-inducing drug streptozotocin with multiple low doses (60 mg/kg) to induce T2DM; group 4 would be injected with iron-dextran at high dose (800 mg/kg) (Yang et al., 2002), and also injected with the diabetes-inducing drug streptozotocin with multiple low doses (60 mg/kg) to induce T2DM; and group 5 would be injected with iron-dextran at high dose (800 mg/kg) (Yang et al., 2002), 400 mg/kg of deferoxamine and also injected with the diabetes-inducing drug streptozotocin with multiple low doses (60 mg/kg) to induce T2DM. After three months blood would be taken (5 ml) to measure iron status, oxidative stress and endothelium parameters, and HbA1c (as described in Chapter 2). Transferrin would be separated from the plasma using an Aurum™ Serum Protein Mini Kit in order to investigate transferrin fragmentation using two dimensional gel electrophoresis and mass spectrometry for all groups. The aims of this study would be:

1) to test whether there is NTBI in normal and/or diabetic rats by comparing the levels in groups 1 and 2; and also comparing other changes in all parameters;

2) to examine the effects of low iron versus high iron (both via injection of iron-dextran) on all parameters;

3) to examine transferrin fragmentation in the four diabetic groups compared with control group to give obtain a clear view on changes to the structure of transferrin.

4) to identify any correlations between NTBI and all parameters in the study; and
5) to identify any correlations between HbA$_{1c}$ and all parameters.

Predicted outcomes of study

1) A significant level NTBI should be found in all diabetic groups (2-5) compared with the control, group 1.

2) All iron parameters in groups 2-5 should change due to the different levels of iron injected, compared with group 1.

3) Due to the high dose of iron injected in group 4 without deferoxamine this group should show high levels of NTBI and general iron status, oxidative stress, elevated E-selectin and cRP and greater fragmentation in transferrin.

4) There should be improvement in HbA$_{1c}$, significant reduction in NTBI and other iron parameters, endothelium parameters (E-selectin and cRP) and oxidative stress parameters when the diabetic groups are injected with deferoxamine.

Because NTBI is heterogeneous in nature and there are different methods for measuring it, comparison of different methods could be included in future studies in order to know the type of iron ligands which predominate in T2DM and obese non-diabetic subjects, in addition to measurement of the labile iron pool which is thought to be more redox active than NTBI (Cabanchik, 2014).
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Appendix 1 correlation of other iron indices with age

Figure A1.1 Correlations between age and total serum iron levels ($P = 0.356$).

Figure A1.2 Correlations between age and % saturation of transferrin ($P = 0.440$).
Figure A1.3 Correlations between age and NTBI levels (P = 0.470).

Figure A1.4 Correlations between age and sTfR (P = 0.616).
Appendix 2 correlation other oxidative stress indices with age

Figure A2.1 Correlations between age and total ascorbate ($P = 0.895$).

Figure A2.2 Correlations between age and ascorbate ($P = 0.985$).
Figure A2.3 Correlations between age and DHA ($P = 0.896$).

Figure A2.4 Correlations between age and total antioxidant capacity ($P = 0.682$).
Appendix 3 correlation between E-selectin and hsC-RP and age

Figure A3.1 Correlations between age and E-selectin levels ($P = 0.0997$).

Figure A3.2 Correlations between age and hs-CRP ($P = 0.221$).
Appendix 4: Correlation between NTBI and iron indices

Figure A4.1 Correlations between NTBI and total serum iron ($P = 0.396$).

Figure A4.2 Correlations between NTBI and TIBC ($P = 0.527$).
Figure A4.3 correlation between NTBI and % transferrin saturation ($P = 0.328$).
Appendix 5 correlation between NTBI and oxidative stress indices

Figure A5.1 Correlations between NTBI and MDA ($P = 0.390$).

Figure A5.2 Correlations between NTBI and total ascorbate ($P = 0.396$).
Figure A5.3 Correlations between NTBI and DHA ($P = 0.865$).

Figure A5.4 Correlations between NTBI and ascorbate ($P = 0.563$).
Figure A5.5 Correlations between NTBI and total antioxidant capacity ($P = 0.593$).
Appendix 6 correlation between NTBI hsC-RP

Figure A6.1 Correlation between hs-CRP and E-selectin ($P = 0.801$).
Appendix 7 Validation of the NTBI method

Table 31. NTBI assay compilation of standards

<table>
<thead>
<tr>
<th>Fe$^{3+}$ μM</th>
<th>Mean</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.006</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0.019</td>
<td>0.008</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>0.035</td>
<td>0.008</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>0.078</td>
<td>0.022</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>0.155</td>
<td>0.051</td>
<td>26</td>
</tr>
<tr>
<td>20</td>
<td>0.315</td>
<td>0.127</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>0.576</td>
<td>0.082</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 39. NTBI assay compilation of standards (mean and SD). For sample size see Table 31.
Table 32. NTBI assay coefficient of variation

<table>
<thead>
<tr>
<th>Ferric iron standard</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM (n=26)</td>
<td>0.019</td>
<td>0.008</td>
<td>0.432</td>
</tr>
<tr>
<td>5 μM (n=32)</td>
<td>0.078</td>
<td>0.024</td>
<td>0.309</td>
</tr>
<tr>
<td>10 μM (n=26)</td>
<td>0.155</td>
<td>0.051</td>
<td>0.332</td>
</tr>
</tbody>
</table>

Figure 40. NTBI assay standards over 24 months
(Mean and SD, Table 32.)
CONSENT FORM
Version 2  28/08/2011

University of Plymouth, School of Health Professions.
Plymouth Hospitals NHS Trust Department of Diabetes and Endocrinology.
Peninsula College of Medicine and Dentistry.

TITLE OF STUDY: THE RELATIONSHIP BETWEEN IRON, OXIDATIVE STRESS, GLYCATED PROTEINS AND THE DEVELOPMENT OF ATHEROSCLEROSIS IN TYPE 2 DIABETES

Name of Researchers:
Dr Keith Collard. University of Plymouth
Prof Jon Pinkney. Department of Diabetes and Endocrinology, Plymouth Hospitals
NHS Trust. Professor of Medicine, Peninsula College of Medicine & Dentistry.
Mr Husam Aliwaid. [PhD Student]. University of Plymouth

Please initial the boxes
1  I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. 

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

3  I am willing to allow access to my medical records by authorized people but understand that strict confidentiality will be maintained. The purpose of this is to ensure that the study is being carried out correctly. 

4  I consent to my blood samples being stored and used in future approved research in the area of diabetes and the blood vessel complications associated with diabetes. 

5  I agree to take part in the above study

Name of patient  Date  Signature

Name of person taking Consent (if different From researcher)

Date  Signature

Researcher  Date  Signature

(One copy for patient, one for researcher, one for medical notes)
Appendix 9 Questionnaire forms

**Control Information Proforma**

Name:

Sex:

Height:

Weight:

Medical history:

Diabetes: Yes/No

Angina: Yes/No

Myocardia infraction: Yes/No

Stroke: Yes/No

Peripheral vascular diseases: Yes/No

High blood pressure: Yes/No

Other factors:

Smoking status: never smoked ☐; ex-smoker ☐; current smoker ☐

Alcohol intake:

Non-drinker ☐ Drinker ☐

If you drink, approximately how many units do you drink in a week?
Medical treatment:

Are you being treated for any other condition? Yes/No

If yes, what condition is being treated and what drugs are being prescribed?

Drug: __________________________ Dose __________________________

Non drug treatment e.g. diet, exercise.
Diabetes Mellitus type 2 Information Proforma

Name:

Sex:

Height:

Weight:

Family history:
Does anyone in your family suffer from diabetes? Yes/No

If the answer to the above is yes, which types of diabetes have been diagnosed? Type 1 C; Type 2 C.

Does anyone in your family suffer from any of the following cardiovascular diseases?

Angina Yes/No

Myocardia infraction Yes/No

Stroke Yes/No

Peripheral vascular diseases Yes/No

High blood pressure: Yes/No
Your Diabetes

How many years have you have you suffered from diabetes? ______ years

Have you been diagnosed with any of the following complications of diabetes?
Retinopathy: Yes/No
Nephropathy Yes/No
Neuropathy Yes/No

Do you suffer from any of the following conditions?
Angina: Yes/No
Myocardial Infarction: Yes/No
Stroke: Yes/No
Peripheral vascular disease: Yes/No

Other factors:
Smoking status: never smoked C; ex-smoker C; current smoker C
Alcohol intake:

Non-drinker C Drinker C

If you drink, approximately how many units do you drink in a week?

How is your diabetes being treated?

Drugs used:

Type of drug: Dose

Non drug treatment e.g. diet, exercise.

Are you being treated for any other condition?

Yes/No

If yes, what condition is being treated and what drugs are being prescribed?

Drug: Dose
Obese Information Proforma

Name:

Sex:

Height:

Weight:

Medical history:

Diabetes: Yes/No

Angina: Yes/No

Myocardia infraction: Yes/No

Stroke: Yes/No

Peripheral vascular diseases: Yes/No

High blood pressure: Yes/No

Sleep Apnoea: Yes/No

Other factors:

Smoking status: never smoked☐; ex-smoker☐; current smoker ☐

Alcohol intake:
Non-drinker □  Drinker □  If you drink, approximately how many units do you drink in a week?

Medical treatment
Are you being treated for any other condition? Yes/No
If yes, what condition is being treated and what drugs are being prescribed?
Drug:        Dose

Non drug treatment e.g. diet, exercise.
### Appendix 10 Anthropometric indices

<table>
<thead>
<tr>
<th>Diabetes</th>
<th>Gender</th>
<th>Age</th>
<th>BMI</th>
<th>W</th>
<th>H</th>
<th>W/H Ratio</th>
<th>Exercise</th>
<th>alcohol</th>
<th>Cigarettes</th>
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<tbody>
<tr>
<td>1 F</td>
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<td>110</td>
<td>113</td>
<td>0.98</td>
<td>27.3</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>2 F</td>
<td>61</td>
<td>34</td>
<td>113.6</td>
<td>122.4</td>
<td>0.929155</td>
<td>30.6</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>3 F</td>
<td>58</td>
<td>32.3</td>
<td>108.167</td>
<td>111.567</td>
<td>0.9688567</td>
<td>39.7</td>
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<td>No</td>
<td></td>
</tr>
<tr>
<td>4 F</td>
<td>52</td>
<td>30.4</td>
<td>92.8</td>
<td>107.667</td>
<td>0.86192</td>
<td>42</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5 M</td>
<td>61</td>
<td>35.1</td>
<td>114.333</td>
<td>120.667</td>
<td>0.94751</td>
<td>29.1</td>
<td>walking</td>
<td>6 units/week</td>
<td>No</td>
</tr>
<tr>
<td>6 M</td>
<td>70</td>
<td>38.6</td>
<td>123.067</td>
<td>130.333</td>
<td>0.90709</td>
<td>43.1</td>
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