OXIDATIVE STRESS AND CARDIOVASCULAR AGEING IN DIABETES

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By

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Oxidative stress and cardiovascular ageing in diabetes
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Abstract
Oxidative stress may be elevated in diabetes and telomeric and mitochondrial DNA are two areas of the genome that may be particularly vulnerable to oxidative stress. This thesis investigates oxidative DNA damage in people with diabetes and its potential role in accelerating vascular ageing.

Methods: 51 patients with diabetes (aged 31-78 years) and 101 healthy controls (aged 19-75 years) were recruited. Pulse wave velocity (PWV), a surrogate for vascular stiffness and age, was measured. Oxidative DNA damage was measured in peripheral blood using the comet assay, and telomere terminal restriction fragment (TRF) lengths and mitochondrial DNA (mtDNA) content were quantified.

Results: PWV increased with age in both study groups (p<0.001) and was significantly higher in the patient group (8.00 ± 2.89 versus 7.29 ± 1.64 m/s; p=0.006), suggesting accelerated vascular ageing in diabetes. This was accompanied by elevated levels of oxidative DNA damage; 25.81 ± 1.18 versus 21.40 ± 0.81 %Tail DNA (p=0.003) in patients versus controls respectively. TRF length inversely correlated with age in both groups (p<0.05), with similar rates of attrition, and although they were shorter in the patients with diabetes, this was not significant (p=0.110). Quantification of mtDNA revealed significantly lower levels in the patients with diabetes compared to the controls (0.014 versus 0.016; p=0.020).

Conclusions: There is accelerated vascular ageing in diabetes, which is associated with enhanced oxidative stress. There is also increased oxidative damage to DNA and a decrease in mtDNA. There was no alteration in TRF length in people with diabetes when compared to a healthy control population, however, larger studies are likely to be required to further investigate this relationship. These studies demonstrate that DNA is an important site of oxidative damage in people with diabetes.
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<th>Definition</th>
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<tbody>
<tr>
<td>8oxoG</td>
<td>8-oxo-7,8,dihydroguanine</td>
</tr>
<tr>
<td>8OhdG</td>
<td>8-oxo-7,8,dihydrodeoxyguanosine</td>
</tr>
<tr>
<td>ACR</td>
<td>albumin to creatinine ratio</td>
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<tr>
<td>AGE</td>
<td>advanced glycation end product</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AU</td>
<td>arbitrary units</td>
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<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>bp/yr</td>
<td>base pairs per year</td>
</tr>
<tr>
<td>bpm</td>
<td>beats per minute</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>C of V</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunoassay</td>
</tr>
<tr>
<td>EDTA</td>
<td>diamino ethanetetra – acetic acid disodium salt</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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FPG formamidopyrimidine DNA glycosylase
GC/MS gas chromatography with mass spectrometry
GPx glutathione peroxidase
GSH reduced glutathione
GSSG oxidised glutathione
HbA1c glycated haemoglobin
HDL high density lipoprotein
HMW high molecular weight
HPLC high performance liquid chromatography
HT hypertension
HUVECs human umbilical vein endothelial cells
IDDM insulin dependent diabetes mellitus
IGT impaired glucose tolerance
Isop isoprostane
Kb kilobases
LDL low density lipoprotein
LMP low melting point
log logarithmic
MBq megabecquerel
mtDNA mitochondrial DNA
NAD nicotinamide adenine dinucleotide
NADP nicotinamide adenine dinucleotide phosphate
nDNA nuclear DNA
NER nucleotide excision repair
NGT normal glucose tolerance
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>NIDDM</td>
<td>non insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>% Tail DNA</td>
<td>percentage of tail DNA</td>
</tr>
<tr>
<td>PP</td>
<td>pulse pressure</td>
</tr>
<tr>
<td>PWV</td>
<td>pulse wave velocity</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SCGE</td>
<td>single cell gel electrophoresis</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDS</td>
<td>sodium diodecyl sulphate</td>
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<tr>
<td>sem</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium sodium citrate</td>
</tr>
<tr>
<td>TAE</td>
<td>tris/glacial acetic acid EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>tris/boric acid EDTA</td>
</tr>
<tr>
<td>Total Chol</td>
<td>total cholesterol</td>
</tr>
<tr>
<td>TRF</td>
<td>terminal restriction fragment</td>
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Chapter 1: Introduction

1.1.1 Overview

Diabetes mellitus is one of the major medical disorders worldwide, and has rapidly increased in recent years [1-3], yet much is still unknown about the progression of the condition and its complications. It is characterised by a state of chronic hyperglycaemia, and classification is based on aetiology, with type 2 diabetes accounting for 90 to 95 per cent of cases worldwide [4]. Whatever the aetiology (type 1 or 2), the complications are similar, with hyperglycaemia thought to play an important role in their development, as it is the main common feature [5, 6]. The complications are predominantly vascular in nature, and cardiovascular disease is the leading cause of morbidity and mortality [7]. In a healthy non-diabetic population, age is the greatest risk factor for cardiovascular disease [8]; therefore, it is suggested that there is accelerated vascular ageing in diabetes that results in premature development of cardiovascular disease.

There have been many proposals for the mechanisms behind hyperglycaemia-induced vascular disease, all of which may be a consequence of oxidative stress [9]. Of particular interest is the proposal that oxidative damage to DNA may affect cell function. Two key areas of interest are telomeres and mitochondrial DNA, which both appear to be more susceptible to oxidant damage [10, 11]. Telomeric DNA, an index of cell age, shortens with replication and this attrition eventually leads to cell senescence [12]. In oxidative stress this process may be accelerated. The mitochondrial genome encodes several components of the electron transport chain, the chief site of energy production [13]. Oxidative damage to the genome may result in decreased energy production and therefore cell function. The combination of
accelerated biological ageing and cellular energy depletion could contribute to many of the pathophysiological features of diabetes, which predominantly affect tissues with high metabolic/energy demands.

1.1.2 Aim of chapter

In this introductory chapter diabetes and its associated vascular complications and the potential role of hyperglycaemia in their development will be discussed. Oxidative stress will then be described with the proposal it is the mechanism underlying pathways of hyperglycaemia-induced vascular complications. Of interest is the oxidative damage to DNA, and in particular telomeric DNA and mitochondrial DNA. Therefore, telomeres will be described with the role of oxidative stress in their shortening with the consequences and their potential role in vascular disease. The final section of the introduction will describe mitochondria and the mitochondrial genome, with reference to mutations and damage that may play a role in the complications of diabetes.

1.2 Diabetes mellitus

1.2.1 Background

Diabetes mellitus is characterised by a state of chronic hyperglycaemia, and classically associated with the symptoms of excessive thirst, increased urine volume, and, in severe cases, weight loss [4]. There are two main forms of diabetes. Type 1 diabetes usually has its onset in children or younger adults and is characterised by an absolute deficiency of insulin due primarily to autoimmune-mediated destruction of the pancreatic \(\beta\)-cells. Type 1 diabetes is fatal without life-long insulin replacement [4]. Type 2 diabetes is very much more common than type 1 diabetes [1-3]. Its onset
is usually in middle age and older. It is associated with obesity and a more sedentary life-style on a background of genetic predisposition [4]. Type 2 diabetes has a more insidious onset than type 1 diabetes and is characterised by a progressive worsening hyperglycaemia, due to increasing resistance to insulin’s action. This resistance, as a result of a post-receptor defect in insulin receptor signalling, causes patients with type 2 diabetes to initially over-produce insulin from their pancreas to overcome this, but eventually pancreatic function wanes and hyperglycaemia ensues. Thus, patients with type 2 diabetes are not insulin-dependent, but they may require insulin to help control their hyperglycaemia [4].

1.2.2 Incidence and prevalence of diabetes

Although diabetes as a whole affects more Asian and African-Caribbean people, Type 1 diabetes is predominantly one of white Caucasian populations and is relatively rare in both oriental and black populations. The incidence varies greatly worldwide, which is not easily explained by genetic or environmental factors, and is slightly more common in males than females. Type 2 diabetes is much more prevalent, accounting for 90-95% of all diabetes cases worldwide, although the values vary greatly depending on the criteria used for definition and the sample populations used for testing [1-3].

There has been an explosive increase in the number of people diagnosed with diabetes in the last 20 years, relating particularly to type 2 diabetes. This increase is set to continue, with projected global figures of people with diabetes rising from the estimated 150 million in 2000, to 220 million in 2010, and 300 million in 2025 [1, 2]. Most cases will be type 2 diabetes, thought to be a result of the changing
environment, behaviour, and lifestyle of humans, leading to obesity, a risk factor for type 2 diabetes.

1.2.3 Morbidity and mortality in diabetes

Whatever the aetiology of the diabetes (type 1 or type 2), the complications are similar, and are predominantly vascular in nature. They can be conveniently divided into macrovascular and microvascular complications that culminate in premature morbidity and mortality, primarily from cardiovascular disease [7]. Microvascular complications comprise retinopathy, nephropathy, and often include neuropathy although it may have a neurological pathogenesis. Examples of the less specific macrovascular complications include stroke, myocardial infarction, ischaemic heart disease, and limb ischaemia.

1.2.4 Cardiovascular disease (CVD)

Cardiovascular disease (CVD), which includes coronary heart disease, cerebrovascular disease, and peripheral vascular disease, is the leading cause of death in developed and developing countries [14]. (It is thought that as communicable diseases are controlled, populations can achieve the age where cardiovascular disease presents itself.) The pathogenesis of CVD is multifactorial, and in a healthy population, risk factors for CVD include hypertension, obesity, hyperlipidaemia, smoking, and sedentary lifestyle; however, age is still the factor that confers the greatest risk [8]. This has led to the proposal that cardiovascular disease is an ageing process, but this does not explain why there are many people who achieve 'old age' without evidence of cardiovascular disease. The true situation is complex and involves many factors including age, genetics, and risk factors. It is still unknown
how these factors interact and influence one another producing pathological conditions in some individuals and not others.

Several studies have investigated the effects of ageing on the cardiovascular system in healthy subjects. Well-documented structural changes in arteries include intimal thickening and increased stiffness accompanied by luminal dilatation (reviewed in [15, 16]). The macroscopic changes that lead to these progressive alterations include increased fibronectin and collagen content with cross-linking of collagen, fragmentation of elastin, and invasion of the intima by vascular smooth muscle cells (reviewed in [17]). Functional changes observed in ageing include altered regulation of vascular smooth muscle tone due in part to endothelial dysfunction. The exact cause of this dysfunction is unknown, but it may affect the production of anticoagulants (e.g. plasminogen activator inhibitor-1) and vasodilators (e.g. nitric oxide, which also restrains smooth muscle cell migration and proliferation) [18]. The subsequent altered regulation in vascular tone can contribute to the previously mentioned structural changes, and both can contribute to the development of atherosclerosis, hypertension, and left ventricular hypertrophy [16].

Atherosclerosis is often the underlying cause of CVD. Figure 1.1 gives an overview of the early processes involved to enable further understanding of how the changes that occur in ageing can contribute to its pathogenesis and progression, and also how diabetes, when discussed later, may interact.
Figure 1.1: Early events in the development of atherosclerosis

Overview of the early events in the atherosclerosis process, where atherogenic stimuli (step 2), e.g. hypertension, lead to endothelial dysfunction (step 3), thus promoting leukocyte adhesion and migration into the artery wall (step 4). These processes lead to the formation of a fatty streak, which initially consists of lipid-laden monocytes and macrophages (foam cells), and T lymphocytes. The release of cytokines and chemokines locally from activated macrophages attenuate these processes (step 5), and also stimulates many others. These include the activation, proliferation and migration of smooth muscle cells into the fatty streak (step 5), thus further contributing to atheroma growth. Adapted from Poch et al[19].

The earliest changes in the atherosclerotic process take place in the endothelium, and are thought to be a response to injury [20]. These changes (reviewed in [21]) include
increased endothelial permeability to lipoproteins and other circulating factors, increased leukocyte adhesion, and migration of leukocytes into the artery wall. Various mediators are involved in each of these processes, including nitric oxide, adhesion molecules, and oxidised lipoprotein. These processes lead to the development of a fatty streak, which initially consists of lipid-laden monocytes and macrophages (foam cells), and T lymphocytes. Smooth muscle cells and platelets join these, and each step again involves many mediators. The result is a progressive narrowing of the lumen. As fatty streaks progress to intermediate and advanced lesions, they undergo structural alterations. A fibrous cap forms over the lesion, covering the mixture of leukocytes, lipids and debris, isolating the lesion from the lumen. The lesion can continue to expand and further occlude the blood vessel by continued leukocyte adhesion and entry, and the core may become necrotic. This is a result of necrosis and apoptosis, increased proteolytic activity, and lipid accumulation, and is characteristic of advanced, complicated lesions. Plaque rupture is a complication of advanced lesions, and this can lead to the adverse outcomes of CVD, for example myocardial infarction.

1.2.5 Diabetes and CVD

CVD is the leading cause of morbidity and mortality in diabetes. In patients with diabetes, the same risk factors for CVD exist as for the general population [22]; however, diabetes itself is an independent risk factor [7, 23, 24], thought to accelerate the process observed with ageing in non-diabetic populations. The majority of studies have investigated coronary heart disease (CHD) as the end-point measured for CVD, and have reported the relative risk of death from CHD ranging from 1.5 to 2.5 times greater in men with diabetes compared to healthy populations, and ranging from 1.7
to more than 4.0 times greater in women with diabetes compared to those without (reviewed in [6]). The majority of studies investigated type 2 diabetes, but type 1 diabetes is also an independent risk factor for CVD, with a relative risk of developing the condition up to 10 times higher compared with non-diabetic persons of a similar age [25, 26].

A number of known risk factors for vascular disease, such as hypertension, dyslipidaemia, and central obesity, are commonly found in patients with diabetes, particularly type 2 diabetes [7, 24]. Moreover, individuals with type 2 diabetes are also more likely to have multiple risk factors for CHD ('clustering') [27], and although these established risk factors confer a similar relative risk of CHD among patients with type 2 diabetes and among healthy controls, they may be additive [24]. There is a lack of these risk factors in many patients with type 1 diabetes, and individually or together, their presence in type 2 diabetes still does not account for all of the excess risk [7, 28]. This has led to the proposal of hyperglycaemia as the major contributing factor in the development of vascular complications in diabetes, as it is the main common feature [5, 6].

1.2.6 Vascular complications and hyperglycaemia

Several studies have investigated the relationship between hyperglycaemia and CVD in diabetes, with some prospective studies linking long-term glucose control (as measured by glycated haemoglobin (HbA1c)) with cardiovascular morbidity and mortality [29, 30], whilst others have found no association [31, 32]. In non-diabetics there is a positive association between HbA1c and CVD, and this appears stronger in women [30].
Intervention trials to improve glycaemic control have been positive for microvascular complications, but inconclusive for large vessel complications. Two seminal studies, the diabetes control and complications trial (DCCT) [33], and the United Kingdom prospective diabetes study (UKPDS) [34], investigated the effects of improved glycaemic control on microvascular complications in type 1 and type 2 patients with diabetes respectively. Both studies assigned participants to conventional therapy, or intensive treatment, with the aim of the latter to achieve as near normal blood glucose concentrations as possible. After a mean follow-up period of 6.5 years, the DCCT found a 76% reduction in risk of developing retinopathy, 54% reduction for albuminuria, and 60% reduction for clinical neuropathy in the intensive treatment group. The UKPDS had less of a difference in HbA1c between the conventional and intensive treatment groups compared to the DCCT (0.9% versus 2%), but still showed a 25% risk reduction in microvascular endpoints (p=0.0099).

Both of the aforementioned studies did not set out to investigate the effects of improved glucose control on macrovascular complications, but subgroup analyses were performed to investigate any relationship. The DCCT showed a 41% reduction in major cardiovascular events in the intensively treated group, but this did not reach significance (p=0.08) [35]. This was probably due to the small number of events overall as a consequence of the relatively young age of the participants. The UKPDS also did not find any statistically significant benefits of tighter glycaemic control on macrovascular endpoints, although the reduced risk for myocardial infarction neared significance (p=0.052) [34]. Jensen-Urstad [36] and colleagues specifically investigated markers for coronary atherosclerosis in 31 patients with insulin-dependent diabetes previously assigned to intensified conventional insulin treatment,
and 28 patients with insulin-dependent diabetes previously assigned to standard insulin treatment. The patients in the intensive arm of the study had significantly lower HbA1c values compared to the standard treatment group after 10 years, (7.1 ± 0.1% and 8.2 ± 0.2 % respectively; p<0.001). They also had less stiff arteries (p=0.011) and thinner intima-media in the left (but not right) common carotid artery (p=0.009). There was no significant difference in endothelial function between the two study groups (p=0.795).

It therefore appears that hyperglycaemia contributes to microvascular complications, but it is not clear if it has a significant role in the development and progression of macrovascular complication in diabetes. It should also be noted that in some of these intervention studies, improvements in other risk factors of cardiovascular disease were also observed in the intensive treatment groups, for example blood pressure [34, 36] and hypercholesterolaemia [33]; therefore, any decrease in vascular complications may not be solely due to the improved glucose control, but also due to a reduction in other risk factors. The previous studies showing diabetes as an independent risk factor [7, 23-26], and the presence of the ‘established’ risk factors not accounting for the overall increase in the risk of developing CVD in diabetes [24, 28], still suggest hyperglycaemia plays a role in the development and progression of CVD in diabetes.

1.2.7 Proposed mechanisms for role of hyperglycaemia in CVD
There have been several mechanisms proposed for the development of CVD by hyperglycaemia. These include endothelial cell dysfunction, increased advanced glycation end products, dysregulation of protein kinase C, and inflammation.
Endothelial cell dysfunction

Endothelial cells are of paramount importance in cardiovascular physiology as their functions include providing a metabolically active interface between blood and tissue that modulates blood flow, nutrient delivery, coagulation, and thrombosis. This interface synthesises important bioactive substances that regulate blood vessel structure and function, including nitric oxide, which is a vasodilator and controls smooth muscle cell migration and proliferation [37]. Taken together, these properties inhibit atherogenesis and protect the blood vessel. *In vitro* studies have shown high glucose causes endothelial cell dysfunction, including decreased proliferation [38] and migration [39], increased collagen [40] and fibronectin synthesis [41], and increased apoptosis [38, 42]. These alterations may then lead to atherosclerosis (figure 1.1) and present as CVD.

Increased advanced glycation end-product (AGE) formation

There is increasing evidence that the spontaneous process of glycation, which is driven by hyperglycaemia and underlies the formation of HbA1c, can modify most molecules in the arterial wall. The products of glycation can then undergo oxidation, or glycoxidation, to yield a host of advanced glycation end-products (AGEs). AGEs have been shown to accumulate with age [43], and are markedly increased in diabetes [44, 45]. Their effects are thought to include lipid peroxidation via their specific receptor (RAGE), platelet aggregation, and inactivation of nitric oxide [46, 47], and therefore may contribute to CVD.
Dysregulation of protein kinase C (PKC)

Protein kinase C is a serine-threonine protein kinase that is relatively abundant in vascular tissue and involved in a multitude of cell functions. Hyperglycaemia causes an up-regulation of PKC [48-52] through the synthesis of diacylglycerol [53]. This up-regulation has been shown to alter vascular smooth muscle cell function and growth [48, 51, 52], alter vascular blood flow [54, 55], thicken the basement membrane [56], and increase permeability [57], all of which may contribute to the development of CVD.

Inflammation

Inflammation plays a key role in the initiation and progression of atherosclerosis [21]. Several inflammatory markers have been identified in atherosclerotic lesions [58], including cytokines and growth factors. Recent studies have also shown elevated inflammatory markers in type 1 [59] and type 2 diabetes [60], which may be due to a greater release of cytokines from activated macrophages through AGEs [47]. Cytokines increase the synthesis of platelet activating factor, stimulate lipolysis, stimulate the expression of adhesion molecules, and upregulate the synthesis and cell expression of procoagulant activity in endothelial cells [61].

As yet, there is no single mechanism identified that causes the increased risk of CVD in diabetes. However, it has been suggested that each of the aforementioned pathogenic mechanisms may reflect a single hyperglycaemia-induced process: of elevated oxidative stress.
1.3 Oxidative stress

1.3.1 Reactive species

Oxidative stress refers to an imbalance in the oxidants and antioxidants in the body in favour of the former. Reactive species are chemical entities that can be classified as: free radicals, chemical species that possess one or more unpaired electrons and are chemically unstable and highly reactive; or non-radicals that are oxidizers or metabolites, equally important in contributing to the overall oxidative status (table 1.1).

<table>
<thead>
<tr>
<th>Radicals</th>
<th>Non-Radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive Oxygen Species (ROS)</td>
<td></td>
</tr>
<tr>
<td>Superoxide</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>Hypochlorous Acid</td>
</tr>
<tr>
<td>Peroxy</td>
<td>HCl</td>
</tr>
<tr>
<td>Alkoxyl</td>
<td>Ozone</td>
</tr>
<tr>
<td>Hydroperoxyl</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>Reactive Nitrogen Species (RNS)</td>
<td></td>
</tr>
<tr>
<td>Nitric Oxide</td>
<td>Hydroxyl alkenals</td>
</tr>
<tr>
<td>Nitrogen Dioxide</td>
<td>Nitrous acid</td>
</tr>
<tr>
<td>Others</td>
<td>Dinitrogen trioxide</td>
</tr>
<tr>
<td>Thiy</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>Trichloromethyl</td>
<td>Alkyl Peroxynitrites</td>
</tr>
</tbody>
</table>

**Table 1.1: Reactive species**

A list of free radical and non-radical reactive species, where R represents an alkyl chain. The superscripted bold dot indicates an unpaired electron and the negative charge indicates a gained electron. Singlet oxygen is an unstable molecule due to the two electrons present in its outer orbit spinning in opposite directions. Adapted from Dhalla et al [62].
Reactive species can originate in various ways including biochemical redox reactions as a part of normal metabolism (producing $O_2^-$, NO', $H_2O_2$), by phagocytes as part of a controlled inflammatory reaction (HOCl, $O_2^-$), from lipid peroxidation (hydroxy alkenals), and occasionally in response to factors such as ionising radiation, UV light, cigarette smoke, hyperoxia, and ischaemia ($O_2^-$, $OH^-$, ROO') [63]. They appear to play a critical role in the normal functioning of a cell, for example in host defence mechanisms, for normal growth of vascular smooth muscle cells, as secondary messengers, and in preventing atherogenesis. However, their ability to damage DNA, lipids, and proteins, as well as activate redox sensitive genes, and modify enzyme function [62] [64], indicates they could have a potentially detrimental effect on the body.

1.3.2. The oxidative stress theory of ageing

The oxidative stress theory of ageing [65] suggests cellular molecules are modified and damaged by free radicals, and this leads to altered cell function. There is an accumulation of damage over time, producing an increasing number of defective cells. This could ultimately lead to cell senescence (the limited ability of human somatic cells to divide, with accompanying changes in cell function, morphology, and gene expression [66]), and then tissue dysfunction. Consistent with this is the direct relationship between the rate of oxidative damage and the metabolic rate of an organism, and the inverse relationship between the rate of oxidative damage and the life span of an organism [67]. Investigations into many pathophysiological conditions, such as cardiovascular disease [62, 68, 69] and cancer [70], also implicate ROS in their progression.
1.3.3 Oxidative damage to DNA

The ability of specific reactive species to damage molecules varies, with hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$) reacting with few molecules at diffusion-controlled rates, whereas the hydroxyl radical (OH\(^{•}\)) reacts at diffusion-controlled rates with almost anything, including RNA and DNA. The less reactive species can only attack DNA via the hydroxyl radical formed from the Haber-Weiss reaction in the presence of transition metal ions [63]:

\[
\text{O}_2^{•-} \xrightarrow{\text{Superoxide dismutase}} \text{H}_2\text{O}_2 \xrightarrow{\text{Transition metal}} \text{O}_2 + \text{OH}^{•} + \text{OH}
\]

When the metal ion is iron (Fe\(^{2+}\)), it is termed the Fenton reaction. An alternative pathway for the less reactive species to attack DNA is via an acute rise in intracellular free calcium (Ca\(^{2+}\)), which may fragment DNA by activating Ca\(^{2+}\)-dependent endonucleases [63].

Reactions of the hydroxyl radical with pyrimidines and purines result in multiple products in DNA (reviewed in [71]). The presence of oxidised base lesions in DNA is well established, and may be formed \textit{in situ} or arise by incorporation from the deoxynucleotide pool. The number of lesions identified is growing and among them 8-oxo-7,8-dihydroguanine (8oxoG; figure 1.2) is the most abundant, and the most frequently studied (sometimes measured as the nucleoside 8OHdG).
Many oxidative lesions are mutagenic, including 8-oxoG, which when formed in situ can anneal with adenine or cytosine nucleotides, and therefore cause G to T substitutions. Less commonly, 8-oxoG may be misincorporated opposite adenine, producing an A to C substitution [72]. As well as mutation, other effects of oxidative DNA damage include blocking replication [73], and microsatellite instability [74]. Microsatellites are repetitive sequences of DNA that are a constant length in normal cells; however, oxidative damage can cause mutations in the repeat sequences and alter their lengths (microsatellite instability). This instability is present in tumour cells and has been linked to some sporadic cancers [74].

1.3.4 Antioxidant defence

Several defence mechanisms exist in the body to counteract and prevent the effects of oxidative stress. The two most prominent are DNA repair and antioxidant defences, the latter of which can be divided into enzymatic and non-enzymatic (table 1.2).
### Enzymatic Scavengers

<table>
<thead>
<tr>
<th>Enzymatic Scavengers</th>
<th>Non-Enzymatic Scavengers</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD - Superoxide Dismutase</td>
<td>Vitamin A</td>
</tr>
<tr>
<td>$2O_2^+ + 2H^+ \rightarrow H_2O_2 + O_2$</td>
<td>Vitamin C (ascorbic acid)</td>
</tr>
<tr>
<td>CAT Catalase (peroxisomal bound)</td>
<td>Vitamin E (α-tocopherol)</td>
</tr>
<tr>
<td>$2H_2O_2 \rightarrow O_2 + H_2O$</td>
<td>β-Carotene</td>
</tr>
<tr>
<td>GPx Glutathione Peroxidase</td>
<td>Cysteine</td>
</tr>
<tr>
<td>$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>$2GSH + ROOH \rightarrow GSSG + ROH + H_2O$</td>
<td>Uric acid</td>
</tr>
<tr>
<td></td>
<td>Flavonoids</td>
</tr>
<tr>
<td></td>
<td>Sulfhydryl group</td>
</tr>
<tr>
<td></td>
<td>Thioether compounds</td>
</tr>
</tbody>
</table>

### Table 1.2: Antioxidants

A list of enzymatic antioxidants on the left hand side of the table, with the reactions they catalyse, and non-enzymatic antioxidants on the right hand side of the table. GSH, reduced glutathione; GSSG, oxidised glutathione; R, alkyl chain. Adapted from Dhalla et al [62].

Superoxide dismutase (SOD), Catalase (CAT), and glutathione peroxidase (GPx) are present throughout the body at varying concentrations depending on the tissue and the current environment [62]. SOD exists in two forms in humans, copper-zinc SOD (CuZnSOD) mainly present in the cytosol, and manganese SOD (MnSOD) primarily in the mitochondria. The enzyme dismutases superoxide anion into hydrogen peroxide, which is further reduced to water by catalase, present in peroxisomes, and by GPx located in the cytosol and mitochondria (table 1.2). These enzymatic scavengers, along with the non-enzymatic antioxidants appear to act together quenching reactive species in the body.

### 1.3.5 DNA Repair

DNA repair (reviewed in [75]) acts protectively to remove damage after it has taken place. Damage can come from various sources such as alkylating agents, UV light, X-
rays, replication errors, and oxidative stress. The consequences of damage are numerous and diverse, from altered DNA metabolism triggering apoptosis, to irreversible mutations contributing to oncogenesis [76]. The repair system in place has different pathways, primarily base excision repair (BER), nucleotide excision repair (NER), recombinational repair, and mismatch repair. BER, the main guardian against oxidative damage from reactive species, involves the removal of non-bulky adducts, and is dependent on lesion-specific enzymes, e.g. glycosylases. The resultant apurinic/apyrimidinic (AP) site is removed by AP endonucleases and the gap filled by a DNA polymerase. NER involves the removal of several nucleotides surrounding a lesion, and is therefore less specific and generally acts as a back up for BER in repair of oxidative damage. Homologous recombination and non-homologous end joining, both mechanisms of recombinational repair, act against double-strand breaks, with the former using the identical sister chromatid as a template and is thus error free, whilst the latter joins the ends of a double-strand break without any template with the possible loss or gain of a few nucleotides. Mismatch repair occurs when enzymes remove a mismatched but undamaged nucleotide opposite a modified base, e.g. an oxidative lesion.

1.3.6 Diabetes and oxidative stress

There has been recent interest in the oxidative stress of hyperglycaemia as a potential mechanism for vascular disease in diabetes, with some evidence of increased markers of oxidative stress found in patients with type 1 and type 2 diabetes compared to controls [77-84]. The findings are not consistent though, which may be attributed to small, heterogeneous populations, different methods, and the evanescence of reactive species.
Several pathways have been suggested for hyperglycaemia-induced oxidative stress, including auto-oxidation of glucose, the polyol pathway, local tissue damage, and changes in antioxidant defence. The list is long, perhaps because each pathway is a different reflection on a common underlying mechanism, or because different tissues are sensitive to different mechanisms. There are several apparent overlaps between the hypotheses, and many that are probably not known as yet.

**Glucose auto-oxidation**

Auto-oxidation refers to the ability of glucose to enolise, in the presence of transition metal ions. Monosaccharides with an α-hydroxyaldehyde structure, like glucose, are subject to enediol rearrangement that results in the formation of a radical ion [85]. This radical ion can then reduce molecular oxygen to form $O_2^-$, and subsequently $H_2O_2$ and $OH^*$, or the dicarbonyl groups formed in the pathway may modify adjacent lysine groups to form AGEs. [9, 86]

**Polyol pathway**

In tissues where glucose uptake is independent of insulin, exposure to elevated glucose causes an increased activity of aldose reductase and sorbitol dehydrogenase. These two enzymes constitute the polyol pathway (figure 1.3), and increases in their activity causes an increase in intracellular sorbitol and fructose levels, a decrease in the ratio of NADPH to NADP+, and increases in the cytosolic NADH-to-NAD+ ratio. The reduction of glucose to sorbitol consumes NADPH, and this is required for the generation of reduced glutathione (GSH), an antioxidant (table 1.2). Therefore this could contribute to an increase in oxidative stress [87].
Aldose reductase catalyses the conversion of glucose to sorbitol, using NADPH as a co-factor. Sorbitol dehydrogenase (SDH) oxidises sorbitol to fructose with the reduction of NAD$^+$ to NADH. The decreased availability of NADPH for glutathione reductase (GR), leads to a decrease in conversion of oxidised glutathione (GSSG) to reduced glutathione (GSH), an antioxidant.

Vascular wall enzymatic sources

Several sources of reactive species have been identified in the vascular wall, and as complications within diabetes are predominantly vascular in nature, they are thought to play an important role.

NAD(P)H oxidase has been proposed to be a major source of superoxide anion in normal and diseased blood vessels. It is a membrane associated enzyme that catalyses the reduction of oxygen to produce superoxide, using NADH or NADPH as the electron donor [88].

\[
\text{NAD(P)H} + 2\text{O}_2 \rightarrow \text{NAD(P)}^+ + \text{H}^+ + 2\text{O}_2^{-}
\]
Superoxide ions are also produced as a by-product of xanthine production from hypoxanthine via xanthine oxidase [89]. This enzyme has similar properties to NAD (P)H oxidase [90] and is involved in ROS induced vascular damage.

Endothelial NO synthase is also suggested as an enzymatic source of oxidants in the vascular wall as it produces NO, which is biodegraded by reactive species, producing more reactive species. The depleted NO, a vasodilator, and increased production of free radicals impart further oxidative injury to the endothelium, and may predispose to atheroma [88].

Decreased antioxidant defence

The previous mechanisms propose increased production of reactive species leading to oxidative stress; however, the increase in oxidative stress could be due to impaired defences. Earlier studies evaluating antioxidant capacity in diabetes by measuring levels of particular scavengers such as vitamin E, vitamin C, and antioxidant enzymes have shown differing results. Studies into antioxidant status in diabetes have shown varying results for specific antioxidants. Akkus et al [91] found lowered vitamin C in leukocytes of type 2 patients, but not lowered SOD or GPx, whereas Atalay et al [92] found decreased levels of erythrocyte SOD and CAT in type 1 patients, and Ruiz and colleagues [93] found no difference in SOD activity but decreased GPx levels in type 1 diabetes. Two separate studies [94, 95] found total radical trapping capacity depressed in type 1 and type 2 diabetics, although it is unknown if a specific scavenger was primarily responsible. Anderson et al [96] contradicted those results, with no significant difference in antioxidant capacity in type 1 and type 2 patients with diabetes. The lack of consistency between studies may be due to different study
populations investigated, varying doses and time period of vitamin supplementation, and/or different outcomes measured. Further studies investigating antioxidant therapy in diabetes have also given varying results. The majority use pharmacological doses of vitamin E, with Sarđaš and colleagues [80] showing a decrease in DNA damage in NIDDM and IDDM after 12 weeks supplementation, but a similar decrease was also observed in the non-smoking placebo group. Sampson and colleagues [97] showed no difference in DNA damage in type 2 patients after 8 weeks, but Engelen et al [98] showed a decrease in lipid peroxidation after 3 months supplementation with vitamin E in patients with type 1 diabetes, and Davi et al [78] also showed a decrease in lipid peroxidation in both IDDM and NIDDM patients after only 2 weeks.

There is very little research in DNA repair in diabetes. Tyrberg et al [99] investigated the DNA repair enzyme 8-oxoG DNA glycosylase (Ogg1) in pancreases from patients with type 2 diabetes and compared it to healthy controls. Ogg1 is the major enzyme involved in removing 8OHdG. The authors found significantly elevated expression of Ogg1 in islet cell mitochondria, and this correlated with the duration of diabetes. It was therefore suggested this up-regulation in repair was a consequence of increased hyperglycaemia-induced oxidative DNA damage. Further studies are required to confirm this increase in repair activity, but it suggests any elevation in oxidative DNA damage in diabetes would not be a consequence of decreased repair.

1.3.7 Oxidative stress and CVD

Oxidative stress is involved in the early events of atherosclerosis, with the uptake of oxidised low-density lipoprotein, and the accumulation of lipid peroxidation products found in lesions [100]. It has also been suggested that oxidative stress may play many
other roles in the development of vascular disease. Li and colleagues demonstrated superoxide induced proliferation, and hydrogen peroxide caused apoptosis of cultured rat vascular smooth muscle cells [101], and therefore can contribute to atherosclerosis. In section 1.1.6 various mechanisms proposed for the development of CVD by hyperglycaemia were presented. Nishikawa and colleagues [9] inhibited the mitochondrial electron transport chain, thus decreasing superoxide production. The authors demonstrated this prevented; glucose-induced activation of protein kinase C, formation of advanced glycation end products, and sorbitol accumulation via the polyol pathway. Oxidative stress can also cause endothelial dysfunction through decreased nitric oxide (NO). Superoxide anion reacts with NO to produce peroxynitrite (ONOO⁻; table 1.1) [102], therefore removing the beneficial effects of NO and also further contributing to further damage through ONOO⁻ [103]. Curcio et al [42] also found that the decreased proliferation of endothelial cells in high glucose was reversed with antioxidants, and Lorenzi and colleagues [104] went on to show high glucose induced DNA damage in endothelial cells and suggested this may lead to decreased proliferation and dysfunction.

Inflammation was also suggested as a contributor to hyperglycaemia-induced CVD in section 1.1.6. Oxidative stress and inflammation are closely linked. Inflammation is characterised by a respiratory burst of activated neutrophils and macrophages that can lead to elevated levels of reactive species [105]. However, it has been suggested that oxidative stress induces the inflammatory response. Different mechanisms have been proposed including the potential role of peroxisome proliferator-activated receptors [106], insulin resistance [107-109], and advanced glycation end products (AGEs) [110]. The last hypothesis was investigated in porcine coronary arteries [110], and
found diabetes increased NAD(P)H oxidase activity and oxidative stress, producing inflammatory responses in porcine coronary media and adventitia. AGEs induced the expression of several inflammatory genes in coronary cells in a redox-sensitive manner, and the authors proposed they might be involved in the development of accelerated coronary atherosclerosis in diabetes. This would suggest that oxidative stress is increased due to hyperglycaemia, and this in turn causes inflammation.

Several pathways are therefore suggested for hyperglycaemia-induced oxidative stress contributing to vascular disease. Given the potential damage to DNA from oxidative stress that can lead to mutation [72], blocked gene expression [73], and microsatellite instability [74], it has been suggested that DNA damage plays a role in vascular damage. Evidence to support this comes from; elevated levels of DNA damage demonstrated in peripheral blood lymphocytes from patients with coronary artery disease [111]; increased levels of 8OHdG in rabbit [112] and human atherosclerotic plaques [113], and microsatellite instability from an increase in mutation rate in human atherosclerotic plaques [114].

A further proposal involves specific areas of DNA that are vulnerable to oxidative stress, exhibiting further damage with detrimental effects on cell function. Two such areas are telomeres and mitochondrial DNA.
1.4 Telomeres

1.4.1. Telomere structure and function

Telomeres are distinctive nucleoprotein structures at the end of chromosomes, where non-coding DNA sequences consisting of repeated units of the hexanucleotide TTAGGG, arranged in tandem. They protect the chromosomes against degradation, rearrangement, and fusion with other chromosome ends, and play multiple roles in spatial organisation of the cell nucleus and in chromosomal separation during cell division. They are also thought to influence the transcription of genes located near chromosome ends, in the so-called sub-telomeric regions [115-117].

Telomeres vary in length between individuals, between some tissues, and between species. The average lengths in humans vary greatly from 15kb in germ line cells, 8-10kb in the somatic cell population of an average young person, to 4kb in senescent cells. [12] The observed shortening of telomeres with increasing number of cell divisions, and therefore age, is termed the 'end replication problem' and although the mechanism involved is not clear, evidence suggests it is due to incomplete synthesis of the telomere in cell replication [116, 118, 119].

1.4.2 End replication problem

DNA replication is bi-directional, however, conventional DNA polymerases can only replicate in the 5'-3' direction and cannot start DNA synthesis de novo. Instead a short RNA molecule primes DNA synthesis, and in the leading strand replication, synthesis is completed to the end of the template. For the lagging strand, several primers are used to start synthesis of Okazaki fragments. These are later joined by removal of the
primers and the consequent gaps filled via a combination of ligases and DNA polymerases [12].

**Figure 1.4: End replication problem**

*Incomplete synthesis of the lagging strand in DNA replication, thought to be the cause of progressive shortening of telomeres with age. Adapted from Shay and Wright [120].*

This creates a problem for linear chromosomes when the terminal RNA primer is removed opposite the 3'-end of the telomere, because the gap cannot be filled; therefore there is incomplete synthesis of the telomere (figure 1.4). This could explain the G-rich overhang found at the 3'end of telomeres (as well as shortening of the telomere with cell replication), which has also been suggested as essential for establishing a stable telomeric structure [12].

Telomeres were thought to be linear but they actually form loop structures from the overhang at the 3'end folding back and tucking inside the double-stranded DNA,
forming a ‘t-loop’ [121]. Where the t-loop invades the duplex DNA and base pairs with one of the strands, a displacement loop is formed, called the D loop. Formation of these structures appears to be dependent on specific telomere binding proteins, particularly the telomeric repeat factors, TRF 1 and TRF 2. TRF 1 is thought to aid in the coiling of the loop, whilst TRF 2 is found bound at the base of the t-loop, presumably where the G strand invades the duplex DNA, and appears to maintain the 3’-overhangs (figure 1.5). These structures could explain how telomeres protect their ends from degradation and DNA-repair activities, and help distinguish them from double-strand breaks. [122]

Figure 1.5: Proposed structure of a telomere

The left portion shows the linear structure of the telomere, displaying the 3’overhang, with the approximate number of hexanucleotide repeats. The dotted line in the centre of the picture represents the whole subtelomeric part of the chromosome. The right portion illustrates the proposed structure of the telomeric loop structure with the telomeric repeat factors, TRF 1 and TRF 2. Adapted from Von Zglinicki [12].
1.4.3 Senescence and the telomere hypothesis of ageing

The incomplete synthesis, and subsequent loss of telomeric DNA with each replication, could be damaging to cells. It has been suggested as the cause of both cell senescence and the Hayflick limit, which refers to the limited replicative potential of normal somatic cells before they enter into senescence. Several mechanisms have been suggested for telomere shortening leading to cellular senescence including:

1. Shortening of one or more telomeres below a critical length will induce cell senescence, possibly via p53 [123].

2. Accumulation of critically shortened telomeres to a threshold level will initiate cell senescence [124].

3. Loss of a specific telomeric structure, rather than a reduction in overall length, e.g. t-loops, meaning they are exposed to end-to-end fusions [122].

4. Alterations in gene expression in those genes in the sub-telomeric regions [12].

As mentioned previously, cellular senescence is a consequence of the inability of cells to divide indefinitely. This irreversible cell cycle block in G0/G1 is triggered via activation of the tumour suppressor genes p53, p21, and p16, although it is unknown the mechanisms behind activation of these proteins [12]. Senescence is also associated with distinctive molecular and morphological alterations such as cellular flattening and increased adherence, over-expression of genes that encode secreted proteins such as inflammatory factors and cytokines, and the expression of senescence-associated-β-galactosidase activity (reviewed in [125]).

The number of divisions that cells complete before they senesce varies greatly depending on the cell type, species, age, and genetics. There are also certain cell types
that never enter senescence e.g. germ-line cells and tumour cells. Senescence is proposed to contribute to ageing, with previous research showing an inverse relationship between the replicative potential of cells in vitro with the tissue donor age [126], and an age-dependent increase in senescent cells in dermal fibroblasts and epidermal keratinocytes in vivo [127].

The suggestions of senescence contributing to ageing, and telomere attrition contributing to senescence, have led to the telomere hypothesis of ageing [12, 124]. Evidence to support this comes with the inverse relationship between donor age and telomere length, and the observed accelerated telomere loss and decreased life-span in genetic syndromes, such as Werner’s and Down’s [128]. General causes of death in individuals with these conditions associated with premature ageing, are premature cancer and cardiovascular disease, the common causes of mortality in the general, elderly population. Also, lengthening telomeres results in cells that have longer replicative limits and are “younger” as assessed by their patterns of gene expression, physiologic markers, and morphology [129].

1.4.4 Telomerase

While human somatic cells seem to gradually lose telomeric repeats, telomere length is maintained in immortal cell populations, including the germ line, by telomerase [130]. This is a reverse transcriptase, which includes a catalytic component and a RNA template, which can anneal to the G-rich strand of the telomere repeat array, and is therefore capable of synthesising telomeric DNA de novo.
Further evidence to support the telomere shortening theory of ageing comes from studies in mice with the knockout of telomerase resulting in amplification of some characteristics associated with systemic ageing in later generations of mice [131]. Also, telomerase is present in about 90% of all malignant tumours, and therefore thought to be fundamental in their immortality by preventing degradation of their telomeres, or at least slowing their attrition [132]. There are some adult somatic cells in humans that express telomerase, for example, T cells when activated to proliferate, and although their telomeres still shorten, they do so at a much slower rate compared to telomerase-negative cells [133]. Thus, in some cases, telomerase is not sufficient to prevent erosion that occurs as a consequence of DNA replication. It is still unknown what determines the ability of telomerase to maintain telomere stability; however, the amount and/or duration of telomerase expression, and the level and activities of telomere associated proteins and regulatory proteins, may influence whether the enzyme can act, e.g. TRF 1 binds to duplex TTAGGG and negatively regulates telomerase activity [134].

1.4.5 Oxidative stress and telomeres

The effect of the end-replication problem is rather small compared to the telomere attrition rates observed in human cells. This has led to further suggestions for telomere shortening, including the action of a C-strand-specific exonuclease [135], and external influences including oxidative stress [12]. Guanine has the lowest oxidation potential of the common DNA bases, rendering telomeres especially sensitive targets to oxidative damage due to the GGG triplet in their hexanucleotide repeat [10]. Further, the t- and D-loops at the end of telomeres may block access for repair, although there is no evidence to support this. A study by Oikawa and
Kawanishi [10] found hydrogen peroxide did cause predominant damage at the 5' site of the 5'-GGG-3' in the telomere sequence, and oxidation of guanine was more efficient in telomeres compared to non-telomeric sequences. They also found that when single-stranded DNA was used, the damage induced by oxidative stress occurred at every guanine.

A further proposal for the increased shortening observed in telomeres is a single-strand break in the lagging strand. Oxidation causes single-strand breaks which halt replication. If the break is in the lagging strand, replication of the leading strand continues, and the stop may not be detected for a long time. If synthesis is near the end of the chromosome, it is proposed that replication of the leading strand could finish without completion of the lagging strand, resulting in an overhang [12].

1.4.6 Diabetes, CVD and telomere attrition

If oxidative stress is paramount in telomere attrition as suggested in previous research, [12] and there are elevated levels of oxidative stress in diabetes, this would suggest accelerated telomere shortening in individuals with diabetes. This in turn may contribute to the vascular complications associated with diabetes, as previous studies have shown shorter telomeres are associated with markers of vascular ageing (increased arterial stiffness) [136, 137], and an increased risk of developing atherosclerosis [138, 139]. Minamino and colleagues [66] also found senescence-associated phenotypes of vascular endothelial cells present in human atherosclerotic lesions, and suggested endothelial cell senescence induced by telomere shortening may contribute to atherogenesis.
Jeanclós et al [140] investigated terminal restriction fragments (TRFs) in white blood cells of 54 patients with insulin dependent diabetes mellitus (IDDM), 74 patients with non-insulin dependent diabetes mellitus (NIDDM), and 106 control subjects. The authors found significantly shorter TRFs in the patients with IDDM compared to the controls, 8.6 ± 0.1 kb versus 9.2 ± 0.1 kb respectively (p = 0.002), but no significant difference between the patients with NIDDM and the controls. This latter finding and the lack of relationship between TRF length and both the duration or complications of diabetes, suggested hyperglycaemia was not the sole cause of shorter TRFs in the IDDM patients. The authors proposed shorter telomeres may be a feature intrinsic to white blood cells of IDDM patients, as these cells are involved in the immune response and IDDM is mediated via immunologic processes, or that TRFs are shorter due to genetic factors in these individuals.
1.5 Mitochondria

1.5.1. Mitochondria function

Mitochondria are the site of cellular respiration, and hence where the majority of adenosine triphosphate (ATP) is produced in the body [141]. Cells contain varying numbers of mitochondria depending on their energy requirements, with high energy tissues, such as the heart and skeletal muscle having several hundred per cell. Cellular respiration is usually split into glycolysis, the citric acid cycle (figure 1.6), and the electron transport chain (ETC, figure 1.7).

![Diagram of glycolysis and the citric acid cycle](image)

**Figure 1.6: Overview of glycolysis and the citric acid cycle**

Glycolysis is the sequence of reactions that converts glucose into pyruvate, in the cytosol of a cell, with the production of a relatively small amount of ATP. Under aerobic conditions, pyruvate enters the mitochondrial matrix, undergoes oxidative carboxylation to form acetyl coenzyme A, which is then completely oxidised to carbon dioxide (CO$_2$) by the citric acid cycle. In this cycle 2 molecules of CO$_2$ leave the cycle,
6 electrons are transferred to nicotinamide adenine dinucleotide (NAD\(^+\)), and 2 electrons are transferred to a flavin adenine dinucleotide (FAD) molecule. These electron carriers are then oxidised in the electron transport chain to produce ATP.

Figure 1.7: Electron transport chain

The electron transport chain consists of 5 complexes. Electrons are transferred from NADH and FADH\(_2\) to complexes I (NADH-Q reductase) and II (succinate-Q reductase), and are then carried to complex III (cytochrome reductase) by ubiquinone (Q). Cytochrome c (Cyt c) shuttles electrons from complex III to IV (cytochrome oxidase), where electrons are transferred to molecular oxygen (O\(_2\)) to reduce it to water (H\(_2\)O). Electron flow within these transmembrane complexes leads to the pumping of protons across the inner mitochondrial membrane by complexes I, III, and IV, thus creating a pH gradient and membrane potential. The flow of protons back to the matrix side of the inner membrane through complex V (ATP synthase) drives ATP synthesis.
In addition to the production of ATP, mitochondria are also involved in apoptosis, glucose-induced insulin production, and oxygen sensing. Apoptosis is programmed cell death that plays essential roles in development, tissue homeostasis, and various diseases including cancer [142] and stroke [143]. Mitochondria contain several pro-apoptotic molecules that activate cytosolic proteins to execute apoptosis, block anti-apoptotic proteins in the cytosol, and directly cleave nuclear DNA. These pro-apoptotic molecules are released from the mitochondria when triggered, and initiate apoptosis (reviewed in [144]).

Glucose-stimulated insulin release from pancreatic β-cells initially involves an increase in ATP as the glucose is metabolised, and this causes closure of ATP-sensitive potassium channels. The subsequent depolarisation of the plasma membrane leads to a calcium influx, which in turn triggers the release of insulin through exocytosis [145]. Noda and colleagues [146] investigated murine pancreatic β-cells with suppressed transcription of mitochondrial DNA, but not that of nuclear DNA. There was a reduction in expression of the electron transport system, which caused NADH accumulation. This in turn halted the citric acid cycle and led to a loss of glucose-induced insulin secretion with concomitant losses of membrane potential depolarisation, and increases in intracellular calcium. These results show the importance of mitochondrial ATP over glycolytic ATP in glucose-induced insulin release.

Hypoxia activates gene transcription for several factors responsive to the lack of oxygen including erythropoietin, vascular endothelial growth factor, and glycolytic
enzymes. Research indicates this up-regulated transcription is mitochondrial-dependent involving increased ROS [147].

1.5.2 Mitochondria and oxidative stress

Oxidative phosphorylation is the process where ATP is generated by a transfer of electrons from reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin adenine dinucleotide (FADH$_2$) to molecular oxygen via the ETC, with the transfer of four electrons producing two molecules of water (figure 1.7).

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

However, partial reduction can lead to superoxide production, and subsequently other reactive oxygen species such as hydrogen peroxide and the hydroxyl radical.

$$O_2 + e^- \longrightarrow O_2^-$$

Mitochondria reduce about 90% of the cell’s oxygen, and of this 1-2% are released as ROS [148, 149]. Mitochondria are therefore a major source of free radicals in the body, and inevitably are a target for oxidative damage. This led Harman to propose the mitochondrial theory of ageing [150], an extension of the free radical theory of ageing. Harman proposed oxidative damage to the mitochondria can lead to a detrimental cycle where the damage causes altered mitochondrial function, this in turn leads to increased ROS production that further damages the mitochondria. The consequence is an accumulation of dysfunctional mitochondria with age, which could lead to decreased energy production and compromised cell function.
1.5.3 Mitochondrial DNA

A topic that has gained a renewed interest recently, is the oxidative damage to mitochondrial DNA (mtDNA), of which there are two to ten copies per mitochondrion, consisting of 16,569 base pairs (bp) in humans, arranged as a double-stranded circular molecule [13] (figure 1.8). The genetic code is different to that of nuclear DNA and encodes 22 transfer RNAs, 2 ribosomal RNAs, and 13 structural genes. These structural genes encode 13 components of oxidative phosphorylation; 7 of the 39 subunits of complex I, 1 of the 10 subunits of complex III, 3 of the 13 subunits of complex IV, and 2 subunits of complex V.

MtDNA is maternally inherited as there is very little mtDNA contributed to the fertilised egg by the sperm [151]. This is because mitochondria in sperm are located in the mid-piece of the tail, and this is eliminated early in the process of fertilisation [152]. Thus while the head of the sperm contributes its nuclear DNA to the fertilised egg, very little mtDNA is contributed and therefore the vast proportion of mtDNA is of maternal origin.
Figure 1.8: The mitochondrial genome

The outer and inner circles represent the heavy (H) and light (L) strand, respectively. The 22 transfer RNA genes are depicted by dots and the single-letter code of the amino acid. The shaded boxes are the genes that encode the ribosomal RNA (rRNA) species and the 13 protein-coding genes for complexes I, III, IV and V of the ETC chain. The positions of the most commonly encountered point mutations, A3243G (in MELAS), A8344G (in MERRF), T8993G/C (in NARP/MILS), and G3460A, G11778A, T14484C and A14495G (in LHON) are indicated. Modified from Nature Reviews Genetics [153].
1.5.4 Oxidative stress and mtDNA

The mitochondrial genome is more susceptible then the nuclear genome to oxidative damage due to the close proximity of free radicals produced via oxidative phosphorylation, and also due to the lack of histone protection [154], insufficient repair mechanisms [11], and its highly compact structure [13], which consists almost entirely of coding regions. Yakes and Van Houten [11] found hydrogen peroxide induced two to three times more damage to mtDNA than nuclear DNA, and a 60-minute exposure to 200μM led to persistent mtDNA damage even though nuclear damage was repaired within 1.5 hours. Richter et al [155] found high levels of 8OHdG in nuclear and mtDNA, with levels of 1 per 130 000 bases in the nuclear genome, and 1 per 8000 bases in the mitochondria. Other studies have challenged cells with oxidative stress using xanthine oxidase [156], t-butylhydroperoxide [157], and tumour necrosis factor-α [158] and shown damage to mtDNA.

To investigate if there is an increase in oxidative damage to mtDNA with ageing, several groups have measured levels of 8OHdG by high performance liquid chromatography (HPLC). Levels have been shown to increase with age in several rodent tissues [159-161], and in the human diaphragm [162] and brain [163]. Previously HPLC has been thought to overestimate oxidative damage due to artefactual damage generated in the process of DNA isolation [164]. This could be especially true for mitochondria, as their isolation provides an extra step for artefactual damage. Anson et al [165] investigated levels of 8OHdG in mtDNA in cell culture using HPLC and Southern blotting with formamidopyrimidine DNA glycosylase (FPG) enzyme, the latter of which does not require the isolation of mitochondria. The authors found the techniques comparable, but approximately three
times higher using HPLC. These results question the validity of the results found in previous studies using HPLC.

1.5.5 MtDNA mutations

Damage to the mtDNA, if not repaired, could lead to mutations during replication. There has been extensive research investigating deletion mutations and point mutations within mtDNA with ageing, with the majority showing an increase in deletions with age [166-169], but varying results for the incidence of point mutations with age [167, 169, 170]. All mutations were at very low levels, generally less than 1% of the total mtDNA content [167-170], and it is unknown the impact such low levels would exert on cell function.

Quantitative investigation of mtDNA in ageing has also given varying results, with a decrease in content in rat liver [171], but either no change in human skeletal muscle and cardiac muscle [168], or increases in human muscle [172], brain tissue [173], and lung tissue [174]. These increases are thought to be a compensatory mechanism for decreased mitochondrial function [171-174]. Activities of the complexes of the ETC have been investigated in ageing, with decreases observed in some of them with age in human skeletal muscle [175, 176]. Studies have also shown an increase in cytochrome c oxidase (complex IV)-deficient fibres in skeletal muscle with age [176, 177], and high levels of mtDNA deletions accompany these, without a decrease in mtDNA copy number [178].

Liu et al [179] investigated the role of oxidative stress in the regulation of mtDNA copy number in ageing. The authors measured mtDNA copy number in human
leukocytes, using quantitative PCR, in 156 healthy subjects aged 25 to 80 years. The study found copy number increased up to approximately 50 years of age and then started to decline. They also found positive correlations between mtDNA copy number and oxidative stress indices including leukocyte 8OHdG content (p=0.003), and a plasma measure of lipid peroxidation after correction for age, sex, and BMI.

1.5.6 MtDNA diseases

The existence of mutant mtDNA with wild-type mtDNA within a cell (heteroplasmy), has led to the theory of a mutation threshold, where once the percentage of mutated DNA reaches a certain level, it becomes noticeable. Many mutations are also involved in the development of various diseases (figure 1.7), and may be heteroplasmic or homoplasmic. For example, the common deletion, the loss of a 4977 bp sequence from nucleotide position 8470 to 13447, has been found in the brains of patients with Parkinson’s disease, an age-associated disease; while the point mutation arising from an A to G substitution at nucleotide 3243 (A3243G) has been found in patients with Mitochondrial Encephalomyopathy Lactic Acidosis (MELAS). There are several other diseases associated with alterations of mtDNA, both from large deletions, in the cases of Kearns-Sayre Syndrome (KSS), Chronic Progressive External Ophthalmoplegia (CPEO); and from point mutations such as LHON (Leber’s Hereditary Opticus Neuropathy), and MERRF (Myoclonic Epilepsy, Ragged Red Fibres) (reviewed in [180, 181]). Contrary to the mutations in ageing, if the mutation is heteroplasmic, a large proportion of mutated mtDNA is observed in the above diseases. Different mutations of the same gene can result in different phenotypes due to the degree of heteroplasmy, the position of the mutation, and environmental influences, such as diet [182].
1.5.7 MtDNA and diabetes

It has been suggested that mtDNA may be involved in the pathogenesis of diabetes mellitus. Evidence leading to this proposal comes from: the strong genetic component apparent with the condition; patients with mtDNA diseases, including mutations and deletions, have a much greater incidence of diabetes [183-186]; and the association of more than 40 different mtDNA mutations with the type 2 phenotype (reviewed in [182]). Specific mutations that have generated a lot of interest include the A3243G mutation, that Gerbitz and colleagues [187] reported is found in approximately 0.5-1.5% of unselected diabetic patients (whether type 1 or 2), increasing up to 10% if patients have a family history of diabetes. Also there is maternally inherited diabetes with deafness, a distinct subtype of diabetes that is associated with and possibly the result of a systemic 10.4kb mtDNA deletion [188]. Van den Ouweland et al [189] also reported maternal transmission of a rare polymorphic mtDNA restriction site in a large family with Type 2 diabetes. These qualitative changes still only account for a very small proportion of patients with diabetes (reviewed in [182, 187]).

The same group has carried out the majority of investigations of quantitative changes in mtDNA in diabetes. Lee and colleagues [190] first reported mean mtDNA content 35% lower in the peripheral blood of 55 patients with non-insulin dependent diabetes mellitus compared to 29 age- and sex-matched controls, using slot-blot analysis. The authors went on to discover a significantly lower mtDNA content in the peripheral blood of 23 subjects who developed NIDDM during a 2-year follow-up period in a prospective population based study. MtDNA content was then quantified using polymerase chain reaction (PCR), and compared to 22 age- and sex-matched controls (p<0.05). Song et al [191], from the same group, later investigated peripheral blood
mtDNA content, using real-time PCR, in the offspring of 82 patients with type 2 diabetes. 52 of the offspring had normal glucose tolerance (NGT), 21 had impaired glucose tolerance (IGT), and 9 had newly-diagnosed type 2 diabetes. The offspring group as a whole had approximately 17% less mtDNA compared to 52 controls. When the offspring were split into NGT, IGT, and those with diabetes, all 3 groups had lower values compared to controls for mtDNA content, but the only difference that was large enough to be statistically significant was between the controls and the NGT group. There was a progressive increase in mtDNA content from the NGT to IGT to diabetes group. The authors suggested that the low level of mtDNA causes development of diabetes and mitochondrial biogenesis is then enhanced to compensate for this.

The only other group to investigate mtDNA content in diabetes, found approximately 50% less mtDNA in the skeletal muscle of 10 type 1 (p=0.006) and 40 type 2 patients (p<0.001), using Southern blotting. The authors also found increases in expression of RNA for some of the genes of the ETC that are encoded by the mitochondrial genome, but this was not due to an alteration in mtDNA copy number [192]. Much is unknown regarding the potential role of mtDNA in diabetes and its associated complications. Further research is required to establish if there are decreases in content, and what the consequences are. This study proposes a decrease in mtDNA in diabetes may be a consequence of hyperglycaemia-induced oxidative DNA damage. This may lead to dysfunctional mitochondria, and therefore a decrease in ATP production that may then affect cell function. Furthermore, because pancreatic β-cell mitochondria play a central role in glucose-stimulated insulin release [193], damage to the β-cell mitochondria will attenuate this response.
1.6 Project aims

This study aimed to investigate the novel hypothesis that increased oxidative stress in diabetes may have two key deleterious effects on DNA; 1) accelerated telomere attrition and thus accelerated ageing and premature senescence, 2) increased oxidative damage to mitochondrial DNA that leads to “drop out” of functional mitochondria, thereby reducing cellular energy generation. The combination of accelerated biological ageing and cellular energy depletion could explain many of the pathophysiological features of diabetes, which predominantly affect tissues with high metabolic/energy demands.

The study focussed on human cells from patients with diabetes mellitus and an age-matched non-diabetic volunteer control population. The cells used for the study were human peripheral blood cells and several techniques were adopted to fulfil the specific aims, which were as follows:

Specific Aims:

- To establish if the vasculature is phenotypically “older” in patients with diabetes compared to the control population by measuring aortic pulse wave velocity, an index of vascular stiffness, and therefore indicative of ‘biological’ age.

- To define whether there is increased oxidative damage to cellular DNA in patients with diabetes when compared to non-diabetic controls. DNA damage was quantified using the comet assay, with and without FPG enzyme,
previously shown to be a reliable, reproducible measure of oxidative DNA damage. The well-established lipid peroxidation measure of urinary isoprostanes was also used as a measure of systemic oxidative stress in the two study groups.

- To measure telomere lengths by Southern blotting, using DNA from human peripheral blood, to compare the age-related rates of telomere attrition in patients with diabetes and age-matched healthy controls, and establish any relationships with markers of vascular ageing and oxidative stress.

- To determine whether mitochondrial DNA content is depleted in people with diabetes as a consequence of oxidative damage by comparing the ratio of mitochondrial DNA to genomic DNA using real-time PCR.
Chapter 2: Introduction to methods

2.1 Introduction

To investigate oxidative stress and cardiovascular ageing in diabetes, and fulfil the aims in chapter 1 (p61), several methods were employed as detailed within the aims. This chapter introduces these methods, and gives justification for their use.

2.2 A marker for cardiovascular ageing

Changes within the cardiovascular system with ageing are well documented and include intimal thickening and increased stiffness accompanied by luminal dilatation (reviewed in [15, 16]). Pulse wave velocity (PWV) is a non-invasive measure of the velocity of the pressure wave that is generated in the heart at the start of systole, to travel between two sites of the arterial system. It is related to the square root of Young's elasticity modulus (stress/strain) and to the thickness/radius ratio (Moens Korteweg equation [194]), and is therefore a marker of aortic stiffness. It was measured in the study participants as an index of cardiovascular ageing.

To calculate PWV the time delay between the pulse pressure waves at two sites, a known distance apart, has to be calculated. An applanation tonometer is used, which compresses a peripheral artery therefore equalising circumferential pressures, allowing a peripheral pressure waveform to be recorded (figure 2.1).
To measure a pressure wave from the artery, the tonometer compresses the artery against the bone, equalising the circumferential pressures and allowing a peripheral pressure waveform to be recorded via the sensor.

The velocity is therefore generated by dividing the distance between the two sites at which the pressure wave is recorded, with the time taken from the first to second site. Although it is a superficial measurement and allows only an estimate of the distance travelled by the pulse, and is a composite measure of the varying stiffness over that distance, studies have found it to be an accurate, reproducible index of arterial stiffness [195], and a powerful independent cardiovascular risk factor in various populations [136, 196-198], including patients with diabetes.

2.3 Determination of oxidative damage to DNA

To investigate if there is increased oxidative damage to cellular DNA in diabetes, Single Cell Gel Electrophoresis (SCGE), or the comet assay, was carried out on peripheral blood cells of patients with diabetes and healthy controls. SCGE provides a rapid, visual method for assessing DNA breakage quantitatively in single cells, and is
based on the ability of strand breaks to relax super-coiled DNA. Essentially a cell suspension is embedded in agarose and lysed to remove cytoplasm, membranes, and most nuclear proteins. This leaves 'nucleoids'; super-coiled DNA arranged in loops, attached to the nuclear matrix, which unwind when placed in alkaline electrophoresis buffer. During electrophoresis those loops containing a break are free to migrate towards the anode, extending from the nucleoid to form a 'tail'. After staining, the cells are viewed by fluorescence microscopy and present as comet-like structures (figure 2.2), and are scored with the intensity of the tail relating to the extent of DNA strand breaks [199, 200].

![Figure 2.2: Peripheral blood 'comets'](image)

**Figure 2.2: Peripheral blood 'comets'**

*Example of the view seen through a microscope of comets showing DNA damage in cells from whole blood. The blue box represents the area of the comet, with the green box on top measuring background levels of fluorescence. The red vertical lines specify where the comet head and tail are according to fluorescence intensities.*

The assay was first introduced by Östling and Johanson [201], and later modified by Singh and colleagues [202] who introduced the alkaline conditions for the
electrophoresis allowing detection of alkali-labile sites, DNA cross-linking, and incomplete excision repair sites. Advantages of the assay include collection of data at the level of the single cell, the need for small numbers of cells per sample, its sensitivity for detecting low levels of DNA damage, flexibility, low cost, and ease of application. However, disadvantages include the lack of specificity, artefactual oxidation may take place, repair may occur during the process, and a portion of the DNA in the tail could be excised DNA from repair processes.

To specifically measure oxidised bases in DNA, lesion specific repair endonucleases can be used in conjunction with the comet assay to introduce breaks at the sites of damaged bases. Foramidopyrimidine DNA glycosylase (FPG) is such an enzyme from E coli. that recognises damaged bases including 8-oxo-7,8,dihydroguanine (8oxoG), 8-oxoadenine, and foramidopyrimidine (Fapy) derivatives such as Fapyadenine or Fapyguanine. The N-glycosylase releases the damaged purines from the double stranded DNA creating an apurinic (AP) site, which is then cleaved by the AP-lyase leaving a one base gap. This introduction of breaks into the DNA therefore increases the amount of DNA in the comet tails (Figure 2.3), and by subtracting the values for FPG buffer alone, the difference will be representative of the number of oxidised purines in that cell population.
Figure 2.3: Peripheral blood ‘comets’ treated with FPG enzyme

Example of the view seen through a microscope of comets showing oxidative DNA damage in cells from whole blood. The cells were incubated with FPG enzyme, which creates strand breaks at oxidised bases, and therefore increases the amount of DNA in the ‘tail’. The blue box represents the area of the comet, with the green box on top measuring background levels of fluorescence. The red vertical lines specify where the comet head and tail are according to fluorescence intensities.

Several studies in the past have compared methods to measure 8OHdG, the nucleoside of 8oxoG that is released during repair, and found large discrepancies. High performance liquid chromatography with electrochemical detection (HPLC/ED) and gas chromatography with mass spectrometry (GC/MS), which are more accurate for the detection of specific bases, show much higher levels of background damage compared with enzymic methods using repair endonucleases, such as the comet assay, alkaline elution, alkaline unwinding, and nick translation [164, 203, 204].
Epe's group [164] directly compared HPLC/ED and alkaline elution with repair endonucleases in various types of cell culture. The enzymic method gave much lower levels of background compared to the HPLC, but both techniques gave similar increases when additionally induced modifications were quantified, suggesting the difference is not due to an underestimate of background damage by alkaline elution but an overestimate by HPLC. This is supported by the different enzymic methods showing similar levels for estimates in background damage [164, 203], although they are essentially indirect. It is also thought that cells have an effective base excision repair process eliminating potentially mutagenic bases as quickly as possible, therefore keeping the steady-state level of oxidative damage low (reviewed in [205]).

2.4 Determination of oxidative damage to lipids

As a further measure of oxidative stress within the study groups, lipid peroxidation was investigated. Isoprostanes (isoPs) are prostaglandin-like compounds formed in vivo from non-enzymatic free-radical catalysed peroxidation of arachidonic acid [206]. They are initially formed in situ in the phospholipid domain of cell membranes, and are subsequently cleared by phospholipases, enter the general circulation, and are excreted in urine [207]. Levels of urinary isoprostanes are therefore a measure of oxidant stress in vivo; however, they are a myriad of species formed in oxidant stress, so studies of the effects of single isoPs may seriously underestimate concentration-effect relationships in vivo. A urinary measure is better than a plasma measure as it prevents measurement of isoPs generated from auto-oxidation of lipid containing samples, and is therefore more stable to storage at −20°C [208]. A disadvantage of using urine is the possibility the results reflect to some extent the formation of isoPs in the kidneys; however, the correlation of concentrations of a specific isoP and its
metabolite in urine suggests it reflects systemic, rather than renal, formation of the compound.

Meagher and Fitzgerald [209] reviewed indices of lipid peroxidation and concluded independent measures of stable products of lipid peroxidation in urine, such as 4-HNE and specific isoPs have particular appeal, which agrees with the findings of Moore and Roberts [210]. The most commonly employed method for measurement of isoPs is a negative chemical ionisation GC/MS assay. This method is highly sensitive and accurate, however it is labour intensive and the technology is not widely available. This has led to the development of immunoassays for specific F2-IsoPs [208], which are more stable than other subgroups e.g. D2/E2-isoPs, and in particular 15-isoP-F2t (also known as 8-epi-PGF2α or 8-iso-PGF2α), now the best characterised isoP. Wang et al [208] investigated urinary 15-isoP-F2t levels in humans by radioimmunoassay and enzyme-linked immunoassay (ELISA), and validated the latter by comparison with GC/MS. The results showed a strong correlation over 9 samples for the two methods with an R-value of 0.99 (p-value < 0.001). Previous studies have carried out further validation showing no significant daily variation in healthy subjects [208], and similar concentrations of urinary isoprostanes on consecutive days [211, 212]. Wang et al [208] and Richelle and colleagues [213] also showed a single sample of urine in the morning adequately represents daily isoP excretion in humans.

2.5 Telomere length measurement

The hypothesis of this study is that there is increased oxidative stress in diabetes, and this causes increased damage to DNA, particularly telomeric DNA. To measure telomere length, Southern blotting using enzymes with restriction sites in sub-
telomeric DNA, yields the mean or median terminal restriction fragment (TRF) length of all the telomeres within the cell population. The resultant blots are hybridised and probed to detect the TTAGGG repeats of telomeres. Figure 2.4 shows an example of TRFs as they present on an autoradiograph. They appear as a smear due to the considerable variability in telomere length within an individual, not only between cells, but also between chromosomes [214]. This robust method is very time-consuming and the variation in amount of sub-telomeric DNA in different individuals could detract from the accuracy. Studies using quantitative fluorescence in situ hybridisation (FISH), where the telomeric repeats are directly labelled and individual cells can be assessed, have correlated well with TRF values obtained from Southern blotting, indicating it is a valid technique. [215]
**Figure 2.4: Autoradiograph of TRFs**

Example of an autoradiograph of a southern blot for TRF lengths after it was hybridised and probed with a radioactively labelled probe. Each lane represents the TRF lengths of all the cells within a DNA sample from the peripheral blood of an individual. 1kb and high molecular weight markers are drawn on after to allow determination of the mean and median TRFs within each lane, and therefore for an individual.

### 2.6 Quantification of mitochondrial DNA

Another target for oxidative damage is mitochondrial DNA. If there is increased oxidative stress in diabetes, this could cause increased damage to mitochondrial DNA, and possibly result in a decrease of mitochondrial copy number in patients with diabetes compared to controls. Southern blotting to generate a ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) is a well-established method for the
quantification of mtDNA [178]. Disadvantages of the method include the large amounts of DNA required, it is time-consuming, and may produce artefact during the process; for example incomplete DNA transfer of the larger mtDNA fragments compared to the nuclear DNA fragments, and contamination of the nuclear DNA radioactive signal with the much stronger mtDNA signal. Real-time PCR is becoming increasingly important for many applications, and has been developed by many groups to quantify mtDNA [178, 216-218].

There are several fluorescent chemistries to detect and quantify gene transcripts, including SYBR Green, a dye that fluoresces when bound to double-stranded DNA. This allows the accumulation of double-stranded DNA to be monitored in real-time. Quantitative assessments based on a single reading taken at the end of the PCR reaction are inherently inaccurate due to the influence of limiting reagents and small differences in reaction components or cycling parameters. Instead, by looking at the threshold cycle (Ct), which is defined as the cycle at which fluorescence is determined to be statistically significant above background, these inaccuracies are absent.
Figure 2.5: Real-time PCR amplification plot

The solid blue line represents the threshold fluorescence, which is used to determine the threshold cycle (Ct). The 5 curves represent standards of increasing template quantity (from right to left), with the no template control represented by the green horizontal line below the threshold fluorescence.

The above graph in figure 2.5 is an example of an amplification plot for the standards used in real-time PCR. It demonstrates that increasing template quantity results in a lower threshold cycle (Ct), and this relationship is expressed mathematically with Ct being inversely proportional to the log of the original quantity.

To quantify mtDNA, a segment of the mitochondrial genome is amplified, and normalised for differences in template DNA by a separate PCR of a segment of a single copy gene in the nuclear genome. Use of a passive reference dye provides a stable baseline to which all standards and samples are normalised. This compensates...
for any variability in fluorescence between wells caused by pipetting errors or instrument variability.

Real-time PCR to quantify mitochondrial DNA is a relatively new technique, but previous studies have shown it to be consistent and reproducible [217, 218], with data concordant between Southern blotting and real-time PCR [178, 216]. Chabi et al [216] also suggested that, in addition to the advantages of time saved and small amounts of DNA required, real-time PCR has a higher sensitivity than southern blot analysis.
Chapter 3: Materials and methods

3.1 Materials

All chemicals were purchased from Sigma (Dorset, UK) or Fisher (Loughborough, UK), unless stated otherwise.

3.2 Patient recruitment

Ethical approval for this study was sought and granted by the Leicestershire Research Ethics Committee (Ref: 5881; Appendix 1).

Patients with hypertension and diabetes were recruited whilst attending outpatient clinics at Leicester Royal Infirmary. Initial screening was carried out by examining patient notes to exclude any inflammatory diseases, chronic renal failure, and newly diagnosed diabetics. Controls were recruited by posting an invitation on the University of Leicester e-bulletin, which is sent to staff and students every week. All interested individuals were given information regarding the study (appendices 3 and 5) and informed consent was obtained from those willing to take part (appendices 4 and 6).

Each individual (patients and controls) was given an hour appointment at the Clinical Research Unit at Leicester Royal Infirmary, where all investigations were carried out. All participants were asked to refrain from caffeine and smoking for two hours prior to their appointment.
3.3 Investigations

Every study participant underwent the following investigations: a history was taken including lifestyle questions and family history (example of proforma in appendix 2); blood was sampled by venipuncture; height, weight, and blood pressure were recorded; a 12 lead ECG was carried out; pulse wave velocity and tonometry were measured; and a urine sample taken. Patient notes were well documented with diabetic complications.

Blood was sent to Leicester Royal Infirmary Chemical Pathology and Haematology departments for determination of urea and electrolytes, glycated haemoglobin (HbA1c), a random glucose, full lipid profile, and full blood count. Urine was checked for pH, and presence of blood, ketones, and glucose. A sample was also sent to Leicester Royal Infirmary Chemical Pathology department for measurement of albumin and creatinine.

For the laboratory measures, approximately 8ml of blood was taken into a lithium heparin coated vacutainer and immediately stored on ice in the dark. From the urine, a further 3 samples of 1ml each were immediately placed on ice prior to freezing and storage at -80°C.

All subjects had been seated at rest for at least 10 minutes prior to blood sampling, and rested supine for 10 minutes prior to blood pressure, and pulse wave velocity recording. Blood pressures were recorded using a semi-automated oscillometric device (Omron 705CP; Omron Healthcare, Milton Keynes, UK). Three measures
were taken, five minutes apart, and the mean value of the last two measurements was taken as representative of brachial blood pressure.

3.4 Pulse wave velocity (PWV) using applanation tonometry

Applanation tonometry to measure pulse wave velocity was carried out by Dr P Lacy immediately following determination of brachial blood pressure. The carotid and femoral artery pressure waveforms were sequentially recorded using a Millar tonometer (SPC-301; Millar Instruments Houston, Texas, USA) and gated to a three lead ECG. Waveforms were processed using dedicated software (SphygmoCor v7, AtCor, Sydney, Australia). The integral system software was used to calculate an average artery waveform after pressure waveforms not achieving the automatic quality controls, specified by the SphygmoCor software, were rejected. The time delay was calculated using a foot-of-the-wave method (intersecting tangents) using proprietary SphygmoCor software. The surface distance from supra-sternal notch to each recording site was measured and the total transit distance calculated by subtracting the notch to carotid distance from the notch to femoral distance. PWV was calculated using the difference in distance divided by the respective time delay. Data from the mean of two PWV measurements was taken for each subject. An example of the output from applanation tonometry is shown in figure 3.1.
Figure 3.1: Applanation tonometry to calculate pulse wave velocity

Sites on body for applanation tonometry to calculate PWV, with an example of the pressure waves gated to a 3-lead ECG for both the carotid and femoral sites, and the subsequent display of results. $d$ represents the distance measured over the surface of the body used in the calculation for PWV.

3.5 The comet assay

The comet assay was performed according to the method developed by Singh and colleagues [202], with minor modifications. All stages prior to neutralisation of the slides were carried out in dimmed light to minimise DNA damage by photosensitisation.

For each sample, 40µl whole blood was mixed with 600µl of 0.6% (w/v) low melting point (LMP) agarose (Invitrogen, Paisley, UK), of which 60µl was laid onto 9
replicate slides previously coated with a layer of 1% (w/v) normal melting point agarose (Invitrogen, Paisley, UK). The slides were left on ice for 5-10 minutes to set, before adding a second layer of LMP agarose. This second layer was also allowed to solidify on ice. The slides were then immediately immersed in chilled lysis solution (2.5 M NaCl, 100 mM Na₂EDTA (Diamino ethanetetra - acetic acid disodium salt), 10 mM Tris, pH 10, with 1% (v/v) Triton X-100 and 10% (v/v) Dimethyl sulphoxide (DMSO) was added just prior to use), in Wilson jars and left at 0-4°C overnight. After lysis, 6 slides were removed from the Wilson jars and washed three times in FPG enzyme buffer (40mM HEPES, 0.1M KCl, 0.5mM EDTA, 0.2mg/ml Bovine serum albumin (BSA), pH 8.0). FPG enzyme (New England Biolabs (UK) Ltd. Hitchin, UK) at a concentration of 1.6 units/ml in enzyme buffer (50µl) was laid onto 3 replicate slides with a coverslip, and 50µl FPG enzyme buffer alone was laid onto the other 3 slides with a coverslip. All 6 slides were then incubated in the dark at 37°C for 30 minutes. After 15 minutes the remaining 3 slides were removed from lysis and washed 3 times with TE buffer (10mM Tris-Cl, 1mM EDTA, pH 7.5). After 30 minutes incubation the slides were taken out, the coverslips removed, and all 9 slides placed in a horizontal gel electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH >13) and left in solution for 30 minutes at 4°C to allow unwinding of the DNA. After electrophoresis at 4°C for 20 minutes at 25V and 300mA, slides were washed three times with neutralising solution (0.4 M Tris-HCl, pH 7.5), exposed to cold 100% ethanol, and air-dried.

When ready to score, 60µl of SYBR Green 1 stain (Molecular Probes, Oregon, USA) was added under a coverslip, and cells were visualised under a green-blue light (wavelength 485nm) using a fluorescence microscope (Nikon Eclipse E400), and then
scored using Komet Analysis 4.0 (Kinetic Imaging Ltd, Liverpool, UK). A total of 50 cells per slide were scored, with 3 replicate slides per condition, i.e. TB buffer, FPG enzyme buffer alone, and FPG enzyme. The mean percentage of tail DNA (% Tail DNA) was taken as a measure of DNA damage, which essentially represents the intensity of the comet tail relative to the intensity of the whole comet. The measure of oxidised purines was calculated by subtraction of the mean tail DNA score with FPG enzyme buffer alone, from that with the FPG enzyme.

3.6 Lipid peroxidation measure

15-isoprostane $F_{2\alpha}$ ($15$-isoP $F_{2\alpha}$) levels were measured using the commercially available Enzyme Immunoassay for Urinary Isoprostanes from Oxford Biomedical Research (Biogenesis Ltd., Dorset, UK). The suppliers have validated the assay by direct comparison with GC/MS, and found a correlation ($R^2$) of > 0.8.

Initially, the supplied 15-isoP $F_{2\alpha}$ standard was diluted in supplied dilution buffer to final concentrations ranging 0ng/100μl to 10ng/100μl. Thawed urine samples (150μL), and standards, were mixed with 150μL enhancing reagent that eliminates interferences due to non-specific binding, and 150μL 15-isoP $F_{2\alpha}$-HRP (horseradish peroxidase). After mixing, 200μL aliquots were transferred to the supplied 96 well microplate, and incubated at room temperature for 2 hours. The 15-isoP $F_{2\alpha}$ in the samples, or standards, competes with the 15-isoP $F_{2\alpha}$-HRP for binding to a polyclonal antibody, specific for 15-isoP $F_{2\alpha}$, coated on the microplate. The plate contents were emptied, and it was washed 3 times with approximately 400μL supplied wash buffer each time. The HRP activity resulted in colour development when 200μL TMB (3,3', 5,5'-Tetramethylbenzidine) substrate was added, which was then stopped after 30
minutes with 50µL of 3N sulphuric acid. The colour intensity developed is proportional to the amount of 15-isoprostane F₂ accounts, and inversely proportional to the amount of unconjugated 15-isoprostane F₂ in the samples, or standards. The plate was then read at 450nm in a Cytofluor plate reader (Applied Biosystems).

The standards were used to generate a standard curve and all values were expressed as a percentage of the zero standard, i.e. the value obtained when only HRP conjugate is present. Final concentrations of isoPs in the samples were then read from the standard curve and corrected by creatinine concentration (determined from the sample sent to the Chemical Pathology Department), to remove any effect of urinary volume. If urine samples lay beyond the range of the standard curve, they were repeated after dilution with the supplied dilution buffer.

3.7 TRF length measurement
3.7.1 DNA extraction
Whole blood was frozen and stored at -80°C, within 2 hours of venipuncture. DNA extraction was carried out using the QIAamp commercial kit (QIAGEN, Crawley, UK). Blood was thawed at room temperature and 200µl was added to an eppendorf containing 20µl protease. Buffer AL (200µl) was then added and the sample was pulse vortexed for 15 seconds, briefly centrifuged, and then incubated at 56°C for 10 minutes. Ethanol (200µl) was added and the sample was pulse vortexed again for 15 seconds, before being transferred to a QIAamp spin column and centrifuged at 12 000g for 1 minute. Washes were then carried out according to the manufacturer’s recommendations, firstly with 500µl Buffer A1 with 1 minute centrifuging at 12 000g, and then with 500µl Buffer A2 with 3 minutes centrifuging at 12 000g. Elution
buffer (55\mu{l}) was then added and the samples left at room temperature for 10 minutes. A final centrifuge at 12 000g for 1 minute left the extracted DNA in the eluate. Two preparations per subject were then pooled and stored at -80°C until ready for quantification.

3.7.2 DNA quantification

DNA was quantified using the PicoGREEN dsDNA quantitation kit from Molecular Probes (Invitrogen, Paisley, UK). The fluorescent dye picoGREEN binds primarily to double-stranded DNA, and the protocol has been developed to minimise the fluorescence contribution of RNA and single-stranded DNA. When excited with 485nm light, the fluorescence emission of the dye at 530nm increases significantly in the presence of DNA.

Lambda DNA standards, diluted in TE at concentrations of 0-1ng/\mu{l}, were assayed in duplicate to generate a standard curve. Samples, from the QIAamp DNA extractions, were diluted 1000-fold and 100\mu{l} was added to a 96 well microplate. Picogreen reagent (100\mu{l}) was then added to each well and incubated at room temperature for 5 minutes. The fluorescence was read in a Cytofluor plate reader (Applied Biosystems) and concentrations were calculated from the standard curve (figure 3.2). The samples were then stored at 4°C until TRF lengths were measured.
Figure 3.2: DNA quantification standard curve

Example of the standard curve generated from the picoGREEN dsDNA quantitation kit. The fluorescent dye picoGREEN binds to double-stranded DNA, therefore increasing fluorescence (y-axis), represents larger quantities of DNA (x-axis).

3.7.3 Restriction enzyme digestion

DNA (1-2µg in 40 µl) was digested with 2µl of the restriction enzymes Hinf I and Rsa I (New England Biolabs (UK) Ltd., Hitchin, UK) at 37°C for at least 3 hours, with the resultant digested DNA resolved by electrophoresis on a 0.5% (w/v) agarose gel. High molecular weight markers (heated at 65°C for 5 minutes prior to loading) and 1kb ladders (Invitrogen, Paisley, UK) were also loaded into vacant wells. Electrophoresis was in 1x TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0) buffer for 1000-volt hours. The gel was stained in 1 litre of distilled water containing 0.5µg/ml ethidium bromide on a rocking platform, and then viewed in the Alphalmager gel documentation apparatus, and an image was taken. The gel was then depurinated for 10 minutes with
agitation in 0.125M HCl, to create breaks in the relatively large DNA fragments to allow easier transfer of the DNA, and then placed in 5 to 10 volumes of alkaline blotting buffer, (0.4M NaOH, 1M NaCl) for 15 minutes with agitation. Fresh buffer was added, and then the gel was agitated for a further 20 minutes.

3.7.4 Southern blotting
The DNA was transferred to positively charged nylon membrane (Hybond-XL; Amersham, Chalfont, UK) by Southern blotting overnight with alkaline blotting buffer (ABB). Nylon membrane and 3MM chromatography paper were soaked briefly in water and in ABB for greater than 5 minutes prior to use, and the blot was constructed as in figure 3.3.

**Figure 3.3: Southern blot apparatus**

A glass plate is placed over a container containing alkaline blotting buffer (ABB). Onto this plate, a wick of 3MM chromatography paper was placed with the edges in the ABB. The agarose gel is placed on to the 3MM, followed by: a positively charged nylon membrane, 2 sheets of 3MM cut to the same size as the gel, 10 sheets of blotting paper, a stack of paper towels, and a small weight.
Blotting was allowed to proceed for more than 16 hours, and then the membrane was placed between 3MM paper and the transferred DNA fixed by baking at 80°C for 2 hours. The blot was then washed in distilled water and stored at 4°C, until ready for hybridisation.

3.7.5 Hybridisation

Blots were hybridised and probed with a $^{32}$P labelled probe. This was made by adding 5 pmol of (TTAGGG)$_4$ oligo nucleotide, 2μl T4 polynucleotide kinase (PNK; New England Biolabs, Hitchin, UK), 5μl 10x PNK buffer (New England Biolabs, Hitchin, UK), and 5μl $\gamma^{32}$P-ATP (9.25MBq; Amersham, Chalfont, UK) to an Eppendorf and incubating at 37°C for 30 minutes. The reaction was then stopped by heating the probe at 80°C for 10 minutes, prior to purification using the QIAquick nucleotide removal kit (QIAGEN, Crawley, UK).

The QIAquick system combines spin column technology with the selective binding properties of a silica-gel membrane, with buffers provided to optimise DNA yield. The protocol removes enzymes, salts, and unincorporated nucleotides, and the pure DNA is eluted with 200μl TE.

Briefly, 10 volumes of Buffer PN were added to the DNA to be purified, and transferred to a spin column. This was then centrifuged at 3000g for one minute and transferred to a new collection tube and washed twice with 500μl Buffer PE. Again, after each wash the column was centrifuged at 3000g for 1 minute, and placed in a new collection tube. A further centrifuge at 12 000g for 1 minute, with nothing added, was carried out prior to the final elution step. Elution buffer (200μl) was added, and
the column was centrifuged at 12,000g for 1 minute, producing purified DNA in the eluate.

High molecular weight (HMW) and 1 kb ladders were dCTP-labelled using Rediprime™ II, a commercial kit available from Amersham (Chalfont, UK). Briefly, 5ng of 1 kb ladder and 15ng of HMW marker (Invitrogen, Paisley, UK) in 45μl of TE were denatured at 100°C for 5 minutes and chilled on ice before adding to a Rediprime reaction tube. A buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow enzyme, and random primers were supplied in the tubes. 5μl of Redivue 32P dCTP (9.25MBq; Amersham, Chalfont, UK) was added after the markers, mixed well, and then incubated at 37°C for 10 minutes. The reaction was stopped with 5μl of 0.2M EDTA, and then purified as above with the QIAquick nucleotide removal kit. The marker probes were then stored at -20°C until ready for use, when they were denatured again by boiling for 5 minutes and chilled. The marker probes were then added to hybridisation buffer (0.5M PO₄, 1mM EDTA, 7% sodium dodecyl sulphate (SDS), 1% BSA, pH 7.2) at a concentration of 0.5μl marker probe/ml of hybridisation solution, along with all 200μl of purified telomere probe. The blot was pre-hybridised for at least 30 minutes in hybridisation buffer at 50°C before replacing with the hybridisation solution (buffer and probes), and placed in the Hybaid oven for at least 2 hours at 50°C. Blots were then washed briefly with 2 x SSC (150mM NaCl, 15mM sodium citrate), 0.1% (w/v) SDS, and excess radio-label was rinsed away, followed by two washes in 1 x SSC, 0.1% (w/v) SDS at room temperature, until the background radioactivity reached a suitably low level (<10 counts per second). Finally, the membrane was exposed to a Kodak X-Omat Ar Film (Sigma) at -80°C and analysed using Alphalmager gel documentation apparatus and Telometric 1.2 software [219].
The software calculates the average grayscale intensity for each row of pixels in the smear. These intensities were then used to obtain relative frequencies for each telomere length (TL). A second data set of relative frequencies was generated from linear interpolation of uniformly spaced telomere lengths. The software then calculated the statistics as below:

\[
\text{Mean} = \frac{\sum_{i=1}^{n} \gamma_i L_i}{\sum_{i=1}^{n} \gamma_i} \quad \text{Median} = L_j \text{ such that } \frac{\sum_{i=1}^{j} \gamma_i}{\sum_{i=1}^{n} \gamma_i} = \frac{1}{2}
\]

Where \( n \) is the total number of uniformly spaced telomere lengths, \( L_i \) is the \( i \)th telomere length, \( \gamma_i \) is the relative frequency of \( L_i \). Two reference samples were run on every gel to check inter-gel variability.

3.8 Real-time PCR

A 222-bp fragment of the mitochondrial genome, accession number J01415, was selected for quantification of mitochondrial DNA (mtDNA), and to normalise for differences in input DNA, a separate real-time PCR to quantify a 75-bp fragment of the single copy human gene 36B4, accession number AC004263, was used. The primers; \text{mt}14620 (5'-CCCAACCAACCCCTTACTAAACCCA-3') and \text{mt}14841 (5'-TTTCATCATGCGGAGATGTTGGATG-3') [220] for the mitochondrial segment, and 36B4u (5'-CAGCAAGTGGGAAGGTGTAATCC-3') and 36B4d (5'-CAGCAAGTGGGAAGGTGTAATCC-3') [221] for the nuclear genome segment, were supplied by Invitrogen (Paisley, UK).

A standard curve was generated by serial dilutions of a reference DNA sample. All dilutions of reference and sample DNA were in TE made with sterile distilled water.
The reference sample was a mixture of equal quantities of whole blood from 4 healthy volunteers, which had been mixed together gently, and the DNA extracted and quantified as described in sections 3.7.1 and 3.7.2. This reference DNA was initially diluted to a stock supply of 10ng/μl, and then serially diluted to give quantities of 3.125ng to 50ng for the nuclear DNA PCR. TE alone was used as the no template control (NTC). The mitochondrial PCR required further dilution of the reference sample to 200pg/μl in TE, due to the greater number of copies of the mitochondrial genome per cell. This was then serially diluted to give quantities of 62.5pg, to 1ng, with TE alone again as the NTC.

All sample DNA had been previously quantified as described in section 3.7.2, and was initially diluted to 3ng/μl, 5μl of which was used for each reaction in the nuclear DNA PCR. For the mtDNA PCR, the samples were further diluted to 50pg/μl, which was then ready for amplification.

Stratagene's Brilliant SYBR Green QPCR Core Reagent Kit was used for all the PCR reagents. Each 25μl reaction contained 5μl of template DNA, 2.5μl 10x Core PCR buffer, 2mM Magnesium Chloride, 0.8mM of each nucleotide (dATP, dGTP, dCTP, dTTP), 0.1μM of both forward and reverse primers, 8% (v/v) Glycerol, 3% (v/v) DMSO, 0.5x SYBR Green, 30nM 6-ROX reference dye, and 1.25 units SureStart Taq DNA polymerase. The quantities were the same for amplification of both the mitochondrial and nuclear DNA fragments.

PCR was carried out in the Stratagene MX4000 quantitative PCR instrument. There was an initial 10 minute denaturing step at 95°C, before 40 cycles of: 30 seconds at
95°C; 60 seconds at 60°C; and 30 seconds at 72°C. The melt curve followed with 1 minute at 95°C, 30 seconds at 55°C, then an increase of 1°C every 30 seconds for 41 cycles. This was the same thermal profile for amplification of the mitochondrial and nuclear DNA segments. Initial quantities were calculated from the standard curve, using the threshold cycle of each sample (figures 3.4-3.7). Samples were performed in triplicate and means were calculated, allowing ratios of mtDNA to nuclear DNA to be generated for every individual.

*Figure 3.4: Mitochondrial PCR standard curve*

Example of the standard curve generated for the polymerase chain reaction (PCR) to quantify mitochondrial DNA. The x-axis is the initial template quantity of DNA within a well, with the y-axis the threshold cycle (Ct), i.e. the cycle that fluorescence goes above the threshold. Ct is inversely proportional to the log of the original quantity.
**Figure 3.5: Mitochondrial PCR standard curve with samples**

Example of the standard curve generated for the polymerase chain reaction (PCR) to quantify mitochondrial DNA as in figure 3.4, with samples also plotted. The solid blue boxes represent the standards, and the open blue boxes represent the samples. The unknowns are plotted on the standard curve according to their threshold cycle (Ct) values.
Figure 3.6: Nuclear PCR standard curve

Example of the standard curve generated for the polymerase chain reaction (PCR) to quantify nuclear DNA. The x-axis is the initial template quantity of DNA within a well, with the y-axis the threshold cycle (Ct), i.e. the cycle that fluorescence goes above the threshold. Ct is inversely proportional to the log of the original quantity.
Figure 3.7: Nuclear PCR standard curve with samples

Example of the standard curve generated for the polymerase chain reaction (PCR) to quantify nuclear DNA as in figure 3.6, with samples also plotted. The solid blue boxes represent the standards, and the open blue boxes represent the samples. The unknowns are plotted on the standard curve according to their threshold cycle (Ct) values.
3.9 Statistics

All statistical analyses were carried out using Minitab Statistical Software release 13.32. All data was tested for normality, using the Anderson-Darling test. Normally distributed data is displayed as mean ± standard error of the mean (sem), and non-normally distributed data as median ± standard deviation (SD). Variance was calculated as the square of SD, and the coefficient of variation as the SD divided by the mean. Direct comparisons between data from patients with diabetes and controls were made using a non-paired student’s t-test or a non-parametric test (Mann-Whitney U-test) where data distribution did not conform to normality. Step-wise multiple regression analysis was used to determine predictors for many of the variables measured. A value of $P<0.05$ was taken as significant.
Chapter 4. Physiological data in both study populations

4.1 Introduction

Diabetes Mellitus is one of the major disorders worldwide, and its prevalence has rapidly increased in recent years [3]. It is characterised by a state of chronic hyperglycaemia, and classically associated with the symptoms of excessive thirst, increased urine volume, and, in severe cases, weight loss. Type 1 diabetes usually has its onset in children or younger adults and is characterised by an absolute deficiency of insulin due to pancreatic failure. Type 2 diabetes usually presents in middle age and older, and is characterised by a progressive worsening hyperglycaemia, due to a resistance to insulin's action. Thus, patients with type 2 diabetes are not insulin-dependent, but they may require insulin to help control their hyperglycaemia.

Whatever the aetiology of the diabetes (type 1 or type 2), the complications are similar. They can be conveniently divided into macrovascular and microvascular complications that culminate in premature morbidity and mortality, primarily from cardiovascular disease [24]. A number of known risk factors for vascular disease, such as hypertension, dyslipidaemia, and central obesity, are commonly found in patients with diabetes, but they only account for about 25% of the excess risk [28]; therefore, hyperglycaemia is suggested as the major contributing factor in the development of vascular complications in diabetes, as it is the main common feature [222].

This study aims to investigate oxidative stress, which may be increased in diabetes as a consequence of hyperglycaemia, and vascular ageing in patients with diabetes. To
establish profiles of the study groups, physiological data were gathered, and any population differences investigated. These data were than available for analysis with further study data to investigate the effects, if any, of physiological variables.

4.2 Aim

This chapter aims to examine the physiological characteristics in the all the volunteers recruited, including any medications being taken at the time of investigation and disease-associated complications, both of which may affect the participants’ physiology. The correlations with age, and any differences between the study populations will be investigated.

4.3 Methods

Recruitment was carried out as described in chapter 3.1. The initial methods of recruiting patients with diabetes whilst attending outpatient clinic, and taking blood and urine samples whilst there, were abandoned. This was largely due to the lack of time available to spend with each patient, and it also required a follow-up appointment to enable pulse wave velocity measurements to be taken. There was low attendance for follow-up appointments and it often meant only one individual a day, every day of the week and this was a very inefficient use of time. This led to a new recruitment strategy whereby we provided information about the study at the outpatient clinic, and asked patients to leave contact details if they were interested in participating. Those interested were then contacted, given any extra information required regarding the study, and then booked in for an appointment if they were still interested. This enabled certain days to be arranged for seeing several patients in one day, and for all investigations for an individual to be carried out within an hour visit
to the clinic research unit. This method of recruitment optimised time, with several
samples being processed at one time, and left other days free for laboratory work.

Blood and urine samples were sent to the Haematology and Chemical Pathology
departments of the Leicester Royal Infirmary for determination of the measures
detailed in chapter 3.2.

4.4 Recruitment of patients with diabetes

After initial screening, 53 diabetics were recruited, 2 of which were excluded: one
suffered from Still’s disease as a child, and has rheumatoid arthritis as an adult and
was unsuitable for pulse wave velocity studies; and the other was in atrial fibrillation
at the time of investigation, which was an exclusion criterion.

<table>
<thead>
<tr>
<th></th>
<th>Type 1 (n=24)</th>
<th>Type 2 (n=27)</th>
<th>All diabetics (n=51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age (years)</td>
<td>46</td>
<td>61</td>
<td>54</td>
</tr>
<tr>
<td>Range (years)</td>
<td>31-67</td>
<td>40-78</td>
<td>31-78</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>19/5</td>
<td>19/8</td>
<td>38/13</td>
</tr>
<tr>
<td>Mean Disease duration (years)</td>
<td>22</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>Range (years)</td>
<td>4-42</td>
<td>1.5-33</td>
<td>1.5-42</td>
</tr>
</tbody>
</table>

*Table 4.1: Demographic characteristics of patients with diabetes*

*Age, gender, disease duration, and numbers of patients with type 1 and type 2 diabetes.*
Demographic characteristics of the 51 patients with diabetes are shown in table 4.1. Those with type 1 diabetes were younger (mean age 41 years versus 61 years) and had longer known disease duration (mean duration 22 years versus 10 years) compared to type 2 patients, due to the earlier onset characteristic of type 1 diabetes. Overall, there were fewer women in the group, only 25.5% overall, and 20.8% and 29.6% in the type 1 and type 2 subgroups respectively.

Cardiovascular disease is the leading cause of morbidity and mortality in patients with diabetes [7, 24]; therefore the majority of long-term complications associated with diabetes are vascular in nature, and are classified as macro- or microvascular. Neuropathy, a further common complication in diabetes, is often grouped with the microvascular complications although the underlying pathology may be neurological or vascular. In table 4.2, the numbers of patients with neuropathy are not included in the microvascular numbers, but are displayed separately.
### Table 4.2: Complications in patients with diabetes

Numbers of macro- and microvascular complications within the patients with diabetes, with neuropathy and hypertension displayed separately. HT = hypertension, BL = borderline hypertension, and wc = white coat hypertension.

The numbers of complications present in the patients with diabetes are displayed in table 4.2. The number of complications within an individual varied greatly, from none of the above (n=8; 15.7%), to all of those in the table (n=4; 31.4%). In both the type 1 and type 2 patients, approximately half had microvascular disease, with another 25% of type 1s and 33.3% of type 2s with neuropathy. The patients with type 2 diabetes had a higher percentage of macrovascular disease (25.9%) compared to type 1 (8.3%).

The patients in this cohort maintained their glycaemic control by insulin, oral hypoglycaemics, or diet control (table 4.3). Predictably, many were also receiving blood pressure and lipid lowering medications with potential cardiovascular effects (table 4.4).
### Table 4.3: Methods of glycaemic control in the patients with diabetes

Each column displays the number and percentage of patients with diabetes using each method of glycaemic control.

<table>
<thead>
<tr>
<th>Glycaemic control</th>
<th>Insulin</th>
<th>Oral hypoglycaemics</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>41 (80.4)</td>
<td>14 (17.6)</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

### Table 4.4: Blood pressure and lipid lowering medication in the patients with diabetes

Each column displays the number and percentage of patients on the respective medication. CCB, calcium channel blockers; ACE I, angiotensin II converting enzyme inhibitors; A II antag, angiotensin II receptor blockers.

<table>
<thead>
<tr>
<th>Diuretic</th>
<th>CCB</th>
<th>ACE I/ A II antag</th>
<th>α-blocker</th>
<th>β-blocker</th>
<th>Statin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>8 (15.7)</td>
<td>7 (13.7)</td>
<td>21 (41.4)</td>
<td>6 (11.8)</td>
<td>7 (13.7)</td>
</tr>
</tbody>
</table>

All further data and analyses refer to all the patients with diabetes grouped together, as the numbers with type 1 and type 2 separately are not sufficient and it is not possible to compare the two groups with diabetes due to their different age ranges. The factor of whether they are type 1 or 2 was entered into multiple regressions for data obtained from the group with diabetes, although numbers may be too small to show differences between them.
4.5 Recruitment of healthy controls

108 volunteers were recruited as healthy controls. On investigation 7 were excluded: 2 due to high blood pressure, 1 with morbid obesity, 2 were potentially diabetic, 1 had rheumatoid arthritis, and 1 due to excessive smoking. The remaining 101 had an average age of 46 years, ranging from 19 to 75 years, and consisted of 59 females (median age 47 years) and 42 males (median age 45 years). There were no significant differences in ages between the sexes, with a p-value of 0.826 when a Mann-Whitney U-test was carried out on the data.

Three of the control volunteers were receiving medication with potential cardiovascular effects. One was taking a diuretic alone, another was on the combined therapy of a calcium channel blocker and an ACE inhibitor, and the third was receiving a statin.

From the 101 controls, 51 were age and sex-matched to the diabetic group, which included the three detailed above on blood pressure and lipid lowering medication.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age (range)</td>
<td>51 (32-75)</td>
<td>55 (31-78)</td>
<td>0.359</td>
</tr>
<tr>
<td>Gender (M/F) n</td>
<td>38/13</td>
<td>38/13</td>
<td>1.000</td>
</tr>
<tr>
<td>Ethnicity (White/Indo-Asian/Other) n</td>
<td>46/3/2</td>
<td>47/3/1</td>
<td>0.654</td>
</tr>
</tbody>
</table>

*Table 4.5: Matching controls to patients with diabetes*

Ages, genders, and ethnicity of the patients with diabetes and their matched controls, with p-values from Mann-Whitney U-tests to determine any differences.
Table 4.5 details the data used to match the controls and patients with diabetes. The control group's ages were not normally distributed; therefore, the Mann-Whitney U-test was used to investigate any differences. This same test was applied to the gender and ethnicity data after it had been ranked. The p-values in the table indicate there were no significant differences between ages (p=0.359) and ethnicity (p=0.654) of the two study populations, and the genders matched identically (p=1.000).

4.6 Smoking history

It is well known that smoking is a risk factor for cardiovascular disease [24, 223], and therefore has detrimental effects on the cardiovascular system. Previous studies have also suggested it increases oxidative stress [224], [225], and may therefore affect the study results.

![Control Population Smoking History](image1)

![Diabetic Population Smoking History](image2)

**Figure 4.1: Smoking history of both study populations**

(a) and (b) illustrate the numbers of current smokers, ex-smokers, and those who have never smoked in the 51 controls and patients with diabetes respectively. The numbers displayed around the outside of the pie chart are the number of individuals that segment represents.
There were larger numbers of current (n=10) and ex-smokers (n=16) in the patients with diabetes (figure 4.1), but this is not significantly different compared to the controls (n=4 and n=13 respectively) when the data were ranked, as determined by the Mann-Whitney U-test (p=0.090).

The original control group comprised of 101 healthy individuals. Studying the physiological characteristics of this group illustrates what happens in healthy individuals as they age, i.e. what occurs in ‘normal’ ageing. By comparing the patients with diabetes with the 51 age and sex-matched controls, any differences between the two groups can be determined.

Predicted differences between the groups are those physiological measures commonly elevated in diabetes including: glycated haemoglobin (HbA1c), albumin to creatinine ratio (ACR) in urine, and blood glucose concentration. These are indicators of long-term blood glucose control, early kidney damage, and a transient measure of glucose control, respectively.

4.7 Glycated haemoglobin (HbA1c)

HbA1c is a measure of one of three fractions of glycated haemoglobin within the blood of an individual, and is characteristically elevated in diabetes due to the chronic exposure of high glucose in the blood. The percentage of haemoglobin A that is glycated is proportional to the time that the red cells have been exposed to glucose and the glucose concentrations [226]. It is routinely used as an indication of the blood glucose control of a patient with diabetes over the preceding weeks, with higher values representing poorer control.
Figure 4.2: HbA1c versus age in both study populations

The relationship of HbA1c with age in 49 of the 101 controls, and 49 of the 51 patients with diabetes. The two groups were not age- and sex-matched. Equations for the regression lines are also displayed.

Glycated haemoglobin showed a significant relationship with age in the control population (p=0.004; figure 4.2). Data were only available in 49 of the 101 control participants, whom had a mean HbA1c ± sem of 5.5 ± 0.4%. HbA1c values were obtained in 49 of the 51 patients with diabetes, with a mean ± sem of 8.6 ± 0.2%. The patients with diabetes had higher values as expected, and HbA1c actually decreased with age (figure 4.2), although this was not significant (p=0.153). Of the 49 controls, 25 were age-and sex-matched to the patients with diabetes.
Table 4.6: HbA1c data in both study populations

HbA1c values in 25 controls and the 25 patients with diabetes to whom they were age- and sex-matched. The p-value from a 2-sample t-test comparing the groups is also shown.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=25)</th>
<th>Diabetic (n=25)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean HbA1c ± sem (%)</td>
<td>5.5 ± 0.1</td>
<td>8.4 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

To formally assess the difference between the populations, a 2-sample t-test was carried out on HbA1c data from the 25 controls with the data from their 25 matched diabetics. As predicted there was a significant difference between the groups (p<0.001; table 4.6). In the group with diabetes, multiple step-wise regressions revealed blood glucose concentration to be a significant predictor of HbA1c (p=0.001). Disease duration and whether type 1 or type 2 diabetes had no effect. Pooled HbA1c data in both study populations had the presence of diabetes as the only significant predictor (p=0.001).

4.8 Blood glucose concentrations

Blood glucose concentrations were available in 51 of the patients with diabetes and 50 age- and sex-matched controls. To test the population differences, only data for the 50 patients matched to the 50 controls with the available glucose data were used. Median ± SD values were 4.4 ± 0.6 mmol/L and 10.4 ± 4.7 mmol/L in the controls and patients with diabetes respectively. Due to the non-normal distribution of the data, the
Mann-Whitney U-test was used to test the difference between the groups, which was found to be significant at \( p < 0.001 \), confirming the difference.

Blood glucose concentration is a transient measure affected by many factors, such as when an individual last ate and what was consumed, also what medication the patients with diabetes were taking and when they took it, and therefore no regression analysis was carried out on the data.

4.9 Albumin to creatinine ratio (ACR)

Urinary albumin excretion rate is a measure of protein (albumin) excreted in urine and is commonly corrected for creatinine to improve sensitivity and specificity of 'spot' urine samples. The result is expressed as an albumin: creatinine ratio (ACR), with low levels from minimal albumin present in urine of healthy controls, but it is commonly elevated in diabetes. This elevation is due to the leakage of protein (albumin) from the blood into the collecting system of the kidneys, as a result of damage to the filtering structures of the kidneys [227]. Early changes in the microvasculature [228] are suggested as the cause of this damage. Several studies have revealed an elevated ACR to be an independent cardiovascular risk factor in people with diabetes and in healthy control populations [229-231].

ACR values were obtained in 49 of the controls and 51 of the patients with diabetes; however, some of these values were deemed "unreliable" due to the presence of blood in the urine. The Chemical Pathology laboratory at Leicester Royal Infirmary routinely tests for haematuria when the urine is sent for determination of ACR. Analyses were carried out on ACR data from 47 patients with diabetes and their
matched controls. There were no relationships with age in either study population alone, however ACR was related to age in the group as a whole, p=0.017. Multiple step-wise regression was carried out with the log-transformed values for ACR, as the residuals were not normally distributed for regression using the raw data. Predictably, the presence of diabetes was also a significant predictor (p<0.001).

<table>
<thead>
<tr>
<th></th>
<th>Control (n=47)</th>
<th>Diabetic (n=47)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median ACR ± SD (AU)</td>
<td>0.5 ± 1.2</td>
<td>1.0 ± 23.1</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Table 4.7: ACR data in both study populations*

ACR values in 47 patients with diabetes and their 47 matched controls, with the p-value from a Mann-Whitney U-test comparing any differences.

The ACR data were abnormally distributed; therefore the medians are displayed in table 4.7 for 47 patients with diabetes who had accurate ACRs and 47 age- and sex-matched controls. Predictably, the patient group had higher ACRs (1.0 ± 23.1 vs. 0.5 ± 1.2) compared to the controls (p=0.002 from a Mann-Whitney U-test).

4.10 Body mass index (BMI)

Body mass index (BMI), a measure of obesity, is known to increase with age [232], and obesity is a risk factor for cardiovascular disease [233] and type 2 diabetes [223, 234, 235].
BMI was determined in all study participants, and had a significant positive relationship with age in the control population; $p=0.001$ when regression analysis was carried out (figure 4.3).
Figure 4.4: BMI versus age in both study populations

The relationship of BMI with age in 51 patients with diabetes and 51 age- and sex-matched controls. Equations for the regression lines are also shown.

In the patients with diabetes, there was a negative relationship between BMI and age (figure 4.4), but it was not significant (p=0.306). The 51 controls age-and sex-matched to the patients with diabetes, still showed a positive relationship for the two variables, but it was not significant with the smaller numbers over a smaller age-range (p=0.327). Multiple step-wise regression was carried out on the BMI data in the patients with diabetes and revealed age (p=0.041), HbA1c (p=0.026), and whether the patients had type 1 or type 2 diabetes (p=0.012) as significant predictors. HbA1c remained a significant predictor if age, and aetiology of diabetes were not accounted for. The duration of diabetes and ACR data had no influence.
The relationship of HbA1c with BMI in 49 of 51 patients with diabetes, with the equation for the regression line.

To illustrate the significant relationship between HbA1c and BMI in the patients with diabetes, the data were plotted against one another. Figure 4.5 shows elevated BMI in those patients with poorer diabetic control; however, it is more likely that an elevated BMI causes increased HbA1c. The Pearson correlation coefficient for the relationship was 0.337 (p=0.022).

The diabetes group were divided into type 1 and type 2 patients to further investigate the differences in BMI between the subgroups, and the relationships with age, which became significant after the aetiology of diabetes was accounted for.
Figure 4.6: BMI versus age in patients with type 1 and type 2 diabetes

The relationship of BMI with age in 24 patients with type 1 diabetes and 27 with type 2 diabetes. Equations for the regression lines are also shown.

The patients with type 2 diabetes had significantly higher BMI values compared to those with type 1 (p=0.012 from multiple regression after accounting for age and HbA1c), with a steeper negative relationship with age (figure 4.5). Due to the smaller numbers in the subgroups of patients with diabetes and relatively large spread of data, there were no significant predictors for BMI when multiple step-wise regression was carried out in either group.
From figure 4.4, the BMI data in the patients with diabetes appears to lie higher compared to the age- and sex-matched controls, and were therefore investigated to formally assess if there was a difference. The data were not normally distributed; therefore median BMI values are presented in table 4.8, with the log-transformed values, which were normal. A 2-sample t-test on the transformed data revealed the patients with diabetes had significantly higher BMI values (p=0.021).

Multiple step-wise regression on BMI data from both study populations together with age, and the presence of diabetes as predictors, revealed the latter as the only significant predictor (p=0.015).

### 4.11 Blood pressure (BP)

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were also measured in all participants, with pulse pressure (PP) calculated as the difference between the two. It is well known that SBP increases with increasing age [236], therefore widening the gap between it and DBP causing a subsequent increase in PP with age.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=51)</th>
<th>Diabetic (n=51)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median BMI ± SD (kg/m²)</td>
<td>25.00 ± 3.22</td>
<td>27.06 ± 3.93</td>
<td></td>
</tr>
<tr>
<td>Mean Log BMI ± sem (AU)</td>
<td>1.41 ± 0.01</td>
<td>1.43 ± 0.01</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Table 4.8: BMI data in both study populations

Median values, and log-transformed values for BMI in 51 patients with diabetes and 51 age- and sex-matched controls.
High blood pressure is also a risk factor for cardiovascular disease and commonly accompanies diabetes [7, 24].

Figure 4.7: Control BP versus age

The relationships between SBP, DBP, and PP with age in 101 controls. Equations for the regression lines are also shown.

Figure 4.7 shows SBP and PP have significant linear relationships with age, increasing with age in the control population (p<0.001). DBP has a significant polynomial relationship, rising with age until approximately 50 years, after which it reaches a plateau.
The relationships in the patients with diabetes were different from those in the controls, as seen in figure 4.8. This is difficult to interpret because of the confounding of concomitant antihypertensive medication. SBP and DBP were not significantly related to age, and DBP actually decreases as the patients age. PP did show a significant positive relationship (p<0.001). Multiple step-wise regression of the patients' BP data investigating; duration of diabetes, aetiology (type 1 or 2), diabetic control (HbA1c), severity (ACR), and age as predictors found significant positive relationships for: duration of diabetes (p=0.022), and HbA1c (p=0.006) for DBP; age for PP as before (p<0.001); and age neared significance for SBP (p=0.067).
Figure 4.9: BP data in both study populations

SBP, DBP, and PP median values with SD error bars in 51 patients with diabetes and 51 age- and sex-matched controls.

The DBP and PP data in the patients with diabetes were abnormally distributed; therefore, median values for all BPs are displayed in figure 4.9. The figure illustrates the differences between the two populations, with the data for statistical analysis in table 4.9.
**Table 4.9: BP data in both study populations**

Mean SBP, and mean values for the log-transformed DBP and PP data, with p-values from 2-sample t-tests.

DBP and PP values were normally distributed after log-transformation, with the transformed data displayed in table 4.9. Two-sample t-tests revealed the patients with diabetes had significantly higher blood pressures for all three measures, p=0.004, 0.030, and 0.033 for SBP, DBP, and PP respectively (table 4.9).

Multiple stepwise regression on the combined BP data from the study populations found both age and the presence of diabetes significant predictors for SBP, (p<0.001 and 0.008, respectively). PP revealed age as the only significant predictor (p <0.001), and neither age nor the presence of diabetes predicted the DBP.

The differences observed in blood pressures between the two populations and the relationships with age, may not only be due to the physiological changes to the vasculature in diabetes, but also due to the medication the patients with diabetes were receiving, many of which were prescribed to control blood pressure (table 4.4, p99).

<table>
<thead>
<tr>
<th></th>
<th>Control (n=51)</th>
<th>Diabetic (n=51)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SBP ± sem (mmHg)</td>
<td>131 ± 2</td>
<td>139 ± 2</td>
<td>0.004</td>
</tr>
<tr>
<td>Mean Log DBP ± sem (AU)</td>
<td>1.88 ± 0.01</td>
<td>1.91 ± 0.01</td>
<td>0.030</td>
</tr>
<tr>
<td>Mean Log PP ± sem (AU)</td>
<td>1.72 ± 0.01</td>
<td>1.76 ± 0.01</td>
<td>0.033</td>
</tr>
</tbody>
</table>
4.12 Heart rate

Heart rate has previously been positively related to age [237], and is also a cardiovascular risk factor [238].

![Control heart rates versus age](image.png)

**Figure 4.10: Control heart rates versus age**

The relationship of heart rate with age in 101 controls

Investigation of heart rate in the control population, revealed a slight increase of 0.062 beats per minute (bpm) per year (figure 4.10); however, this was not near significance as illustrated in the figure with the shallow slope of the regression line.
Figure 4.11: Heart rate versus age in both study populations

The relationship of heart rate with age was even less apparent in the patients with diabetes, with an almost horizontal regression line on figure 4.8. There were also no associations with blood glucose control, aetiology, severity, or duration of diabetes. It is also clear from the figure that those individuals with diabetes had higher heart rates at any age, with a mean ± sem of 68 ± 1 bpm compared to 61 ± 2 bpm in the control group, although the gap decreased with age. A 2-sample t-test revealed this was significantly higher (p=0.002). Multiple step-wise regressions found the presence of diabetes to be a significant predictor for heart rate in the whole study group (p=0.044).
4.13 Lipids

Lipid concentrations measured in the participants' serum were total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides. Alterations of lipid measures have previously been investigated with age and BMI [239-242], and in pathological conditions including diabetes [7, 24]. High levels of total cholesterol, LDL cholesterol, and triglycerides, and low levels of HDL cholesterol are risk factors for cardiovascular disease [243-246], and dyslipidaemia is common in diabetes, with the changes generally adverse [7, 24, 235].

Total cholesterol and triglycerides were obtained in 99 of the controls, with the additional HDL values, and therefore the ratio of total cholesterol to HDL in 59 of those. LDL cholesterol was also available in 55 of the controls i.e. full lipid profiles.
Figure 4.12: Control lipid measures versus age

The relationships with age in controls for: total cholesterol (cholesterol), n=99; LDL cholesterol (LDL), n=55; and triglyceride levels, n=99. Equations for the regression lines are also displayed.

Figure 4.12 illustrates the relationships between total cholesterol, LDL cholesterol, and triglyceride levels with age in control subjects, all of which were significant (p<0.001, =0.006, and <0.001 respectively). The residuals for the regression between the triglycerides and age were not normal, however log transformation of the triglyceride values, which normalised the data and the residuals, gave the same relationship with a p-value of 0.001. The lipid measures of HDL cholesterol and the ratio of total cholesterol to HDL cholesterol did not show any significant relationships with age.
Further regression was carried out to look at the effects of BMI on lipids in the whole control population.

![Graph](image.png)

**Figure 4.13: Control lipid measures versus BMI**

The relationships with BMI in controls for: HDL cholesterol (HDL), n=59; the ratio of total cholesterol to HDL cholesterol (Total Chol: HDL), n=59; LDL cholesterol (LDL), n=55; and triglyceride levels, n=99. Equations for the regression lines are also displayed.

All the lipid measures in figure 4.13 were found to be significantly predicted by BMI, but as BMI increases with age (figure 4.3), step-wise multiple regression was carried out to find out which measures were predicted by BMI following correction for age.

HDL cholesterol, and the ratio of total cholesterol to HDL cholesterol were significantly related to BMI alone, with p-values of 0.003 and <0.001 respectively. LDL cholesterol, which is significantly related to age (p=0.031) when BMI is
included in the regression, revealed a borderline significance level with BMI (p=0.051). The triglyceride data were also significantly related to age (p=0.009) and BMI (p=0.022). Age remained the only predictor for total cholesterol.

Full lipid profiles were obtained for 49 of the patients with diabetes, and a full lipid profile without LDL was obtained for 1 patient. In this group, there were no significant relationships between any of the lipid measures and age (figure 4.14); however, this may be confounded by the fact that 19.6% of the people with diabetes were receiving lipid lowering therapy, i.e. statins (table 4.4).

**Figure 4.14: Lipid measure versus age in the patients with diabetes**

The relationships with age in the patients with diabetes for: total cholesterol (Cholesterol), n=50; HDL cholesterol (HDL), n=50; LDL cholesterol (LDL), n=49; triglycerides, n=50; and the ratio of total cholesterol to HDL cholesterol (Total Chol: HDL), n=50.
Figure 4.14 illustrates the lack of relationship between any of the lipid measures and age in the patients with diabetes, with nearly horizontal regression lines. BMI was investigated to determine if it was a predictor for any of the lipids in the same group.

There were significant associations between total cholesterol \((p=0.011)\), the ratio of total cholesterol to HDL \((p=0.010)\), and triglycerides \((p=0.002)\) with BMI in the patients with diabetes (figure 4.15). When multiple step-wise regressions were carried out including BMI, HbA1c, ACR, whether type 1 or type 2 diabetes, and diabetes duration as predictors, BMI was still the only significant predictor for total cholesterol.
and the ratio of total cholesterol to HDL cholesterol. The triglyceride data showed a significant positive relationship with HbA1c (p=0.014), and a barely significant positive relationship with BMI (p=0.048). These associations between BMI and lipid abnormalities in diabetes are remarkably well preserved, mindful of the fact that 19.6% of the patients studied were receiving lipid lowering therapy (statins; table 4.4).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Cholesterol ± sem (mmol/L)</strong></td>
<td>5.0 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>0.140</td>
</tr>
<tr>
<td><strong>Median HDL ± SD (mmol/L)</strong></td>
<td>1.3 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td><strong>Mean Log HDL ± sem (AU)</strong></td>
<td>0.13 ± 0.02</td>
<td>0.04 ± 0.02</td>
<td>0.007</td>
</tr>
<tr>
<td><strong>Mean LDL ± sem (mmol/L)</strong></td>
<td>3.2 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>0.036</td>
</tr>
<tr>
<td><strong>Median Total Chol:HDL ± SD (mmol/L)</strong></td>
<td>3.8 ± 1.0</td>
<td>4.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td><strong>Mean Log Total Chol:HDL ± sem (AU)</strong></td>
<td>0.58 ± 0.02</td>
<td>0.60 ± 0.02</td>
<td>0.390</td>
</tr>
<tr>
<td><strong>Median Triglycerides ± SD (mmol/L)</strong></td>
<td>1.2 ± 0.6</td>
<td>1.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td><strong>Mean Log Triglycerides ± sem (AU)</strong></td>
<td>0.07 ± 0.03</td>
<td>0.12 ± 0.04</td>
<td>0.320</td>
</tr>
</tbody>
</table>

*Table 4.10: Lipid data in both study populations*

Data in patients with diabetes and matched controls for: total cholesterol (Cholesterol), n=50; HDL cholesterol (HDL), n=33; LDL cholesterol (LDL), n=30; the ratio of total cholesterol to HDL cholesterol (Total Chol: HDL), n=33; and triglycerides, n=50. P-values from 2-sample t-tests, to formally test any differences between the groups, are also displayed.
Table 4.10 displays the mean ± sem values for normally distributed lipid data. If the data did not follow a normal distribution, the median ± SD values are displayed with the mean ± sem of the log-transformed values below as this normalised the data in each case. Differences between the groups were formally tested using 2-sample t-tests.

Of the 51 age- and sex-matched controls, total cholesterol and triglyceride data were available for 50 of them. The additional data for HDL and therefore the ratio of total cholesterol to HDL were obtained in 33 controls, and only 30 had LDL data giving a full lipid profile. When comparing lipid measures where results were only available in some of the controls, only data for those patients matched to the controls with the available data were included. On comparison between the patients with diabetes and controls, HDL cholesterol and LDL cholesterol were significantly higher in the controls, with p-values of 0.007 and 0.036 respectively (table 4.10).

As the patients with diabetes had a significantly greater BMI compared to the controls, and HDL cholesterol had a significant negative relationship with BMI in the control group (figure 4.13), multiple stepwise regression analysis was carried out on the HDL cholesterol data with age, BMI, and the presence of diabetes as predictors. The results revealed the latter two were significant; \( p = 0.003 \) for BMI and \( p = 0.028 \) for if the individual was a diabetic or control. Multiple regression analyses of all the other lipid data with the same variables entered in the model found: age (\( p = 0.032 \)), presence of diabetes (\( p = 0.027 \)), and BMI (\( p = 0.028 \)) to be significant predictors for total cholesterol; only the presence of diabetes influenced LDL cholesterol (\( p = 0.019 \)); and BMI alone showed very significant relationships (\( p < 0.001 \)) with both the ratio of total
cholesterol to HDL cholesterol and triglyceride concentrations. The differences observed between the groups for lipid measures, and the relationships between these measures and age and BMI, may again be due to the changes in physiology and also the presence of lipid lowering drugs in the patients with diabetes. The cause of the unexpected higher levels of LDL cholesterol in the controls, and the presence of diabetes as the only significant predictor causing a decrease, is almost certainly a consequence of lipid lowering therapy in the patients with diabetes.
4.14 Gender differences

The control group was divided into males and females, and then each parameter was investigated for any effect gender may have.

<table>
<thead>
<tr>
<th></th>
<th>Male (n=42)</th>
<th>Female (n=59)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age ± SD (years)</td>
<td>45 ± 18</td>
<td>47 ± 13</td>
<td>0.826</td>
</tr>
<tr>
<td>Mean BMI ± sem (kg/m²)</td>
<td>25.25 ± 0.47</td>
<td>24.74 ± 0.54</td>
<td>0.471</td>
</tr>
<tr>
<td>Mean heart rate ± sem (bpm)</td>
<td>58 ± 1</td>
<td>65 ± 1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean SBP ± sem (mmHg)</td>
<td>128 ± 2</td>
<td>123 ± 2</td>
<td>0.074</td>
</tr>
<tr>
<td>Mean DBP ± sem (mmHg)</td>
<td>75 ± 2</td>
<td>75 ± 1</td>
<td>0.727</td>
</tr>
<tr>
<td>Median PP ± SD (mmHg)</td>
<td>50 ± 8</td>
<td>48 ± 10</td>
<td></td>
</tr>
<tr>
<td>Mean Log PP ± sem (AU)</td>
<td>1.72 ± 0.01</td>
<td>1.67 ± 0.01</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Table 4.11: Physiological data in males and females

Numbers, age, BMI, heart rate, SBP, DBP, and PP values for both males and females in the 101 controls, with p-values from statistical tests to investigate differences.

Table 4.11 contains the mean values ± sem for normally distributed data, and the median values ± SD for those not normally distributed. If log transformation normalised the data, the mean log values are presented below the medians. All normal distributions were tested using a 2-sample t-test, and the non-parametric Mann-Whitney U-test was applied to non-normally distributed data. There were no significant differences between the male and female groups for age (p=0.826), body mass index (BMI; p=0.471), systolic (SBP; p=0.074), and diastolic blood pressures
The male group did have a significantly higher pulse pressure (PP; \( p=0.004 \)) and a significantly lower heart rate (\( p<0.001 \)) compared to the female group.

There were also some differences in lipid profiles between the genders.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=25)</td>
<td>(n=34)</td>
<td></td>
</tr>
<tr>
<td>Median HDL ± SD (mmol/L)</td>
<td>1.2 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Median Total Chol: HDL ± SD (AU)</td>
<td>3.9 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Mean Log Total Chol: HDL ± sem (AU)</td>
<td>0.58 ± 0.02</td>
<td>0.51 ± 0.02</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Table 4.12: Gender differences in HDL and Total Chol: HDL

Data in males and females of the control population for HDL cholesterol (HDL), total cholesterol (Total Chol), and the ratio of total cholesterol to HDL cholesterol (Total Chol: HDL). P-values from statistical tests to determine differences are also displayed.

Table 4.12 displays the median ± SD values for HDL cholesterol and the ratio of total cholesterol to HDL cholesterol, as the raw data were not normally distributed. Log-transformation normalised the ratio data, therefore the log values are also in the table with the p-value obtained from a 2-sample t-test. The result shows the males had significantly higher ratios compared to the females (\( p=0.018 \)). Transformation did not normalise the HDL cholesterol raw data and therefore the non-parametric Mann-Whitney U-test was used to determine any differences between the men and women. The results showed the men had significantly lower HDL values (\( p=0.001 \)).
Table 4.13: Gender differences in total cholesterol and triglyceride data

Data in males and females of the control population for total cholesterol (Cholesterol) and triglyceride data, with p-values from statistical tests.

<table>
<thead>
<tr>
<th></th>
<th>Male (n=40)</th>
<th>Female (n=59)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Cholesterol ± sem (mmol/L)</td>
<td>4.8 ± 0.2</td>
<td>5.1 ± 0.1</td>
<td>0.120</td>
</tr>
<tr>
<td>Median Triglycerides ± SD (mmol/L)</td>
<td>1.2 ± 0.6</td>
<td>0.9 ± 0.4</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Log transformation did not normalise the triglyceride data, therefore the non-parametric Mann-Whitney U-test was again used, and revealed the males had significantly greater concentrations of triglycerides compared to the females (p=0.007; table 4.13). There were no significance differences between the genders for total cholesterol after a 2-sample t-test was carried out (p=0.120; table 4.13). LDL cholesterol also showed no differences between the men and women, with mean ± sem values of 3.0 ± 0.2mmol/L (n=24) and 3.1 ± 0.1mmol/L (n=31) respectively (p=0.510 from a 2-sample t-test).

Step-wise multiple regressions for each of the lipid measures in the whole control group using age, sex, and BMI as predictors found: age alone to be a significant predictor of total cholesterol (p<0.001) and LDL cholesterol (p=0.031); age and sex to be significant predictors for triglycerides (p=0.003 and 0.009 respectively); and BMI and sex to be significant predictors for HDL cholesterol (p=0.003 and 0.032 respectively), and also for the total cholesterol to HDL cholesterol ratio (p=0.002 and <0.001 respectively).
Due to the small numbers of females in the participants with diabetes, it was not possible to look at gender differences in the group.

4.15 Discussion

After initial slow recruitment of patients with diabetes, a change in method resulted in greater numbers recruited, with higher attendance rates for appointments, and therefore more efficient use of time and facilities. The latter method involved approaching patients with diabetes, and providing information regarding the study, whilst they attended outpatient clinic. If they were interested in participating in the study, they left contact details and were later phoned, given any extra information required, and invited to attend an appointment at the Clinical Research Unit.

From the 101 controls recruited, 51 were successfully age- and sex-matched to the patients with diabetes (table 4.5). There were also no significant differences in ethnic origin (table 4.5) or smoking history (figure 4.1) between the two study groups.

The majority of patients with diabetes attending outpatient clinics are referred from their general practitioner due to poor control of diabetes. As such, many had complications associated with the condition (table 4.2), with a higher percentage of microvascular compared to macrovascular damage. Prospective studies have linked HbA1c with cardiovascular morbidity and mortality [29, 30]; however, although intervention trials have suggested blood glucose control decreases the incidence of vascular disease, they have not been conclusive, with either no significant beneficial effect on cardiovascular end points [247, 248] or a positive effect that was not consistent in all groups [34, 36, 249]. The inconsistencies between studies, and the
diversity of incidence and severity of vascular disease within an individual and across individuals with diabetes, suggests the extent of tissue damage depends on the interaction of hyperglycaemia with other factors, including genetic, biochemical, and environmental factors, which may be individual- and tissue-specific.

In this study HbA1c was significantly elevated in diabetes as expected (table 4.6), beyond 4.0 to 6.1%, the normal range reported by the Chemical Pathology department at Leicester Royal Infirmary. In the control population there was a significant positive relationship between HbA1c and age (figure 4.2). This agrees with the findings of Yates and Laing [250], who also showed a positive relationship in 121 healthy female and 71 healthy male individuals. The authors concluded that glycaemic control deteriorates with age in healthy, non-diabetic individuals due to deteriorating pancreatic β-cell function. An earlier, larger study [251] measured HbA1c in 399 patients undergoing routine oral glucose tolerance test (OGTT), of which 127 were normal, 94 had impaired glucose tolerance (IGT) and 178 had diabetes. The study found no significant correlations between age and HbA1c in any of the groups, and suggested conflicting studies used older populations, which included individuals with unrecognised diabetes, or glucose intolerant patients, thereby giving a false relationship. The control group studied in this thesis covered a wide age range, and although they did not undergo an OGTT, plasma glucose and HbA1c were measured to exclude volunteers with undiagnosed diabetes.

Significantly higher ACR values in the patients with diabetes compared to the controls was demonstrated, as expected. There is great variation in ratios within the individuals with diabetes, reflecting individual differences in kidney damage. The
increasing values represent greater damage, and are therefore an index of severity of microvascular disease within the kidney. Previous studies have also shown increased albumin excretion rates to reflect generalised vascular dysfunction, and are an independent risk factor for cardiovascular morbidity and mortality in diabetes and in healthy controls [229-231].

BMI increased with age in the control population (figure 4.3), which agrees with previous findings [232], and excess weight has been shown to be a risk factor for the development of cardiovascular risk factors and cardiovascular disease itself in the Framingham study [233].

BMI was significantly elevated in the patients with diabetes compared to the control population (table 4.8). This elevation in diabetes was dependent on whether the patients had type 1 or type 2 diabetes, with values higher in type 2 as expected (figure 4.6). Previous studies have shown obesity to be an independent risk factor for type 2 diabetes [234, 235]. Unusually, a negative relationship between BMI and age in the type 2 patients was suggested (figure 4.6), but this was not significant probably due to the smaller numbers investigated. This decrease in BMI may be due to dietary control, and may either contribute to, or be influenced by, improved glucose control. Figure 4.5 shows poorer diabetic control correlates with increasing BMI, although it may be that increasing BMI causes an increase in HbA1c. Therefore the gradual decrease in BMI with age in all the patients with diabetes (figure 4.4) may cause the similar decline in HbA1c with age in the same group (figure 4.2). This would suggest the greater decline in BMI with age in type 2 diabetes, may result in a greater decline in HbA1c with age. Step-wise multiple regression carried out on the HbA1c data found
no effect of the aetiology of diabetes, but this may be due to too few numbers being investigated.

Several studies have investigated the effects of ageing on the cardiovascular system in healthy subjects. Well-documented changes in the arteries include intimal thickening and increased stiffness accompanied by luminal dilatation (Reviewed in [15, 16]). The increased stiffness leads to a decreased arterial compliance and increased speed of pulse wave transmission. Reduced compliance will increase PP, while the increased pulse wave velocity will increase systolic pressure augmentation, and therefore increase SBP and PP. These findings were shown in the control population (figure 4.7), and agree with results from the Framingham study [236]. Generally, DBP does not increase after middle age, and may decrease in older individuals due to the decrease in conduit artery compliance [236]. This is also shown in the controls (figure 4.7), with a polynomial relationship with DBP increasing until approximately 55 years of age, where it plateaus, and then possibly decreases after 60 to 70 years. This decrease is not obvious in this study due to the small numbers above that age. In the Framingham study, increases in SBP, DBP, and PP were all found to be risk factors for cardiovascular disease [252], with PP suggested as the best (also reviewed in [15]).

All measures of blood pressure were elevated in the patients with diabetes compared to age- and sex-matched controls (figure 4.9 and table 4.9), despite many of them taking antihypertensive drug therapy. Only PP remained related to age (figure 4.8), with duration of diabetes and HbA1c predictors of DBP. These higher blood pressures, and lack of relationship with age, suggest other variables were affecting
blood pressure in this group with diabetes. Many of the patients had vascular complications (table 4.2) that may have resulted from the elevated blood pressures, and may now contribute to worsening the pressures. Other risk factors for high blood pressure present in the patients with diabetes, including obesity, may also contribute.

Heart rate has previously been shown to increase with age [237], and although the trend is seen in the control population, it was not a significant relationship (figure 4.10). Studies have shown higher heart rates are associated with increased mortality in the general population as well as cardiovascular disease [253-256]. There were significantly elevated heart rates in the patients with diabetes compared to controls (figure 4.11). It known that elevated heart rate can be a manifestation of parasympathetic cardiac autonomic dysfunction in patients with diabetes [257], and this may have been the cause of the higher heart rates in the patients with diabetes compared to the control group in this study.

Alterations in lipid profile in the control population were observed with ageing (figure 4.12), and there were some associations with BMI (figure 4.13). These findings are similar to the previous studies [239-242], with increases in total cholesterol, LDL cholesterol and triglycerides with age, an increase in the ratio of total cholesterol to HDL with BMI, and a decrease in HDL cholesterol with BMI. Increases in total cholesterol, the ratio of total cholesterol to HDL cholesterol, LDL cholesterol, and triglycerides, and a decrease in HDL cholesterol are all known as risk factors for cardiovascular disease [243, 245, 246].
There were no associations between any of the lipid measures and age in the patients with diabetes (figure 4.14), but BMI did show significant positive associations with total cholesterol, the ratio of total cholesterol to HDL cholesterol, and triglycerides in the patients with diabetes (figure 4.15). Table 4.10 displays the differences in lipid profiles between the two study populations, with multiple step-wise regression confirming the presence of diabetes a significant predictor for decreasing HDL cholesterol, LDL cholesterol, and total cholesterol. Type 1 and type 2 diabetes are both commonly accompanied by lipid disorders [4]. In poorly controlled patients with type 1 diabetes, hypertriglyceridaemia is the most obvious mainly due to increased LDL cholesterol production by the liver. Other lipid changes may include increased HDL cholesterol. Dyslipidaemia in patients with type 2 diabetes is often subtle with decreased HDL cholesterol, similar or slightly decreased LDL, increased ratio of LDL to HDL cholesterol, and modest rises in triglyceride levels compared to people without diabetes. These abnormalities are due to excessive production of very low-density lipoprotein by the liver, due to insulin resistance [4, 258]. In this study, there were 27 type 2 patients and 24 type 1 patients, and although entering the aetiology of diabetes as a predictor had no significant effect on lipid measures this may be due to the small numbers. The lack of significantly higher triglyceride levels and lower levels of total cholesterol in the patients with diabetes, may be a consequence of lipid lowering medication.

The large control population (101 individuals) of mixed gender, with no significant difference in age between the men and women, gave the opportunity to study gender differences in a healthy population (table 4.11 – 4.13). Gender differences of haemodynamic variables were only seen in pulse pressure and heart rate. These are
well known, and Dart and Kingwell in their review [15] suggested that the relation between central pressure augmentation and height could produce male-female differences, and the somewhat faster heart rate in women will, to an extent, counteract the effects of shorter stature. However, the female population in this study had a lower pulse pressure, suggesting although they were significantly shorter (data not shown), their faster heart rate completely counteracted the effects, or more likely, other factors influence the variables.

The only significant differences in lipid profiles between the genders were a decreased HDL cholesterol, increased ratio of total cholesterol to HDL cholesterol, and increased triglycerides in the men. Pre-menopausal women are less prone to cardiovascular diseases than men, but then tend to catch up with men post-menopause [259]. This cardio protection is thought to be partly due to their different lipid profile [240], which agrees with the observations in this study.

4.16 Conclusions
In a healthy control population, there were several significant physiological changes with age. Glucose tolerance decreased (indicated by an increase in HbA1c), BMI increased, SBP and PP increased, DBP increased up to 50 years, after which there was little change, and there was an increase in the lipid measures of total cholesterol, LDL cholesterol, and triglycerides. Each of these alterations is adverse, and significantly elevated values are independent risk factors for cardiovascular morbidity and mortality. This agrees with age as the largest risk factor predictive of cardiovascular disease.
Gender differences were also observed with a significantly higher PP, and significantly lower heart rate in the men. Also, HDL cholesterol was lower, and the ratio of total cholesterol to HDL cholesterol and triglyceride levels were higher in the males compared to the females.

In diabetes, the majority of the relationships between the physiological variables and age were lost, and there were significant differences compared to age- and sex-matched controls. Aetiology of the condition only became a significant factor for BMI, with higher values in type 2 diabetes compared to type 1. As expected HbA1c, blood glucose, and ACR were all elevated, with the last variable indicating microvascular damage. BMI, all blood pressure measures, and heart rate were also significantly higher in patients with diabetes. The lipid data showed the patients with diabetes had significantly lower HDL cholesterol and the unexpected result of lower LDL cholesterol, but these may be influenced by lipid lowering medication. The data suggest that many of the physiological changes that occur with age in a healthy population, many of which are risk factors for cardiovascular disease, are accelerated in diabetes whether type 1 or type 2, and therefore may contribute to the pathogenesis and progression of vascular complications.
Chapter 5. Arterial stiffness in ageing and diabetes

5.1 Introduction

Cardiovascular diseases, including hypertension, coronary disease, congestive heart failure, and stroke, occur at increasing rates with advancing age (reviewed in [8]). This has led to the proposal that cardiovascular disease is an ageing process, but this does not explain why there are many people who achieve ‘old age’ without evidence of cardiovascular disease. The true situation is complex and involves many factors including age, genetics, and risk factors. It is still unknown how these factors interact and influence one another producing pathological conditions in some individuals and not others.

Several studies have investigated the effects of ageing on the cardiovascular system in healthy subjects. Well-documented changes in arteries include intimal thickening and increased stiffness accompanied by luminal dilatation (Reviewed in [15, 16]). The macroscopic changes that lead to these progressive alterations include increased fibronectin and collagen content with cross-linking of collagen, fragmentation of elastin, and invasion of the intima by vascular smooth muscle cells (reviewed in [17]).

Increased stiffness leads to a decreased arterial compliance and increased speed of pulse wave transmission, the latter due to its dependence on vessel wall properties (Moens Korteweg equation [194]). Reduced compliance will increase pulse pressure, while the increased pulse wave velocity (PWV) will increase systolic pressure augmentation, and therefore increase systolic blood pressure and pulse pressure.
In chapter 4, there was an increase in pulse pressure with age in healthy controls (figure 4.7) and patients with diabetes (figure 4.8), and the patients with diabetes had significantly higher pulse pressures compared to age- and sex-matched controls (figure 4.9). In this chapter, a measure of arterial stiffness will be investigated.

PWV, a non-invasive measure of arterial stiffness, increases with increasing age in healthy individuals [136]. It has also been shown to be a powerful predictor of both cardiovascular and all-cause mortality in many patient groups [260-264]. Stiffening of the arteries occurs in diabetes [265-269], and previous research has shown PWV is increased in patients with diabetes compared to age-and sex-matched controls [197, 270, 271], and has also been shown to predict premature mortality in these patients [197]. This suggests there is accelerated vascular ageing in diabetes and this may contribute to the vascular complications common in those individuals with diabetes.

5.2 Aim

This chapter will investigate the relationship of PWV, a measure of arterial stiffness, with age in a healthy population, and then in patients with diabetes. Any relationships between the marker of stiffness and other physiological variables will be investigated, as will any differences between the populations to determine if there is accelerated vascular ageing in diabetes.

5.3 Methods

Carotid to femoral PWV was determined in 101 healthy controls using applanation tonometry as described in chapter 3. Demographic and physiological characteristics of the controls are detailed in chapter 4. The same method was applied to 51 patients
with diabetes and the results compared to 51 age-and sex-matched controls. Patient demographics and physiological characteristics are also detailed in chapter 4.

5.4 Results

![Graph showing the relationship of PWV with age in controls. The equation for the regression line is given as y = 0.0848X + 3.001 with R² = 0.6702.]

**Figure 5.1: Control PWV versus age**

The relationship of PWV with age in 101 controls, with the equation for the regression line. Velocity is measured in metres per second (m/s).

PWV increased with age in the 101 healthy controls, with a mean ± sem of 6.88 ± 0.16 m/s. The regression equation gives an increase in PWV of 0.08 m/s per year, and has a high R² value (0.670; p<0.001). The increase in PWV with ageing meant that PWV had approximately doubled after 55 years of ageing.
PWV positively correlated with heart rate, systolic blood pressure, and pulse pressure, although step-wise multiple regressions revealed only age (p<0.001), heart rate (p=0.002), and systolic blood pressure (p=0.025) as significant predictors.

To investigate any gender effect within the control group, data for the males and females were compared.

<table>
<thead>
<tr>
<th></th>
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<th>Female</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(n=42)</td>
<td>(n=59)</td>
<td></td>
</tr>
<tr>
<td>Median age ± SD (years)</td>
<td>45 ± 18</td>
<td>47 ± 13</td>
<td>0.826</td>
</tr>
<tr>
<td>Median PWV ± SD (m/s)</td>
<td>6.87 ± 1.74</td>
<td>6.57 ± 1.47</td>
<td></td>
</tr>
<tr>
<td>Mean Log PWV ± sem (AU)</td>
<td>0.84 ± 0.02</td>
<td>0.82 ± 0.01</td>
<td>0.486</td>
</tr>
</tbody>
</table>

**Table 5.1: PWV data in males and females**

Median ages and PWVs of the males and females of the control population, with the log-transformed values for the PWV data. P-values from statistical tests to formally investigate differences are also displayed.

Table 5.1 displays the median ± SD values for age and PWV, as the raw data were not normally distributed. Log-transformation normalised the PWV data, therefore the log values are also displayed in the table. The Mann-Whitney U-test and a 2-sample t-test were used to formally assess differences between the genders for the age data and log-transformed PWV data respectively. P-values, displayed in the table, show there were no significant differences in either ages or PWV between the genders.
PWV was determined in 47 of the 51 patients with diabetes. The results were compared to their 47 age- and sex-matched controls.

![Figure 5.2: PWV versus age in both study populations](image)

The relationship of PWV with age in 47 patients with diabetes and 47 age- and sex-matched controls, with equations for regression lines displayed.

The patients with diabetes have a steeper regression line on figure 5.2, with a slope representing an increase of 0.13 m/s in PWV with every year of age, compared to the 0.09 m/s increase in the controls. The diabetes group also had the same predictors for PWV reaching significance, as the control group, when multiple step-wise regressions were carried out, i.e. age (p<0.001), systolic blood pressure (p=0.001), and heart rate (p=0.009). There were no relationships with measures of diabetic control, disease duration, type of diabetes, or albumin to creatinine ratios.
The PWV data in the patients with diabetes were not normally distributed, even if transformed; therefore, the non-parametric Mann-Whitney U test was used to formally assess if there was a difference between the two groups. Median PWV ± SD values for the controls and patients with diabetes respectively, were 7.29 ± 1.64 m/s and 8.00 ± 2.89 m/s. The test reached significance with a p-value of 0.006.

Step-wise multiple regression on all the PWV data from both study populations together revealed; age (p< 0.001), systolic blood pressure (p< 0.001), heart rate (p= 0.003), and the presence of diabetes (p=0.037), to be significant predictors for PWV.

5.5 Discussion

PWV increases in humans as they age [136], and is a well-established index of arterial stiffness. In this study, there was an increase of 0.08 m/s per year in PWV in 101 controls aged 19 to 75 years (figure 5.1), indicating an increase in arterial stiffness with age in this group. Previous studies have shown significant relationships between PWV and age in healthy controls, but have not published the rate of increase [197, 271]. Cameron and colleagues [272] investigated 112 healthy subjects, aged 34 to 90 years and showed an increase of 0.12 ± 0.02 m/s per year in carotid to femoral PWV. This higher rate of increase of PWV compared to the 0.08 m/s per year observed in this study may be due to the older age range of the participants, and/or due to other differences in the study populations or methodology. The authors found age and systolic blood pressure as significant predictors for PWV as in this study, but did not investigate heart rate.
It is difficult to compare absolute mean or median values for PWV between studies due to the different age ranges investigated. Taniwaki and colleagues [271] studied 285 healthy subjects with a mean age of 49.8 years, and showed they had a mean PWV of 7.19 m/s. This is similar to the 7.29 m/s observed in the 47 controls of a similar age (median 51 years) in this study.

Benetos et al investigated 193 healthy subjects (120 men, 73 women), with a mean age of 56 ± 11 years. The mean age-adjusted PWV in the whole group was 11.0 ± 0.2 m/s, and the men had significantly higher values compared to the women (11.4 ± 0.2 m/s versus 10.4 ± 0.3 m/s respectively, p<0.05). Multivariate analysis in the gender groups separately found: age (p<0.0001), mean arterial pressure (MAP) (p<0.0001), and TRF length (p<0.05) significant predictors in the men; and age (p<0.01), MAP (p<0.001), and heart rate (p<0.01) for the women. Table 5.1 shows the men had slightly higher PWVs compared to the women in this study, but this was not significant. Mitchell et al [273] investigated PWV in 188 healthy men and 333 healthy women in the Framingham Heart Study offspring cohort. The authors found gender was not a significant predictor of PWV in this group, although the women had larger reflected waves, which could not be wholly explained by height differences. Oestrogens are known to have acute effects on arterial stiffness [274], and pre-menopausal women are less prone than men to cardiovascular diseases [275]. The female control population in this study, and probably in the Framingham offspring cohort, included women of pre- and post-menopausal ages, and this may explain the lack of significant difference between the genders.
Cruikshank and colleagues [197], Cameron and colleagues [272], and Taniwaki and colleagues [271] also investigated PWV in type 2 diabetes and found higher values in patients with diabetes compared to controls. The first study mentioned [197], reported a rate of increase of 0.22 m/s per year in PWV in 178 patients with type 2 diabetes, with a mean age of 62.4 years. This again is a greater rate of increase compared to the 0.13 m/s per year in the patients with diabetes (median age 54 years) in this study (figure 5.2). In the same study, the mean PWV of the white European group with diabetes was 12.0 m/s compared to 10.1 m/s in 54 age- and sex-matched white European controls. Median PWV ± SD values for the controls and patients with diabetes in this study were 7.29 ± 1.64 m/s and 8.00 ± 2.89 m/s, (p= 0.006). The differences between studies may again be due to the older age of the participants in Cruikshank and colleagues’ study and/or other differences in the subjects or methods used.

Cameron et al reported a mean PWV of 13.60 ± 0.31 m/s in 57 patients with type 2 diabetes compared to 12.77 ± 0.27 m/s in 112 controls. This was not significant and differences in age groups and gender distributions (the controls were significantly older and a had a larger proportion of females compared to the patient group), makes it difficult to draw conclusions on the effects of diabetes.

Tanawaki and colleagues investigated type 2 diabetes in 271 patients aged 16 to 75 years (mean 51.2 years). The authors reported a mean PWV of 9.02 m/s compared to 7.19 m/s in 285 age-matched controls (p<0.0001). On all study participants, the authors found diabetes, age, smoking, and hypertension risk factors for increased PWV. In the patients with diabetes, duration of the condition was an independent risk
factor for elevated PWV. In the same study, intimal media thickness (IMT) of the carotid artery was also measured in the same subjects. The authors found increased thickening with age in controls and patients with diabetes, and this was significantly greater in the individuals with diabetes. IMT significantly positively correlated with PWV, although there were variations in the changes of the two variables, and in their risk factors.

The evidence from this study and those mentioned above indicates accelerated vascular ageing in diabetes. Within this study, the PWV of a 45-year-old with diabetes approximates to the same PWV as a 57-year-old non-diabetes, i.e. a diabetes-associated vascular ageing effect of about 8 years. A number of mechanisms linking diabetes with increased arterial stiffness have been suggested. Hyperglycaemia can cause glycation of the proteins in the arterial wall, and these glycosylated proteins are elevated in diabetes [276] and have been associated with ageing [43] and atherosclerosis [276-278], and therefore may contribute to the increased arterial stiffness in these conditions. However, there is no evidence linking blood glucose or glycated haemoglobin (a measure of long-term glycaemic control) with measures of arterial stiffness. Insulin resistance may also contribute through a decreased ability of insulin to elicit endothelium-dependent vasodilation [279]. This lack of vasodilation may increase the possibility of injury to the vessel wall and stiffness, as the blood vessels will be unable to counteract increases in blood pressure by dilation. There is also the possibility of an inflammatory response increasing the movement of immune cells into the artery wall leading to wall stiffening [280]. Markers of inflammation are elevated in type 1 [59] and type 2 diabetes [60], and have been associated with the development of type 2 diabetes in middle-aged adults [281]. Further research is
required to determine the exact mechanism that leads to the elevated PWV, and therefore increased arterial stiffness in diabetes. If discovered there is potential for the development of therapeutic interventions that may help prevent or slow the progression of vascular complications in diabetes.

5.6 Conclusions

Arterial stiffness increases with age in healthy humans, and this relationship is accelerated in diabetes as evidenced by increased pulse wave velocity. The mechanism for this accelerated vascular ageing is unknown, although several theories have been proposed including protein glycation, decreased vasodilation through insulin resistance, and an inflammatory response. The stiffer arteries will increase systolic blood pressure, and pulse pressure, and may contribute to the pathogenesis of vascular complications common in diabetes. Further research is required to elucidate the mechanism, as this may lead to therapeutic developments to prevent or slow the progression of vascular complications in diabetes.
Chapter 6. DNA damage and oxidative stress in ageing and diabetes

6.1 Introduction

Oxidative stress is an imbalance of oxidants and antioxidants in favour of the former, and has been implicated in ageing [282], and various pathological conditions including cardiovascular disease [62, 68] and diabetes [77, 283-285]. This has led to the development of many techniques to quantify reactive oxygen species generation \textit{in vivo}, although this is made difficult due to their evanescence. Reactive oxygen species can attack all molecules in the body, including DNA, and can cause structural alterations such as base pair mutations, rearrangements, deletions, and insertions (reviewed in [71]).

The most widely used methods for detection of DNA damage are single cell gel electrophoresis (SCGE), also called the comet assay, and high performance liquid chromatography (HPLC) measurement of selected oxidised bases [286]. The comet assay detects DNA strand breaks in individual cells, and can be used with the addition of lesion specific repair endonucleases to introduce breaks at the sites of damaged bases [203, 287]. The percentage of tail DNA (% Tail DNA) in the comet is representative of the level of DNA damage, with larger ‘tails’ representing more damage [199, 200]. Advantages of the comet assay over HPLC include increased sensitivity for low levels of damage, small quantities of sample required, and ease of application [286]. Previous studies investigating oxidative stress, using the comet assay in patients with diabetes, have yielded inconclusive results [77, 79-82, 96, 97], perhaps due to different methodology, or different patient populations. This led to
determination of oxidative DNA damage, using the comet assay, in the patients with diabetes in this study.

Earlier studies investigating oxidative stress focussed most commonly on lipid peroxidation, including direct measures of products or breakdown products, or indirect measures, for example exhaled pentane (reviewed in [209, 210]). Isoprostanes, an example of the more recently discovered anylates formed in vivo, are chemically stable, free radical-catalysed products of arachidonic acid. Gas chromatography with mass spectrometry has been used to enable detection of low levels in both plasma and urine [288]; however this is expensive, and technically difficult and has led to the development of immunological assays [208]. As a further measure of systemic oxidative stress in the patients with diabetes, a commercially available enzyme-linked immunoassay (ELISA) for urinary isoprostanes was used in this study to detect levels of lipid peroxidation products in vivo. Previous research has found increased urinary isoprostanes in diabetes [78], and perhaps in ageing [208] although this latter finding has not been confirmed by other studies [289, 290].

6.2 Aim

The aim of this chapter was to investigate if oxidative stress is increased in patients with diabetes using the comet assay to detect levels of DNA damage, and an ELISA for urinary isoprostanes to quantify lipid peroxidation. The results were compared with a control population to determine any differences, and any possible relationships with the physiological variables in chapter 4 were also investigated.
Results from validation and optimisation experiments of the comet assay are presented before the study population data, and the question of increased oxidative stress with increasing age was investigated.

6.3 Methods

Initial validation and optimisation experiments were carried out for the comet assay on blood sampled from healthy volunteers, and an initial pilot study was undertaken on small numbers of patients with diabetes, patients with essential hypertension, and healthy controls to enable sample size to be determined from a power calculation.

The comet assay was carried out as described in chapter 3, with the addition of formamidopyrimidine DNA glycosylase (FPG) enzyme to detect oxidised purines. To determine the levels of oxidative stress in patients with diabetes, blood was sampled from 48 of the 51 outpatients recruited at Leicester Royal Infirmary and their age- and sex-matched controls. Urine samples were also obtained from 43 of these patients and their controls for determination of urinary isoprostane concentrations, an index of lipid peroxidation. The method for measuring urinary isoprostane concentrations is detailed in chapter 3.

6.4 Comet assay validations and optimisation

6.4.1 Comet assay variation

To determine the reproducibility of the comet assay, inter- and intra-assay variation were studied. Two healthy, male volunteers (aged 28 and 40 years) had blood samples drawn into Lithium Heparin coated vacutainers, and the comet assay was carried out as described in chapter 3.
Figure 6.1: Comet intra-assay variation

Mean % Tail DNA from 50 comets on 13 replicate slides within one electrophoresis assay to determine intra-assay variation.

Intra-assay variation was determined from 13 replicate slides within one electrophoresis tank, using blood sampled from one individual, and repeated for a separate volunteer. Each bar of figure 6.1 represents the mean % Tail DNA (± sem) of 50 comets on one slide, with the 13 bars representing the 13 slides. The mean (± sem) % Tail DNA for the 13 slides was 19.9% (± 1.0%), with a variance within the tank of 13.4%, and a coefficient of variation of 18.4%. The results were similar for the second subject with a mean (± sem) % tail DNA of 22.5% (± 0.9%), and 11.9% for variance and 15.3% as the coefficient of variation.
Inter-assay variation was investigated by repeating the comet assay on the same sample, on four separate occasions. For each assay, 3 replicate slides (150 comets) were scored that were in the same position in the tank, and this was again carried out in the two healthy volunteers.

One-way analysis of variance found there were no significant differences between the four assays in either individual (p=0.718 and 0.800 for the 40 and 28 year olds respectively). The coefficients of variation, calculated from the standard deviations of the mean values of the four assays, were 8.4% in the older subject and 8.8% in the younger subject.

**Figure 6.2: Comet inter-assay variation**

Each bar of the figure represents the mean % Tail DNA (± sem) from 150 comets on 3 replicate slides within a single electrophoresis assay, with the four bars representing four separate assays to calculate inter-assay variation.
Human samples are subject to variation from external and internal influences, the latter of which is termed physiological variation. This is investigated by keeping all external variables and study conditions identical, therefore exposing the physiological variation. It is useful to investigate these changes to help distinguish true differences between study groups or conditions from 'normal' variation.

Figure 6.3: Intra-individual variation in oxidative DNA damage

Mean % Tail DNA (± sem) of four separate days in 5 individuals (Ss1-Ss5) to determine intra-individual variation in DNA damage

In this study, the physiological variation of DNA damage within peripheral blood cells was determined using blood sampled from healthy individuals on four separate days. Samples were taken at the same time each day to rule out any effects of circadian rhythm, and subjects were asked to refrain from caffeine, smoking, or exercise two hours prior to sampling. Values for each separate day were calculated.
from the mean of 150 comets on 3 replicate slides (50 per slide), and each bar of figure 6.3 represents the mean % Tail DNA (± sem) of four days for each of the 5 volunteers (Ss1 – Ss5).

<table>
<thead>
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<th>Subject (Ss)</th>
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<th>3</th>
<th>4</th>
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<tr>
<td>C of V (%)</td>
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<td>21.5</td>
<td>28.0</td>
<td>22.3</td>
<td>28.0</td>
</tr>
</tbody>
</table>

Table 6.1: Intra-individual variation in DNA damage

Basic characteristics of the 5 individuals used to determine intra-individual variation in DNA damage, with the values for variance and coefficients of variation (C of V) for % Tail DNA in each individual over the four days.

Table 6.1 displays the intra-individual variation in each of the 5 volunteers. The median variance ± SD for the 5 subjects was 16.7 ± 3.8%, and the median coefficient of variation ± SD was 27.4 ± 3.2%.
Situations may arise when blood cannot be immediately processed. The effects on DNA damage of storing the blood on ice in the dark were therefore investigated.

![Figure 6.4: Effect of short-term storage on DNA damage](image)

**Figure 6.4: Effect of short-term storage on DNA damage**

*Each bar of the figure represents the mean % Tail DNA (± sem) of 14 blood samples stored on ice in the dark for 0 to 3 hours to investigate the effect of short-term storage on DNA damage.*

Blood was taken from 14 healthy subjects and either immediately put on slides and then into lysis overnight, or stored on ice. After 1, 2 and 3 hours samples were taken and processed in exactly the same manner. The mean % Tail DNA was calculated from 150 comets on 3 replicate slides per individual, with the mean of the 14 individuals calculated for each storage time. One-way analysis of variance revealed no significant differences between storage times (p=0.874). This agrees with previous research [291], and indicates there will be little or no artefactual DNA damage induced by storing the blood samples for up to 3 hours on ice in the dark.
6.4.2 Formamidopyrimidine DNA Glycosylase (FPG)

Formamidopyrimidine DNA Glycosylase (FPG), a bacterial repair enzyme, was introduced to the comet assay to create breaks at oxidised purines and formamidopyrimidine lesions. The use of FPG with the comet assay is widespread; however, due to different methodologies and sources of the enzyme, concentrations used vary widely. A titration experiment was carried out to determine the optimal concentration of FPG for use on whole blood within this study.

![FPG titrations](image)

**Figure 6.5: FPG titrations**

Mean % Tail DNA ($\pm$ sem) from a single blood sample when slides were incubated with TE buffer alone, FPG buffer alone, and FPG enzyme diluted in FPG buffer at increasing concentrations. Each bar of the figure represents the mean of 4 identical experiments.
The titration experiment was carried out on blood sampled from a single healthy volunteer and was repeated a further 3 times using blood freshly sampled from the same individual. Mean % Tail DNA of 150 comets on 3 replicate slides was calculated for each study condition (TE buffer alone, FPG buffer alone, and each concentration of FPG enzyme) in each experiment, and a mean of the 4 repeated experiments was calculated for each condition. Figure 6.5 shows results similar to that found by the European Standards Committee on Oxidative DNA Damage (ESCODD) in their progress report for the year ended 31st of January 2002, with an initial increase in damage, leading to a plateau before a further increase. It is suggested an optimum concentration lies on the plateau as beyond this there is non-specific DNA strand cleavage or nuclease activity, and before this plateau many sites may go undetected due to the slower rate of reaction. It is not possible to increase the incubation time and use lower concentrations as FPG has a short-half life with 30 minutes incubation optimal [287]. It was therefore decided to use a 1 in 5000 dilution for subsequent experiments.

6.5 Pilot comet data for power calculations
Prior to the use of FPG enzyme, pilot comet data was generated from blood samples collected from hypertensives, diabetics, and age- and sex-matched controls. The mean % Tail DNA was calculated for each individual from 150 comets on 3 replicate slides incubated in TE buffer.
Figure 6.6: Pilot comet data

Mean % Tail DNA (± sem) in 20 patients with diabetes, 20 patients with hypertension, and 10 controls. *P<0.05 from one-way ANOVA and Tukey's pairwise comparisons.

Figure 6.6 illustrates differences in DNA damage in blood sampled from the three groups investigated. There were no significant differences between the gender distributions, or mean ages of the groups; 45.8 ± 2.1, 45.8 ± 1.8, and 43.1 ± 4.0 years in the hypertensive patients, those with diabetes, and the controls respectively. The patients with diabetes consisted of 13 patients with type 2 diabetes mellitus, of which 8 were hypertensive, and 7 patients with type 1 diabetes mellitus, of which 2 were hypertensive. One-way analysis of variance revealed a significant difference between the groups, with a p-value of 0.002. Tukey’s pairwise comparisons showed the patients with diabetes had significantly greater DNA damage, as measured by the comet assay, with confidence intervals for pairwise differences between level means.
of 0.41 to 6.28%, and 1.72 to 8.91% compared to the hypertensive and control groups respectively. There were no significant differences between the hypertensives and controls.

From the results it is apparent the pilot study already reached significance, therefore a power calculation was not necessary. Larger numbers than the pilot study were recruited into the study due to the lack of pilot data and therefore power calculations for some of the other variables measured, e.g. TRF length measurement.

6.6 Oxidative damage in study populations – comet data
Comet data were obtained for 48 out of 51 of the diabetics recruited, and all 51 of their matched controls. For each individual a mean % Tail DNA was calculated from 150 comets on 3 replicate slides incubated in TE buffer, FPG buffer alone, and FPG enzyme at a concentration of 1 in 5000 in FPG buffer. The difference in means of the latter 2 conditions was calculated for each individual, representing the lesions excised by FPG.
Figure 6.7: Comet data for controls and patients with diabetes

The bars labelled TE are the mean % Tail DNA ($\pm$ sem) for the 48 controls and 48 patients with diabetes at baseline. The bars on the right hand side of the figure are the means ($\pm$ sem) of the difference in % Tail DNA after the addition of FPG enzyme for the same individuals. *$P = 0.001$ for TE and 0.003 for FPG from 2-sample t-tests.

Figure 6.7 clearly illustrates the patients with diabetes had higher DNA damage compared to the control group with values of 12.7% vs. 10.7% for slides incubated in TE buffer, and 25.8% vs. 21.4% after the addition of FPG. To formally test this 2-sample t-tests were performed and revealed significantly greater damage in those with diabetes at baseline, i.e. when the slides were incubated with TE alone ($p=0.001$); and a significantly greater number of lesions excised by FPG in those with diabetes compared to the controls ($p=0.003$).
On investigation, there were no significant relationships for comet data with any of the physiological variables measured (age, BMI, heart rate, blood pressures, lipid measures, PWV), in either of the study populations. The increased DNA damage in diabetes in this study was not related to control or duration of diabetes, presence of protein in urine, or whether they had type 1 or type 2 diabetes. Multiple step-wise regression found the only significant predictor for the pooled comet data from both study groups, and any of the physiological variables, was the presence of diabetes for the FPG comet data ($p=0.001$). This was a positive relationship with an increase in the % Tail DNA with FPG in diabetes compared to healthy controls.

6.7 Oxidative damage in study populations – lipid peroxidation data

A further measure of systemic oxidative damage, of lipids and not DNA, was measured in the same subjects. This measured the concentration of 15-isoprostane $F_2\alpha$ in the urine of patients and their matched controls by ELISA. Data were only obtained from 43 of the patients with diabetes due to haematuria or no urine sample being given. The Chemical Pathology laboratory at Leicester Royal Infirmary detected haematuria when the urine samples were sent for determination of albumin to creatinine ratios, and may give possible false results for those ratios. The isoprostane concentrations are normalised by creatinine values; therefore, the data from those samples with haematuria were excluded.
Figure 6.8: Urinary isoprostane standard curve

Example of the standard curve generated from the 15-isoprostane F$_{2t}$ ELISA kit for the determination of urinary isoprostane levels. The y-axis represents the absorbance measured by a spectrophotometer as a percentage of the zero standard (BO). BO is the value when only the supplied 15-isoprostane F$_{2t}$-horseradish peroxidase was present to bind to the antibody, and therefore no other source of isoprostane. Full methods are detailed in chapter 3.

Urinary isoprostane concentrations were determined from a standard curve generated using a supplied standard of 15-isoprostane F$_{2t}$ (figure 6.8). $R^2$ values for the 3 assays required to process all the samples were 0.978, 0.977 and 0.978, indicating little deviation of the standards from the straight line, and consistency between the assays.
Figure 6.9: Lipid peroxidation data for controls and patients with diabetes

Median (± SD) values for urinary isoprostane concentrations ([Isop]) in 43 patients with diabetes and their 43 age- and sex-matched controls.

A normality test revealed the isoprostane data were not normally distributed, but were found to be normal after log-transformation. Figure 6.9 therefore displays the median (±SD) values, illustrating the patients with diabetes had higher concentrations of urinary isoprostanes (316.3 ± 605.1 ng/mmol creatinine versus 129.5 ± 546.8 ng/mmol creatinine), with the data for statistical analysis in table 6.2.
controls
(n=43)

Diabetics
(n=43)
p-value

Mean Log [Isop] ± sem (AU) 2.1 ± 0.1 2.4 ± 0.1 0.011

Table 6.2: Lipid peroxidation data for controls and patients with diabetes

Log transformed concentrations of urinary isoprostanes ([Isop]) in controls and patients with diabetes, with a p-value from a 2-sample t-test.

To formally assess the differences in urinary isoprostanes between the study groups, a 2-sample t-test was carried out on the log-transformed data showing the patients with diabetes had significantly higher values compared to the controls for that particular measure of lipid peroxidation (p=0.011, table 6.2).

On investigation, there were no relationships between the isoprostane data and any physiological measures (including age) in the control group; however, the isoprostane data in the patients with diabetes correlated with ACR values. Both sets of data were log-transformed to normalise the residuals before correlation. The Pearson correlation co-efficient gave a p-value of 0.031. In both study populations together, multiple step-wise regressions found only the presence of diabetes neared significance as a predictor for isoprostane concentrations (p= 0.071).
The comet and isoprostane assays both found an increase in oxidative stress in patients with diabetes compared to the control group, suggesting there is increased oxidative stress \textit{in vivo} in diabetes. As they are both measuring oxidative stress, the two methods were plotted against one another.

\textbf{Figure 6.10: Correlation of two markers of oxidative stress}

The mean percentages of tail DNA induced by FPG enzyme in the comet assay were ranked and plotted on the y-axis, with the concentrations of urinary isoprostanes [Isop] from the same individuals, also ranked, plotted on the x-axis.

Figure 6.10 illustrates there was no relationship between the two measures of oxidative stress as measured in 43 diabetics and their 43 matched controls. The lack of correlation may be due to the different molecules investigated (DNA versus lipids) and/or the different samples (blood versus urine), or due to different pathways of formation.
6.8 Discussion

The comet assay is increasingly used in genotoxicity testing [292] as well as human biomonitoring studies [293, 294]. Previous studies investigating DNA damage by SCGE in human subjects have shown great variation between studies [77, 79-82, 96, 97]. This may be attributed to different methodology, heterogeneous populations, and true physiological variation. To test the reproducibility of the assay, intra-, inter-assay, and intra-subject variability were investigated in this study. The coefficients of variation were 18.4% and 15.3% for intra-assay variation in two subjects (figure 6.1), which were between 11.6% and 21.3% previously reported by Collins et al [294] and Holz and colleagues [295] respectively. The inter-assay variation showed much lower values between 8 and 9% (figure 6.2) compared with Holz and colleagues (18.1%). Collins' and colleagues recorded the inter-assay variation as a correlation, and found there to be good agreement between the means of identical lymphocyte samples processed on two separate occasions in six subjects ($r = 0.97$).

As with measuring any physiological parameter in humans, there will be a degree of variation within subjects. Holz and colleagues studied 10 subjects on two separate days and showed a mean (± SD) coefficient of variation of 42.2% (± 18.6%) with a range of 18 to 72% [295]. This again was much higher compared to the median result in 5 subjects on four separate days in this study (table 6.1). Collins et al also showed a higher mean intra-individual variance of 20.9% in 6 subjects, compared with the variance for the 5 healthy volunteers used here.

The authors from both previous investigations concluded that the significant differences within and between individuals are a major source of variability. This
agrees with the results in this chapter, whereas the variability of the assay gives lower values indicating it is a relatively robust method.

Collins et al went on to investigate lymphocyte tail DNA using the comet assay in 10 insulin dependent diabetes mellitus (IDDM) subjects compared to 8 controls, and found a significant difference; 26.2 ± 7.4 vs. 11.8 ± 2.4 % (mean ± SD) respectively, p<0.05. The authors concluded that although there is considerable intra-individual variation, differences between groups of individuals could be readily measured, given only a sufficient number of individuals per group.

The pilot data in the patient populations in this study showed a significant increase in DNA damage, as measured by the percentage of DNA in the comet ‘tail’, in the patients with diabetes compared to controls (figure 6.6). This was then confirmed in the study data (figure 6.7). There was also a specific increase in oxidative damage to DNA in diabetes, indicated by the FPG-induced damage in blood cells, compared to the controls (figure 6.7).

Previous studies investigating DNA damage using SCGE in patients with diabetes have yielded varying results. Collins et al [77] and Hannon-Fletcher and colleagues [79] both investigated IDDM. The Collins study found significant differences between basal levels of tail DNA in isolated lymphocytes of 10 patients with diabetes and 10 control subjects (25.0 ± 7.5% vs. 10.9 ± 3%, p<0.003), but no significant difference in the number of FPG sites. The latter group carried out a similar study with larger numbers, and also investigated sub-populations of blood cells. The authors found no differences in basal levels of tail DNA in whole blood, isolated
lymphocytes, or monocytes in 23 patients with IDDM compared to 32 controls. When they investigated neutrophils in 50 individuals with IDDM and 50 controls, there were significant differences; 8.40 ± 0.83% versus 4.34 ± 0.27% in the IDDM group and controls respectively.

Dinçer and colleagues [82] studied 45 patients with type 1 diabetes, and found they had significantly greater basal levels of DNA damage and significantly greater FPG-sensitive sites compared to 40 controls. The FPG sensitive sites were significantly correlated with HbA1c (r=0.59, p<0.01), glucose (r=0.45, p<0.02), and the antioxidant enzyme superoxide dismutase (r=-0.50, p<0.02). The authors concluded impaired antioxidant defence in patients with type 1 diabetes may be one of the mechanisms responsible for increased DNA damage in those patients.

An earlier publication from the same group [81] showed significantly more FPG sites in 63 subjects with type 2 diabetes compared to 41 controls. The patient group was then further divided, and higher values were shown in the poorly controlled group (HbA1c ≥ 6.2%) compared to the well controlled (p<0.001). The values for men were 144 ± 29, 174 ± 26, and 213 ± 40 (AU) for the controls, the well-controlled diabetics, and the poorly controlled diabetics respectively, and 128 ± 17, 171 ± 9, and 211 ± 31 (AU) in the female populations. The FPG sensitive sites were correlated with reduced glutathione (r=-0.62, p<0.001), glucose (r=0.49, p<0.01), and HbA1c (r=0.69, p<0.001) in the patients with diabetes, and with reduced glutathione (r=-0.52, p<0.01) and HbA1c (r=0.56, p<0.005) in the controls. Again the authors postulated the decreased antioxidant defence may contribute to the increased DNA damage, and also suggested hyperglycaemia may induce oxidative damage in type 2 diabetes.
A study published in 2001 [97] also investigated type 2 diabetes. They studied olive tail moment, the product of tail length and tail intensity, in isolated lymphocytes of 31 patients with type 2 diabetes and found this was not significantly different to the smaller control population (n=16). The authors then treated the cells with hydrogen peroxide, and found the increase in strand breaks was greater in the individuals with diabetes compared to the controls; 0.098 ± 0.08 (AU) vs. 0.004 ± 0.03 (AU) respectively, (p = 0.005). The authors suggested that lymphocyte DNA in type 2 diabetes has an increased susceptibility to oxidative damage.

Şardaş and colleagues [80] visually scored comets as either showing migration or no migration at all from 63 patients with IDDM and 48 patients with NIDDM and found the 2 groups together had a significantly greater number of cells with damage compared to 30 controls (p<0.05). Those with NIDDM showed higher values of 41.1 ± 19.7 (AU) compared to 29.3 ± 16.7 (AU) in the IDDMs, (p<0.05).

The variation between different studies, including the present study, may be due to different methodologies, or different study populations. The results from the comet assay in this study suggest there is increased DNA damage in patients with type 1 and type 2 diabetes, and that there is a specific increase in oxidative damage to DNA. The elevated urinary isoprostane concentrations, an index of lipid peroxidation, observed in the same study participants (figure 6.9), provides further evidence to support the increased oxidative damage in vivo in those individuals with diabetes.

Previous studies investigating isoprostanes have found them elevated in hypertension [289], hypercholesterolaemia [296], and diabetes [78]. As commonly seen in diabetes,
the patients in this study had higher blood pressure and dyslipidaemia (chapter 4) compared to the controls. These differences may cause the observed elevation of isoprostanes, although there were no relationships between the isoprostane concentrations and blood pressure or any lipid measures. Davi et al [78] investigated urinary isoprostanes by radioimmunoassay in 62 NIDDMs, 23 IDDMs, and age- and sex-matched controls. Isoprostanes were significantly elevated in both groups of patients with diabetes compared to their matched controls, and this was independent of the presence of macrovascular complications, hypertension, and hypercholesterolaemia. Aggressive control of hyperglycaemia in these patients suppressed the elevation in isoprostanes, as did treatment with vitamin E, although both of these sub-studies involved small numbers (21 NIDDMs and 10 NIDDMs respectively). This data suggests the increased lipid peroxidation in diabetes is due to increased oxidative stress induced by hyperglycaemia.

Previous studies investigating isoprostane concentrations in urine showing no effect of lipid content of the diet [213], or of increased substrate availability in hypercholesterolaemia [296], support their use as an index of oxidative stress. Also, evidence from elevated levels of isoprostanes in situations of increased oxidative stress without hyperlipidaemia, such as smoking [212, 224], and the suppression of elevated isoprostanes by antioxidants [78, 212], implicates oxidative stress as the mediator of increased lipid peroxidation.

Lipid peroxidation products can damage DNA (reviewed in [297]), and may therefore contribute to the damage observed in the comet assay. Figure 6.10 clearly illustrates no correlation between urinary isoprostane concentrations in urine and DNA damage
in peripheral blood cells. This could be due to the different molecules (DNA versus lipids) and/or different human samples investigated (blood versus urine), or different pathways of oxidative stress leading to the DNA damage and isoprostane formation.

In the patients with diabetes in this study, lipid peroxidation levels positively correlated with ACR, an indication of protein leakage in the kidneys, and therefore severity of kidney damage ($p=0.031$). This suggests that the complications of diabetes might elevate oxidative stress, and/or a role for oxidative stress in the pathogenesis of complications. Previous research has investigated oxidative stress early in the progression of diabetes, and in the absence of complications to answer this. Martin-Gallán and colleagues [84] showed elevated lipid peroxidation and protein oxidation in type 1 patients with diabetes, with and without microangiography. The same group [83] also investigated 54 patients with type 1 diabetes (aged 2 to 22 years), 24 of which were evaluated 7 to 10 days after clinical onset of the condition. The remaining 30 were free of neuropathy, nephropathy, and retinopathy. Lipid peroxidation and protein oxidation markers were increased in all the patients compared to the 60 healthy age- and sex-matched controls. Both of these studies and the lack of elevated oxidative stress in hypertension [298], suggest oxidative stress is not a result of the complications in diabetes. This suggests oxidative stress may play a role in the development of complications, and has been suggested before in the review by Giugliano et al [222].

It seems apparent hyperglycaemia plays a role in the complications associated with diabetes [299], with tight control of blood glucose reducing the risk of development and severity [34, 247, 249]. There are several different hypotheses for hyperglycaemia
inducing oxidative stress. These are detailed in chapter 1, and include auto-oxidation of glucose, the polyol pathway, and local tissue damage. Previous studies have shown oxidative stress measures correlate with HbA$_{1c}$ and/or blood glucose concentrations [77, 78, 81, 82], and a reduction in oxidative stress with improved glycaemic control [78]. This is not always the case, as in this study and others [79, 80, 97, 300]. It may be that oxidative stress occurs as a result of many different pathways, which contribute at different levels at different times throughout the progression of diabetes, or the oxidative stress is induced by another mechanism.

In the control population in this study, HbA$_{1c}$ increased with age (figure 4.2) without a corresponding increase in oxidative damage to DNA or lipids. This questions the theory of hyperglycaemia induced oxidative damage, although oxidative stress has been implicated in ageing [301]. A previous study [208] has shown a relationship between age and isoprostane levels ($r=0.81$) in 20 healthy subjects. This was largely determined by data obtained from the three oldest patients and has not been confirmed in this study or others [289, 290]. It is not possible to discard the oxidative theory of ageing [65] on this lack of relationship, as it proposes an accumulation of damage over time that would not be observed in peripheral blood cells due to their relatively rapid turnover, or cause an increase in excreted lipid peroxidation products. Instead the steady state of oxidative stress maintained in the body as humans age, may lead to accumulation of oxidative damage in molecules within tissues where there is little or no cell turnover.

Alternative hypotheses for the increased oxidative stress observed in diabetes from early in the disease progression, and independent of complications, include
inflammation, and decreased antioxidant defence. Markers of inflammation are elevated in type 1 [59] and type 2 diabetes [60], and have been associated with the development of type 2 diabetes in middle-aged adults [281]. Inflammation is characterised by a respiratory burst of activated neutrophils and macrophages that can lead to elevated levels of reactive species. However, reactive species themselves are thought to act indirectly as cellular mechanisms and elicit an inflammatory response [302, 303], and this may cause the observed elevation in inflammatory markers mentioned previously. Various hypotheses for hyperglycaemia induced oxidative stress have already been mentioned. Hypotheses for an inflammatory response induced by oxidative stress early in diabetes are emerging, including the potential role of peroxisome proliferator-activated receptors [106], insulin resistance [107-109], and advanced glycation end products (AGEs) [110]. The last hypothesis was investigated in porcine coronary arteries [110], and found diabetes increased NAD(P)H oxidase activity and oxidative stress, producing inflammatory responses in porcine coronary media and adventitia. AGEs induced the expression of several inflammatory genes in coronary cells in a redox-sensitive manner, and the authors proposed they might be involved in the development of accelerated coronary atherosclerosis in diabetes. This would suggest that oxidative stress is increased due to hyperglycaemia, and this in turn causes inflammation.

Alterations in antioxidant status have previously been reported in diabetes, although the results have been inconsistent [91-96], suggesting there may be disturbances in antioxidant defences in some patients with diabetes, but they may be a consequence of the elevated oxidative stress and not the cause. Furthermore, intervention studies investigating antioxidant supplementation in patients with diabetes have shown
varying results [78, 80, 97, 98]. This could be due to different methodology, or perhaps the use of single antioxidants provides only a partial picture, and individuals may react differently to different antioxidants.

6.9 Conclusions

Oxidative stress is elevated in diabetes, as illustrated here by increased oxidative DNA damage measured by the comet assay with FPG, and increased urinary isoprostane concentrations as an index of lipid peroxidation.

The cause of the elevated oxidative stress is unknown. Hyperglycaemia has been suggested, and in previous studies has been related to measures of oxidative DNA damage, although this was not the case in this study. Alternative hypotheses include inflammation, decreased antioxidant defence, and the presence of complications. The last proposal is unlikely with oxidative stress actually proposed as a contributor in the development of complications. Studies investigating inflammation suggest elevated oxidative stress induces the inflammatory response; therefore the initial increase in oxidative stress is the consequence of another pathway. Further research is required investigating antioxidant status and antioxidant intervention in diabetes. As yet, the results have been disappointing, suggesting if there is a decrease in antioxidant defence in some individuals with diabetes, it is perhaps a consequence of the oxidative stress and not the cause.

Oxidative damage may be the result of different sources of reactive species acting in the body at the same time, and the contribution of each of these sources may vary
over the progression of diabetes. Further research is required to elucidate the mechanisms of increased oxidative stress within diabetes.
7.1 Introduction

Telomeres are specialised structures at chromosomal ends composed of a variable number of tandem repeats of the DNA sequence TTAGGG. Their functions include protection of chromosomes from degradation, prevention of end-to-end fusions, and positioning of chromosomes within the nucleus [304, 305]. In human somatic cells the length of telomeres decreases with age in vivo [119] and with cell division in vitro [306], and this shortening has been suggested as a mitotic clock, eventually leading to cell senescence and possibly death [12, 124].

Von Zglinicki and colleagues [12] suggested the observed attrition rates for telomeres in vivo are too rapid to be explained by the incomplete synthesis of the telomere in cell replication alone (the end replication problem), and proposed oxidative stress as an external influence. Therefore in situations of increased oxidative stress, for example diabetes as addressed in chapter 6, there may be increased telomere attrition.

A previous study [140] investigating telomeres in patients with diabetes found those with insulin dependent diabetes mellitus (IDDM; n=54) had significantly shorter telomeres compared to non-diabetics (n=42), and this was not related to the duration or complications of IDDM. They also studied non-insulin dependent diabetes mellitus (NIDDM; n=74), but found no difference in telomere length compared to controls (n=80).

Other studies have found shorter telomeres are associated with pulse pressure and pulse wave velocity (PWV) [136, 137], clinical markers of large artery stiffness that
increase with age. It was therefore hypothesised that telomere shortening may contribute to vascular ageing and vascular disease. Brouilette and colleagues [139] demonstrated a relationship between telomere length in peripheral blood leukocytes and the risk of developing atherosclerosis, whilst Minamino and colleagues [66] found senescence-associated phenotypes of vascular endothelial cells present in human atherosclerotic lesions, and suggested endothelial cell senescence induced by telomere shortening may contribute to atherogenesis.

7.2 Aim

Southern blotting is the traditional method for telomere length measurement. There are many variations used for this method, therefore the technique was developed prior to measurement in the study groups to optimise the conditions.

The hypothesis is that oxidative stress is increased in diabetes, and this accelerates telomere attrition, which may then contribute to vascular ageing and cardiovascular disease, a common complication in diabetes. The aim of this chapter was to investigate any differences in length, or rate of loss of telomeres with age between the patients with diabetes and controls. The relationships of telomere length with oxidative stress and markers of vascular ageing were also investigated.

7.3 Methods

Southern blotting was used to determine a terminal restriction fragment (TRF) length for each sample investigated. Initial methods were similar to those described in chapter 3, with the following differences. Lymphocytes were isolated and embedded in agarose plugs before lysis. The plugs were then washed, digested with restriction
enzymes, and resolved on a 0.5% agarose gel in 0.5x tris/boric acid EDTA (TBE) buffer, with 0.5x TBE as the electrophoresis buffer. The DNA was transferred to a positively charged nylon membrane by Southern blotting with 20 x saline sodium citrate (SSC; 0.3M Trisodiumcitrate, 3M NaCl) as the transfer buffer. After sufficient time was allowed for transfer of the DNA onto the membrane, the DNA was fixed with UV light. Blots were hybridised and probed with a $^{32}$P labelled probe.

The following results indicate the experiments leading to the changes in methodology, culminating in the final method for determining TRF length as described in chapter 3. This was the method used for obtaining TRF lengths in 51 patients with diabetes and their age- and sex-matched controls. Details of patient and control demographics are in chapter 4.

Median TRF lengths, as calculated by Telometric software using the equation in section 3.4, are displayed for the method development data, as they are suggested as more reproducible than mean values (equation also in section 3.4) in this study (table 7.1), and others [219]. To enable comparison with other studies, both mean and median TRF values are presented for the study populations.

7.4 Method development for determination of TRF length

7.4.1 TAE versus TBE buffer

The initial method for Southern blotting used tris/boric acid EDTA (TBE) buffer as the electrophoresis buffer. Previous research has shown TBE buffer may produce spurious artefacts for electrophoresis of large DNA molecules, and faster electrophoretic mobilities perhaps due to the formation of non-specific DNA-borate
complexes [307]. These findings were compared to tris/glacial acetic acid EDTA (TAE) buffer, which gave more reproducible results with fewer artefacts for DNA fragments between 400 base pairs and 48.5 kilobase pairs. Human TRFs lie within this range, therefore it would suggest TAE buffer should be used to cast the gel, and as the electrophoresis buffer in determining TRF lengths from human DNA. To investigate if TBE buffer would give significantly different results compared to TAE buffer, blood was sampled from 5 healthy volunteers, and isolated lymphocytes were embedded in 2 agarose plugs per sample. The methods were carried out on one plug as detailed in section 7.3, and then repeated on the second plug with the replacement of TBE buffer with TAE.

![Figure 7.1: Effect of different electrophoresis buffer on TRF length](image)

*Figure 7.1: Effect of different electrophoresis buffer on TRF length*

Mean (± sem) of five median TRF lengths from 5 healthy volunteers using TAE and TBE buffers. *P<0.05 from a paired t-test.
Figure 7.1 indicates TBE gave higher values for TRF length compared to TAE buffer in the same samples. To formally test this difference a paired t-test was performed on the data and found to be significant (p=0.006). The higher values obtained using TBE are probably less accurate than those for TAE due to the size of the TRF DNA and the optimal resolution of the two buffers. Thereafter TAE was used and any previous data using TBE buffer was not included with the latter results.

7.4.2 Effect of different DNA preparation techniques

Isolating lymphocytes and embedding them in agarose plugs, as the method of DNA extraction prior to digestion with restriction enzymes, is very time-consuming and technically problematic. This led to the use of QIAamp, a commercial kit from QIAGEN, to extract DNA from whole blood. The methods are detailed in chapter 3, but essentially DNA was extracted from whole blood using the QIAamp kit, quantified, and then digested with restriction enzymes.

To compare if the different methods of DNA preparation had any effect on TRF length, blood was sampled from 8 healthy volunteers and DNA was either extracted from whole blood using QIAamp preparations (QIAamp preps), or lymphocytes were isolated, and agarose plugs containing the cells were made. After the plugs were lysed and washed, the methods were essentially the same.
Figure 7.2: Effect of DNA preparation technique on TRF length

Each bar of the figure represents the mean (± sem) of 8 median TRF lengths from the same 8 healthy volunteers. For each individual, DNA was prepared either by isolation from whole blood using QIAamp preps, or was embedded in agarose plugs from isolated lymphocytes.

TRF lengths were slightly longer, although not significantly as determined by a paired t-test, when the plug method was used (figure 7.2). This could be due to the different cells present in the whole blood, and may reach significance if larger numbers were investigated. As there was no significant difference indicated here between the methods, and the QIAGEN kit is less time-consuming, it became the method of choice for the study.

The optimised methods are described in chapter 3 (p81-6), but briefly DNA extracted using a QIAGEN kit, TAE buffer for the gel electrophoresis, and alkaline blotting
buffer (ABB) as the transfer buffer were used. The last alteration in method was introduced because the ABB transferred the DNA quicker and more successfully compared to SSC. These methods were applied for calculating inter-assay variability (section 7.5) and the subject data (section 7.6).

7.5 Inter-assay variability
For determination of TRF lengths in all the samples, 6 gels were required. As a precaution, controls were processed on the same gel as their age- and sex-matched patients with diabetes, and a range of ages were processed on each gel, therefore minimising a potential batch effect. Inter-assay variation was also calculated from repeated measures of two reference samples (Ref 1 and Ref 2), which were processed on the same gels as the study samples. The samples were analysed in an identical manner to the diabetic and control samples.
### Table 7.1: Inter-assay variance for TRF lengths

Mean, median, variance, and coefficient of variation (C of V) values for the mean and median TRF lengths for 2 reference samples (ref 1 and ref 2), repeated six times on different gels.

Inter-assay variation for determining TRF length shows the variance is less for the median TRF values compared to the means for both reference samples (table 7.1). These were calculated by Telometric software using the equations in section 3.4. This indicates median TRF lengths may be more reproducible and therefore precise, as suggested previously [219]. Overall inter-assay variability gave low values, indicating data from separate gels can be analysed together.

#### 7.6 Subject data

TRF lengths were calculated in 51 diabetics and their 51 age and sex-matched controls.
<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Diabetics</th>
<th>p-value</th>
</tr>
</thead>
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<td>Median age (range) years</td>
<td>51 (32-75)</td>
<td>55 (31-78)</td>
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<td>Mean of means ± sem (kb)</td>
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<td>7.65 ± 0.11</td>
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</tr>
</tbody>
</table>

Table 7.2: TRF data from peripheral blood in patients with diabetes and controls.

Median ages and means of median and mean TRF lengths are displayed, in patients with diabetes and age- and sex-matched non-diabetic controls.

Table 7.2 displays the TRF lengths for the study populations, indicating patients with diabetes have shorter telomeres compared to their matched controls, although this is not significant with the p-values from 2-sample t-tests displayed in the table.

Telomeres are known to decrease in length as humans get older [119], and therefore this relationship was investigated in both study groups.
Figure 7.3: Relationship of TRF length with age in both study populations

Median TRF length plotted against age in patients with diabetes and age- and sex-matched controls

Figure 7.3 displays the regression equations and significance levels for median TRF lengths with age, indicating a loss of 18.6 base pairs per year (bp/yr) in the controls and 17 bp/yr in the patients with diabetes. Similar relationships were seen with the mean TRF lengths, with the equations $y = -0.026x + 11.3$ ($p=0.030$) for the controls, and $y = -0.027x + 11.1$ ($p=0.038$) for the patients with diabetes. The base pair (bp) loss per year is very similar in both groups as illustrated by the similar slopes of regression lines on figure 7.3, but the line is about 200 - 300 bp lower for those individuals with diabetes. This suggests, at a given age patients with diabetes have shorter telomeres compared to healthy controls, which was indicated in table 7.2 although this was not significant.
Multiple step-wise regressions with median TRF length as the response, and age and the presence of diabetes as predictors found only age, as expected, to be significant (p=0.001).

Oxidative stress is thought to contribute to telomere attrition [12], and was therefore investigated in the TRF data from both study groups. The above regression was repeated with baseline comet damage (TE buffer), FPG induced damage, and urinary isoprostane concentrations as additional predictors. Age remained the only significant independent variable, although comet damage with TE buffer alone neared significance (p=0.054) if there were four or less predictors entered.

PWV, a measure of arterial stiffness and therefore vascular ageing, has previously been related to TRF length [136], and was therefore investigated in the study groups.
Figure 7.4: Relationship of PWV and TRF length in the study populations

PWV data plotted against median TRF lengths in patients with diabetes and control subjects.

The relationship between TRF length and PWV was very significant in the control group, as shown in figure 7.4; however, it was not apparent in those individuals with diabetes due to the greater spread of data.

Multiple step-wise regression analysis for pulse wave velocity (PWV), as carried out previously in chapter 5, was repeated with median TRF length as an additional predictor. The results were the same as before with age, systolic blood pressure, heart rate, and the presence of diabetes as significant predictors. This suggests alterations in median TRF do not affect PWV and the relationship in the control group was only an
age effect, or the relationship is only in healthy controls and this is masked by the data from the patients with diabetes when studying the two groups together.

Benetos and colleagues [136] also related pulse pressure to median TRF length. In this study, median TRF negatively correlated with pulse pressure, but again when multiple regression was carried out, median TRF was not a predictor of pulse pressure. This suggests the negative relationship observed in this study between pulse pressure and median TRF length was because they are both related to age, and not predictive of one another.

7.7 Discussion

It is well established that telomeres decrease in length with increasing age in humans, and this study confirms that (figure 7.3). The observed attrition rates are similar to those previously reported by Von Zglinicki and colleagues [308], investigating TRF lengths in peripheral blood mononuclear cells in vascular dementia patients. They showed losses of 20 bp/yr in the patients with vascular dementia and the control population, but at any age the patients’ telomeres were approximately 440 bp shorter. Elisabeth Jeanclos and colleagues [137] investigated TRF length in diabetes, and reported losses of 27 bp/yr in the control group, 30 bp/yr in IDDM, and 18 bp/yr in NIDDM, with no significant differences between these slopes for declining TRF length.

There is great variation in published telomere attrition rates from 14 bp/yr in 143 healthy individuals aged 60-97 years [221], to 38 bp/yr in 120 healthy men and 36 bp/yr in 73 healthy women [136]. These differences between studies may be
attributed to several factors including different methodology and/or different populations investigated. This is especially true for looking at different age groups and age ranges, as studies have shown the attrition rate varies with age in humans [119, 309, 310], therefore the lower rate of loss of 14 bp/yr reported by Cawthorn and colleagues may be due to their older age range (60 - 97 years). Table 7.3 at the end of this chapter (p195-7), summarises previous studies investigating TRF lengths in humans, along with the present results to provide a clearer view of how the data presented here relates to previous findings.

Comparing the absolute value for mean or median TRF lengths between studies is more difficult, due to the different age groups studied. The majority use mean TRF length as indicative of telomere length; however, in this study, repeated mean TRF values showed greater variation compared to median values (table 7.1). This suggests median TRF lengths are more precise and reproducible, which agrees with Grant and colleagues [219].

Frenck and colleagues [309] studied several generations within families, with the grandparents sharing a similar mean age (50.4 years) to the median age of the control population in this study (51 years). They reported a mean (± SD) TRF length of 9.6 ± 0.8 kb in the grandparents, which is slightly lower than the 9.98 ± 0.19 kb reported earlier in the controls in this study (table 7.2). These differences may be due to the range of ages in this study extending 10 years younger than Frenck and colleagues’ grandparent group. Again, table 7.3 (p195-7) provides a clearer view of the present results in relation to previous studies.
The heterogeneous nature of telomere length also makes investigations more difficult, with large numbers required to show relationships. At any given age in humans, there is considerable variation in telomere size in peripheral blood cells [310, 311]. This is true even in neonates [312], although adults show greater variation compared to children [309].

Twin studies have revealed telomere length is largely determined by genetic factors, which may explain a large component of the variation in telomere length in individuals of the same age. In 1994 [313], TRF lengths in 123 twin pairs aged 2 to 95 years were studied. The authors found heritability accounted for 78% of the variance of TRF lengths in 3 cohorts grouped by age. They also found a large batch effect, and the different age cohorts were processed in different batches. When the authors corrected for age and batch effects, there were strong correlations between twins and this was greatest in monozygotic twins. Rufer and colleagues [310] also showed stronger correlations in telomere lengths in monozygotic twins compared to dizygotic, and no correlation in unrelated individuals of the same age. A study in 2004 [314] went on to investigate the mode of inheritance of TRF length, and suggested from their results it is X-linked. These findings would suggest that TRF length is more likely to determine diabetes, rather than diabetes influencing TRF length.

Other determining factors for telomere length include the presence of active telomerase, and external influences such as oxidative damage. Telomerase, a ribonucleoprotein enzyme, synthesises telomeric repeats and therefore maintains telomere length and halts senescence in human cells [315]. It is present at low levels in normal peripheral blood and bone marrow cells [316, 317], and its activity appears
to vary with age [119]. The significance of telomere activity in subpopulations of leukocytes and haematopoietic progenitors is not clear, as the telomeres of these cells shorten as a function of donor age in vivo [309, 318]. Weng and colleagues [319], found telomerase activity increased upon activation of T cells. This was found to be transient and insufficient to maintain telomere length over the replicative life span of T cell cultures by another group [315]. Yui and colleagues also suggested the telomerase activity in haematopoietic cells was insufficient to prevent the replication dependent loss of telomeric DNA in those cells.

Oxidative stress increases the attrition rate of telomeres [12], and as found in this study (chapter 6) and others [77, 283-285], there is increased oxidative stress in diabetes. This leads to the hypothesis that patients with diabetes will have shorter telomeres as a consequence of the increased oxidative stress they are exposed to. The only previous study investigating TRF length in white blood cells of 74 NIDDM and 54 IDDM patients found, after age-adjustment, those patients with IDDM had significantly shorter TRFs compared to controls [137]. There was no significant difference between the NIDDM group and controls, or in the rate of loss between the three groups. The authors concluded that these observed shorter TRFs likely reflected a marked reduction in the TRF length of subsets of white blood cells that play a role in the pathogenesis of IDDM. They proposed that the shortening may have happened prior to the clinical manifestation of the disorder, or it could be that the reduced length is determined by genetic factors that establish the expression and function of those subsets of blood cells involved in the pathogenesis of IDDM.
In this study, the patients with diabetes had slightly shorter TRFs, but this was not significant (table 7.2), and similar attrition rates compared to the controls (figure 7.3). Therefore although the patients with diabetes had significantly greater oxidative stress (Chapter 6), and this is thought to increase telomere loss, this did not affect TRF length or attrition rate in this study. This may be due to the small numbers involved and therefore lack of power. Using the difference in TRF length observed from the current data, a sample size of 216 is required to show a difference with 90% power at the 0.05 significance level. There may be other explanations such as increased antioxidant defence and/or DNA repair in the patients with diabetes, counteracting the increased oxidative stress. There is also some evidence that certain medication for the treatment of diabetes has antioxidant properties, although this is also inconclusive [320, 321]. These suggest no increase in oxidative stress would be observed, which was not the case. There was elevated oxidative DNA damage to the nuclear genome, as measured by the comet assay, in the same individuals (chapter 6). A final alternative explanation is that oxidative stress does not cause accelerated telomere attrition in peripheral blood cells.

Other studies have found shorter telomeres are associated with markers of vascular ageing, such as PWV [136, 137], and have proposed shorter telomeres may contribute to vascular ageing and disease. This does not seem apparent in this study, as the patients with diabetes had significantly higher PWVs, yet no significant difference in TRF lengths compared to the controls. Figure 7.4 confirms the association of pulse wave velocity (PWV) with TRF length in the controls as found earlier by Benetos and colleagues [136] in 120 healthy men and 73 healthy women. After adjustment for age and mean arterial pressure (MAP), the authors found TRF length accounted for 2% of
variability in PWV in men, but this was not significant in women. In the present study, the relationship between PWV and TRF length was only apparent in the controls, and only because they are both significantly related to age, and not predictive of one another. This was also the case for pulse pressure (PP), which again disagrees with Benetos and colleagues who found TRF length accounted for 11.9% of the variability in PP, after adjustment for age and MAP, in men only. Jeanclos and colleagues [137] also found TRF length predictive of PP in 98 healthy twins, although they did not find a relationship between age and TRF length. This is unusual given the genetic component of TRF length, the large numbers studied, and the wide age range of the twins (18-44 years).

Further research has found shorter telomeres present in atherosclerosis [322], in vascular endothelial cells that are under greater haemodynamic stress [116], and atherosclerotic plaques [66]. Various hypotheses have been proposed to explain these observations, with oxidative stress and inflammation the most prominent. Oxidative stress is implicated in atherosclerosis [18]; therefore the decreased telomere length observed in the condition may be attributed to the increased oxidative damage. This contradicts the findings presented here, but this may be attributed to the different cells investigated. Inflammation, is also increased in the pathogenesis of coronary atherosclerosis [21], and may lead to the shorter telomeres through increased cell replication or by increasing oxidative stress. Inflammation, as the cause of increased telomere shortening, could also explain the shorter TRFs in the IDDMs in the study by Jeanclos and colleagues [137], due to the inflammatory response through activation of subsets of white blood cells in IDDM.
There may be increased loss of telomere length early in the life of individuals who develop diabetes. This is partially supported by the slope of decline in TRF length in the patients with diabetes in this study lying lower than the slope for the control group (figure 7.3). Although this was not a significant difference, this may be due to the small numbers involved. It suggests genetically short telomeres may predispose to diabetes, or a period of increased telomere attrition in patients with diabetes prior to investigation. It is unlikely that short telomeres cause diabetes, because patients with ataxia-telangiectasia [323] and dyskeratosis [324], both inherited disorders exhibiting signs of premature ageing and characterised by abnormally short telomeres, have no reported excess of diabetes. The latter theory of a previous period of increased attrition poses the question of when this increased shortening happened. The Barker hypothesis proposes low birth weight babies have a period of catch up which is linked to cardiovascular disease, and may be a risk for diabetes [325]. An alternative is that increased attrition may happen prior to the clinical manifestation of the disease, but when the individual is exposed to hyperglycaemia, or possibly heightened inflammation or levels of oxidative stress. It may take time for the body to counteract these conditions, and in that time the telomeres may be prematurely shortened.

7.8 Conclusions

In this study, TRF length in peripheral blood cells was slightly but not significantly shorter in patients with diabetes compared to age- and sex-matched controls. This was despite observed increased oxidative stress in peripheral blood cells from the same samples. The lack of significance may be due to the small numbers investigated, or oxidative stress may not accelerate telomere attrition in peripheral blood cells.
TRF length was not predictive of markers of vascular ageing, and is therefore thought not to contribute to the vascular complications common in diabetes, or vascular ageing in healthy controls.

Further studies investigating TRF length and oxidative stress are required in large numbers of patients with diabetes to establish if the slightly shorter TRF length observed in this study is representative of a diabetic population. Moreover, methods are being developed to use real-time PCR to more accurately determine TRF length. The application of such techniques to this kind of study would be interesting. Ideally markers of antioxidant status, DNA repair activity, inflammation, and telomerase activity would also be investigated, and the population would include sufficient numbers of untreated patients, type 1 and type 2 patients, and those without complications. In addition, it is worth reflecting on the fact that we have studied peripheral blood cells as a surrogate for generalised cellular ageing in diabetes. It is conceivable that the effects of enhanced oxidative stress in diabetes, may be more pronounced in other cell types such as endothelial cells, cardiac myocytes etc. Whilst these would be more relevant to the vascular ageing hypothesis, they are less accessible for study.
<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Mean age ± SD (range) (years)</th>
<th>Mean TRF ± sem (kb)</th>
<th>Attrition rate (bp/year)</th>
<th>Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slagboom et al</td>
<td>123 twin pairs 4 age cohorts</td>
<td>(2-95)</td>
<td>4.15 ± 1.4</td>
<td>8.3 ± 0.64 (SD)</td>
<td>31.0</td>
</tr>
<tr>
<td>1994 [313]</td>
<td></td>
<td>17.1 ± 2.4</td>
<td>7.8 ± 0.56 (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>43.7 ± 5.79</td>
<td>7.3 ± 0.76 (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>79 ± 7.82</td>
<td>5.6 ± 0.40 (SD)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TRF length inversely correlated with age in the group as a whole (p&lt;0.005). No independent effect of sex. 78% heritability for mean TRF length.</td>
</tr>
<tr>
<td>Jeanclos et al</td>
<td>54 IDDM 42 controls 74 NIDDM 80 controls 106 controls</td>
<td>30.4 ± 7.7 (17-48) similar age range</td>
<td>8.6 ± 0.1®</td>
<td>30 ± 15 (SD)</td>
<td>*p = 0.002 compared to age-matched controls.</td>
</tr>
<tr>
<td>1998 [140]</td>
<td></td>
<td>59.5 ± 8.0 (34-75) similar age range</td>
<td>9.2 ± 0.1®</td>
<td>18 ± 14 (SD)</td>
<td>TRF length inversely correlated with age in all groups, (p&lt;0.0001 for 106 controls). No relationships with duration or complications of diabetes.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51.5 ± 18.1 (20-87)</td>
<td>8.1 ± 0.1®</td>
<td>27 ± 5 (SD)</td>
<td></td>
</tr>
<tr>
<td>Frenck et al</td>
<td>12 newborns 24 parents 35 grandparents 4 great grandparents</td>
<td>&lt; 1 hour (0-1 hour)</td>
<td>16.4 ± 1.2 (SD)</td>
<td></td>
<td>Greater attrition of telomeres when younger.</td>
</tr>
<tr>
<td>1998 [309]</td>
<td></td>
<td>26.5 (20-36)</td>
<td>11.6 ± 1.2 (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50.4 (42-72)</td>
<td>9.6 ± 0.8 (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 (62-82)</td>
<td>8.0 ± 1.1 (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rufer et al</td>
<td>301 healthy individuals granulocytes lymphocytes</td>
<td>(0-90)</td>
<td>39</td>
<td>TRF length inversely correlated with age (p&lt;0.0001). No significant difference between males and females. Strong correlations between TRF lengths of monozygotic twins, and less so between dizygotic twins. No correlation between unrelated individuals.</td>
<td></td>
</tr>
<tr>
<td>1999 [310]</td>
<td></td>
<td></td>
<td>59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.3 – refer to page 197 for legend.
<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Mean age ± SD (range) (years)</th>
<th>Mean TRF ± sem (kb)</th>
<th>Attrition rate (bp/year)</th>
<th>Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeanclous et al</td>
<td>98 twins</td>
<td>36.98 ± 7.95 (18-44)</td>
<td>8.73 ± 0.79 (SD)</td>
<td></td>
<td>No significant relationship with age, but TRF length inversely correlated with pulse pressure (p=0.0032), and women had longer TRF lengths than men.</td>
</tr>
<tr>
<td>2000 [137]</td>
<td>38 men 60 women</td>
<td>37.52 ± 6.51 36.63 ± 7.7</td>
<td>8.51 ± 0.88 (SD)</td>
<td>8.87 ± 0.71 (SD)</td>
<td></td>
</tr>
<tr>
<td>Benetos et al</td>
<td>120 men</td>
<td>55 ± 11</td>
<td>8.37 ± 0.07**</td>
<td>38</td>
<td>*p=0.016 compared to women. TRF length inversely correlated with age in both genders (p=0.0001), and with pulse wave velocity in men (p&lt;0.05) after adjustment for age and mean arterial pressure.</td>
</tr>
<tr>
<td>2001 [136]</td>
<td>73 women</td>
<td>56 ± 11</td>
<td>8.67 ± 0.09®</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Samani et al</td>
<td>10 CAD patients</td>
<td>(42-72)</td>
<td></td>
<td>35 ± 4 (sem)</td>
<td>TRF length inversely correlated with age (p&lt;0.001). Mean TRF lengths 30 ± 90 (sem) bp shorter compared to controls after age and sex adjustment (p=0.002).</td>
</tr>
<tr>
<td>2001 [322]</td>
<td>20 controls</td>
<td>(39-72)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cawthorn et al</td>
<td>143 healthy individuals</td>
<td>(60-97)</td>
<td></td>
<td>Approx. 14</td>
<td>Association between TRF length and age (p=0.0074), and TRF length and mortality (p=0.021 for those aged 60-74 years; p=0.086 for those ≤ 75 years). No independent effect of sex.</td>
</tr>
<tr>
<td>2003 [221]</td>
<td>71 women, 72 men</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brouilette et al</td>
<td>203 premature MI cases</td>
<td>46.8 ± 6.2</td>
<td></td>
<td>28.3 ± 7.0</td>
<td>*p&lt;0.0001 when all cases were compared to all controls after adjusting for age and sex.</td>
</tr>
<tr>
<td>2003 [139]</td>
<td>173 men, 30 women</td>
<td></td>
<td></td>
<td></td>
<td>TRF inversely correlated with age in both groups (p=0.0001). No independent effect of sex.</td>
</tr>
<tr>
<td></td>
<td>180 controls</td>
<td>47.2 ± 5.9</td>
<td></td>
<td>24.8 ± 9.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>155 men, 25 women</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nawrot et al</td>
<td>119 men</td>
<td>Approx (15-80)</td>
<td>6.77 ± 0.05**</td>
<td>24</td>
<td>*p=0.028 when compared to women. Sex, age, and smoking were significant determinants of TRF length.</td>
</tr>
<tr>
<td>2004 [314]</td>
<td>152 women</td>
<td>Approx (15-80)</td>
<td>6.92 ± 0.05®</td>
<td>19</td>
<td>Strong intrafamilial correlations, best explained by X-linked inheritance of TRF length.</td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Mean age ± SD (range) (years)</td>
<td>Mean TRF ± sem (kb)</td>
<td>Attrition rate (bp/year)</td>
<td>Correlations</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>--------------------------------</td>
<td>---------------------</td>
<td>------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>This study</td>
<td>51 patients with diabetes 51 controls</td>
<td>35 (31-78) 51 (32-75)</td>
<td>9.62 ± 0.18 (sem) 9.98 ± 0.19 (sem)</td>
<td>17.0 18.6</td>
<td>TRF inversely correlated with age in both the patients with diabetes (p=0.046), and the controls (p=0.010).</td>
</tr>
</tbody>
</table>

@ mean TRF length age-adjusted. ¹ telomere length calculated using fluorescence in situ hybridisation. ² mean TRF calculated using quantitative PCR. ³ median age.

**Table 7.3: Studies investigating TRF lengths in humans**

Previous studies investigating TRF lengths in peripheral blood of humans are listed in chronological order, detailing subjects studied, and if available; mean age and range, mean TRF length, rate of attrition, and any correlations or further findings. The last entry in the table in bold are the results from this study to allow comparison with the previous research. All methods involved Southern blotting, unless noted otherwise. Abbreviations used: IDDM, insulin dependent diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus; CAD, coronary artery disease; MI, myocardial infarction; Approx., approximately.
8.1 Introduction

Mitochondria are a major site of superoxide production in the body [148, 149], and the mitochondrial genome is believed to be especially susceptible to oxidative damage [11, 155] and more so than the nuclear genome. The mitochondrial genome is especially vulnerable for several reasons including; i) its close proximity to the site of ROS production, ii) the lack of histone protection [154], iii) insufficient repair mechanisms [11], and iv) its highly compact structure with few non-coding regions [13]. The mitochondrial theory of ageing [326] proposes that oxidative damage to the mitochondria will adversely affect its function, and that this in turn will lead to the generation of more ROS, that will cause more damage. This vicious circle will result in an accumulation of dysfunctional mitochondria with age, which could eventually lead to cell loss, dysfunctional tissue, and ageing. This theory has developed further, proposing that it is oxidative damage to the mitochondrial DNA (mtDNA) that leads to dysfunctional mitochondria and therefore ageing [155].

There is considerable data regarding qualitative changes in mtDNA in ageing, with an increase in deletions with age observed in many tissues [166-169], but inconclusive results from investigations into the incidence of point mutations with age [167, 169, 170, 327]. Quantitative investigation of mtDNA in ageing has also provided contradictory data, with a decrease in content in rat liver [171], but either no change in human skeletal muscle and cardiac muscle [168], or increases in human muscle [172], brain tissue [173], and lung tissue [174]. These increases are thought to be a compensatory mechanism for decreased mitochondrial function [171-174]. A study
investigating mtDNA content in leukocytes [179], found increases up to a plateau in middle age, where the copy number then started to decline. The authors found the content correlated with oxidative stress indices. These studies suggest that the mitochondrial mass is not static but may be responsive to the local metabolic environment, whereby a reduction in available energy or reduced mitochondrial mass in some way leads to an increase in mitochondrial number.

Qualitative changes in mtDNA, such as mutations and deletions, have been implicated in the pathogenesis of diabetes mellitus, but explain only a very small proportion of the case of diabetes mellitus (reviewed in [182, 187]). Quantitative changes in mtDNA have also been investigated in diabetes. Studies have found decreased levels of peripheral blood mtDNA in non-insulin dependent diabetes mellitus (NIDDM) [190, 191], preceding the onset of NIDDM [190], and in the offspring of patients with type 2 diabetes [191]. A decrease in mtDNA content has also been observed in skeletal muscle of type 1 and type 2 patients, with an increase in mitochondrial gene expression [192]. Chapter 6 of this study, and other research groups have shown increased oxidative stress in patients with diabetes compared to controls [77-84]. This increase in oxidative stress could lead to increased damage in mtDNA, and this lead to the proposal that decreased mtDNA content in diabetes is a consequence of the increased oxidative stress. Furthermore, because pancreatic β-cell mitochondria play a central role in glucose-stimulated insulin release [193], damage to the β-cell mitochondria will attenuate this response.
8.2 Aim

The hypothesis is that oxidative stress is increased in diabetes and this leads to increased damage to the mitochondrial genome, which in turn decreases mtDNA copy number. This chapter aims to quantify mtDNA in patients with diabetes and compare the values with age- and sex-matched controls. Any relationships between mtDNA content and oxidative stress and any of the physiological variables investigated in chapter 4 will also be studied.

8.3 Methods

MtDNA content was quantified in peripheral blood cells of 51 patients with diabetes and 51 age- and sex-matched controls. Using real-time PCR, a segment of the mitochondrial genome is amplified. This is then normalised for differences in template DNA by a separate PCR of a segment of a single copy gene in the nuclear genome. Full methods are detailed in chapter 3, and demographic and physiological characteristics of the patients with diabetes and the controls are detailed in chapter 4.

It was necessary to carry out four separate PCR assays for both the mitochondrial and nuclear PCR reactions (i.e. 8 in total), due to the number of samples being investigated in triplicate. As a precaution to avoid any batch effect, patients with diabetes and age- and sex-matched controls were processed in the same assay, and a wide age range was included in each assay.

8.4 PCR assay variation

To investigate the reproducibility of the real-time PCR method, variability within and between separate assays was investigated.
### Table 8.1: Mitochondrial PCR intra-assay variation

Each column displays the mean variance and coefficient of variation (C of V) of 26 samples in a single assay, with the four columns representing the four assays (mtPCR 1-4) required to process all the samples. Individual sample variance was calculated from the quantities in three replicate wells.

Intra-assay variance for the mtDNA PCR was calculated for each sample from the quantity of mtDNA in three replicate wells. It was therefore possible to calculate variance for every sample measured, and then calculate the mean intra-assay variance from all the samples. Table 8.1 displays the mean intra-assay variance and coefficient of variation for four mitochondrial PCR assays, with 26 samples in each assay. The values are very low: 0.13%, 0.01%, 0.02%, and 0.02% for the mean variance of the 4 assays; and 1.17%, 0.41%, 0.47%, and 0.37% for the mean coefficient of variation of the 4 assays. These results indicate very little intra-assay variance, and suggest the method is reproducible.

<table>
<thead>
<tr>
<th></th>
<th>mtPCR 1</th>
<th>mtPCR 2</th>
<th>mtPCR 3</th>
<th>mtPCR 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Mean variance % (range)</td>
<td>0.13 (0.00-1.35)</td>
<td>0.01 (0.00-0.07)</td>
<td>0.02 (0.00-0.14)</td>
<td>0.02 (0.00-0.15)</td>
</tr>
<tr>
<td>Mean C of V % (range)</td>
<td>1.17 (0.04-5.43)</td>
<td>0.41 (0.10-1.11)</td>
<td>0.47 (0.07-1.66)</td>
<td>0.37 (0.05-1.67)</td>
</tr>
</tbody>
</table>
Table 8.2: Nuclear PCR intra-assay variation

Each column displays the mean variance and coefficient of variation (C of V) of 26 samples in a single assay, with the four columns representing the four assays (nPCR 1-4) required to process all the samples. Individual sample variance was calculated from the quantities in three replicate wells.

Intra-assay variance for the nuclear DNA (nDNA) PCR was calculated for each sample from the quantity of nDNA in three replicate wells. Again, it was possible to calculate the mean intra-assay variance from all the samples. Table 8.2 displays the mean intra-assay variance and coefficient of variation for four nuclear PCR assays, with 26 samples in each assay. The values are again very low: 0.02% for the mean variance of each of the 4 assays; and 0.50%, 0.49%, 0.49%, and 0.41% for the mean coefficient of variation of the 4 assays. This again indicates very little intra-assay variance, and suggests the method is reproducible.

To investigate the inter-assay variation, the threshold cycles (Ct) of the standards were compared in each assay, as these were identical samples treated in exactly the same manner.

<table>
<thead>
<tr>
<th>n</th>
<th>nPCR 1</th>
<th>nPCR 2</th>
<th>nPCR 3</th>
<th>nPCR 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Mean variance % 0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>(range) (0.00-0.11) (0.00-0.10) (0.00-0.09) (0.00-0.10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean C of V % 0.50</td>
<td>0.49</td>
<td>0.49</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>(range) (0.11-1.38) (0.11-1.35) (0.05-1.27) (0.04-1.30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 8.3: Mitochondrial PCR inter-assay variation

<table>
<thead>
<tr>
<th></th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mean Ct ±</td>
<td>25.74 ±</td>
<td>24.62 ±</td>
<td>23.28 ±</td>
<td>22.23 ±</td>
<td>22.32 ±</td>
</tr>
<tr>
<td>sem</td>
<td>0.59</td>
<td>0.69</td>
<td>0.59</td>
<td>0.48</td>
<td>1.12</td>
</tr>
<tr>
<td>Variance %</td>
<td>1.38</td>
<td>1.93</td>
<td>1.39</td>
<td>0.90</td>
<td>5.01</td>
</tr>
<tr>
<td>C of V %</td>
<td>4.56</td>
<td>5.64</td>
<td>5.07</td>
<td>4.27</td>
<td>10.02</td>
</tr>
</tbody>
</table>

Each column displays the mean threshold cycle (Ct) ± sem, the inter-assay variance, and the coefficient of variation (C of V) for individual standards (Std 1-5) in the four mitochondrial PCR assays.

The same five standards were used for each of the four mitochondrial PCR assays. Inter-assay variation was calculated for each of the standards, and table 8.3 displays the results. The variances over 4 assays for standards 1 to 5 were 1.38%, 1.93%, 1.39%, 0.90%, and 5.01%, with a mean value of 2.12 ± 0.74%; and the coefficients of variation over 4 assays for the same standards were 4.56%, 5.64%, 5.07%, 4.27%, and 10.02%, with a mean value of 5.91 ± 1.05%. These are relatively low values, again suggesting the method is reproducible, and samples can be compared across different assays.
Table 8.4: Genomic PCR inter-assay variation

Each column displays the mean threshold cycle (Ct) ± sem, the inter-assay variance, and the coefficient of variation (C of V) for individual standards (Std 1-5) in the four nuclear PCR assays.

<table>
<thead>
<tr>
<th></th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mean Ct ±</td>
<td>26.94 ±</td>
<td>25.79 ±</td>
<td>24.56 ±</td>
<td>23.48 ±</td>
<td>22.26 ±</td>
</tr>
<tr>
<td>sem</td>
<td>0.24</td>
<td>0.24</td>
<td>0.26</td>
<td>0.27</td>
<td>0.21</td>
</tr>
<tr>
<td>Variance %</td>
<td>0.23</td>
<td>0.22</td>
<td>0.26</td>
<td>0.29</td>
<td>0.18</td>
</tr>
<tr>
<td>C of V %</td>
<td>1.78</td>
<td>1.82</td>
<td>2.08</td>
<td>2.28</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Inter-assay variance was calculated in the same manner for the nuclear PCR assays. Again the same five standards were used for each of the four nuclear PCR assays, and the inter-assay variation for each of the standards is in table 8.4. The variances over 4 assays for standards 1 to 5 were 0.23%, 0.22%, 0.26%, 0.29%, and 0.18%, with a mean of 0.24 ± 0.02%; and the coefficients of variation over 4 assays for the same standards were 1.78%, 1.82%, 2.08%, 2.28%, and 1.92%, with a mean of 1.98 ± 0.09%. Again, these are relatively low values, suggesting the method is reproducible, and samples can be compared across different assays.

To further assess reproducibility and inter-assay variation, the standard curves generated to calculate the sample concentrations were examined. The $R^2$ values for the four standard curves for the mitochondrial PCR assays were: 0.991, 0.998, 1.000, and 0.998. This indicates very little deviation of the standards from the straight line,
and consistency between assays. The $R^2$ values for the four standard curves for the nuclear PCR assays were: 1.000, 0.999, 0.999, and 0.998, again indicating very little deviation of the standards from the straight line, and consistency over the four separate assays.

8.5 Subject data

Ratios of mitochondrial to genomic DNA were calculated for 50 out of 51 of the patients with diabetes (due to lack of sample for one patient), and all 51 of the matched controls.

![Figure 8.1: MtDNA content data in both study populations](image)

Median ratios with SD error bars of mitochondrial to nuclear DNA ($mtDNA$: $nDNA$) in 50 patients with diabetes and 50 age- and sex-matched controls.

The data were not normally distributed; therefore, figure 8.1 displays the median values for the ratio of mitochondrial DNA to nuclear DNA ($mtDNA$: $nDNA$) in the 50
patients with diabetes and their matched controls, with the data for statistical analysis in table 8.5.

<table>
<thead>
<tr>
<th></th>
<th>Controls n=50</th>
<th>Diabetics n=50</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean log mtDNA: nDNA ± SD (AU)</td>
<td>-1.84 ± 0.01</td>
<td>-1.79 ± 0.02</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Table 8.5: MtDNA content data in both study populations

Log-transformed ratios of mitochondrial to nuclear DNA (mtDNA: nDNA) in patients with diabetes and age- and sex-matched controls, with the p-value from a 2-sample t-test

After log-transformation, the mtDNA: nDNA ratios did fit a normal distribution. A two-sample t-test was carried out on the transformed data and revealed the patients with diabetes had significantly lower values for the ratio compared to age- and sex-matched controls (p=0.020), indicating they had less mtDNA (table 8.5).

Previous research has shown conflicting results with the relationship of mtDNA content with age in healthy controls [168, 172-174, 190, 191], and there is no evidence of a relationship in diabetes [190, 191]. This was therefore investigated in the study populations.
The ratio of mitochondrial to nuclear DNA showed a slight but not statistically significant decrease with age in both the patients with diabetes and controls, as illustrated in figure 8.2. Statistics were carried out on the log-transformed data for mtDNA content, as the residuals were not normally distributed for the raw data. The slopes for both study groups were similar, although the regression line for the patients with diabetes was lower, indicating at a given age they have less mitochondrial DNA compared to healthy controls as shown in table 8.5.

There were no other correlations between mtDNA content and any of the physiological variables investigated in chapter 4 for either group. The following
parameters of; duration of diabetes, blood glucose control, extent of protein leakage in the kidneys, and whether patients had type 1 or type 2 diabetes had no effect on mtDNA content in the patients. When the two populations were grouped, and multiple step-wise regression was carried out, the presence of diabetes was the only (p=0.031) significant predictor for mitochondrial DNA content, although age neared significance with a p-value of 0.061.

Figure 8.3: MtDNA content versus oxidative damage in both study populations

The relationship between the log-transformed ratios of mitochondrial DNA to nuclear DNA (log10mt:nDNA ratio) and oxidative DNA damage in 50 patients with diabetes and 50 age-and sex-matched controls. The measure of the percentage of 'tail' DNA in the comet assay after the addition of formamidopyrimidine DNA glycosylase (FPG) enzyme was used as an index of oxidative nuclear DNA damage (refer to chapter 6 for further information). Equations for the regression lines are also shown.
Oxidative stress was increased in the patients with diabetes (chapter 6), and was therefore proposed to damage the mtDNA, and may perhaps lead to the observed decrease in content. Figure 8.3 shows there are no relationships between the log-transformed data of mtDNA content and oxidative DNA damage in both the control population (p=0.423), and in the patients with diabetes (p=0.292). The figure displays the data from the comet assay as a measure of oxidative stress (refer to chapter 6 for further details). Similar results were found when urinary isoprostanes were investigated as an index of systemic oxidative stress (refer to chapter 6 for further details). Multiple step-wise regression was repeated for all the subjects’ mtDNA content data pooled, with the addition of the oxidative stress indices of percentage tail DNA in the comet assay, and urinary isoprostanes as predictors. Again, the presence of diabetes was the only significant predictor for mtDNA content.

8.6 Discussion

Real-time PCR is a relatively new technique for the quantification of mtDNA, but previous studies have shown it to be consistent and reproducible [217, 218], with data concordant between Southern blotting and real-time PCR [178, 216]. To test the reproducibility in this study, intra- and inter-assay variability were investigated (tables 8.1-8.4). Previous studies have reported coefficients of variation within an assay of: 1.09% for the mean of 6 dilutions of a plasmid standard repeated 10 times [218]; <2.5% for 2 separate templates within one blood sample repeated 3 times [178]; 2% for the mean of 5 dilutions of a plasma standard carried out in duplicate [217]; and 11.3% for the mean of 11 peripheral blood samples also in duplicate [217]. The intra-assay coefficients of variation in this study for both the mitochondrial and nuclear PCRs were calculated from 26 samples carried out in triplicate from peripheral blood
of the study participants. The ranges for the coefficients of variation displayed in tables 8.1 and 8.2 indicate that even the highest value is less than the mean reported by Gahan and colleagues for samples from peripheral blood [217], suggesting relatively low intra-assay variation in this study.

Inter-assay coefficients of variation previously reported were: 3.7% (1:1000 dilution) and 5.78% (1:100 dilution) for the means of 4 peripheral blood samples repeated in 5 separate assays [218]; 3% for the mean of 7 plasma standards for mtDNA quantification over 8 assays [217]; and 2.6% for the mean of 5 plasma standards for nDNA quantification over 8 assays [217]. These are similar results to those observed in this study, calculated from the mean of 5 standards (5 dilutions of the same sample) over 4 assays.

The intra- and inter-assay variations in this study were higher for the mitochondrial PCR compared to the genomic PCR (tables 8.1-8.4), which was also found by Gahan et al [217]. This could be explained by the further dilution of the samples for determining mtDNA content that is necessary due to multiple copies of the mitochondrial genome within a cell.

Overall, the relatively low values for assay variability, including the high $R^2$ values for the standard curves that remain consistent between assays, indicate the technique used in this study for quantification of mtDNA is reproducible.

There was a decrease in peripheral blood mtDNA in patients with diabetes compared to age- and sex-matched controls (figure 8.1 and table 8.5), as found by a group in
South Korea [190, 191]. Lee and colleagues [190] first reported mean mtDNA content 35% lower in 55 patients with non-insulin dependent diabetes mellitus (NIDDM) compared to 29 age- and sex-matched controls using Southern blot analysis. The authors also found in a prospective population based study, that 23 subjects who developed NIDDM during a 2-year follow-up period had a significantly lower mtDNA content (by PCR) prior to clinical manifestation of the condition, compared to 22 age- and sex-matched controls (p<0.05). Song et al [191], from the same group, later investigated peripheral blood mtDNA content, using real-time PCR, in the offspring of 82 patients with type 2 diabetes. 52 of the offspring had normal glucose tolerance (NGT), 21 had impaired glucose tolerance (IGT), and 9 had newly-diagnosed type 2 diabetes. The offspring group as a whole had approximately 17% less mtDNA compared to 52 controls. When the offspring were split into NGT, IGT, and those with diabetes, all 3 groups had lower values compared to controls for mtDNA content, but the only significant difference was between the controls and the NGT group. The implications of these decreases are unknown; however, studies investigating cells depleted of mitochondrial DNA indicate the mitochondria not only supply ATP to cells, but also have distinct roles in apoptosis, glucose-induced insulin secretion, and oxygen sensing (reviewed in [193]).

Figure 8.2 shows there are slight, but not significant decreases in mtDNA in peripheral blood with age in both study populations. Previous studies have been inconclusive with Song et al [191] showing a significant negative relationship in the control group (mean age ± sem was 41.8 ± 0.9 years), but not in the offspring of the patients with diabetes to which they were age-and sex-matched. Lee and colleagues [190] reported no relationship with age in either the 51 NIDDM patients (mean age ±
SD was 50 ± 11 years) or 29 age- and sex-matched controls. Lui et al [179] also investigated mtDNA content, using real-time PCR, in the leukocytes of 156 healthy subjects aged 25 to 80 years, and actually showed an increase in content up to middle age, where it reached a plateau before decreasing. Studies investigating mtDNA content with age in other tissues have shown a decrease in content in rat liver [171], but either no change in human skeletal muscle and cardiac muscle [168], or increases in human muscle [172], brain tissue [173], and lung tissue [174]. These increases are thought to be a compensatory mechanism for decreased mitochondrial function [171-174]. The conflicting results from studies investigating mtDNA content with age may be due to different methodologies, and/or different study populations investigated, or it may be that other factors play a more important role in determining mtDNA copy number.

On investigation of oxidative stress indices and mtDNA content, there were no significant relationships in this study (figure 8.3). This agrees with previous studies investigating healthy subjects, where there was an increase in mtDNA content with age, and an increase in oxidative stress but the two parameters did not correlate [172, 174]. Liu et al [179] however, found significant positive correlations between mtDNA content in leukocytes and thiobarbituric active reactive substances (a measure of lipid peroxidation) in plasma (p=0.001), 8OHdG content in leukocytes (p=0.003), and incidence of the common deletion (p=0.016). These positive associations between mtDNA content and oxidative stress are supported by the cell culture work of Lee and colleagues [328], where human lung fibroblasts were treated with hydrogen peroxide (H$_2$O$_2$). The authors found an increase in mitochondrial mass and mtDNA content after treatment with H$_2$O$_2$, and the resultant mitochondria from the induced oxidative
stress were functional. Measuring oxidative damage within mitochondria is not easy, as the isolation itself can cause damage \[164, 165\], therefore it is difficult to determine the exact effects of increased oxidative stress to mtDNA.

In this study there is increased oxidative stress in the patients with diabetes (chapter 6), yet they have lower quantities of mitochondrial DNA compared to age-and sex-matched controls (figure 8.1 and table 8.5). This would seem to contradict the evidence suggesting a positive relationship between the two variables. Both the increased oxidative stress \[77-84\] and lower mtDNA content \[190, 191\] agree with previous studies in diabetes, although they have not been investigated together before. The presence of lower mtDNA quantities prior to the clinical manifestation of type 2 diabetes \[190\] suggests it is not a consequence of the condition, but perhaps a contributor in the pathogenesis. Type 1 diabetes is almost certainly caused by autoimmune destruction of the pancreatic \(\beta\)-cells, but a decrease in mtDNA within those cells may make them more susceptible. The cause of the decrease is unknown although the presence of lower mtDNA content in the offspring of patients with type 2 diabetes suggests it is inherited \[191\]. In this study there was no influence on mtDNA content if the patients had type 1 or 2 diabetes. An alternative hypothesis implicates insulin resistance as the cause of depleted mtDNA, with the previously mentioned study by Song and colleagues \[191\] correlating some indices of insulin resistance (log-transformed insulin sensitivity index, \(p<0.05\); and fasting C-peptide concentration, \(p<0.05\)) with mtDNA content. However, other indices including fasting insulin and the acute insulin response to glucose did not correlate. As mentioned earlier, mitochondria play an important role in glucose induced insulin secretion.
[193]; therefore, the decrease in mtDNA quantity is more likely to cause insulin resistance, than the insulin resistance causing a decrease in mtDNA.

If oxidative stress does cause an increase in mtDNA, it would suggest an increase in mtDNA in diabetes after onset of the condition. This agrees with the previously mentioned findings of Song et al [191], investigating 82 offspring of patients with type 2 diabetes. When the separate groups of offspring (NGT, IGT, and diabetes) were investigated, only those with NGT had significantly less mtDNA compared to the controls. There was an increase in mtDNA in IGT compared to NGT, and in diabetes compared to IGT, although all the groups still had lower values compared to the controls. It could be that a progressive increase in oxidative stress from NGT to IGT, and from IGT to diabetes, caused a progressive increase in mtDNA quantity, which was no longer significantly lower compared to the control group. This is a simplistic view without evidence to support it as oxidative stress was not measured, and given the lower contents of mtDNA in diabetes observed in this study and others [190, 191], it is unlikely. An alternative hypothesis could be a decrease in mtDNA precedes diabetes; the subsequent increase in oxidative stress in diabetes may cause an initial increase in mtDNA. The persistent oxidative stress may cause qualitative damage to the mitochondrial genome, leading to dysfunctional mitochondria and this may lead to a decrease in mtDNA. Lee et al [174] investigated mtDNA in human lung tissue of 49 subjects and found it increased with age (p<0.005), accompanied but not correlated with an increase in oxidative damage to nuclear DNA. The authors went on to discover a further small increase in light smokers, but a significant decrease in heavy smokers. Smoking is known to increase oxidative stress [212, 224, 225] and therefore the slight increase in the light smokers may have caused an increase in
mtDNA content in those individuals, but in the heavy smokers it was too much stress and resulted in a decrease in mtDNA. Unfortunately the authors do not mention differences in oxidative stress between the non-, light, and heavy smokers in the study, so there is no evidence to support the hypothesis.

8.7 Conclusions

MtDNA content, measured with real-time PCR, is decreased in patients with diabetes in this study compared to age-and sex-matched controls. Oxidative stress measures did not correlate with mtDNA content, and there was also no relationship between the quantity of mtDNA and age in healthy controls or patients with diabetes. Evidence from other studies suggests heredity as the major factor influencing mtDNA content, with other factors, such as oxidative stress or insulin resistance, perhaps contributing throughout life in unknown quantities.

Further studies are required to quantify mtDNA and determine mitochondrial function in different tissues in ageing and in oxidative stress, to investigate if there are any relationships. Further investigations in patients with diabetes are also required to confirm if the decrease precedes the onset of the condition, and to elucidate the cause of the decreased quantity of mtDNA and whether it has any significance with regard to cellular energetics. Also of interest is the effect of elevated oxidative stress on quantity and quality of mtDNA after the onset of diabetes.
Chapter 9. Summary and Conclusions

The aim of this thesis was to investigate oxidative stress and cardiovascular ageing in humans with diabetes mellitus. Chapter 1 detailed background information for the study, and presented the hypothesis and study aims. Briefly, hyperglycaemia is thought to contribute to the vascular complications commonly associated with diabetes [5, 6]. Age is the greatest risk factor for cardiovascular disease in the general population [8]; therefore, it is suggested that diabetes leads to premature ageing, predominantly in the vascular system.

Oxidative stress may be elevated in diabetes as a consequence of hyperglycaemia [77-84], and may be an important factor to explain the relationship between hyperglycaemia and vascular ageing. Oxidative stress can damage many biomolecules, including DNA [71]; which led to the proposal that DNA damage may be increased in diabetes. Two particular areas of the genome of interest in this regard are the telomeric and mitochondrial DNA, as they may be more susceptible to oxidative damage [10, 11]. Increased telomere attrition, as a consequence of elevated oxidative stress may cause premature cell senescence and therefore ageing [12]. This, along with elevated levels of oxidative damage to the mitochondrial genome that may affect mitochondria function and therefore cell energy production [150, 176], are proposed to contribute to the development of premature vascular ageing in diabetes.
9.1 Summary of results

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Diabetics</th>
<th>p-value</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean HbA1c ± sem (%)</td>
<td>5.5 ± 0.1</td>
<td>8.4 ± 0.3</td>
<td>&lt;0.001</td>
<td>104</td>
</tr>
<tr>
<td>Median glucose ± SD (mmol/L)</td>
<td>4.4 ± 0.6</td>
<td>10.4 ± 4.7</td>
<td>&lt;0.001</td>
<td>104</td>
</tr>
<tr>
<td>Median ACR ± SD (AU)</td>
<td>0.5 ± 1.2</td>
<td>1.0 ± 23.1</td>
<td>0.002</td>
<td>106</td>
</tr>
<tr>
<td>Mean Log BMI ± sem (AU)</td>
<td>1.41 ± 0.01</td>
<td>1.43 ± 0.01</td>
<td>0.021</td>
<td>111</td>
</tr>
<tr>
<td>Mean SBP ± sem (mmHg)</td>
<td>131 ± 2</td>
<td>139 ± 2</td>
<td>0.004</td>
<td>115</td>
</tr>
<tr>
<td>Mean Log DBP ± sem (AU)</td>
<td>1.88 ± 0.01</td>
<td>1.91 ± 0.01</td>
<td>0.030</td>
<td>115</td>
</tr>
<tr>
<td>Mean Log PP ± sem (AU)</td>
<td>1.72 ± 0.01</td>
<td>1.76 ± 0.01</td>
<td>0.033</td>
<td>115</td>
</tr>
<tr>
<td>Mean heart rate ± sem (bpm)</td>
<td>61 ± 2</td>
<td>68 ± 1</td>
<td>0.002</td>
<td>117</td>
</tr>
<tr>
<td>Median PWV ± SD (m/s)</td>
<td>7.29 ± 1.64</td>
<td>8.00 ± 2.89</td>
<td>0.006</td>
<td>140</td>
</tr>
<tr>
<td>Mean Log HDL ± sem (AU)</td>
<td>0.13 ± 0.02</td>
<td>0.04 ± 0.02</td>
<td>0.007</td>
<td>123</td>
</tr>
<tr>
<td>Mean LDL ± sem (mmol/L)</td>
<td>3.2 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>0.036</td>
<td>123</td>
</tr>
<tr>
<td>Mean % Tail DNA ± sem TE</td>
<td>10.71 ± 0.50</td>
<td>12.77 ± 0.36</td>
<td>0.001</td>
<td>159</td>
</tr>
<tr>
<td>Mean % Tail DNA ± sem FPG</td>
<td>21.40 ± 0.81</td>
<td>25.81 ± 1.18</td>
<td>0.003</td>
<td>159</td>
</tr>
<tr>
<td>Mean Log [Isop] ± sem (AU)</td>
<td>2.12 ± 0.10</td>
<td>2.45 ± 0.08</td>
<td>0.011</td>
<td>163</td>
</tr>
<tr>
<td>Mean Log mtDNA: nDNA ± sem (AU)</td>
<td>-1.84 ± 0.01</td>
<td>-1.79 ± 0.02</td>
<td>0.020</td>
<td>206</td>
</tr>
</tbody>
</table>

Table 9.1 Summary of population differences

All significant differences in variables investigated, in chapters 4 to 8 inclusive, between 51 patients with diabetes and 51 age- and sex-matched controls are displayed. Data were not always available in all study participants, and therefore numbers investigated may be less than 51 in each group. Normally distributed data are displayed as the mean ± sem. Data that did not follow a normal distribution, were log-transformed with the subsequent data presented in the table. Two-sample t-tests
formally tested the differences between the study populations for the normally distributed data, with p-values in the table. If the data did not conform to a normal distribution after transformation, the median ± SD values are presented, and p-values are from a Mann-Whitney U-Test.

Abbreviations used: HbA$_{1c}$, glycated haemoglobin; ACR, albumin to creatinine ratio; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; PWV, pulse wave velocity; HDL, HDL cholesterol, LDL, LDL cholesterol; % Tail DNA, percentage of tail DNA in the comet assay; TE, Tris EDTA buffer; FPG, formamidopyrimidine glycosylase; [Isop], urinary isoprostane concentration; and mtDNA, mitochondrial DNA. For further details of each variable, including raw data and numbers investigated, refer to the page number in the last column.

Chapters 4-8 inclusive, detail results from the study, each addressing individual aims. There were 101 healthy control volunteers recruited, ranging in age from 19 to 75 years. Investigating this group alone, revealed the characteristics of ageing in a healthy population i.e. ‘normal’ ageing. From this group, individuals were age- and sex-matched to patients with diabetes, and this allowed any differences between the two study groups, and any differences in ageing between the two populations, to be determined.

In chapter 4, physiological profiles were gathered for both study populations, and the data showed the two groups were well matched in terms of age, gender, ethnic origin, and smoking history. In the healthy controls, there were several significant increases
in physiological variables with age, including HbA1c, BMI, systolic blood pressure, pulse pressure, total cholesterol, LDL cholesterol, and triglycerides. Each of these increases is a risk factor for cardiovascular disease, and agrees with age conferring the greatest risk [8]. The size of the control population and the gender distribution also enabled differences between men and women to be investigated, with elevated pulse pressure and lipid alterations between the genders implying a greater risk of cardiovascular disease in the males, which is well known from large population based studies [259].

In the patients with diabetes, the majority of correlations between the physiological variables and age were lost, only pulse pressure remained significant with a positive relationship. Other relationships that appeared included a decrease in blood glucose control, and adverse alterations in lipids with increasing BMI. The aetiology of diabetes only became a significant factor for BMI, with higher values in type 2 diabetes as expected. Overall, the cohort of patients with type 1 and type 2 diabetes appear representative of a population with diabetes, with physiological profiles similar whether type 1 or type 2. As the complications in type 1 and type 2 diabetes are similar, they were investigated together in this study. If there had been more time for recruitment of greater numbers, type 1 and type 2 diabetes would have been investigated separately to determine any differences.

On comparison of physiology between the controls and patients with diabetes, glycated haemoglobin (HbA1c), blood glucose, and protein excretion from the kidneys (ACR), were all elevated (table 9.1) as predicted. There were also significantly higher values for BMI, blood pressure, and heart rate, and significantly lower values for
HDL and LDL cholesterol (table 9.1). The elevated HbA1c, ACR, BMI and blood pressures each confer a greater risk for cardiovascular disease in diabetes [22]. These observations are consistent with the clustering of many cardiovascular risk factors within patients with diabetes [27], and could be a consequence of accelerated ageing. The lack of relationship between the physiological variables and age in diabetes may be due to the values reaching a plateau, where it is not possible for them to go any higher without a further insult, or more likely, it could be confounded by therapeutic manipulation of the risk factors with drug therapy.

Due to the nature of diabetes, many of the patients recruited had vascular complications, and were receiving blood pressure and lipid lowering medications with potential cardiovascular effects, as well as insulin and/or oral hypoglycaemics to control their blood glucose. If the patients recruited had been untreated, population differences between the patients with diabetes and age- and sex-matched controls may not have been observed due to the shorter exposure time to hyperglycaemia. Also, recruitment would have been difficult, as it would have required screening of large numbers of people to identify those with previously unidentified diabetes.

The initial aim of the study (p61) was to establish if the vasculature is biologically older in patients with diabetes. Chapter 5 investigated biological ageing by measuring pulse wave velocity (PWV), an index of vascular stiffness [136]. With age, arteries become stiffer, which was confirmed by the studies in a “healthy” control population. This vascular ageing effect is pathological and most likely represents an increase in collagen content, collagen cross-links and fragmentation of elastin in conduit arteries [17]. In addition, impaired endothelial function may contribute to this functional
stiffening of the conduit arteries, resulting in an increase in PWV. This relationship between age and arterial stiffness was also confirmed in the patients with diabetes, with significantly higher values in those patients compared to age- and sex-matched controls (table 9.1). These higher values were a consequence of accelerated stiffening with age in diabetes as witnessed by the steeper regression line compared to the control population. The mechanism of this accelerated stiffening is unknown, although collagen protein glycation [44, 45], inflammation [59, 60], and endothelial dysfunction [38-42] as suggested above, are all likely to have been enhanced in people with diabetes, perhaps as a result of hyperglycaemia. These pathological changes within blood vessels are likely to be an important factor in the premature development of macrovascular disease events in people with diabetes – a conclusion supported by the observation that PWV is an independent predictor of increased cardiovascular morbidity and mortality in people with diabetes [197].

Several mechanisms have been proposed for the role of hyperglycaemia in cardiovascular disease [38-42, 44-52] and a further proposal suggests oxidative stress as the single hyperglycaemia-induced process that underlies them [9]. Therefore, after showing accelerated vascular ageing in a representative population of patients with diabetes, oxidative DNA damage was investigated, using the comet assay, in peripheral blood cells of the same individuals, and compared to age- and sex-matched controls.

Peripheral blood cells are relatively advantageous due to their quick and easy obtainment, with little discomfort to the participant. Also, as this study is interested in the vascular system, results from the parameters investigated in peripheral blood cells
may reflect what happens to the blood vessel cells in vivo, due to their proximity as they circulate through the vasculature. Disadvantages of peripheral blood cells include their rapid turnover, and therefore damage and accumulation of damage may not be found in these cells although it would occur in other tissues with a slower turnover, e.g. vascular endothelial cells.

The comet assay results fulfilled the second aim of this thesis (p61) and revealed significantly elevated levels of DNA damage, and specifically oxidative DNA damage, in the peripheral blood of patients with diabetes, compared to age-and sex-matched controls (table 9.1). This suggests an increase in oxidative stress in diabetes, which was confirmed by elevated urinary isoprostanes (table 9.1), a well-recognised measure of systemic oxidative stress.

The mechanism for this elevated oxidative stress is unknown, although hyperglycaemia is often cited. Suggested mechanisms of ROS production by hyperglycaemia include auto-oxidation of glucose [85], the polyol pathway [87], and local tissue damage [88, 89]. The lack of relationships between oxidative stress measures and blood glucose control in this study and some others [79, 80, 97, 300], questions the theory of hyperglycaemia as the cause. However, it may be that the measure of oxidative damage in DNA in peripheral blood is reflective of several days’ exposure to high glucose, but there is no measure of blood glucose control over several days. HbA1c reflects blood glucose control over the preceding few weeks and blood glucose concentrations are transient. Other studies have shown correlations between indices of oxidative stress and HbA1c and/or blood glucose [77, 78, 81, 82], and in vitro studies from the same research group as this study have shown elevated
oxidative DNA damage in human umbilical vein endothelial cells (HUVECs) treated with high glucose. This increase in damage was counteracted by the addition of antioxidants, indicating high glucose does cause an increase in oxidative damage \textit{in vitro}. Intervention studies investigating antioxidant supplementation in patients with diabetes have shown varying results [78, 80, 97, 98]. This could be due to different methodology, or perhaps the use of single or inadequate doses of antioxidants which thus provide only partial inhibition of oxidative stress. Moreover, individuals may vary in their response to antioxidants.

Alternative hypotheses to hyperglycaemia for the induction of oxidative stress include inflammation, decreased antioxidant defence, and the presence of complications. The last is unlikely with oxidative stress elevated in patients with diabetes who do not have complications [83, 84], and is therefore proposed as a contributor in the development of complications rather than a consequence [222]. Studies investigating antioxidant status in diabetes have been inconsistent [91-96], suggesting there may be disturbances in antioxidant defences in some patients with diabetes, but they may be a consequence of the elevated oxidative stress and not the cause. Also, as mentioned previously, antioxidant intervention trials in patients with diabetes have also been inconsistent. The proposal that inflammation leads to oxidative stress has gained recent attention, as studies have shown elevated inflammatory markers in type 2 [60, 281] as well as type 1 diabetes [59]. However, recent studies investigating inflammation, suggest elevated oxidative stress induces the inflammatory response [110, 302, 303]; therefore, the initial increase in oxidative stress is the consequence of another pathway.
Proposed consequences of the elevated oxidative DNA damage include accelerated vascular ageing in diabetes. Several mechanisms have been suggested including increased telomere shortening, leading to premature cell senescence and ageing [12], and decreased mitochondrial DNA content resulting in dysfunctional mitochondria, and therefore energy depletion and cell dysfunction [150, 176]. These two areas of DNA are of particular interest as they are thought to be more susceptible to oxidative damage [10, 11]. The final two aims of this thesis (p62) were to measure telomeres in patients with diabetes, and compare the age-related attrition rates with those of healthy controls, and to determine if mitochondrial DNA content is depleted in diabetes as a consequence of elevated oxidative stress.

Terminal restriction fragments (TRFs) shorten with cell replication [306], and in humans with age [119], and are therefore proposed as a molecular marker of cellular ageing. In chapter 7, both study populations showed significant negative relationships between TRF length and age, with similar slopes for regression lines. The data suggested shorter TRF lengths in diabetes, but it did not reach significance when compared to the control population. There was an association between TRF length and pulse wave velocity in the control group, i.e. arterial stiffness and vascular ageing, but this appeared to be a consequence of the strong associations for both variables with age.

Therefore, in this study there was an increase in oxidative damage to DNA in the patients with diabetes (chapter 6), but this did not appear to accelerate telomere attrition in those individuals. Cell culture work in the same research group has recently shown accelerated telomere shortening in HUVECs treated with high
glucose, accompanied by a more rapid entry into senescence. A previous study *in vivo* found significantly shorter telomeres in patients with type 1 diabetes compared to healthy controls, but without an increase in attrition rate [140]. This led to the proposal of a period of accelerated shortening prior to the time of investigation. Suggestions include a period of accelerated growth in low birth weight babies, or exposure to a damaging agent, (e.g. hyperglycaemia, inflammation, or increased oxidative stress), prior to clinical manifestation of the condition. This latter theory suggests a delayed initiation of the necessary heightened defences or repair mechanisms to counteract the increased stress(es). This is unlikely as there was increased DNA damage in peripheral blood cells of the same individuals (chapter 6), contradicting a sufficient increase in defences to prevent damage to the nuclear DNA. Also, *in vitro* work within this research group found a decrease in DNA repair in HUVECs treated with high glucose. The lack of increased attrition rate in peripheral blood cells in the patients with diabetes in this study suggests that oxidative stress may not accelerate telomere shortening in peripheral blood cells *in vivo*. This does not exclude accelerated shortening and therefore premature senescence in other tissues of the same patients, for example in cells of the blood vessels where the impact on vascular ageing would be greater.

Another target for reactive oxygen species is the mitochondrial genome. The elevated oxidative stress in diabetes could cause increased damage to mitochondrial DNA and this in turn may affect its copy number [190-192]. Chapter 8 revealed the patients with diabetes had significantly lower levels of mitochondrial DNA compared to the control population (table 9.1). It is unknown when this decrease in content occurred, but the similar slopes for the regression lines relating content to age in the patients
with diabetes and the control population suggest it happened earlier in life, or perhaps was inherited. Ageing has also been proposed to alter mitochondrial DNA through oxidative stress [179]; however, there were no relationships between oxidative stress and age in this study, which may explain the lack of relationship of mitochondrial DNA content with age. The consequences of the decrease in mitochondrial DNA are unknown, although it is proposed it will decrease energy production, which may lead to cell dysfunction.

The studies described fulfilled the initial aims of the thesis (p61-2), with the results showing accelerated vascular ageing in diabetes, with elevated levels of oxidative stress and decreased mitochondrial DNA content, although no change in telomere length. It is unknown what role oxidative stress and mitochondrial DNA content may play in the development of vascular complications in diabetes, but hypotheses are proposed for both.

The lack of relationships between any of the differences observed in diabetes compared to the control population with glycaemic control, suggests hyperglycaemia may not be the cause of these differences, or perhaps not the only cause. Inflammation and insulin resistance have both been suggested as alternative hypotheses, although inflammation and oxidative stress are closely linked with an increase in oxidative stress thought to mediate the inflammatory response. The presence of many factors may mask the relationship between hyperglycaemia and markers of vascular ageing, oxidative stress, and mitochondrial DNA content.
9.2 Further research

The results have given rise to many questions, and further research is clearly required. The mechanism behind the elevated oxidative stress in diabetes is not definitely known, although several pathways have been suggested for high glucose increasing ROS, with *in vitro* studies supporting this. Antioxidant trials and studies of antioxidant status in patients with diabetes have been inconclusive; however, further studies may reveal this is due to individual specific needs for antioxidant supplementation. Furthermore, within an individual, differences in antioxidant capacity may also be tissue specific. Investigations into the specific pathways for high glucose leading to elevated oxidative stress may reveal the particular mechanism or several mechanisms, perhaps leading to research to try and block these with implications for therapeutic intervention.

Further research with larger numbers would establish if there is a decrease in telomere length in patients with diabetes compared to healthy controls. It may be that telomeres are not shorter in peripheral blood cells of patients with diabetes compared to healthy controls, and telomere length should perhaps be investigated in blood vessel cells of healthy arteries, and those from patients with diabetes. Problems related to this include the invasive procedure required, and either a large amount of material would be required to obtain enough cells, or the cells would have to be cultured which then questions if the findings occur *in vivo*. The suggestion of an earlier period of accelerated attrition in diabetes should be investigated. Ideally, telomere length would be measured in individuals at risk of developing diabetes.
Measuring mitochondrial DNA content in pre-diabetic individuals would also help to confirm if the decrease in content precedes diabetes. Very little work has been carried out on mitochondrial DNA content in diabetes. The cause and consequence of the decreased content is as yet unknown, and needs to be investigated, perhaps along with studies quantifying mitochondria themselves, and mitochondrial function in diabetes. Also, further work investigating ATP production would help establish if there is an energy crisis in cells that may contribute to premature ageing in diabetes.

Overall, the situation in diabetes is complex, with many hypotheses proposed for the accelerated vascular ageing observed in the condition. Hyperglycaemia appears to play a role, and may act through oxidative stress, although further studies are required to confirm this. Investigating single mechanisms is unlikely to provide all of the answers. However, this study has been the most detailed thus far, to evaluate the capacity of diabetes to induce oxidative DNA damage in humans. This is notable despite the fact that many of the patients with diabetes were receiving multiple drug therapies to reduce their cardiovascular risk. Moreover, DNA is ordinarily efficiently repaired and the measured DNA damage is a net effect of the balance between damage and repair and most likely underestimates the extent of DNA damage. Further studies are essential to better define the consequences of a chronic enhancement of nuclear and mitochondrial DNA damage in diabetes. DNA is fundamental to life and its preservation and repair is a default position for cell survival. Mindful of this, it seems likely that people with diabetes are likely to expend considerable energy repairing DNA and as such, this may represent a fundamental mechanism to account for cellular energy deficit in diabetes. Further studies are ongoing to evaluate this intriguing hypothesis.
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SPECIAL NOTE

THE FOLLOWING IMAGE IS OF POOR QUALITY DUE TO THE ORIGINAL DOCUMENT.

THE BEST AVAILABLE IMAGE HAS BEEN ACHIEVED.
Leicestershire, Northamptonshire and Rutland
Strategic Health Authority

Our Ref: pgr/sl/335
29 November 2002

Professor B Williams
Faculty of Medicine and Biological Sciences
PO Box 65
Leicester Royal Infirmary

Dear Professor Williams

The relationship between accelerated vascular ageing and vascular matrix synthesis in hypertensive and diabetic patients – our ref: 5881

I am in receipt of a letter from Dr Lacey dated 16th October 2002. This is a protocol amendment application to this study to allow consent to be obtained by Dr Lacey and Ms Dewer.

By chairman’s action this protocol amendment is approved.

I have also received a revised version of the patient information sheet, control information sheet and consent forms for this study. These are approved subject to modifying the ‘control consent form’ where it says ‘signature of control’ it should read ‘signature of research participant’ (or just signature).

Yours sincerely

P. G. Rabey
Chairman
Leicestershire Research Ethics Committee

(NB All communications relating to Leicestershire Research Ethics Committee must be sent to the Committee Secretariat at Leicestershire, Northamptonshire and Rutland Health Authority. If, however, your original application was submitted through a Trust Research & Development Office, then any response or further correspondence must be submitted in the same way.)
# Clinical Studies Proforma

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Would the patient agree to their GP receiving details? No ^  Yes f

### Past Medical History:
- Hypertension †
- Diabetes: Type I † Type II †
- IHD † MI †
- Asthma † COPD †
- Arthritis † specify: ________
- Liver disease †
- Other significant: ____________________

### Family History:

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<td>Father</td>
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### Medication: (Include OTC and OCP as appropriate)

- ACE I:  
- AIIA:  
- CCB:  
- Thiazide:  
- Loop diuretic:  
- β-blocker:  
- α-blocker:  
- Statin:  
- Insulin:  
- Oral Hypogls:  

Other: ____________________

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**Clinical Studies Proforma**

**Lifestyle:**

**Occupation**

**Diet**

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<th></th>
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<tr>
<td>Salt</td>
<td>□</td>
<td>□</td>
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Fruit/Vegetables ________ portions/day

**Exercise**

Type

Duration ________ minutes

Frequency ________ /week

**Tobacco:**

Current † Ex † Never †

______ Pack Years Years since quit: ______ years

**Alcohol:** ________ Units per week

**Blood Pressures:** (Sitting after 5 minutes rest)

1. _____ / _____ mmHg  2. _____ / _____ mmHg  3. _____ / _____ mmHg

**Investigations**

**Urinalysis:**

Blood † Protein †

Glucose † pH † ACR:

**Anthropometry:**

Weight: ________ Kg Height: ________ m BMI: ________ kg/m²

**ECG**

Rate: ________ bpm

Sinus Rhythm † Atrial Fibrillation † Other: †

Bundle Branch Block: No † Yes † Right † Left † ICVD †

Left Ventricular hypertrophy No † Yes † Cornell † S-Lyon †

**ABPM**

Mean Day BP: _____ / _____ mmHg %>130/80 _____/ _____ HR______ bpm

Mean Night BP: _____ / _____ mmHg %>110/75 _____/ _____ HR______ bpm
# Clinical Studies Proforma

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<td>K⁺ ________</td>
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## Applanation Tonometry & Pulse Wave Velocity

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<td>Pulse Wave Velocity</td>
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## Analysis of DNA Damage

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<tr>
<td>Urinary Isoprostanes</td>
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CONTROL INFORMATION SHEET

The relationship between accelerated vascular ageing and vascular matrix synthesis in hypertensive and diabetic patients.

You are invited to take part in the following study:

1. **What is the purpose of the study?**

As we get older, our blood vessels become stiffer and less elastic resulting in a greater likelihood of our developing conditions such as elevated blood pressure (hypertension), stroke and myocardial infarction (heart attack). This is a result of the effects of mechanical strain on the vessel wall together with a progressive age-related thickening due to biochemical changes. This thickening happens much earlier and is more severe in patients with diabetes or pre-existing high blood pressure.

We are interested in finding out how diabetes and hypertension might affect these processes and influence the way in which blood vessels work. These findings might enable us to develop new ways of detecting and monitoring changes occurring in the blood vessels in patients with high blood pressure or diabetes, so that hopefully we will be able to intervene earlier and prevent some of the complications of these common diseases. In order to investigate these processes we need to make comparisons against people with no known cardiovascular disease. Therefore, we are asking you to consider volunteering as a healthy control for these studies.

2. **What will be involved if I take part in the study?**

This study will involve a single visit to the Clinical Research Unit at the Leicester Royal Infirmary. This visit would last roughly one hour. During this visit some or all of the following would be performed:

- Blood tests (See below)
- Heart Tracing (ECG)
- Blood pressure recording
- Height and weight
- Urine sample taken for analysis
- Pulse wave analysis and pulse wave velocity (See below)
The blood we will take at this visit will be used to check for all the things we would routinely check for in patients with cardiovascular disease. These include your blood sugar, cholesterol, kidney function, liver function and haemoglobin levels. In addition to this we will be checking for markers of blood vessel wall thickening and assessing markers of damage to cells that make up the vessel walls.

In addition to taking your blood pressure with a normal device, it will also be measured using a novel system, comprising a small pressure sensor, placed on the skin near the wrist. This sensor is known as a tonometer and it will be used to measure the normal fluctuations in pressure that occur in the arterial system with each heartbeat. We will then make an assessment of the elasticity of your arterial system by measuring how quickly your pulse travels with every heartbeat. This is done using the sensor that was used on the artery at your wrist only this time a measurement is taken at two separate sites, the large arteries at the side of your neck and at the top of your leg in the groin area. Your heart rate will also be monitored continuously during this second measurement. All these measurements are painless and a female nurse will be present when female patients are tested. These procedures will take approximately 20 minutes in total and are known as pulse wave analysis and estimation of pulse wave velocity.

3. Will information obtained in the study be confidential?

All control information will be treated with the usual degree of confidentiality under the data protection act.

Once the results of your investigations are available you will be informed of the results. Should any abnormal results be found then you would be contacted with these and advised to visit your GP to discuss them in more detail.

4. What if I am harmed by the study?

Medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

5. What happens if I do not wish to participate in this study or if you wish to withdraw from the study?

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision.

The Principal Investigator for this study is Professor Bryan Williams.

You may contact Dr Adrian Stanley, Clinical Research Fellow to Professor Bryan Williams or Dr Peter Lacy, Research Scientist to Professor Bryan Williams on 0116 258 6909 with any questions you may have regarding the above study.

Thank you for agreeing to participate in this study.
CONTROL CONSENT FORM

"The relationship between accelerated vascular ageing and vascular matrix synthesis in hypertensive, diabetic, and renal patients".

Principle Investigator's Professor Bryan Williams.

This form should be read in conjunction with the Control Information Leaflet.

I agree to take part in the above study as described in the Control Information leaflet – Version 5 (15/10/02).

I understand that I may withdraw from the study at any time without justifying my decision and without affecting any future care I may require.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all information will be treated as confidential. This would only be required if abnormal results were found.

I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

I have read the Control information leaflet on the above study and have had the opportunity to discuss the details with ................................................... and ask any questions. The nature and the purpose of the tests have been explained to me and I understand what will be required if I take part in the study.

Signature ..........................................................Date................................................

Name in block letters ..........................................................................................................

I confirm that I have explained the nature of the Trial, as detailed in the Control Information Leaflet, and in terms which in my judgement are suited to the understanding of the Control.

Signature of Investigator..............................Date................................................

Name in block letters..........................................................

Version 3 (15/10/02) 278
The relationship between accelerated vascular ageing and vascular matrix synthesis in hypertensive and diabetic patients

You are invited to take part in the following study:

1. What is the purpose of the study?

As we get older, our blood vessels become stiffer and less elastic resulting in a greater likelihood of our developing conditions such as elevated blood pressure (hypertension), stroke and myocardial infarction (heart attack). This is a result of the effects of mechanical strain on the vessel wall together with a progressive age-related thickening due to biochemical changes. This thickening happens much earlier and is more severe in patients with diabetes or pre-existing high blood pressure.

We are interested in finding out how diabetes and hypertension might affect these processes and influence the way in which blood vessels work. These findings might enable us to develop new ways of detecting and monitoring changes occurring in the blood vessels in patients with high blood pressure or diabetes, so that hopefully we will be able to intervene earlier and prevent some of the complications of these common diseases.

2. What will be involved if I take part in the study?

This study will involve a single visit to the Clinical Research Unit at the Leicester Royal Infirmary. This visit would last roughly one hour. During this visit the following would be performed:

- Blood test (see below)
- Heart tracing (ECG)
- Blood pressure recording
- Height and weight
- Urine sample taken for analysis
- Pulse wave analysis and estimation of pulse wave velocity (see below)

The blood we will take at this visit will be used to check for all the things we would routinely check in patients with diabetes or hypertension. This includes your blood sugar, cholesterol and kidney function. In addition to this we will take a sample of blood to assess markers of vessel wall thickening and cellular aging together with assessing markers of damage to cells that make up the vessel wall.
In addition to taking your blood pressure with a normal device, it will also be measured using a novel system, comprising a small pressure sensor, placed on the skin near the wrist. This sensor is known as a *tonometer* and it will be used to measure the normal fluctuations in pressure that occur in the arterial system with each heartbeat. We will then make an assessment of the elasticity of your arterial system by measuring how quickly your pulse travels with every heartbeat. This is done using the sensor that was used on the artery at your wrist only this time a measurement is taken at two separate sites, the large arteries at the side of your neck and at the top of your leg in the groin area. Your heart rate will also be monitored continuously during this second measurement. All these measurements are painless and a female nurse will be present when female patients are tested. These procedures will take approximately 20 minutes in total and are known as *pulse wave analysis and estimation of pulse wave velocity*.

3. **Will information obtained in the study be confidential?**

All patient information will be treated with the usual degree of confidentiality under the data protection act.

Whilst the results of the tests in this study are not of current diagnostic benefit, information obtained will be used in a scientific study and will contribute to advancing our understanding of the progression of hypertension and diabetes. This information may also help to influence the way in which patients will be treated in the future.

Your General Practitioner will be made aware of your participation in the study and a copy of your test results will be sent to them for their records. Should any abnormal results be found then you would be contacted with these and advised to visit your GP to discuss them further.

4. **What if I am harmed by the study?**

Medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

5. **What happens if I do not wish to participate in this study or wish to withdraw from the study?**

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so at any time, without justifying your decision. Your future treatment will not be affected by any such decision.

The Principal Investigator for this study is *Professor Bryan Williams*.

You may contact Dr Adrian Stanley, Clinical Research Fellow to Professor Bryan Williams or Dr Peter Lacy, Research Scientist to Professor Bryan Williams on 0116 258 6909 with any questions you may have regarding the above study.
PATIENT CONSENT FORM

"The relationship between accelerated vascular ageing and vascular matrix synthesis in hypertensive and diabetic patients".

Principal Investigator's Professor Bryan Williams.

This form should be read in conjunction with the Patient Information Leaflet.

I agree to take part in the above study as described in the Patient Information leaflet – Version 4 (8/7/02).

I understand that I may withdraw from the study at any time without justifying my decision and without affecting any future care I may require.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all information will be treated as confidential. This would only be required if abnormal results were found.

I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

I have read the Patient information leaflet on the above study and have had the opportunity to discuss the details with .......................................................... and ask any questions. The nature and the purpose of the tests have been explained to me and I understand what will be required if I take part in the study.

Signature .....................................................................Date ..............................................

Name in block letters ...........................................................................................................

I confirm that I have explained the nature of the Trial, as detailed in the Patient Information Leaflet, and in terms which in my judgement are suited to the understanding of the Control.

Signature of Investigator.................................Date....................................................... 

Name in block letters...........................................................................................................