The influence of exercise, smoking cessation and ageing on the level of advance glycation endproducts

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Abstract

Advanced glycation endproducts (AGEs) play a crucial role in the pathogenesis of diabetes and its complications such as retinopathy, nephropathy, neuropathy and atherosclerosis. AGEs may also contribute to the age-related debilitation of skeletal muscles. Glycation of myosin, for instance, does reduce myosin function. Moreover, glycation is accompanied by the generation of free radicals through autoxidation of glucose and glycated proteins. The accumulation of AGEs can be caused by various factors including diet, obesity and smoking, while regular physical activity and altered diet may reduce the levels of AGEs.

The aim of this research was to study the effect of 8 weeks aerobic exercise, smoking and 2 weeks of smoking cessation on the level of serum AGEs in healthy participants and the effects of ageing on glycation of skeletal muscle fibers.

Serum AGEs were determined using an enzyme-linked immunosorbent assay (ELISA) and by their autofluorescence. Oxidative stress biomarkers were measured using a colometric method. Circulating inflammatory cytokines were examined using flow cytometry. In vivo glycation of muscles from young (5 months), old rats (25 months) and very old rats (32 months) were evaluated by immunohistochemistry.

There were no changes in serum AGEs and oxidative stress biomarkers after 8 weeks aerobic exercise. Smokers, however, had elevated serum AGEs, oxidative stress biomarkers and levels of the cytokines IL-2 and TNF-α. Although 2 weeks of smoking cessation had a negligible effect on the level of AGEs and oxidative stress biomarkers, it returned the levels of TNF-α and IL-2 to normal levels. We showed for the first time glycation was increased with age in gastrocnemius muscle. The age-related increase in muscle glycation may contribute to the slowing of the muscle in old age.
Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university, to the best of my knowledge and belief, contains no material previously published or written by another, except where due reference has been made in the text. In addition, no parts of this thesis have been copied from other sources. I understand that any evidence of plagiarism and/or the use of unacknowledged third party data will be dealt with as a very serious matter.

Signature: Hakima Oushah

Date: 10/03/2016
Dedication

This work is dedicated to my mother and father; they have been a great support throughout the journey.
Acknowledgment

The successful completion of this thesis has allowed me to expand my scientific knowledge. The ability for me to pursue would not have occurred without the help and inspiration from many supporters.

Initially, I would like to send my appreciation to my Director of studies Dr. Nessar Ahmed; this thesis would never be completed without his help, support, patience and constant advice. I am also truly thankful for the guidance and endless support from my second supervisor Dr. Hans Degens; he has ensured that the completion of this thesis was a success. I am extremely grateful to Dr. Gethin Evans, for his help and advice throughout.

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Finally, I would like to thanks those whom I have forgotten to mention.
Conference presentations


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<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>3BHB</td>
<td>3-β-hydroxybutyrate</td>
</tr>
<tr>
<td>AFGPs</td>
<td>Alkyl formyl glycosyl pyrroles</td>
</tr>
<tr>
<td>AGE-R1</td>
<td>Oligosaccharyl transferase-48</td>
</tr>
<tr>
<td>AGE-R2</td>
<td>80K-H phosphoprotein</td>
</tr>
<tr>
<td>AGE-R3</td>
<td>Galectin-3</td>
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<tr>
<td>AGEs</td>
<td>Advanced glycation endproducts</td>
</tr>
<tr>
<td>ALI</td>
<td>Arginine-lysine imidazole</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CEL</td>
<td>Carboxyethyllysine</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CML</td>
<td>Carboxymethyllysine</td>
</tr>
<tr>
<td>COHb</td>
<td>Caboxyhemoglobin</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>DLC</td>
<td>Differential leukocyte count</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GOLD</td>
<td>Glyoxal-lysine dimer</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRAC</td>
<td>Hydroxyl radical antioxidant capacity</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-12P70</td>
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<tr>
<td>IL-6α</td>
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</tr>
<tr>
<td>INF-γ</td>
<td>Interferon gamma</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MOLD</td>
<td>Methylglyoxal-lysine dimer</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear Factor-KappaB</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO'</td>
<td>Nitric oxide radical</td>
</tr>
<tr>
<td>O₂'</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>OH'</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline-Tween</td>
</tr>
<tr>
<td>RDB</td>
<td>Reagent dilution buffer</td>
</tr>
<tr>
<td>ROO'</td>
<td>Peroxy radical</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>sRAGE</td>
<td>Soluble receptor for advanced glycation endproducts</td>
</tr>
<tr>
<td>Streptavidin-PE</td>
<td>Streptavidin-phycoerythrin</td>
</tr>
<tr>
<td>TAS</td>
<td>Total antioxidant status</td>
</tr>
<tr>
<td>TNF-β</td>
<td>Tumor Necrosis Factor - Beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>Vfin</td>
<td>Final volume</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1:

Introduction
1. Introduction
Lack of physical activity and a diet consisting of high levels of sugars and fats have been found to be related to obesity in both adults and children (Watts et al., 2005). Obesity is becoming a global pandemic in the developed and developing world. Furthermore, studies have found that obesity and lack of physical activity can contribute to the formation of type 2 diabetes (Sikaris, 2004). The incidence of obesity is increasing and since 1980, the proportion of the population that is obese has doubled. According to the WHO, more than 1.4 billion adults were overweight in 2008 (Halicioglu, 2013; WHO, 2014). Lifestyle factors such as smoking are also risk factors for diabetes (Radzeviciene and Ostrauskas, 2009). There are 60 million people with diabetes in the European Region with about 10.3% of men and 9.6% of women are aged between 25 years and over. Worldwide, approximately 3.4 million people die annually due to diabetic complications (WHO, 2014).

Both obesity and smoking promote the formation of free radicals. Free radicals are atoms or molecules with unpaired electrons. The unpaired electrons make the highly reactive and oxygen radicals can cause oxidative stress (Prasad et al, 2010; Dambal et al, 2011). Oxidative stress is a physiological state characterized by unbalanced quantities of antioxidants and pro-oxidants, which can be the result of inflammation and vice versa (Savini et al.,). Obesity can induce inflammation through a number of mechanisms, one of which is the presence of increased lipid content, due to adipose tissue dysfunction (Crujeiras et al., 2013). Other studies have also found that a diet high in AGEs can increase inflammation (Van Puyvelde et al., 2014). Furthermore, inflammation can also be induced by glycation, which is an important biochemical modification that is accompanied by diabetes mellitus (Basta et al., 2004).
Diabetes is a multifactorial disease, which results from insulin resistance or lack of insulin production or both, and is characterised by hyperglycemia and is a major risk factor of micro and macrovascular diseases (Negre-Salvayre et al., 2009). It was also found that smoking is a risk factor for the development of insulin resistance (Bergman et al., 2012). However, the mechanism by which smoking causes insulin resistance remains unclear.

There is evidence that hyperglycaemia is the initial cause of tissue damage in diabetes. Furthermore, studies have found that an excessive accumulation of the glycated biomolecules and advanced glycation endproducts contributes to tissue damage caused by hyperglycaemia in diabetes (Negre-Salvayre et al., 2009).

1.1 Glycation (Maillard) reaction

In 1912, Louis-Camille Maillard gave the first description of the reaction between protein and carbohydrates. This discovery occurred when he was heating reducing sugars with amino acids as he observed the formation of a yellow-brown product (Maillard, 1912). This reaction was then referred to as the Maillard reaction. After this finding, the Maillard reaction was predominantly used within the food industry as a flavour enhancer. However, in the last two decades the improved understanding of the Maillard reaction has veered away from the food industry and towards the medical field. This was due to the discovery of the structural heterogeneity of human haemoglobin A due to non-enzymatic glycation (Rahbar et al., 1969). Since then, this discovery has promoted further medical research interest as this reaction has both functional and pathological impacts on human tissue and various applications in life sciences (Gerrard, 2006; Ulrich and Cerami, 2001).
1.2 Glycation chemistry

The general mechanism through which the glycation reaction takes place is outlined in Figure 1.1. The whole process of glycation can be divided into three phases: the early phase, intermediate phase and late-phase of the reaction.

1.2.1 Early phase of glycation

The glycation reaction begins with the nucleophilic addition reaction that takes place between the carbonyl groups of reducing sugars and amino groups of macromolecules such as proteins, nucleic acids and lipids resulting in the formation of a freely reversible Schiff base as shown in Figure 1.1. The initiation of this first step occurs when there is a high level of glucose and can take place within a few hours (Brownlee, 1994; Neglia et al., 1983). The reaction is reversible if the concentration of glucose decreases. The chemical rearrangement of the Schiff base results in the formation of the initial glycation products known as Amadori products (Figure 1.1). These are more stable compounds and this reaction can take place within a matter of days or weeks (Yaylayan and Huyghues-Despointes, 1994).

1.2.2 Intermediate phase of glycation

During the intermediate phase, the degradation of the Amadori products occurs through a chain of chemical rearrangements, dehydration and oxidation reactions resulting in the formation of several different dicarbonyl compounds that behave as precursors of the reaction (Baynes and Monnier, 1989). 3-deoxyglucosones, glyoxal and methylglyoxal are the most well characterised dicarbonyl molecules generated in the glycation reaction (Figure 1.1). The dicarbonyl compounds show more reactivity compared to their originators. Their interaction with monoacids produces advanced glycation endproducts.
(AGEs) (Thornalley, 2005). Moreover, they are capable of reacting with extracellular proteins that have been released from the cell through diffusion (Kellow and Savige, 2013).

### 1.2.3 Late phase of glycation

The dicarbonyl molecules formed in the intermediate phase react with free amino groups. This amino-carbonyl reaction system follows a complicated pathway involving repeated reactions of dehydration, condensation, fragmentation, oxidation and cyclisation (Schleicher et al., 2001) that lead to the formation of a highly heterogeneous set of compounds called AGEs (Figure 1.1) (Baynes and Thorpe 1999; Miyata et al., 2000).
Figure 1.1: An illustration showing the glycation formation from protein and sugar. The whole process of glycation can be divided into three phases: The early phase which involves reversible Schiff base formation that rearranges to Amadori product, this product then degrades during the intermediate phase forming dicarbonyl intermediates such as 3-DG. In the final phase known as late phase, the dicarboxyl molecules reacts with free amino acids resulting in formation of AGEs (Adapted from Lapolla et al., 2003).
1.3 Alternative pathways for AGE formation

Further to the previous mechanism, AGEs can also be generated through pathways such as glucose autoxidation (also known as metal-catalysed glucose autoxidation), as shown in Figure 1.2. It has been established that chemical modification of proteins during the glycation reaction is greatly affected by the dicarbonyl compounds formed through glucose autoxidation (Wolff and Dean., 1987; Thornalley et al., 1999). Additionally, glucose can also gradually become autoxidised by a reaction catalysed by transition metals thereby generating an enediol radical, which in turn, simultaneously causes a reduction of molecular oxygen to form oxidising intermediates. These oxidising intermediates such as H$_2$O$_2$, OH’, α-ketoaldehydes and O$_2$’ can cause damage to vital molecules such as nucleic acids, lipids and proteins (Baynes and Thorpe, 1999). When ketoaldehydes react with amino groups of protein molecules, extremely reactive ketoimines are formed and then can ultimately result in the production of AGEs (Ahmed, 2005).

Namiki and Hayashi (1983) have also proposed an alternative mechanism for the generation of AGEs according to which the Schiff base fragmentation may take place before the generation of Amadori products that produces dicarbonyl compounds that lead to the formation of free radicals as shown in Figure 1.2. It is worth mentioning that methylglyoxal and glyoxal are formed when fragmentation of the Schiff base occurs via the Namiki pathway (Ferreira et al., 2003; Monnier, 2003). Moreover, both methylglyoxal and glyoxal can also be produced exogenously (food and smoking) as illustrated in Figure 1.2.

AGEs can also be formed by the autoxidation of Amadori products and are typically known as glycoxidation products yet this term was originally coined to describe the
products generated by sequential glycation and oxidation reactions. As illustrated in Figure 1.2, protein dicarbonyl molecules are generated from Amadori products through a protein enediol that simultaneously produces free radicals. This reaction takes place in the presence of oxygen and under conditions, which support the accelerated glycoxidation reaction catalysed by transition metals. The consequential generation of glycation-derived free radicals has a significant contribution to the causation of complications associated with diabetes (Solnica, 2006).

Figure 1.2: The alternative pathways for glycation reaction involves glucose autoxidation; glucose gets autoxidised producing enediol radical, this causes reduction of molecular oxygen forming oxidising intermediates, such as α-ketoaldehydes, these react with amino acid forming ketoimine, which ultimately result in AGES production. Schiff base fragmentation; produces dicarbonyl compounds that lead to the formation of free radicals, methylglyoxal and glyoxal are also formed and Amadori products (glycoxidation); protein dicarbonyl molecules are generated through a protein enediol, simultaneously producing free radicals Methylglyoxal and glyoxal are not only endogenous metabolites but can also be generated exogenously such cigarette smoke and food. Glycotoxins (not included in diagram) are also exogenous which promote the formation of AGES, as a resultant of reacting with serum proteins.
In addition to protein glycation, lipids and DNA can also be prone to glycation. The glycation of lipids may alter the function of membranous lipids, such as phosphatidylethanolamine (PE) and phosphatidylserin, hence negatively affecting their role in maintaining cellular integrity and survival (Oak et al., 2000). Lipid glycation involves the Maillard reaction between amino phospholipids such as phosphatidylethanolamine and glucose. The reaction involves Schiff base formation and rearrangement to a PE-linked Amadori product (Lertsiri et al., 1998; Bucala et al., 1993). In vitro studies have suggested the formation of reactive oxygen species (ROS) due to Amadori product autoxidation, These ROS are capable of causing unsaturated fatty acid peroxidation that reside membrane lipids. This allows for the propagation of free radical reactions and lead to hydroperoxide formation (Miyazawa et al., 2012). As well as lipid glycation, DNA can also interact with reducing sugars leading to the formation of several DNA-bound AGEs (Seidel et al., 1998; Frischmann et al., 2005). The identification and characterisation of DNA-derived AGEs is still under progress, since these products may induce modifications in DNA, hence have negative functional impact on cellular behaviour and processes. Indeed, a study has shown that DNA-glycation results in cross-linking of nucleotides (Kasai et al., 1998), and destabilises the N-glycosidic bond that links DNA bases to the sugar-phosphate backbone. This was suggested to lead to depurination, phosphate backbone cleavage, local distortion in the DNA dynamics (Baynes, 2002; Cuniasse et al., (1990), fragmentation of the double helix and genetic mutations (Chandra GK et al., 2015).
1.4 Factors affecting glycation
The reaction between the carbonyl group of reducing sugars and the amino groups of proteins can be influenced by the pH, metal ions, oxygen as well as the electrophilicity of the sugar carbonyl carbon (Bunn and Higgins, 1981). Temperature, water activity (Davies et al., 1998) and the quantity of the reducing sugar in the cyclic form (Yaylayan et al., 1999) are also factors that can have an impact on the rate of the glycation reaction. The pKa of the protein amino groups and their locations within the protein structure (Yaylayan et al., 2003) have an effect on the rate of Schiff base formation. The location of the lysine residues (on the molecular surface and the histidine residue near the lysine residue) can accelerate the rate of glycation (Dai et al., 2007).

*In vivo*, studies have shown that the half-life of protein, the extent and the period of hyperglycaemia and the reactivity of certain amino groups affect the degree of glycation (Ahmed, 2005; Wautier and Schmidt, 2004).

1.5 Characterisation of AGEs
Some important characteristic features of AGEs include their ability to crosslink amino groups and yield a yellowish-brown fluorescent colour (Bousova et al., 2005). Even though our knowledge regarding the chemistry of these compounds is still lacking, several AGE structures have been detected *in vitro* and only a small number of structures have been identified *in vivo*. The AGE structures are categorized into four classes based on their physical and chemical properties (Ahmed, 2005).

These 4 classes of AGEs are as follows:

- Fluorescent crosslinked AGEs
- Non-fluorescent crosslinked AGEs
- Fluorescent non-crosslinked AGEs
- Non-fluorescent non-crosslinked AGEs
1.5.1 Fluorescent crosslinked AGEs

Approximately 1% of crosslinking structures comprise the fluorescent AGE crosslinks (Dyer *et al.*, 1991). Researchers have detected fluorescent crosslinked AGEs *in vitro* under physiological conditions, such as pentosidine (Figures 1.3a) and crossline, (Figures 1.3b) (Obayashi *et al.*, 1996; Ulrich and Cerami, 2001).

![Figure 1.3: Schematic structure of fluorescent crosslinked AGEs under physiological conditions such as a) pentosidine and b) crossline.](image1)

1.5.2 Non-fluorescent crosslinked AGEs

Nearly all of the protein crosslinking *in vivo* is caused by non-fluorescent crosslinked AGEs. The imidazolium dilysine crosslinks, also known as methylglyoxal-lysine dimer (MOLD; Figure 1.4 a) or glyoxal-lysine dimer (GOLD; Figure 1.4 b) mainly constitute this group of AGEs (Frye *et al.*, 1998). In addition, arginine-lysine imidazole (ALI; Figure 1.4 c) (Al-Abed and Bucala, 2000) and alkyl formyl glycosyl pyrroles (AFGPs; Figure 1.4 d) (Farmar *et al.*, 1988) are considered to be representative non-fluorescent crosslinked AGEs.
Figure 1.4: Chemical structures of four types of non-fluorescent crosslinked AGES: (a) methylglyoxal-lysine dimer (MOLD) (b) glyoxal-lysine dimer (GOLD) (c) arginine-lysine imidazole (ALI) (d) alkyl formyl glycosyl pyrrole (AFGPs).
1.5.3 Fluorescent non-Crosslinked AGEs

This group of AGE structures includes argpyrimidine as shown in Figure 1.5. Researchers have generated argpyrimidine in vitro using glycation by methylglyoxal (Shipanova et al., 1997).

![Chemical structures of fluorescent crosslinked AGEs argpyrimidine](image)

*Figure 1.5: Chemical structures of fluorescent crosslinked AGEs argpyrimidine*

1.5.4 Non-fluorescent non crosslinked AGEs

A number of non-fluorescent non-crosslinked structures of AGEs have also been characterized under physiological conditions in addition to crosslinked AGEs. Pyralline (Hayase et al., 1989) and carboxymethyllysine (CML) (Ahmed et al., 1986) are considered to be typical examples of AGEs belonging to this group, as shown below in Figures 1.6 a and b.

![Schematic structure of non-fluorescent crosslinked AGEs under physiological conditions such as a) pyralline and b) carboxymethyllysine.](image)

*Figure 1.6: Schematic structure of non-fluorescent crosslinked AGEs under physiological conditions such as a) pyralline and b) carboxymethyllysine.*
From the above mentioned AGEs, pentosidine and Carboxymethyllysine (CML) are of greater significance as they can be employed as indicators of AGE build-up during the life span, particularly in diabetes (McCance et al., 1993).

1.6 Intracellular glycation and AGE formation

Although initial reports considered glucose to be the chief precursor for the generation of AGEs, a number of non-sugar substances and reducing sugars are now considered to be more reactive compared to glucose (Sady et al., 2000). An upsurge in the levels of intracellular sugars occurs during hyperglycaemia (Baynes, 2001). Additional quantities of AGEs are generated from intracellular carbonyl precursors as compared to glucose. Hence, the intracellular generation of AGEs is believed to be the major source of extracellular as well as intracellular AGEs (Babaei-Jadidi et al., 2004). The findings of a study are in agreement with this view as it was demonstrated that thiamine and benfotiamine regulate the levels of AGE in the plasma of diabetic rats by reducing the intracellular levels of cytosolic triose phosphates and making substrates inaccessible for dicarbonyl generation (Babaei-Jadidi et al., 2004). The α-dicarbonyl molecules such as 3-deoxyglucosones, glyoxal and methylglyoxal are essential to the AGE generation because of their increased affinity for arginine residues and the presence of two carbonyl groups (Turk, 2010).

Numerous substances such as nucleic acids and lipids with free amino groups can also act as substrates for the glycation reaction (Mironova et al., 2001). AGEs can also be derived from sugar intermediates of polyol and glycolytic pathways such as fructose-3-phosphate, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Moreover, the production of AGEs from these precursors is quicker than from glucose (Sharma et al., 2002).
1.7 Exogenous sources of AGEs

In addition to the endogenous generation of AGEs, AGEs as well as their originators are acquired from exogenous sources such as foods that are cooked at high temperature and processed foods. In general, food is browned to bring about improvement in colour, aroma, flavour and quality (Tessier and Birlouez-Aragon, 2012). The following factors promote AGE production in foods; a high concentration of protein and lipid, the use of extremely high temperatures for a short span of time as well as a high pH and low amount of water during cooking. It has been established that the treatment of food with dry heat such as baking, roasting and barbecuing results in an increased production of AGEs compared to prolonged cooking at low temperatures with high moisture content such as stewing, steaming and boiling (Uribarri et al., 2005). It has been established through kinetic studies that around 10-30% of ingested AGEs are absorbed in the intestine (Faist et al., 2001).

Another example of the production of AGEs via oral routes is smoking tobacco, (Nicholl et al., 1998). Studies show that smokers have higher serum levels of AGEs compared to non-smokers irrespective of diabetes (Cerami et al., 1997). Smoking tobacco results in a generation of extremely reactive glycotoxins that invade the blood through lungs and speed up the generation of AGEs. Furthermore, smoke produced from cigarettes is rich in methylglyoxal and glyoxal. Thermal saccharide decomposition results in the formation of methylglyoxal and for that reason saccharides are perceived as important originators of AGEs when smoking (Fujioka and Shibamoto, 2006; Talhout et al., 2006).
1.8 Cytotoxicity of AGEs

The modification and aggregation of protein during the formation of AGEs causes proteins to lose their function and become resistant to proteolysis (Edeas et al., 2010). These alterations are considered to be major factors in the contribution to various pathological processes and subsequently lead to tissue damage (Vitek et al., 1994). Various mechanisms are found to cause tissue damage for instance, through the formation of protein cross-links (Bach et al., 2005), interaction with specific receptors for AGEs (RAGE) found on surfaces of certain cells leading to inflammation and oxidative stress (Yan et al., 1994) and intracellular glycation. AGEs are known to be involved in diabetic complications, muscular dysfunction and age related diseases (Stitt, 2001; Negre-Salvayre et al., 2009). These complications are explained in more details in section (1.11)

1.9 Receptors for AGEs

It has been established that the interaction of AGEs with their receptors has a significant contribution to the pathogenesis of complications linked with diabetes as well as inflammation.

A number of AGE receptors have been identified such as oligosaccharyltransferase- 48 (AGE-R1), galectin-3 (AGE-R3), macrophage scavenger receptors types I and II. Presently, the most intensely studied AGE receptors are RAGE (Thornalley, 1998). RAGE is a multi-ligand receptor, which belongs to the immunoglobulin superfamily of molecules. These types of receptors are expressed on the surface of smooth muscle cells, astrocytes, endothelial cells and macrophages.

RAGE consists of an extracellular domain of 332-amino acids arranged as a single “V”-type immunoglobulin-like (variable) domain with subsequent two “C”-type (constant)
domains (Sparvero et al., 2009). The RAGE-related V and C1 domains act as a single structural unit for the binding of some ligands, whereas C2 RAGE domain act independently from the VC1 complex while remaining attached to it through a flexible hinge figure (Koch et al., 2010).

RAGE is fixed in the cell membrane through a fourth transmembrane domain which itself is linked to a charged fifth domain that is located intracellularly. The fifth domain allows the interaction of RAGE with the transduction molecules situated in the cytoplasm. Previously, RAGE was recognized as an AGE receptor synthesized either in vitro or extracted from diabetic patients. Furthermore, RAGE is known to act as signal transduction receptor for AGEs. Moreover, other substances such as fibrillar proteins, amphoterin and pro-inflammatory cytokines (S100-calgranulins) can stimulate RAGE (Hudson et al.; 2003). During the course of inflammation and diabetes, the level of RAGE expression is increased. The interactions between AGE and RAGE may induce a number of signalling pathways, leading to cellular dysfunction. For example, AGEs activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can enhance the production of reactive oxygen species (ROS) (Bierhaus et al., 1997), which were demonstrated to modify the structure of cellular proteins, lipids and nucleic acids (Oka et al., 2000). It was also shown that the AGE/RAGE interaction can increase the phosphorylation of p21ras, the mitogen-activated protein kinases (MAP), extracellular signal-regulated kinase (Erk)-1/2 and p38 (Staal et al., 1990). Erk and p38 can induce the activation and translocation of nuclear factor (NF-κB) from cytoplasm to the nucleus thereby stimulating the gene expression of a number of targets involved in inflammation and atherosclerosis (Bierhaus et al., 1997; Manna et al., 2000). The latter targets include; intercellular adhesion molecule-1, vascular cell adhesion molecule-1, VEGF, endothelin-1, tissue factor, thrombomodulin and proinflammatory cytokines, including
interleukin (IL)-1α, IL-6 and tumor necrosis factor-α (Sen et al., 1990; Iimuro et al., 2000).

Subsequently resulting in vascular permeability. Figure 1.7 summarizes the intracellular signalling pathways, which occur after interaction between AGEs and RAGE.

Figure 1.7: A representation for AGE/RAGE interaction on the cell surface leading to transduction of a signalling cascade; activates nicotinamide adenine dinucleotide phosphate oxidase and enhancing ROS production and phosphorylate p21 RAS and MAPKs. AGE/RAGE interaction induces the activation of p38 MAPK. NF-κB upon AGE/RAGE interaction can translocate to the nucleus causing gene expression.
1.10 Oxidative stress, free radicals and glycation

A physiological state characterized by unbalanced quantities of antioxidants and pro-oxidants reactive oxygen species (ROS) is termed as oxidative stress. A number of different reactive oxygen species are generated in living systems including free radical $\mathrm{O}_2^\cdot$, non-radical $\mathrm{H}_2\mathrm{O}_2$, peroxyl radical (ROO$^\cdot$), nitric oxide (NO$^\cdot$) and hydroxyl radical (OH$^\cdot$). All these reactive oxygen species (ROS) participate in oxidative stress (Farahmand et al., 2003; Valko et al., 2007). In increased concentration, the ROS may cause damage to vital biomolecules lipids, DNA and proteins (D'Souza et al., 2009).

Oxidative stress can induce lipid peroxidation giving rise to malodialdehyde and unsaturated aldehydes (Esterbauer et al., 1984). This can lead to the rearrangement of the membrane lipid bilayer and hence inactivating membrane-bound receptors and enzymes by forming protein cross-linkages (Esterbauer et al., 1984), ultimately increasing tissue permeability (Giugliano et al., 1996). Chronic oxidative stress are capable of modifying DNA, through the degradation of bases, single- or double-stranded DNA breaks, purine, pyrimidine or sugar-bound modifications, mutations, deletions or translocations, and thus cross-linking with proteins (Sies et al., 1985).

At the protein level, oxidative stress can cause disruption of the GSH/GSSG ratio, leading to activation of redox sensitive transcription factors. For instance, NF-κB and hypoxia-inducible factor 1 (AP-1). Other targets of ROS include; tyrosine kinase receptors, most of the growth factor receptors such as epidermal growth factor receptor, vascular endothelial growth factor receptor, and receptor for platelet-derived growth factor as well as protein tyrosine phosphatases and serine/threonine kinases [Sun et al., 1996]. The phosphorylation of NF-κB by ROS through the IκB$\alpha$s domain at serine residues, frees NF-κB to enter the nucleus to activate gene transcription (Perkins, 2007), NF-κB activation via ROS, may induce antioxidant defense-related genes to participate
in immune response are using IL-1β, IL-6, tumor necrosis factor-α, IL-8, and several adhesion molecules (Gilmore, 2006).

Various endogenous redox molecules and antioxidant enzymes regulate the concentration of ROS in normal physiological conditions. Therefore, oxidative stress is not only characterized by high concentrations of ROS, but also by disturbance in the pathways of antioxidant defence (Bonnefont-Rousselot, 2000).

Enzymatic or non-enzymatic antioxidant systems can be utilized in order to protect cells from the hazardous activities of free radicals. Several enzymes such as glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase (SOD) constitute the enzymatic antioxidant system demonstrate their antioxidant activity either by inhibiting activities of the ROS or by eliminating these reactive species (Sies, 1997). In contrast, endogenous scavenging species such as uric acid and glutathione or scavenging molecules derived from dietary components such as carotenoids, lipoic acid and vitamins A, C and E constitute the non-enzymatic system. Co-factors such as vitamins B₁, B₂, B₆, folic acid and antioxidant minerals can also offer defence against adverse effects of free radicals (Comhair and Erzurum, 2002).

The causes behind oxidative stress still need to be determined. However, researchers have proposed different mechanisms involving the generation of free radicals in diabetic patients. These mechanisms include polyol pathway activation, autioxidative glycation (Giugliano et al., 1996) and glycoxidation (Elgawish et al., 1996). In addition to these mechanisms, tissue damage induced by oxidative stress may also comprise interaction between the AGEs and the RAGE receptors (Yan et al., 1994). According to Baynes and Thorpe (1999), oxidative stress can be induced by AGEs and *vice versa*. Hence, it has been proven that free radicals are formed by a process requiring glycated proteins.
and this formation can result in the acceleration of the glycation reaction (Coughlan et al., 2008).

1.11 Diabetic complications and glycation
It is assumed that chronic hyperglycaemia increases the production and accumulation of AGEs; these substances have a significant role in the development of diabetic complications. The formation of AGEs can be limited by controlling the factors that cause hyperglycaemia, thus ultimately reducing the risk of diabetic complications diabetics (Ahmed and Thornalley, 2007). Diabetic complications are commonly categorized into two groups; microvascular and macrovascular complications.

1.11.1 Microvascular complications
Microvascular diseases are common complications of diabetes mellitus, which include a wide range of dysfunctional alterations of the microvascular structure of various organs and tissues. There are a number of pathologies associated with diabetes including retinopathy, nephropathy and neuropathy (Peppa et al., 2003). The overall changes in the microarchitecture of specific organs lead to characteristic alterations in organ function and integrity. In general, affected organs may become exposed to an increase in vascular permeability and a prothrombotic state, the thickening of the basement membrane and decreased blood flow (Chappey et al., 1997).

1.11.1.1 Diabetic retinopathy
One of the most severe diabetic complications involves the microvasculature of the retina. This is known as retinopathy and occurs as a consequence of damage caused to the small blood vessels of the retina. This type of complication can cause visual disturbances ultimately leading to visual loss (Lu et al., 1998). The underlying cause of
such pathology is thought to arise from high levels of circulating AGEs, which provide pools of AGEs within both the intra- as well as extravascular spaces (Sato et al., 2006). The mechanism of action of retinal damage directly influences the endothelial layer of the small vessels as well as its capacity to produce NO (Hogan et al., 1992; Treins et al., 2001). Therefore, factors such as nitric oxide synthase (eNOS) inhibition and/or abnormal expression patterns and endothelial cell abnormal proliferation status may result from the damage caused (Chakravarthy et al., 1998). AGEs also have an inhibitory effect on pericytes and may lead to pericyte loss. These can act as a potential histological hallmark indicating the presence of diabetic retinopathy. Due to endothelial cell loss from small vessels, AGEs may indirectly cause a disruption in vascular tone in favour of vascular constriction leading to a tendency of producing basement membranes with thicker walls. This may ultimately induce a breakdown of the inner blood-retinal barrier (Chibber et al., 1997).

1.11.1.2 Diabetic neuropathy
One of the most common complications associated with diabetes is neuropathy, which occurs in over 50% of individuals suffering from diabetes. It is caused by the progressive deterioration of the peripheral autonomic and sensory nervous system (Magalhães et al., 2008). It has been established that the AGE generation is the most crucial pathway that contributes to the development of diabetic neuropathy (Sugimoto et al., 2008). Moreover, abnormal axonal transport and axonal atrophy in diabetic neuropathy are caused by the modification of tubulin, actin, myelin, neurofilament and other chief axonal cytoskeletal proteins by AGEs (Vlassara et al., 1981). Similarly, the thickness of basement membrane and electrical charge are modified due to the glycation of laminin and collagen. This may result in disturbed regenerative activity (Tarsio et al., 1985). In addition, it is thought that peripheral nerve myelin altered by AGE is more
vulnerable to phagocytosis and this plays a role in segmental demyelination (Williams et al., 1982).

It has been demonstrated by a growing number of researchers that AGE interaction with RAGE contributes to nerve dysfunction. Even though the exact mechanism is still not known, it is assumed that they may have an inflammatory function in peripheral nerve deterioration (Huijberts et al., 2008). Moreover, the degree of expression of inducible nitric oxide synthase (iNOS) is altered by AGEs. This may lead to a reduction in the flow of blood to nerves thereby inducing hypoxia (Wada and Yagihashi, 2005).

1.11.1.3 Diabetic nephropathy
Another common complication associated with diabetes is nephropathy. Around 40-50% of diabetic individuals have diabetic nephropathy, which is considered to be the leading cause of end-stage renal disease across the world. Furthermore, it has contributed considerably to mortality and morbidity in diabetic individuals (Yamagishi et al., 2007). Mesangial expansion together with tubular and glomerular basement membrane thickening are characteristic features of diabetic nephropathy and these changes lead to fibrotic changes, microvacular deterioration and vascular occlusion. Together, these phenomena result in the development of glomerulosclerosis (Coughlan et al., 2005). It has been established through clinical and animal studies that AGE production is the main cause behind the renal deterioration observed in hyperglycaemia (Brownlee et al., 1988; Makino et al., 1995). It is worth mentioning that AGE build up in nephropathic patients can be caused by its reduced removal in kidneys, instead of its augmented synthesis via glycation (Shimoike et al., 2000).
1.11.2 Macrovascular complications

A wide range of pathological changes, influencing major blood vessels and causing structural as well as functional impairments are collectively termed as “macrovascular complications”. Long structural modifications are largely caused by the glycation of different constituents of the wall, whilst functional modifications are caused by endothelial dysfunction, which can lead to either a decrease in vascular compliance or an increase of the stiffness of the arterial wall. In the case of diabetes, macrovascular complications contribute significantly to the development of atherosclerosis, which is the leading cause of a number of vascular disorders such as myocardial infarction, peripheral vascular disorders and stroke (Rahman et al., 2007).

1.11.2.1 Diabetes and atherosclerosis

Atherosclerosis, a complex multifaceted disorder, develops due to accumulation of lipids in the vascular wall thereby impeding the flow of blood in vessels (Mallika et al., 2007). In clinical terms, atherosclerotic arterial disorders can be regarded as a cardiovascular disease (CVD). Reports indicate that diabetic patients are more vulnerable (2-4 times) to the development of CVD compared to non-diabetics (King et al., 2005). Various mechanisms have been implicated in the development of accelerated atherosclerosis in diabetic individuals, including the formation of AGEs (Jandeleit-Dahm and Cooper, 2008; Yamagishi et al., 2005).

It has been established that atherosclerosis can be accelerated by AGEs through numerous mechanisms. For instance, atypical lipoprotein metabolism, alterations in matrix constituents and crosslinking of proteins (Goh and Cooper, 2008). The AGEs have been detected in low-density lipoproteins (LDL) and play a crucial role in altering LDL clearance from the body. It has also been shown that macrophages can engulf
glycated LDL resulting in the development of foam cells; an identifying feature of atherosclerosis lesions (Basta et al., 2004; Brown et al., 2005). Moreover, vasoconstriction is influenced by AGEs by making NO inaccessible (Pandolfi and De Filippis, 2007). AGE generation can result in neointimal proliferation, augmented plaque destabilisation, inhibition of vascular repair and increased endothelial dysfunction via interaction with RAGE.

1.12 Cancer and AGEs
Various studies illustrate the relationship between RAGE expression and a variety of cancers in diabetic patients (Abe et al., 2008) such as renal cell carcinoma (Miki et al., 1993). Studies also concluded that targeting the AGE/RAGE system could be a promising therapeutic approach (Kim and Kim, 2003). It has been reported that oxidative stress caused by AGEs via RAGE can cause mutation of DNA (Setlow et al., 1993) and induce tumour angiogenesis via an increase of vascular permeability, the induction of cytokine secretion and the stimulation of the cellular response to exogenous pro-angiogenic growth factors (Takeuchi et al., 2010).

1.13 Influence of AGEs on skeletal muscles
Skeletal muscle is indispensable for locomotion, posture and breathing. The basic building block of the muscle is the sarcomere. Each sarcomere contains actin filaments, attached to the z-disk and myosin filaments that partly overlap the actin filaments (Figure 1.7). In addition, there are cytoskeletal proteins in the sarcomeres, such as titin, that keep the sarcomere aligned. Bundles of sarcomeres in series form a myofibril. An individual muscle fibre contains many parallel myofibrils and is surrounded by endomysium (Figure 1.7). Fibres in turn are grouped in fascicles and are wrapped in a connective tissue layer described as perimysium. Incorporated within the perimysium
are the blood vessels and nerves. Finally, the whole muscle structure is enveloped with the epimysium that anchors the muscle to the skeleton (Figure 1.7) (Jones and Round, 1990; McNally et al, 2006).

Figure 1.8: Schematic diagram of the skeletal muscle structure, (image adapted from Carrim (2010) Available at: (http://samedical.blogspot.co.uk/2010/07/contraction-of-skeletal-muscle.html).

Myosin is the major muscle protein and makes up 15–25% of the total body protein (Ramamurthy et al., 2001). It interacts with actin and during the power stroke, by hydrolysis of ATP, pulls the actin along; the actin, as it were, slides along the myosin, hence the sliding filament theory of muscle contraction (Huxley and Hanson 1954).

In addition to contractile functions, muscle is also a metabolically active tissue. For instance, it plays an important role in regulating blood glucose levels by taking up glucose via the glucose transporter GLUT4 that translocates from intracellular vesicles to the muscle sarcolemma under the influence of insulin (Cassese et al., 2008). GLUT4
translocation is also stimulated by elevated intracellular [Ca\textsuperscript{2+}] during contractile activity (Richer and Hargreaves, 2013). It is thus not surprising that a number of studies have demonstrated that GLUT4 expression can be induced during acute exercise via the AMPK and CAMKII signalling pathway. An interesting study reported that inflammatory cytokines produced by the adipose tissue, such as TNF-\(\alpha\) (tumor necrosis factor-\(\alpha\)) and interleukin-6 (IL-6) are related to a reduction of GLUT4 expression which subsequently attenuated glucose uptake by muscle. Glycation is a well-known factor that can induce inflammatory cytokines, such as TNF-\(\alpha\) and interleukin-6 (IL-6), by AGE/RAGE interaction (Leguisamo \textit{et al.}, 2012).

Studies have shown that glycation can impair skeletal muscle function by several mechanisms (Cassese \textit{et al.}, 2008). For instance, it was demonstrated that the impairment of insulin function by AGEs in the muscles is a result of AGE / RAGE interaction (Cassese \textit{et al.}, 2008). They found that mice fed with a high AGE diet demonstrated a significant impairment of insulin sensitivity. Furthermore, it has been reported that oxidative stress and inflammation are associated with insulin resistance (Unoki and Yamagishi, 2008). Moreover, studies have related reduced insulin sensitivity with AGEs formation (Cai \textit{et al.}, 2012). Additionally, AGEs can cause skeletal muscle dysfunction by causing post-translational modifications of myosin. It has been shown, for instance, that incubation of myosin with glucose reduced the velocity at which myosin could propel actin in an \textit{in vitro} motility assay (Ramamurthy \textit{et al.}, 2001). Indeed, myosin has a long half-life of approximately 30 days, is rich in lysine residue, and therefore makes them susceptible to the glycation reaction. Ramamurthy \textit{et al.} (2001) reported that lysine side chains of myosin due to modification by glycation; were not cleaved by endoproteinase.
The potential significance of myosin glycation is highlighted by a number of skeletal muscle diseases that are associated with AGEs (Ramamurthy et al., 2003). Also, sarcopenia, the age-related loss of muscle mass is thought to be associated with an increased abundance of glycate myosin (Syrovy and Hodny, 1992; Haus et al., 2007). Sarcopaenia is a significant problem that refers to loss of strength and mass of muscles (Degens and McPhee, 2013) and affects two third of the men and one third of the women aged 60 or more in the United States (Doherty et al., 2003). Sarcopenia exhibits a complex pathogenesis involving inflammation, oxidative stress, hormonal changes, inactivity (Degens, 2010; Sakuma et al., 2014) and modifications in vasculature (Degens, 1998).

In a study on 559 women with moderate or severe disability, aged 65 or above (Dalal et al., 2009) grip strength of subjects with increased CML concentration was less than those with lower concentrations of CML. It was concluded that women with elevated levels of AGEs experience greater muscle weakness (Dalal et al., 2009). Suggesting a link between sarcopenia and AGEs. Hence, additional studies are required to investigate this health problem.

1.14 Measurement of glycation reaction products

Studying the glycation reaction is complicated since AGEs exist in a number of structural forms. For these and other reasons, the quantification of AGEs has proved to be an intricate task. An increased level of reproducibility and accuracy is needed by quantitative assays however; inadequate endogenous standards render assays prone to errors. Researchers have always found it challenging to investigate protein modifications (Smit and Lutgers, 2004).
The formation of AGEs has been studied through qualitative as well as quantitative assays. Fluorescence spectroscopy is to be an important technique for the quantification of AGEs in vivo and in vitro since AGEs exhibit particular fluorescence properties (Vigneshwaran et al., 2005). AGEs can be measured with high specificity and sensitivity using immunochemical techniques such as ELISA that make use of monoclonal and polyclonal antibodies and are specific to different structures of AGEs (Lin et al., 2002). Methods involving the separation of different molecules by for instance, SDS-PAGE have also been used to study glycation products (Ahmed et al., 2007). In recent years, glycation studies have extensively used mass spectrometry. An efficient tool for the quantification of particular protein glycation products in vivo as well as in vitro is the matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Lapolla et al., 2000).

Products formed during the glycation reaction have been grouped into three main classes (early glycation products, intermediate glycation products and advanced glycation products) in order to facilitate the analysis of glycation reaction products in biological and experimental samples.

1.14.1 Measurement of early glycation products
Early stage glycation products are also termed Amadori products and are generally quantified to assess metabolic control in individuals with diabetes. In this setting, glycated serum proteins and glycated haemoglobin (HbA1c) are predominantly measured.

1.14.1.1 Glycated haemoglobin
Glycated haemoglobin (HbA1c) can be quantified using different techniques. Methods such as affinity chromatography involve utilising the differences in the structure of
glycated and non-glycated haemoglobin, whilst others such as ion exchange chromatography exploit the differences in the ion charge for the measurement of HbA1c molecules. The concentration of HbA1c has been employed as an efficient diagnostic marker as it indicates the degree of exposure to glucose during the past four to eight weeks (Kislinger et al., 2004; Schwartz, 1995). The quantification of HbA1c is carried out in clinical laboratories as a routine practice for analysing glycaemic control in diabetes.

1.14.1.2 Glycated serum proteins
The quantification of glycated serum proteins such as fructosamine is a well-known method for evaluating the time-averaged control of glycaemia in individuals with diabetes. In contrast to HbA1c, the quantity of fructosamine indicates the concentration of glucose in blood over the past ten to twenty days, thereby demonstrating a more rapid response to alterations in the treatment of diabetes. In general, the use of colorimetric techniques is preferred for fructosamine quantification (Arsie et al., 2000; Lapolla et al., 2005).

1.14.2 Measurement of intermediate glycation products
The accumulation of AGEs is accompanied by an increase in dicarbonyl compounds like 3-deoxyglucosone, methylglyoxal and glyoxal in diabetic patients (Ahmed and Thornalley, 2003). The determination of the concentration of dicarbonyl compounds can facilitate the analysis of the oxidation processes taking place in glycated proteins. Mass spectrometry can be used to determine quantities of these compounds (McLellan et al., 1992).
1.14.3 Measurement of advanced glycation products

From the outset, quantities of AGEs were determined by fluorimetric and spectroscopic techniques. However, these techniques do not demonstrate sufficient specificity and only give a measure indicating the overall route followed by the glycation reaction (Brownlee et al., 1988). Therefore, researchers have devised ELISA making use of mono and polyclonal antibodies for the detection of AGEs (Turk, 2001).

Initially, the GC/MS had been used to determine the levels of pentosidine and CML however; the CML can now be quantified using anti-CML monoclonal antibodies by ELISA (Gerdemann et al., 2000).

1.15 Natural defence mechanisms against glycation

Numerous defence mechanisms protect human tissues from AGE production and glycation. It has been established that macrophages express certain receptors that allow the detection and removal of glycated proteins through endocytosis (Matsumoto et al., 2002). In addition, a wide range of enzymes, for example, glyoxalase I and II, aldose reductase and oxoaldehyde reductase can eliminate reactive dicarbonyl molecules and diminish AGE generation in a sequential way (Thornalley, 2003). Furthermore, Amadoriases, a group of enzymes, are capable of catalysing the deglycation of Amadori products. The elimination of a sugar moiety from a molecule can be catalysed by fructose lysine 3-phosphokinase and fructose lysine oxidase. This makes the free amino groups available again thereby averting AGE production (Monnier and Wu, 2003).

In addition to the above mentioned enzymes, endogenous antioxidants can also protect the living system from glycation-derived free radicals (Ceriello, 2003; Kil et al., 2004). Similarly, numerous plasma amines can react with Amadori carbonyl groups and sugar resulting in a reduced AGE generation. Certain transport proteins like caeruloplasmin
are also capable of binding with transition metals, thereby making them unavailable for glycoxidation and autoxidative glycation reactions (Ahmed, 2005).

1.16 Pharmacological Interventions

Researchers have determined that various pharmacological agents can inhibit either the crosslinking of AGEs or their activities. These studies have been conducted on cell lines, human and animal models (Luevano-Contreras and Chapman-Novakofski, 2010). Examples of these agents include metformin, benfotiamine, inhibitors of a renin-angiotensin system, aspirin and aminoguanidine. It has been shown that the administration of candersatan for one year caused a decrease in urine concentration of CML in individuals with diabetic kidney disease (Saha et al., 2010).

Others have reported that the AGE generation can be inhibited by aminoguanidine, an agent that scavenges α-dicarbonyl groups (Thornalley, 2003). Nevertheless, a similar clinical trial involving diabetic subjects was discontinued because of safety risks and efficacy issues. Subjects exhibited secondary effects like atypical results for liver function, flu-like symptoms and gastrointestinal disturbances (Thornalley, 2003).

Administration of metformin in diabetic patients has resulted in lower levels of circulating AGEs and reduced NFkB activity (Isoda et al., 2006). Other research involving twenty-two females with polycystic ovary disease (PCOS) has shown that administration of metformin for half a year caused decrease in the serum AGE levels (Diamanti-Kandarakis et al., 2007a). Similarly, the administration of orlistat, a lipase inhibitor in 21 PCOS patients resulted in reduced absorption of the AGEs. This was indicated by low levels of the AGEs in serum following the consumption of a high AGE meal (Kalofoutis et al., 2006). Having investigated the effect of orlistat administration and calorie restriction a research group reported a decrease in serum AGE levels among
both obese and the PCOS subjects, irrespective of the changes in body mass index (Diamanti-Kandarakis et al., 2007b). A number of pharmaceutical agents are still under evaluation in preclinical or clinical phase. However, utilisation of these agents for blocking or lowering the effect of AGEs could be made possible after a long time.

Many studies have investigated the level of AGE in smokers, but studies on smoking cessation and exercise on AGEs levels are scarce, also, study on the effects of AGEs in muscle are poorly investigated.
1.17 Aim and Objectives
The aim of the current study is to investigate the effect of smoking, smoking cessation and exercise on AGEs. Furthermore, the question of whether level of AGE accumulates in skeletal muscle chronologically will also be examined. This study will also determine the benefits of smoking cessation on health and risk factors for chronic diseases in non-symptomatic smokers.

The main objectives for this current study are:

• To investigate the effect of eight weeks exercise endurance training on the level of circulating AGEs, blood glucose, biomarkers of oxidative stress (TAS and MDA) and lipid profile (cholesterol, triglycerides, high-density lipoprotein (HDL) and LDL) in the serum of healthy participants before and after training.

• To determine the effect of smoking and two weeks smoking cessation on the level of a number of parameters including; circulating AGEs, blood glucose, total protein, albumin and biomarkers of oxidative stress (TAS and MDA).

• To determine the effect of smoking and two weeks smoking cessation on white blood cell, differential leukocyte count (DLC), haemoglobin, haematocrit and circulating cytokines.

• To determine whether muscle protein glycation occurs in old age through examining the abundance of AGEs in rat gastrocnemius muscle of old than young male Wistar rats.
CHAPTER 2:

Methods
2. Materials & Methods

(For material, see Appendix 1A)

2.1 Detection of AGEs

2.1.1 Measurement of serum protein concentration using the Biuret method

Protein concentrations in serum samples were measured by using RandoxRX Daytona analyser (Randox Laboratories Ltd). Freshly thawed batches of serum samples were analysed using a Biuret reagent Randox kit. The ‘serum level 3 kit’ was used to calibrate the system. This assay is based on the interaction of cupric ions in an alkaline medium with protein peptide bonds that results in the formation of a coloured complex with absorption at 550 nm (Gornall et al., 1949).

2.1.2 Measurement of serum LDL, HDL, cholesterol and triglycerides

The samples that were used for lipid profile characterisation were carefully placed on iced box and transferred to Manchester Royal Infirmary Hospital (MRI) for analysis. The analysis was conducted using the automated analyser (Advia 2400, Siemens Healthcare Diagnostics) to determine the levels of serum total cholesterol, HDL, LDL and triglycerides according to NHS standards. The analysis was based on colorimetric assays performed according to the manufacture instructions.

2.1.3 Measurements of Low molecular weight AGEs by their fluorescence

Before fluorescent measurements, the glycated samples were thawed at room temperature and diluted 20-fold in distilled water. Two hundred μl of the sample was pipetted in duplicate in the wells of the 96-well plate and then analysed for fluorescence. To separate low molecular weight AGEs (LMW-AGEs), 20 μl serum was deproteinized with 0.15 M trichloroacetic acid (TCA). Then 100 μl chloroform was
added and vortexed for 60 seconds followed by centrifuged at 14,000 rpm for x min. Two hundred μl of the aqueous supernatant were also pipetted in duplicate in the 96-well plate and analysed for their fluorescence. The concentrations of both AGEs and LMW-AGEs were analysed using a spectrophotofluorimeter (BioTek, USA). Results were expressed as arbitrary units (AU) per mg of protein. Fluorescence calibration curves were made with quinine sulfate as standard, which has similar excitation and emission spectra as AGEs (340 and 440 nm, respectively) (Sharp et al., 2003).

2.1.4 Measurement of AGEs using ELISA

For rapid detection and quantification of AGE protein adducts, enzyme-linked immunosorbent assay (ELISA) was performed using a Cell Biolabs AGE ELISA kit. Briefly, a series of AGE-BSA standards was prepared according to Table 2.1. Reduced AGE and AGE-BSA standards were diluted to 10 μg/mL in PBS. To each well of a 96-well plate 100 μL sample was incubated in duplicate for 2 hours at 37 °C. After incubation, the wells were washed with 250 μL PBS. This was followed by blocking with 200 μL blocking solution for 1-2 hours at 37°C on an orbital shaker. After washing, 100 μL of diluted primary antibody was added for 1 hour at 37°C on an orbital shaker. Secondary HRP-conjugated antibody was added for 1 hour at 37°C on an orbital shaker. After washing, 100-μL substrate solution was added at 37°C for up to 30 minutes. The enzymatic reaction was stopped with 100μL stop solution and the absorbance at 450 nm measured using a microplate reader (Lin et al., 2002). The quantity of AGE adducts in protein samples was calculated from the AGE-BSA calibration curve according to table 2.1. The protocol is summarized in the figure 2.1.
Table 2.1: Preparation of AGE-BSA standard calibration curve

<table>
<thead>
<tr>
<th>Standard Tubes</th>
<th>10 μg/mL AGE-BSA (μL)</th>
<th>10 μg/mL Reduced BSA (μL)</th>
<th>AGE-BSA (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>200</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>300</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>450</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>475</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>12.5</td>
<td>487.5</td>
<td>0.25</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2.1 assay summary of advanced glycation endproducts by indirect ELISA.
2.1.5 Detection of Cross-linked AGEs using SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2.2) was used to determine the degree of cross-linking of glycated proteins extracted from muscle. Electrophoresis was performed using the mini-Protean®3 apparatus. The protein samples were denatured in 0.5 M Tris-HCl buffer (pH 6.8) containing 1% SDS (w/v), 1% 2-mercaptoethanol (v/v) and 20% glycine (v/v) and boiled for approximately 5-10 minutes. Before the samples were loaded in the gels 3 µL Bromophenol blue was added to track the proteins on the gel. The sample was then separated in either 7% SDS-PAGE at 4°C or 10% SDS-PAGE at room temperature (for myosin and actin, respectively) alongside with a standard molecular weight marker. After running for 12 hours for myosin and 1 hour for actin, the gels were stained with silver staining to detect the proteins. Briefly, the gels were fixed in 40% methanol/10% acetic acid for 30 minutes. Followed by staining with silver stain (Bio-Rad Silver Stain) according to the instructions of the manufacturer. Lastly, the gels were analysed using an image analysis system G-Box. The level of cross-linking of glycated proteins on the gels was detected as bands, and the intensity of the bands were measured using Image J software.

![Image of the SDS PAGE instrument used for analysis.](image)

**Figure 2.1.2:** Image of the SDS PAGE instrument used for analysis.
2.2 Detection of markers of oxidative stress

2.2.1 Measurement of lipid peroxidation

Malondialdehyde (MDA), a marker of lipid peroxidation was measured in serum samples using a Lipid peroxidation kit (Oxford biomedical research, UK). Briefly, reagent 1 (R₁; N-methyl-2-phenylindole in acetonitrile) was diluted 3:1 with diluents (Ferric Chloride Hexahydrate in Methanol). The MDA standard (1, 1, 3, 3-tetramethoxypropane (TMOP) in Tris-HCl) was provided as a 10 mM stock solution and a diluted to 20 µM for use in the assay. The MDA standards were prepared as shown in table 2.2. For the analysis, 200 µL sample was added to 650 µL diluted R₁ reagent and mixed gently. Then, a 150 µL of reagent 2 (R₂) containing methane sulphonic acids (MSA) was added to the mixture. The tubes were incubated at 45°C for 1 hour. The samples were centrifuged at 15,000 x g for 10 minutes and the absorbance of the supernatant was measured at 586 nm. All tests were carried out in duplicate. Sample blank was made by adding 650 µL of 75% acetonitrile and 25% diluent to a microcentrifuge tube (Esterbauer et al., 1990). A standard calibration curve was prepared and the MDA sample concentration calculated from the standard curve.

Table 2.2: Preparation of MDA standard calibration curve

<table>
<thead>
<tr>
<th>Standard concentration</th>
<th>MDA concentration (µM)</th>
<th>Volume of distilled water or buffer to add (µL)</th>
<th>Volume of 20 µM MDA stock (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₀</td>
<td>0</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>S₁</td>
<td>2.5</td>
<td>175</td>
<td>25</td>
</tr>
<tr>
<td>S₂</td>
<td>5</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>S₃</td>
<td>10</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₄</td>
<td>15</td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>S₅</td>
<td>20</td>
<td>-</td>
<td>200</td>
</tr>
</tbody>
</table>
2.2.2 Measurement of serum total antioxidant status

The total antioxidant status (TAS) of the serum samples was carried with an automated colorimetric measurement method using the RandoxRX Daytona analyzer (Randox Laboratories Ltd). The assay is based on the formation of the ABTS•+ cation [2, 2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] oxidant formation when sample constituents that generate a blue-green colour which is then measured at 600nm (Miller et al., 1993).

2.3 Glucose and albumin concentration

The total protein concentration in serum was determined as described in section 2.2.1.1. The glucose and albumin concentration in the serum were measured by colorimetric methods, according to the manufacturer’s instructions for RandoxRX Daytona Analyzer (Randox Laboratories Ltd., Belfast, Ireland). The glucose concentration was determined after enzymatic oxidation in the presence of glucose oxidase. The measurement of serum albumin concentration was based on its quantitative binding to the indicator 3, 3’, 5, 5’-tetrabromo- m cresol sulphonephthalein, which has absorption at 510 nm.

2.4 Haematological Analysis

Blood samples were collected in tubes containing an anticoagulant Ethylenediaminetetraacetic acid (EDTA) and were further analysed. The haemoglobin concentration was determined immediately after sampling with a HemoCue (HemoCue® Hb 201+ System). According to the HemoCue 2015 manual, the blood sample was loading on a microcuvette, which is placed in the HemoCue Analyzer. The haematocrit was assessed by filling a capillary tube with blood within 1 to 2 cm of the end. The unfilled end of the tube was then sealed and the capillary tube centrifuged for
5 minutes. The haematocrit was measured on a reading device that gives the height of the red blood cell column as % Hct. The total white blood cell (WBC) count was done with a Counting Chamber. The method involves diluting the sample with Turk solution at a ratio of 1:20 and loading it in the haemocytometer chamber. The differential leukocyte count (DLC) was obtained by a stained blood smear previously fixed with methyl alcohol for 2 minutes and stained with Giemsa stain for 8 -10 minutes (Walker et al., 1990).

2.5 Anti-inflammatory cytokine measurement

The following sections will provide details of steps carried out for the preparation of measurements of anti-inflammatory markers.

2.5.1 Multiplex procedure

The human Th1/Th2 11plex kit (eBioscience flow cytomix BMS810FF) was used to carry out the flow-cytometry multiplex procedure. Multiplex bead immune assays relies on the labelling of beads with two fluorescent dyes, one labelling the capture antibody and the second labelling the detection antibody. Following the capturing of analyte by beads, a biotinylated detection antibody is added. Hence, the ratio of signals emitted by the two-labelled antibodies can help to deduce analyte concentration. The reaction mixture is incubated with the molecule streptavidin- PE conjugate to complete the reaction on the surface of each bead. The ratio of the fluorescent signal emitted from each discrete antibody couplet is scanned by two lasers. Where the first laser (green colour) excites the streptavidin-PE dye of the assay, while the second laser (red colour) is used to excite the dyes inside the beads the principle is summarised in figure (2.3)
2.5.1.1 Preparation of standard curve

To quantify the abundance of proteins of interest, lyophilized standard vials were spun for 5 seconds in a microcentrifuge. This lyophilized standard was then reconstituted by the addition of distilled water (according to the label on the standard vial as described on the Th1/Th2 11plex kit instruction). Ten µL of each reconstituted standard was added to a vial labelled standard 1. These vials were made up to a final volume of 200 µL with the provided assay buffer. Then, 100 µL of the same assay buffer solution (1x) was added to 6 tubes labelled standard 2 to 7. An amount of 50 µL of standard 1 was transferred to tube 3. This procedure was repeated creating seven standard dilutions to produce the standard curve for each cytokine as shown in Figure 2.4.

*Figure 2.3: Diagram illustrating the principle of the fluorescent bead immunoassay (BD biosciences, Immunoassays, Multiplexed assays, 2012 Thermo Fisher Scientific Inc). Each bead is coated with a capture antibody. The streptavidin-PE is the detection antibody and binds to this specific capture antibody on the bead, the ratio of signals emitted by the two-labelled antibodies can help to deduce analyte concentration by flow cytometry.*
2.5.1.2 Preparation of bead mixture
The bead mixture volume required was equal to the number of tests multiplied by 25 µL; for 96 tests this means 2400 µL. Reagent dilution buffer (RDB) was then added to give a final volume of 3000 µL. Each individual bead vial was vortexed for 5 seconds and 150 µL was pipetted into a vial (150 µL x10 (number of cytokines) = 1500 uL) labelled “Bead Mix. Then 1350 µL of RDB was added into the bead mixture and was centrifuged at 3000 × g for 5 minutes. Following centrifugation, 2950 µL of the excess fluid was then removed, leaving 100 µL in the tube and replaced by RDB (2950 µL).

2.5.1.3 Preparation of biotin–conjugate mixture
For each test, 50 µL of biotin-conjugate mixture was required and for a 96-well plate, the volume of biotin-conjugate-mixture was 4800 µL. This was brought up to a of 6000 µL (Vfin) with RDB. From the final volume (Vfin), 1/20 (300uL) was taken and added to a vial labelled “Biotin-Conjugate Mix”. Therefore, 300 µl of each biotin-conjugate
was added to a vial (300 × 10 = 3000 µl). Finally, each vial was filled up with 2700 µL of RDB.

2.5.1.4 Preparation of sample for test procedure
The frozen serum samples were placed in a refrigerator to thaw overnight before use in the analysis. Once thawed, 25 µL of each of the standard mixture dilutions (1 to 7) as described in section (2.5.1.1) was added to their corresponding labelled test tube. Then, 25 µL of the assay buffer (1×) was added to the blank tubes and acted as the negative control. A sample was needed for the cytometer setup (positive control) so 25 µL of the standard mixture dilution 1 was then added to tube 8. Next, 25 µL of each serum sample was added to its designated sample tubes and 25 µL of bead mixture was then added to all tubes including the blank tubes. Following the addition of the bead mixture, 50 µL of biotin-conjugate mixture was then added to all tubes including the blank tubes. The contents of each tube was mixed well, and then incubated in the dark at room temperature (18-25°C) for 2 hours. Next, 1 ml of assay buffer (1x) was added to all tubes and these were spun at 200 x g for 5 minutes. The supernatant from each tube was then carefully aspirated leaving 100 µL of liquid in the tubes. Then, 50 µL of streptavidin-phycoerythrin (Streptavidin-PE) solution was added. The contents of each tube were mixed well and incubated at room temperature in the dark (18 - 25°C) for 1 hour. Following the incubation period, 1 ml of assay buffer (1x) was added and the samples spun at 200 × g for 5 minutes. The supernatant from each tube was carefully aspirated and the 100 µL of liquid in each tube was collected. Then 300 µL of assay buffer (1x) was added and the samples mixed in the vortex for 3-5 seconds.
2.5.1.5 Flow cytometry analysis

Firstly, the positive and negative controls were used to set up the cytometry before the sample analysis. Briefly, the controls were placed in the flow cytometer (BD FACScalibur, Becton Dickinson Company) and analysed using the high flow setting (FL2 channel), using Cell Quest Pro software and then by flow cytomix software, on a Becton Dickinson FACScalibur. The principle of flow cytometry is illustrated in Figure 2.5. It involves exciting labelled cells with a laser light source, as they flow through a fluid stream. This stream uses hydrodynamic focusing to ensure that only one cell is detected at a time. Moreover, the software translated the flow cytometric results into cytokine concentrations (pg/ml) and as Dot plots shown on Figure 2.6. Graphs for statistical analysis were produced using the GraphPad Prism 6 software programme (GraphPad Software. Inc, 2012).

![Figure 2.5: Diagram illustrating the principle of flow cytometry](image-url)
Figure 2.6: Dot plots showing the cytokines been gated as A) region 1 (R1: Beads population with 5.5 µM) and region 2 (R2: Beads population with 4.4 µM) referring to an area drawn on a plot displaying flow cytometry data. Each cell population were seen as B&D) red and C&E) green clusters. A negative and positive control was used to ensure that sample data obtained was within the correct range.
2.6 Detection of in vitro glycation

2.6.1 Protein extraction of skeletal muscle

Frozen muscle cross-sections (10-µm) were cut in a cryostat and a total mass of 10-20 mg was collected in a microcentrifuge tube. The tissue was then grinded to allow greater yield of protein extraction homogenized in 600 µL PRO-PREP solution (Pro-Prep Protein Extraction Solution; Intron Biotechnology, Gyeoggi-Do, Korea). The homogenate was then incubated for 20-30 minutes on ice to induce cell lysis. The lysed tissue was then centrifuged at 13,000 rpm at 4°C for 5 minutes to pellet non-dissolvable protein. The supernatant was then transferred to a clean Eppendorf tube and stored at -20 ºC for further analysis and the pellet was discarded.

2.6.2 Measurement skeletal muscle protein concentration with the Bradford method

The amount of protein extracted from the skeletal muscle was determined by a Bradford assay, which based on the classic method of Bradford (1976). A 1-mg/ml BSA stock in distilled water was used as a standard; a further serial dilution of 0 mg to 30 mg/mL of the BSA was prepared according to table 2.3. Ten µL protein lysates were made up with dH₂O up to 100 µL. The Bio-Rad protein dye was diluted 1:5 in distilled water and 2 mL dye was added to the standard and sample tubes and incubated for 5 min at 25°C. The absorbance was measured at 595 nm. The amount of the protein was determined using the standard curve as shown in Figure 2.7.
Table 2.3: Dilution of BSA used for protein estimation

<table>
<thead>
<tr>
<th>Stock 0.1% BSA (µL)</th>
<th>Distilled water (µL)</th>
<th>Bio-Rad reagent (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 2.7: Standard curve for protein estimation

\[ y = 0.0206x + 0.0284 \]
\[ R^2 = 0.9907 \]
2.6.3 In vitro glycation of extracted skeletal protein by methylglyoxal and glucose

*In vitro* glycation was first optimized with lysozyme as described before (Brownlee *et al.*, 1986). Briefly, lysozyme (10 mg/ml) was incubated with glucose (0.5 M) or methylglyoxal (0.1 M) in 0.1 M sodium phosphate buffer containing 3 mM sodium azide, pH 7.4 at 37 °C for different time intervals up to 7 days. The control samples (without the addition of sugars) were incubated under the same conditions. Aliquots were removed at given time intervals and stored at -20 °C until analysis. The same procedure was carried out on the extracted muscle protein. The level of AGEs was then determined by their auto fluorescence and ELISA as described in section 2.2.1.2 and section 2.2.1.3, respectively.

2.7 Determining the effect of age on the AGE formation in skeletal muscle

2.7.1 Immunohistochemistry study

Sections of skeletal muscle were cut at 8-µm thickness and were fixed in 4% paraformaldehyde for 5 minutes. They were rinsed once in 1x PBS tween for 5 minutes, followed by blocking in the diluted blocking buffer (10% serum diluted with PBST) for 30 minutes. The sections were then rinsed once with 1x PBS tween for 5 minutes and incubated in primary antibody (Rabbit polyclonal to AGE antibodies 1:400 (TransGenic Inc, Japan) at room temperature for 1 hour. Then the slides were rinsed 3 times 3 minutes with 1x PBS tween. This time the sections were incubated with (Goat anti – rabbit IgG 1:200) diluted secondary antibody in blocking buffer for 60 minutes in room temperature. The sections were then rinsed 3 times for 3 minutes with PBS and the excessive liquid was dried off, according to instructions of (vectastain Elite ABC kit,
Vector). The ABC kit system involves formations of active enzyme molecules from Adivin/Biotinylated enzyme complex, as a result, of mixing two reagents. The presence of peroxidase (HRP) in the Adivin/Biotinylated enzyme complex allows the Vector® SG Substrate to produce a blue-gray reaction product. Finally, the sections were mounted with Vectashield mounting medium with DAPI. The sections were photographed with a mono camera (black and white) under a microscope (Zeiss M1 wide field) at 10X magnification and then converted to 8 bit (black and white). The depth of stain on the cells in images were then measured by image J, which processes and analyses binary images. The intensity of the individual traces of cell were obtained and the average (mean grey value of cell) was taken as the reading.

2.8 Statistical analysis
All data are expressed as mean ± SEM. All statistical analysis was carried out using the statistical package for the social sciences (SPSS; version 19). Comparisons between groups were made with paired and unpaired student t-Test. Differences were considered significant at p<0.05.
CHAPTER 3:

Influence of exercise on circulating AGEs
3.1 Introduction

A small number of studies have been published which demonstrate a connection between the AGEs and exercise. One of the earliest studies reported that exercise in diabetic rats reduced the concentration of AGEs in comparison with inactive diabetic rats, and claimed that this was related to the enzyme-regulated metabolism and non-enzymatic phenomena including protein glycation (Panteleeva and Rogozkin, 2001). Researchers have further investigated the effect of exercise on age-related increases in with non-enzymatic cross-linking of myocardial collagen in rats (Choi et al., 2009). Interestingly, the study found that exercise improved cardiac efficiency. In addition, the exercise training reduced myocardial stiffness. They demonstrated that the worsening of the cardiac contractility was linked with a reduction in pathologic myocardial collagen cross-linking (AGE cross-linked collagen) in elderly rats (Choi et al., 2009). In another study by Boor et al., (2009), it was found that the concentration of the CML in glomeruli renal cortex and plasma was lower in trained than non-trained Zucker rats.

Studies on the impact of exercise on AGEs in humans are sparse. Goon et al., (2009) determined the impact of Tai Chi on AGEs in Malaysian individuals aged > 45 years. The individuals were randomly divided into test and control groups. It was found after 1 year Tai Chi the levels of MDA and AGEs were reduced. Danzig et al., (2010) investigated the effect of exercise in individuals with coronary artery disease and reported that, the concentration of RAGE was not influenced by the training program.
Yoshikawa et al., (2009) investigated the changes in levels of AGEs in 17 healthy women aged between 30 and 60 years. The subjects took part in a lifestyle modification program for 3 months. The objective of the program was to enhance the physical activity of the participants. Furthermore, they attended an awareness session in the start followed by a weekly supervised sessions. The study demonstrated a reduction in the CML concentration amongst the participants compared to the control and the negative correlation was found be linked with the steps performed daily.

These data suggest that regular exercise reduces circulating AGES. It is known, however, if such an effect of exercise is also seen in young adult healthy individuals with low levels of circulating AGEs. The aim of this study was to assess the effects of an endurance training programme on circulating AGEs and to what extent such changes are associated with alter oxidant and inflammatory statues.
3.2 Aims and objectives
The aim of this study is to investigate the effect of eight weeks exercise endurance training on the level of AGEs, blood glucose, biomarkers of oxidative stress and lipid profile in healthy participants.

To investigate this, the following objectives were applied:

- To determine circulating AGEs in serum of healthy participants before and after training.
- To examine the effect of eight weeks exercise on blood glucose level from healthy subjects.
- To determine the levels of serum cholesterol, triglycerides, high-density lipoprotein (HDL) and LDL after eight weeks of exercise
- To identify the levels of TAS and MDA (biomarker of oxidative stress) in healthy subjects following 8 weeks of exercise.
3.3 Methods

3.3.1 Subjects

Investigators from within our team were responsible for recruiting, follow-up and collecting samples on this study, they kindly provided samples for further studies. The detail of the study design and follow ups are described below.

From 29 subjects recruited in this current study, only 16 participants successfully completed the study. The inclusion criteria included the selection of healthy males aged from 18 to 35 years. The exclusion criteria included cigarette smokers, patients who had surgery involving metal implants as well as people with cardiovascular disease, diabetes, immunological disorders, and numbness in extremities or asthma. All participants attended a screening session. They were instructed to arrive in fasted state (12 hours) also having abstained from exercise, alcohol and caffeine for 24 hours prior the testing session. They were comprehensively briefed on the study protocol, and fully informed of all procedures that were to follow. All questions were answered to ensure that participants were satisfied before continuing with the study. Following this, the consent form was signed by the participants. Measurements of height (cm), body mass (kg) were taken using an electronic scale and a tape measure, approximately 10 mL blood sample was collected before and after the exercise intervention by a phlebotomist. The blood samples collected were then separated by centrifugation and then stored at -80°C. Participants attended the laboratory three times a week for eight weeks of aerobic exercise, consisting of 30 minutes of cycle exercise session, under supervision of students from our research group in the laboratory. Every two weeks, they were required to complete a questionnaire highlighting their up to date, nutritional habits and exercise routine. The ethical approval for human study was obtained from MMU Ethics Committee (See appendix 1B).
3.3.2 Experiential procedure

Serum glucose was analysed onsite by an automated glucose analyser from blood obtained by finger prick. LDL, HDL, cholesterol and triglycerides were analysed by Manchester the Royal Infirmary Hospital according to NHS standards (Protein concentrations in serum samples were measured as described in section (2.2.1.1). The LMW AGEs were assessed by their characteristic fluorescence as mentioned in section (2.2.1.2), the total AGEs were assessed using ELISA according to section (2.2.1.2).

Serum MDA, a marker of lipid peroxidation was quantified spectrophotometrically and assessed according to section (2.2.2.1). The TAS of the serum samples was measured using an automated colorimetric method as described in section (2.2.2.2.).

All values are expressed as means ± SEM. Comparison of the groups was performed using a paired Student t-test. In this study, the value P < .05 is taken as statistically significant.
3.4 Results

3.4.1 Body mass, BMI and standard biochemistry

The lipid profile of participants including the body mass, body fat, serum triglyceride, serum cholesterol, HDL and LDL were not altered significantly after training (Table 4). Also serum parameters (cholesterol, triglycerides, HDL, LDL, serum glucose, and total protein) were not significantly altered by training.

Table 3.1: Participant characteristics and blood biochemistry profile pre and post exercise

<table>
<thead>
<tr>
<th></th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (Kg)</td>
<td>79.22 ± 5.88</td>
<td>78 ± 5.95</td>
<td>0.5</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>25.09 ± 1.60</td>
<td>25.23 ± 1.82</td>
<td>0.7</td>
</tr>
<tr>
<td>Body fat %</td>
<td>20.85 ± 2.15</td>
<td>21.07 ± 2.12</td>
<td>0.2</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>4.36 ± 0.20</td>
<td>4.339 ± 0.27</td>
<td>0.8</td>
</tr>
<tr>
<td>Serum triglyceride (mmol/L)</td>
<td>0.92 ± 0.11</td>
<td>0.96 ± 0.19</td>
<td>0.9</td>
</tr>
<tr>
<td>Serum glucose (mmol/L)</td>
<td>4.89 ± 0.15</td>
<td>4.89 ± 0.08</td>
<td>0.5</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.31 ± 0.09</td>
<td>1.41 ± 0.08</td>
<td>0.7</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.63 ± 0.16</td>
<td>2.10 ± 0.30</td>
<td>0.8</td>
</tr>
<tr>
<td>TP (gm/L)</td>
<td>68.75 ± 1.68</td>
<td>71.14 ± 1.57</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The characteristics and blood biochemistry profile of healthy subjects before and after eight weeks of exercise (n=16). The results are represented as mean ± SEM. Where p=NS represents no significant difference. The abbreviations stands for BMI; body mass index, HDL; high-density lipoprotein, LDL low density lipoprotein and TP; total protein.
3.4.2 Determining serum LMW AGEs

The levels of serum LMW AGEs in healthy volunteers before and after exercise are shown in Figure 3.1. After eight weeks of exercise (4.16 ± 0.18 AU), there was no significant difference in fluorescent AGEs compared to the samples before exercise (4.2 ± 0.22 AU) (P= 0.9).

Figure 3.1: Measurement of serum LMW AGEs concentrations in healthy subjects before and after eight weeks of exercise. The excitation and emission wavelengths were assessed at 350 nm and 420 nm respectively, the results are represented as mean ± SEM (n=16). There was no significant difference between the fluorescent AGEs concentrations before and exercise (P=0.9). Y-axis represents LMW AGEs in (AU), while X-axis represents the 8 weeks exercise period.
3.4.3 Determining the serum level of AGEs using ELISA

The effects on the level of serum AGEs before and after 8 weeks of exercise (10.8 ± 0.71; 10.12 ± 0.57 µg/mL) are shown in Figure 3.2. There was no significant change in the AGEs after exercise ($P = 0.4$).

![Figure 3.2: Serum AGE concentration in healthy subjects before and after eight weeks of exercise, the results are represented as mean ± SEM (n=16. There was no significant difference between the AGEs level before and after exercise ($P=0.4$). Y-axis represents AGEs concentration in (µg/mL), while X-axis represents the 8 weeks exercise period.](image-url)
3.4.4 Measurement oxidative stress markers

3.4.4.1 Measurement of serum MDA concentration

Serum MDA concentration was assessed in 16 participants before and after 8 weeks of exercise (5.4 ± 0.77; 6.04 ± 0.80 µm; Figure 3.3). There was no significant change in MDA concentration after exercise ($P = 0.5$).

![Figure 3.3: Serum MDA concentrations in healthy subjects before and after exercise, the results are represented as mean ± SEM (n=16). No overall changes can be seen between the MDA concentration before and after exercise ($P=0.5$). Y-axis represents MDA concentration in (µM) while X-axis represents the 8 weeks exercise period.](image)
3.4.4.2 Measurement of total antioxidant status

The TAS in serum samples obtained from healthy participants before and after 8 weeks exercise (1.56 ± 0.03; 1.62 ± 0.02 mmol/L) is shown in Figure 3.4. After eight weeks of exercise, there was no significant change in the levels of TAS ($P = 0.1$).

*Figure 3.4: Serum total antioxidant measurement in healthy subjects before and after eight weeks of exercise, the results are represented as mean ± SEM (n=16). There is no significant difference in the level of TAS before and after exercise ($P=0.1$). Y-axis represents TAS concentration in (mmol/L) while X-axis represents the 8 weeks exercise period.*
3.4. Discussion

Various studies have examined the effect of exercise on hyperglycaemia (Solomon et al., 2013); where as other studies have highlighted the effect of exercise on biochemical parameters (cholesterol, triglycerides, HDL, LDL, serum glucose, and total protein), AGEs and oxidative stress (Macías-Cervantes et al., 2014). This current study investigated the influence of a regular 8 weeks aerobic exercise-training programme on the formation and/or accumulation of AGEs and oxidative stress by measuring total antioxidant status and lipid peroxidation. The main observation is that 8 weeks exercise had no significant effect on the biochemical parameters. This is in contrast with Aguilo et al. (2003) who found that LDL decreased whilst the level of triglycerides increased in well-trained professional cyclists after the cycling stage, but not in amateur cyclists, where a mild increase in total cholesterol after maximal and submaximal exercise was observed and also a rise in the level of HDL was detected. Aguilo et al. (2003) study suggests that different intensity of exercise and different training status can influence the cholesterol profile.

The serum glucose level in the present study was not affected by training. Many studies have similarly found that exercise does not affect blood glucose level in healthy subjects (Richard et al., 2010; Babraj et al., 2009). Using saliva samples to analyse total protein Walsh et al. (1999) found that after 5 minutes exercise, there was a significant increase in total protein. These findings are incompatible with the present study, moreover, the total protein was analysed from blood samples. This is maybe due to different intensity of exercise used in our study compared to Walsh et al. (1999).
In addition, the subjects in the Walsh study were exposed to moderate exercise; this explains the differences in total protein observation between our study and Walsh et al. (1999).

AGEs are highly heterogeneous set of compounds that are formed from a reaction between proteins or lipids after exposure to reducing sugars. The level of AGEs can be reduced or increased via different factors such as lifestyle (diet, smoking and exercise). Yoshikawa et al. (2009) investigated the changes in levels of the AGEs in 17 healthy women aged between 30 and 60 years and found a reduction in the CML concentration after training.

Research in influence of exercise on AGE levels in humans are limited (Delbin et al., 2012). In this study, we focus on the effect of regular exercise on the AGE levels and found that the regular exercise does not significantly affect the AGEs levels. Surprisingly, prolonged exercise in normal healthy subjects has been found to increase the level of 3BHB (a ketone body) (Bohlooli et al., 2014), an increase of the ketone body can inhibit AGEs formation by reducing AGEs. In our study the subjects were exposed to 8 weeks, exercises compared to that of prolong exercise. Furthermore, the variation in the exercise type may influence the production and/or accumulation of AGEs. Using obese Zucker rats as model, Boor et al. (2009) indicated that moderate regular exercise can inhibit the formation of AGEs in type 2 diabetic rats, but they have elevated AGEs to start with in contrast to our healthy participants. In line with this, it has been suggested that AGE formation is greatly enhanced by hyperglycaemia (McCance et al., 1993). Exercise causes an increase of 3-β-hydroxybutyrate (3BHB), which reduced AGEs in diabetic patients (Bohlooli et al., 2014). Whereas, our study found that exercise had no influence on the level AGEs, nevertheless, we used non-diabetic subjects.
Interestingly, neither the biochemical parameters nor AGEs were affected after 8 weeks exercise. Similarly, (Chang et al., 2011) found that there was a relation between AGE and lipid profiles of middle aged and elderly diabetic patients. In contradiction, using alcoholic subject (Kalousova et al., 2004) found that there was no relationship between biochemical parameters and AGEs. However, this difference may be attributed to the type of subjects used in our study.

The effect of exercise on oxidative stress biomarkers was also investigated in the present study, as there is evidence suggesting that the level of AGEs can elicit oxidative stress and vice versa. For instance, Scivittaro et al. (2000) found that AGEs caused mesangial oxidative stress. Therefore, a number of oxidative stress biomarkers were examined. MDA is a major endproduct of oxidation of polyunsaturated fatty acids and has frequently been examined as a marker of lipid peroxidation and oxidative stress in vivo (Dalle-Donne et al., 2006). The result in our study shows that there is a no change in in MDA after exercise. This finding was consistent with that by Moflehi et al. 2013, who found no significant differences between the effect of both moderate and high intensity levels of aerobic and resistance exercise on MDA.

Other studies, however, did show an increase in MDA after exercise and other exercises (back squat, bench press, lat pull-down, standing calf raise, arm curl and leg press)(Apple et al., 1983; Moflehi et al., 2012). While Goon et al. (2009) determined the impact of Tai Chi on Malaysian individuals aged > 45 years and observed that after one year of exercise the levels of MDA and the AGEs were reduced. The differences between the type of activity and the magnitude of physical exertion throughout the exercise might cause the differences in the results. Factors including the excessive period and type of exercise can affect the MDA levels as well as AGEs.
The TAS level in our study was similar in both groups (before and after exercise) is similar to the observation of Afzalpour et al. (2008), who demonstrated no differences in the anti-oxidative status in voluntary and untrained healthy subjects after exercise (walking at brisk and very brisk speeds and stepping up and down stairs).

Both findings do not comply with results from the Berzosa et al. (2011), which found a significant increase in the levels of TAS in human blood after single session of exercise. The latter study was confirmed by Robert et al. (1998), which showed an elevation of TAS in a venous blood immediately before and after a simulated half-marathon run. Our study assumes that factors such as the length of the exercise period, sample storage and the time taken to analyse the samples, may give rise to the variation in the outcomes from the studies mentioned above and cause a variation in the concentration of TAS assayed.

In conclusion, our study demonstrate that 8 weeks endurance training in healthy young men is insufficient to induce changes in serum lipid profiles, AGEs or oxidative stress. Hence, longer exercise duration may account for some alterations.
CHAPTER 4:

Influence of smoking and smoking cessation on AGEs
4.1 Introduction

Smoking is a common addiction and a significant risk factor for many chronic diseases including cardiovascular disease (CVD), atherosclerosis and chronic obstructive pulmonary disease. It is estimated that 36% of the cases of CVD is linked to smoking (Rosamond et al. 2007). According to the World Health Organization (WHO), by 2030 around 10 million deaths per year will be related to smoking cigarettes. Smoking cessation is highly effective reducing morbidity and mortality (Rodrigues et al., 2014).

The health risks of smoking are attributable to the more than 7000 compounds in cigarettes of which many are toxic and/or carcinogenic (Abdul-Rasheed, 2013). This includes reactive glycation compounds, known as glycotoxins that can rapidly react with proteins, such as serum proteins, to form advanced glycation endproducts (AGEs) (Cerami et al., 1997). Chronic smoking can also impair glucose tolerance and decrease insulin sensitivity, and is associated with increased serum cholesterol and triglyceride levels (Frati et al., 1996). Decreased insulin sensitivity can lead to glycation of myofibrillar proteins, as observed in diabetic subjects (Syrovy and Hodny, 1992). It has been reported that glycation impairs the function of myosin (Ramamurthy et al., 2001), which may have important implications for skeletal and cardiac muscle function. In addition, Rom et al. (2012) reported that cigarette smoking also increases the risk of sarcopaenia in elderly.

Cigarette smoke also contains compounds that induce an excessive production of free radicals and oxidative stress (Abdul-Rasheed et al., 2013) as reflected among others by lipid peroxidation (Lykkesfeldt et al., 2004; Morrow et al., 1995; Rajeswari et al., 1991), which may contribute to the increased risk of CVD (Bloomer, 2007). Furthermore, smoking is associated with increased numbers of circulating blood cells.
and elevated levels of inflammatory cytokines, such as interleukin-1 (IL-1), Interleukin-6 (IL-6), and tumour necrosis factor α (TNF-α), that all may contribute to the formation of atherosclerosis and development of CVD (Aula and Qadir, 2013).

Smoking cessation may be an important first step in reversing many of the detrimental changes induced by smoking. Some reported benefits of smoking cessation are an increase in HDL-cholesterol, which was, however, not accompanied with an improvement in glucose tolerance (Nilson et al., 1996) and it remains to be seen to what extent smoking cessation can reverse the smoking-induced reduction in insulin sensitivity. Braber et al. (2010) found that in the bronchoalveolar lavage fluid of mice levels of IL-1α and TNF-α induced by 20 weeks smoking were back to baseline levels after 8 weeks smoking cessation. It remains to be seen whether such benefits of smoking cessation also occur in humans and to what extent the glycation of serum proteins, as a result of smoking, is reversible by smoking cessation. We hypothesised that the elevated levels of circulating cytokines, white blood cells and indicators of oxidative stress and AGEs in smokers are readily reversible by 2-weeks smoking cessation.
4.2 Aims and objectives

The aim of this study is to investigate the benefits of smoking cessation on health and risk factors for chronic diseases in non-symptomatic smokers.

The study had the following objectives:

- To determine the level of circulating AGEs before and 2 weeks after smoking cessation.
- To examine the effect of smoking and two weeks smoking cessation on blood glucose, total protein and albumin levels.
- To identify biomarkers of oxidative stress by measuring the TAS and MDA before and after two weeks smoking cessation.
- To determine the effect of smoking and two weeks smoking cessation on white blood cell, differential leukocyte count (DLC), haemoglobin and haematocrit.
- To investigate the effect of smoking and two weeks smoking cessation on circulating cytokines.
4.3 Methods

4.3.1 Subjects
Both male (21) and female (8) participants were recruited for the study. Twenty participants (17 male and 3 female) were moderate to heavy smokers and nine (4 male and 5 female) were non-smokers. Participants were between 18 and 38 years old. Their height and mass were measured with electronic scales and tape measurement respectively. The body mass index was calculated as body mass: height squared. All participants were free of signs and symptoms of chronic diseases. The smoking participants had smoked a minimum of 8-10 cigarettes per day for at least 3 years. The study was approved by the Faculty Academic Ethics Committee of the Manchester Metropolitan University, and all participants provided informed consent before participation in the study.

4.3.2 Experimental procedure
Venous blood was collected from the antecubital vein before and after 2 weeks of smoking cessation. The blood was collected in 4-mL vacutainers without anticoagulants. The blood samples were allowed to clot for 15 minutes and serum was separated from whole blood by centrifugation (20 min; 500 x g) at room temperature. Following centrifugation, the serum was aliquotted in 1-mL microcentrifuge tubes and frozen and stored at -80°C until further analysis.
Serum protein, glucose and albumin concentrations were measured colorimetrically. Haemoglobin concentration was determined with a HemoCue as described in section (2.4). Serum malondialdehyde (MDA), a marker of lipid peroxidation, was quantified spectrophotometrically (section 2.2.2.1). Measurement of Total Antioxidant Status (TAS) in serum was analysed using total Antioxidant Status kit (Randox Labs, Crumlin, UK) as described by the manufacturer (section 2.2.2.1). The abundance of low
molecular weight (LMW) AGEs was assessed by fluorescence (section 2.2.4), whereas the total AGEs was assessed using ELISA (section 2.2.5). The human Th1/Th2 11plex kit (eBioscience flow cytomix BMS810FF, Bender MedSystems, Vienna, Austria, and Europe) was used to determine levels of circulating inflammatory markers according to the instructions of the manufacturer (section 2.5).

4.3.3 Statistical Analysis

All statistical analysis was carried out using the statistical package for the social sciences (SPSS; version 19). Comparisons between smokers with non-smokers, after with smokers and after with non-smokers were made unpaired student t-Test. Differences were considered significant at p<0.05.
4.4 Results

The age, body mass and height of the participants are reported in table 4.1. The smokers were younger than non-smokers and smoked 12.80 ± 1.09 cigarettes per day. The BMI of smokers was less than that of and non-smokers (p<0.05).

Table 4.1: Characteristics of smokers and non-smokers (controls). BMI (body mass index) of the participants was calculated as mass/height².

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-smokers (n=9)</th>
<th>Smokers (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37.7 ± 2.1</td>
<td>29.6 ± 1.9</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.63 ± 0.04</td>
<td>1.72 ± 0.02</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>73.1 ± 3.3</td>
<td>72.0 ± 2.4</td>
</tr>
<tr>
<td>BMI (Kg·m⁻²)</td>
<td>27.7 ± 1.53</td>
<td>24.3 ± 0.60</td>
</tr>
<tr>
<td>cigarettes per day</td>
<td>0</td>
<td>12.80 ± 1.09</td>
</tr>
</tbody>
</table>

Results are given as mean ± SEM. Where BMI (body mass index).
4.4.1 Total protein, albumin and glucose serum concentrations

The total protein concentration in serum was significantly higher in smokers (65.99 ± 0.78 g/L) than non-smokers (65.37 ± 0.78 g/L; *P* = 0.007; Figure 4.1A), but returned to normal levels after 2 weeks smoking cessation (64.50 ± 0.60 g/L). The albumin levels were, however, significantly lower in smokers (44.4 ± 0.35 g/L) than non-smokers (45.6 ± 0.78 g/L; *p* = 0.01; Figure 4.1B), which slightly increased in levels after 2 weeks smoking cessation (44.57 ± 0.54 g/L). The serum levels of glucose did not differ significantly between smokers (5.06 ± 0.21 mmol/L) and non-smokers (5.14 ± 0.25 mmol/L) and did not significantly change with smoking cessation (5.33 ± 0.23 mmol/L; *P* = 0.1) (Figure 4.2).

*Figure 4.1: Concentrations of A) Total serum protein and B) Serum albumin in non-smokers (control, *n*=9) before smoking cessation and after 2 weeks of smoking cessation (*n*=20). *P* = 0.007(Total serum protein) and *p*=0.01 (Serum albumin) when compared to control. Results are represented as mean ± SEM.*
Figure 4.2: Serum glucose concentrations in non-smokers (control, n=9) and before and after 2 weeks smoking cessation (n=20) \((P = 0.1)\). Results are represented as mean ± SEM.
4.4.2 Measurement of Haematology parameters

The haemoglobin concentrations and blood cell count were measured before and after smoking cessation. This included white blood cell count (WBC) and the five types of white blood cells (neutrophils, lymphocytes, monocytes, eosinophils and basophils). The haemoglobin concentration and haematocrit level were significantly higher (15.5 ± 0.19 mg/dl; 46.5 ± 0.58 % respectively; Table 4.2) in smokers than non-smokers (13.68 ± 0.59 mg/L; 41.03 ± 1.53 p=0.01, 0.007 respectively), which was somewhat reduced after 2 weeks smoking cessation (15.28 ± 0.19 mg/L; 45.58 ± 0.58 p=.004, .008 respectively), but remained elevated above control levels (Table 4.2; control vs after; p=0.02, 0.01 respectively). Smokers had a significantly higher (60.3 ± 2.7 x10³ /µL) neutrophil count than control group (54.6 ± 1.6 x 10³ /µL; p=0.04; Table 4.2). This difference had not disappeared after 2 weeks smoking cessation (58.9 ± 1.9 x 10³ /µL) (smoking cessation vs. control p=0.04). The eosinophil count was significantly lower in smokers (2.24 ± 0.23 x 10³ /µL) than control (3.44 ± 0.29 x 10³ /µL; P= 0.01) and remained so after 2 weeks smoke cessation (2.25 ± 0.22 x 10³ /µL; p=0.002).
Table 4.2: The effect of smoking and two weeks of cessation on haematological parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-smokers (Controls)</th>
<th>Smokers (pre)</th>
<th>Smokers (post)</th>
<th>Statistical evaluation (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>control vs before</td>
</tr>
<tr>
<td>WBC (X10^3 /µL)</td>
<td>6.70 ± 0.72</td>
<td>6.98 ± 0.44</td>
<td>6.89 ± 0.38</td>
<td>0.39</td>
</tr>
<tr>
<td>Neutro (%)</td>
<td>54.6 ± 1.6</td>
<td>60.3 ± 2.7</td>
<td>58.9 ± 1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Lympho (%)</td>
<td>34.3 ± 1.2</td>
<td>30.5 ± 2.5</td>
<td>32.2 ± 1.8</td>
<td>0.09</td>
</tr>
<tr>
<td>Mono (%)</td>
<td>6.0 ± 0.41</td>
<td>5.58 ± 0.31</td>
<td>5.58 ± 0.29</td>
<td>0.21</td>
</tr>
<tr>
<td>Eosino (%)</td>
<td>3.44 ± 0.29</td>
<td>2.24 ± 0.23</td>
<td>2.25 ± 0.22</td>
<td>0.01</td>
</tr>
<tr>
<td>Baso (%)</td>
<td>1.67 ± 0.29</td>
<td>1.17 ± 0.21</td>
<td>1.42 ± 0.19</td>
<td>0.09</td>
</tr>
<tr>
<td>HB (mg/dL)</td>
<td>13.7 ± 0.6</td>
<td>15.5 ± 0.2</td>
<td>15.3 ± 0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>41.03 ± 1.53</td>
<td>46.5 ± 0.58</td>
<td>45.58 ± 0.58</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Results are given as mean ± SEM, The abbreviations stands for wbc; white blood cell, neutro; neutrophil, lympho;Lymphocyte, mono; Monocyte , baso; basophil, esion; esionphile, HB ; heamoglobin;Hct,Heamatocrit
4.4.3 Effect of smoking and 2 week of smoking cessation on oxidative stress biomarkers

4.4.3.1 Measurement of total antioxidant status

Smokers had a lower total antioxidant status (TAS) (1.26 ± 0.01 mmol/mL) than non-smokers (1.49 ± 0.04 mmol/mL; Figure 4.3; \( p = 0.0001 \)), which was somewhat reversed after 2 weeks smoking cessation (1.28 ± 0.01 mmol/mL; \( p = 0.0002 \)), but remained lower than control levels (control vs after; \( p = 0.0005 \)).

*Figure 4.3: Serum total antioxidant status (TAS) in non-smokers (control, \( n=9 \)) before and after 2 weeks of smoking cessation (\( n=20 \)), \( p = 0.0001 \) compared to control. Results are represented as mean ± SEM.*
4.4.3.2 Lipid peroxidation (Malondialdehyde)

The level of Malondialdehyde (MDA) in serum of smokers is significantly higher ($3.34 \pm 0.10 \, \text{Mm}; \, 2.15 \pm 0.26 \, \text{Mm}$) than that in the control group (Figure 4.4; $p=0.001$), after 2 weeks smoking cessation remained elevated above control levels ($3.13 \pm 0.09 \, \text{Mm}$) (control vs after; $p=0.002$).

![Figure 4.4: Serum MDA concentrations in non-smokers (control, n=9), before and after 2 weeks smoking cessation (n=20). Control vs before smoking cessation $p = 0.001$; control vs 2 weeks smoking cessation; $p=0.002$. Results are represented as mean ± SEM](image)
4.4.4 Advanced glycation endproducts

Measurements of AGEs using fluorescence spectrophotometry showed that the levels of AGEs were significantly higher in smokers than non-smokers (12.38 ± 0.36; 11.71 ± 0.42 AU; Figure 4.5 A; \( p=0.02 \)) and remained elevated after 2 weeks smoking cessation (11.86 ± 0.28 AU), but not significantly. Determination of AGEs with ELISA confirmed this pattern; smokers having significantly high levels of AGEs in their serum (0.27 ± 0.04 µg/mL) than non-smokers (0.10 ± 0.05 µg/mL; Figure 4.6 B; \( p=0.009 \)), with no significant change after 2 weeks smoking cessation (0.25 ± 0.03 µg/mL; \( p=0.4 \)), remaining higher compared to control (\( p=0.01 \)).

![Figure 4.5: Serum AGE levels measured using A) fluorescence and B) ELISA in non-smokers (control, \( n=9 \)). Fluorescence AGEs Control vs before smoking cessation \( p = 0.02 \); AGEs with ELISA Control vs before smoking cessation \( p = 0.009 \); control vs 2 weeks smoking cessation; \( p=0.01 \) Results are represented as mean ± SEM.](image-url)
4.4.5 Levels of serum cytokines

The levels of different serum cytokines were quantified with flow cytometry. Table 4.3 shows that the serum level of TNF-α in smokers was higher than that in non-smokers (p<0.01). Two weeks smoking cessation resulted in a reduction in the concentration of TNF-α (p<0.05), which was, however, still higher than in non-smokers but not significant (p=NS). There were no significant differences in circulating TNF-β between smokers and non-smokers. Neither did smoking cessation have a significant effect on circulating TNF-β. Again, there is no significant difference in Interferon gamma INF-γ level between smokers and non-smokers.
Table 4.3: The effect of smoking and two weeks of cessation on levels of circulating cytokines

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Non-smokers (Controls) Pg/ml</th>
<th>Smokers (pre) Pg/ml</th>
<th>Smoker (post) Pg/ml</th>
<th>Statistical evaluation (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>control vs before</td>
</tr>
<tr>
<td>TNF-α</td>
<td>24.47±11.19</td>
<td>70.61 ±1.32</td>
<td>51.47 ± 27.21</td>
<td>0.01</td>
</tr>
<tr>
<td>TNF-β</td>
<td>82.91±31.22</td>
<td>108.97 ± 34.01</td>
<td>109.10 ± 46.75</td>
<td>0.29</td>
</tr>
<tr>
<td>INF-γ</td>
<td>139.36± 82.05</td>
<td>144.10 ± 56.99</td>
<td>161.02 ± 55.13</td>
<td>0.48</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100.4 ±53.02</td>
<td>109.38 ± 38.41</td>
<td>94.05 ± 41.34</td>
<td>0.45</td>
</tr>
<tr>
<td>IL-2</td>
<td>32.02±20.01</td>
<td>260.05 ± 58.42</td>
<td>199.59 ± 41.184</td>
<td><strong>0.0006</strong></td>
</tr>
<tr>
<td>IL-4</td>
<td>26.03± 17.97</td>
<td>71.32 ± 25.42</td>
<td>43.94 ± 12.74</td>
<td>0.08</td>
</tr>
<tr>
<td>IL-5</td>
<td>109.42± 89.33</td>
<td>125.44 ± 56.79</td>
<td>131.93 ± 29.50</td>
<td>0.39</td>
</tr>
<tr>
<td>IL-6</td>
<td>16.94±11.21</td>
<td>44.75 ± 20.33</td>
<td>23.75 ± 20.33</td>
<td>0.12</td>
</tr>
<tr>
<td>IL-10</td>
<td>32.02±20.01</td>
<td>71.68 ± 33.88</td>
<td>46.29 ± 26.84</td>
<td>0.17</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>15.37± 8.11</td>
<td>6.91 ± 5.39</td>
<td>13.64 ± 2.92</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Results are given as mean ± SEM, abbreviations stand for TNF-α; tumour necrosis factor-alpha, TNF-β; tumour necrosis factor-beta, INF-γ; interferon-gama, IL-1β; interleukin-1alpha, IL-2, IL-4, IL-5, IL-6, IL-10; interleukin-6, IL-12p70; interleukin-12p70.

The level of IL-1β did not differ significantly between smokers and non-smokers and did not change significantly after 2 weeks smoking cessation. The IL-2 serum concentration was higher in smokers and non-smokers (p<0.001) and did not decrease significantly after 2 weeks smoking cessation remaining elevated above control levels (p<0.001).

The serum concentrations of IL-4, IL-5, IL-6, IL-10 and IL-12p70 did not differ significantly between smokers and non-smokers and was not significantly altered with 2 weeks smoking cessation.
4.5 Discussion

Many studies have examined the effect of cigarette smoking on blood parameters, but few have investigated to what extent and how quickly smoking cessation can reverse many of the detrimental effects of smoking. Here it was observed that many of the abnormal haematological parameters of smokers can be reversed by a period of smoking cessation as short as 2 weeks. Such rapid and measurable normalisations of haematological parameters that have a significant impact on overall health can be a potent stimulus to assist in efforts to stop smoking.

The smokers in our study had a lower BMI than non-smokers. Previous studies have also shown that smokers weigh less and have less body fat than non-smokers (Vanni et al., 2009). It has been suggested that part of this lower body mass maybe related to upregulation of AZGP1 in the airway epithelium, a gene linked to weight loss (Vanni et al., 2009).

The present study showed elevated serum concentrations of total protein, similar to observations in other studies (Alhemieri, 2008). They concluded that an increase of serum total protein may be due to a difference in dietary style amongst the subjects this may also be the case for our subjects. Yet, the levels of albumin in smokers where reduced in our study, similarly to Alsalhen et al. (2014). Albumin represents an important circulating antioxidant (Roche et al., 2008); as albumin has ligand-binding capacities and free radical-trapping properties (Taverna et al., 2013). The reduced albumin may thus aggravate the oxidative stress caused by cigarette smoke constituents in smokers.

Haemoglobin was elevated in smokers and remained so even after 2 weeks smoking cessation. Patel et al. 2010 reported that the increase in haemoglobin amongst smokers is due to the increased inhaled carbon monoxide, which combines with haemoglobin to
form carboxyhemoglobin (COHb). COHb reduces the oxygen carrying capacity of the blood and haemoglobin level is increased to compensate for this reduced oxygen carrying capacity. Even though COHb levels has a half-life of 24 hrs, the level of haemoglobin was only little reduced after smoking cessation, indicating that erythrocytes have a longer half-life than 24 hours.

Smokers may develop insulin resistance and therefore we measured the glucose concentrations in serum. The present study and others (Alhemieri, 2008) show, however, that smokers and non-smokers had similar levels of serum glucose. It is still possible, however, that smoking does impair glucose tolerance and Nilson et al. (1996) found that smoking cessation did not affect glucose tolerance even after 16 weeks smoking cessation.

An impaired glucose control may result in increased levels of AGEs in the blood (Singh et al., 2014). Here, we demonstrated that smokers had significantly increased levels of AGEs when compared to control, which was not returned to normal levels after two weeks cessation. This supports the observation that smoking impairs glucose control, which is not readily reversible by smoking cessation (Nilson et al., 1996). It should be noted, however, that not only impaired glucose control, but also many compounds in cigarette smoke can induce reactive glycation compounds, known as glycotoxins that can rapidly react with proteins, such as serum proteins, to form AGEs (Cerami et al, 1997). Elevated AGEs may thus well be result from a combination of impaired glucose control and circulating cytotoxins. Smoking cessation for 2 weeks may have not been a long period to reduce AGEs, due to the stability of AGEs.

Another factor that can lead to increased levels of circulating AGEs is oxidative stress. Free radicals and the other toxins in cigarette smoke can cause lipid peroxidation, which
will be reflected by increased circulating MDA levels. The present study shows that smokers had indeed higher levels of the oxidative stress biomarker MDA in their serum. Others have also observed indications of increased oxidative stress in smokers, such as a rise in lipid peroxidation that may be associated with atherosclerosis (Aula and Qadir, 2013; Rajeswari et al. 1991; Lykkesfeldt et al. 2004). Here we extend these observations and show that the MDA levels remained elevated after 2 weeks smoking cessation, whereas Polidori et al. (2003) found that MDA levels were reduced after 4 weeks smoking cessation. MDA is an aldehyde compound and these compounds tend to have a long life span (Naudí et al., 2013). It may thus be that 2 weeks smoking cessation did not influence MDA levels significantly in our study, even though lipid peroxidation had normalised.

Mahmood et al. (2007) suggested that measuring TAS is more effective than measuring individual antioxidant activity, as it is time consuming to measure all the known antioxidants in biological fluid and most likely many antioxidants are yet to be discovered. Similar to the observation of Mahmood et al. (2007) the TAS level was lower in smokers than non-smokers in our study. Durak et al. (2002) and Lykkesfeldt et al. (2004) demonstrated that lower antioxidants result in enhanced lipid peroxidation of smokers. Therefore, increase in TAS after 2 weeks (our study) or 4 weeks (Polidori et al. 2003) smoking cessation, supports the notion that the elevated levels of MDA after 2 weeks smoking cessation is indeed more a reflection of the longevity of MDA than a continued elevated oxidative stress.

It is well known that cigarette smoke is associated with increased risk for infection and inflammation, which can lead to many complications, such as cardiovascular disease (Aula and Qadir, 2012). The mechanisms by which cigarette smoke causes harm are not fully understood. However, previous studies have suggested that cigarette smoke may
activate leukocytes and/or macrophages. The activation of these cells may result in the release of cytokines. Cytokines secreted from inflammatory cells, play major roles in the immune system and in turn stimulate the production of macrophages and leukocytes (Newton and Dixit et al, 2012; Karimi et al, 2006).

Cytokines can be divided into interleukins (IL) and interferons (INF), which are further classified as pro-inflammatory, or anti-inflammatory. An imbalance between pro- and anti-inflammatory cytokines can result in harmful effects (Bijjiga and Marino, 2013).

As discussed above, cigarette smoking is associated with oxidative stress. Oxidative stress can increase the expression of Toll-like receptors (TLRs) that mediate NF-κB activation in neutrophils (Asehnoune et al, 2004; Tharappel et al., 2010; Roses et al, 2008; Nadigel et al, 2011), that in turn stimulates the release of cytokines (Preciado et al, 2010). Even low concentrations of cigarette smoke can induce NF-κB activation in lymphocytes (Hasnis et al., 2007).

We demonstrated that cigarette was associated with elevated levels of TNF-α and IL-2. Surprisingly, cigarette smoke did not have a significant effect on the levels of INF-γ. Our results also demonstrated that cigarette smoking caused the levels of neutrophils to significantly increased, but reduced the level of eosinophils.

A report by Byrne and Reen, (2002) indicated that TNFα production by monocytes is rapidly stimulated by cigarette smoking that then induces the release of IL-1β and IL-6. Here we also observed an increase in TNFα, but not IL-6 in smokers. While IL-6 may be stimulated by, TNFα, IL-6 does attract neutrophils, which are essential for TNFα production (Scheller et al., 2011: Demirjian et al., 2005). TNFα and IL-6 thus may stimulate each other via a positive feedback loop. Furthermore TNF-α, is reported to be produced by a variety of cells including neutrophils, T-cells, macrophages, monocytes, epithelial cells, fibroblasts and smooth muscle cells (Demirjian et al., 2005). The
increased abundance of those cells in the blood of smokers may thus underlie the increased TNFα observed in our study, without a concomitant elevation of IL-6.

The benefits of smoking cessation are, however, evident in the reduction of the pro-inflammatory TNF-α after 2 weeks smoking cessation. Similarly, Rodrigues et al, (2014) found that after 30 days the level of TNF-α was decreased.

Ouyang et al. (2000) found that the concentrations of hydroquinone, found in medium-tar cigarettes, could block the production of IL-2. In this study, the present study subjects smoked various types of cigarettes and this may have caused the level of IL-2 to increase in comparison to the study of Ouyang et al. (2000). IL-2 plays a role in growth and activating factors for Th1 and Th2 cells. The Th2 cells can influence the influx of eosinophil. This was demonstrated by the presence of IL-4 and IL-5 cytokines (Ceyhan et al, 2004; Teng and Gao, 2014), that are mediators in the production of eosinophil. We found no difference in the level of the IL-4 and IL-5 in our smokers and non-smokers, which may have contributed to the lower numbers of eosinophils. In contrast, Nordskog et al. (2005) and Byron et al. (1994) found that cigarette smoking increased the level of IL-4. IL-4 expression can be inhibited by IFN-γ via IgE (Teixeira et al, 2005). IFN-γ release is stimulated by IL-12p70, which is the biologically active form of IL-12 (Feinberg et al, 2004). Levels of IFN-γ were not altered in the smokers in our study, however the levels was not affected significantly after 2 weeks smoking cessation.

A recent study has shown that smoking can reduce the expression of the maturation marker CD83 on the surface of myeloid DCs (Stoll et al, 2014). These DC are important for regulating and producing IL-12(Roses et al, 2008). Stoll et al, (2014) found a reduced level of CD83 in smokers with COPD compared to former smokers with COPD. In line with this, we found a slight increase in the level of IL-12 after smoking
cessation compared to smokers, which indicates that the expression of CD83 on DC in smokers may be, impaired consequently suppressing IL-12.

Ouyang et al., 2000, however, found that cigarette smoke contains potent inhibitors of cytokine production and suggested that CS suppressed the level of IL-1β, IL-2, TNF-α and IFN-γ, but this maybe dependent of the type of cigarettes (Lee et al, 2012).

Quitting smoking interrupts the exposure to chemicals in cigarette smoke (Rodrigues et al, 2014). Indeed smoking cessation can affect the level of cytokines. For example, Braber et al. (2010) found that in the bronchoalveolar lavage fluid of A/J mice the elevated IL-1α and TNF-α after 20 weeks smoking were back to baseline levels after 8 weeks smoking cessation. According to Damiá et al. 2011, cigarette smoking is a potent stimulator of chronic inflammation, which can continue even after smoking cessation. This notion fits the observation of significant reduction of cytokines (IL-2 and TNF-α), levels after 2 weeks smoking cessation in our study. However the levels of TNF-β, INF-γ, IL-1β, IL-4, IL-5, IL-6 IL-10 and IL-12P70 were not significantly affected due to reduction of immunosuppressive agents from the cigarettes smoking in the serum of the participants after 2 weeks of smoking cessation (Ouyang et al., 2000).

In conclusion, smoking is associated with increased levels of oxidative stress and inflammation, which can largely be reversed by as short a period of smoking cessation as 2 weeks. This may provide a potent stimulus for attempts to quit smoking.
CHAPTER 5:

The effects of ageing on skeletal muscle glycation
5.1 Introduction

The main contractile proteins of skeletal muscle are actin and myosin. By hydrolysing ATP; it forms a cross-bridge with actin and pulls the actin filament along. During this process, the muscle produces force and shortens; hence, an adequate function of the myosin and actin filament is crucial for appropriate contractile function. Researchers have found that different sugars can alter muscle structure and affect their function through glycation. For instance, incubation of myosin with glucose alters the myosin structure and the actin–myosin interaction, resulting in a reduced speed at which actin filaments can be propelled by myosin in an in vitro motility assay (Ramamurthy et al., 2003). Such a change in vivo would result in slower muscle contractile properties.

In this context, it is interesting to note that during ageing the contractile properties of muscle fibres become slower, independent of changes in myosin heavy chain composition in both rat and human (Degens et al., 1998; Li and Larsson, 1996). This and sarcopenia or loss of muscle strength and muscle mass, as previously mentioned in chapter 1 section 1.13, are important factors underlying mobility difficulties, such as slow walking speed in older adults (Lauretani et al., 2003). A further slowing of the muscle independent of changes in myosin composition or muscle mass would aggravate the muscle weakness that already occurs during ageing because of muscle atrophy and reductions in the force generating capacity of the remaining muscle (Degens et al., 2009). Studies have demonstrated that oxidative stress may alter skeletal muscle function (Callahan et al., 2001; Gilliver et al., 2010). The age-related reduction in muscle contractility may thus be due to oxidative modifications of skeletal muscle proteins and glycation (Lowe et al., 2001; Ramamurthy et al., 2001). Studies have shown that glycation also contributes to musculoskeletal complication of diabetes mellitus (Kim et al., 2001). While there are some studies that have shown increased,
glycation of muscle in aged skeletal muscle (Syrovy and Hodny, 1992; Snow et al., 2005), the degree of glycation of aged muscle proteins is unknown, also because it is difficult to quantify the degree of glycated myofibrillar proteins. Here, we sought to obtain first insight in how glycated myosin can be detected and whether the latter is elevated in old muscle. To do so, we incubated muscle protein extracts with methylglyoxal, a strong glycating agent. It mainly binds to the lysine, cysteine and arginine residues in protein (Thornalley et al., 1999; Goldin et al., 2006). Using these positive controls, we then set out to assess the degree of glycation in young and old rodent muscle and the degree of muscle glycation.
5.2 Aim and objectives
The aim of this study is to determine whether muscle protein glycation occurs in old age. The objectives were:

A) To artificially glycate rat plantaris muscle with different glycation agents (glucose and methylglyoxal) to obtain positive controls.

B) To examine whether AGEs were more abundant in rat gastrocnemius muscle of old than young male Wistar rats.
5.3 Methods

5.3.1 Animals
For the in vitro glycation studies, plantaris muscles excised from 17-week-old male Wistar rats were used. Animals were obtained from Radbond University Nijmegen and protocols for animal welfare were conducted according to their regulations (see appendix 1B). To assess the effects of ageing on muscle protein glycation 5, 25 or 32 months old male Wistar rats were used. Experiment, were housed in pairs with free access to food and water. The environment was maintained at 22°C on a 12-h light/12-h dark cycle. The Wistar rat has a median lifespan of 21.5 months (Harlan Laboratories, Indianapolis, Indiana) and a mean life expectancy of around 24 months. The 5-month-old (young adult) rats have ended their main growth spurt and are mature, whereas the 25 and 32 month-old rats are considered old and very old, respectively. The animals were euthanized by an intraperitoneal injection of an overdose of pentobarbital sodium, and the plantaris muscles were quickly excised, blotted dry, and weighed on an analytical balance. Muscles were then slightly stretched, pinned on cork, frozen in liquid nitrogen, and stored at -80°C until analysis. All procedures were approved by the animal ethics committee of the Radbond University Nijmegen.
5.3.2 Experimental procedure

For in vitro glycation, protein was extracted from the plantaris muscle by homogenising the muscle in the protein extraction solution described in section 2.6.1. The concentration of the extracted protein was measured using the Bradford method (section 2.6.2). The extracted protein was then incubated with glucose (0.5 M) or methylglyoxal (0.1 M) in 0.1 M sodium phosphate buffer containing 3 mM sodium azide, pH 7.4 at 37 °C for different incubation times (0, 1, 3, 5, and 7 days). Studies have found that AGEs can be formed within 7 days; also, the highest level of glycation is achieved within this time period of incubation (Nagaraj et al., 2010; Khan et al., 2015). Similarly, we also found AGEs formed within 7 days incubation with glyoxal, methylglyoxal. Shorter incubation time (2-7 days) generally favours protein rich in amadori compound whereas longer incubation time (7+days) lead to extensive browning and oxidation (Cutler and Rodrigues, 2003). The formation of AGEs in extracted skeletal muscle proteins was determined by their characteristic fluorescence, which was measured using the fluorescence spectrophotometer as illustrated in section 2.2.1.2. ELISA was also employed to quantify the AGEs concentrations as described in section 2.2.1.3. SDS-PAGE followed by silver staining was used to detect the cross-linking of glycated myosin as described in section 2.2.1.4.

Finally, Immunohistochemical analysis was used to detect the accumulation of AGEs in cross-sections of gastrocnemius muscle described in section 2.7.1.
5.4 Results

5.4.1 Effects of period of incubation on fluorescence AGEs

There was a gradual increase in the fluorescence intensity with the duration of incubation with glucose (0.5 M) and methylglyoxal (0.1 M) in the extracted plantaris muscle (Figure 5.1). This is indicative of increased glycation with increased duration of incubation in glucose and methylglyoxal ($p < 0.001$) (Figure 5.1), but more so after incubation with methylglyoxal ($p < 0.001$) (see appendix 1C; Table 1).

Figure 5.1: The effect of duration of incubation with glucose or methylglyoxal on advanced glycation endproduct (AGE) formation in extracted skeletal muscle protein. Extracted skeletal muscle protein (3.15 mg/ml) was incubated with glucose (0.5 M) and methylglyoxal (0.1 M) in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 7 days and autofluorescent determined. $p < 0.001$ compared to control, after 7 days incubation with methylglyoxal. Each value represents the mean ± SEM ($n = 3$) from three independent experiments. Where *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ represents significant difference from control.
5.4.2 Effect of incubation time on formation of AGEs measured by ELISA

ELISA was used to detect AGE levels of plantaris muscle protein extracts glycated \textit{in vitro} as described above. There was an incubation-duration-dependent increase in the level of AGEs after incubation with glucose (0.5 M) but more so after incubation with methylglyoxal (0.1 M) (Figure 5.2) \((p < 0.001)\) (see appendix 1C; Table 2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure52.png}
\caption{The effect of incubation time on level of AGEs in the extracted skeletal muscle protein. Extracted skeletal muscle protein (3.15 mg/ml) was incubated with glucose (0.5 M) and methylglyoxal (0.5 M) in 0.1 M sodium phosphate buffer of pH 7.4 at 37 \degree C for 7 days. \(P < 0.001\) compared to control, after 7 days incubation with methylglyoxal. Each value represents the mean \pm SEM (\(n = 3\)). Where \(*p < 0.05\) and \(**p < 0.01\) represents significant difference.}
\end{figure}
5.4.3 Determining the effect of different incubation time on methylglyoxal-derived cross-linked AGEs formation in vitro

Extracted skeletal muscle protein incubated with methylglyoxal produced cross-linked AGEs as revealed by dimers in gels run for 1 hour (Figure 5.3A), also dimers of actin proteins with molecular weight of approximately 80 kDa were observed in the gel at each incubation period at 37°C (Figure 5.3A: Lane b-f). The degree of glycation also in this case increased with incubation time (Lane f).

On the other hand, myosin glycation was observed after the gel was left running for 12 hour (Figure 5.3B and Figure 5.3.1B); after 7 days incubation, there was more cross-linked AGEs, as determined by the intensity of the band (Lane f) compared to the control (native proteins) (Lane a).
Figure 5.3: The effect of different incubation time on methylglyoxal-derived cross-linked AGEs formation in vitro after A) actin B) myosin: The cross-linked AGEs were determined by SDS-PAGE and stained with silver; the image represents the gel, which indicates cross-linked AGEs formation in vitro after incubation of extracted skeletal muscle proteins (3.15 mg/ml), with 0.1M methylglyoxal at different incubation time. Where (a) Control (non-glycated protein) (b) 0 day (c) 1 day (d) 3 days (e) 5 days (f) 7 days in the presence of 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 7 days.
Figure 5.3.1: The effect of different incubation time on methylglyoxal-derived cross-linked AGEs formation in vitro of A) actin and B) myosin. The graph represents the effect of extracted protein (3.15 mg/ml) after incubation with methylglyoxal at different time (0 – 7 days) in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C. Each value represents the mean ± SEM (n = 3) from three independent experiments.
5.4.4 Determining the effect of different age groups on AGEs formation \textit{in vivo}

There was an age-related increase in the intensity of AGE staining in rat plantaris muscle \textit{in vivo} (5, 25 and 32 months) (Figure 5.4; 5.14±0.10 AU; P<0.01). This is indicative of increased glycation with increased age (exact p-value see appendix 1C; Table 5).

\textit{Figure 5.4: Immunohistochemistry on the plantris muscle from young (5 month), old (25 month) and very old (32 months) Wistar rats. A) 5-month-old rat, negative control for advanced glycation endproduct (AGE). B) 25 months old rat, negative control for advanced glycation end-product (AGE) immunolabeling C) 32 months old rat, negative control for advanced glycation end-product (AGE) D) 5 month, immunolabeling for AGE (arrow); E) 25-month-old rat, immunolabeling for AGE (arrow) and F) 32-month-old rat immunolabeling for AGE (arrow). p<0.01 when old muscle is compared to young.}
Figure 5.4.1: The graph represents the effect of different aged rats (5, 25 and 32 months) on the level of AGES in plantaris muscle. Results are represented as mean ± SEM (n=3). $p<0.01$ (25 months) and $p<0.0001$ (32 months) compared to (5 months).
5.5 Discussion

Accumulation of AGEs are tissue specific (Snow et al., 2007) and are characterised by intra and inter protein crosslinking that alter the structure and function of proteins (Wu et al., 2009). In some studies, AGE complexes are used as biomarkers of oxidative stress and tissue damage (Fu et al, 1996). It is suggested that the accumulation of crosslinking AGEs is linked with ageing and diabetes (Nishigaki et al., 2008).

The present study indicates that AGE formation in skeletal muscle proteins increases with the duration of incubation with methylglyoxal or glucose. Methylglyoxal causes glycation by specifically targeting the arginine residue (Thornalley et al., 1999) as well as lysine residues in protein to form N(epsilon)-(carboxyethyl)lysine (CEL) and the imidazolium crosslink, methylglyoxal-lysine dimer (MOLD) (Degenhardt, 1998). The properties of methylglyoxal for forming AGE are due to the presence of two carbonyl groups on the structure. Methylglyoxal alters the proteins at the functional sites by inducing a loss of positive charge, leading to denaturation of the protein structure and further causing protein inactivation (Kimzey et al., 2011).

Other studies reported that reducing sugars other than glucose can precipitate glycation much faster than glucose (Ahmed, 2005), where glucose is the least reactive of all sugars. In line with this, we found that glucose led to a lower fluorescence intensity compared to methylglyoxal at the same incubation period.

Skeletal muscle is made up of several proteins, such as troponin, actin, titin and myosin (Jones and Round, 1990; Mcnally et al, 2006). Myosin accounts for 15– 25% of the total body protein, has a half-life of 29–30 days and is the key component in contractility. It consists of two heavy chains, which form tail region, and two heads, which can bind with actin (Ramamurthy et al., 2001; Schiaffino and Reggiani, 1996).
Myosin has lysine and arginine rich regions at the actin-binding site, which are potential targets for modification by glycation (Ramamurthy et al., 2001). It has been reported that the degree of glycated myosin is greater in skeletal muscles of aged than young rodents (Snow et al., 2005).

AGEs show autofluorescence and this autofluorescence can be used to determine the degree of glycation of tissue in skeletal muscle (Nomoto et al., 2013). However, this is not always a reliable method to detect all AGEs as not all AGEs produce fluorescence, where the major AGE structures are even reported to be non-fluorescent (Ahmed, 2005). To accommodate this, ELISA was also employed in our study. Our observation of ELISA also shows that AGE formation is time dependent, both after incubation with methylglyoxal and glucose.

Our study shows that the levels of glycation of myosin protein from plantaris muscle gradually increases with the duration of incubation with methylglyoxal and glucose, where the highest levels of glycation was detected after 7 days incubation. Previously mentioned, the methylglyoxal modifies lysine and arginine residues at a faster pace than other sugar (Rabbani et al., 2014) hence, this leads to excessive protein cross-linking. It has been found that AGEs can be formed within 7 days incubation, with the maximum level of glycation achieved within this time period of incubation (Nagaraj et al., 2010; Khan et al., 2015). We studied shorter incubation time (2-7 days) as it generally favours protein rich in amadori compound as longer incubation time (7+ days) lead to extensive browning and oxidation (Cutler and Rodrigues, 2003).

Studies have demonstrated that AGE accumulation is linked with chronological age. For instance, Ramamurthy and Larsson, (2013) found an increase in AGEs in the fibers of 27–30 month-old healthy rats; likewise, we show an increase in AGEs in gastrocnemius
muscles of old rats. Snow et al. (2007) found an increase in AGEs formation in extensor digitorum longus muscle of old age rats (months) and studying collagen of diabetic rats in vivo, Turk et al.(2010) found a greater formation of AGEs in 20 weeks diabetic rats compared to 4 weeks. Both studies show that the degree of AGE formation is greater in diabetic compared to non-diabetic rats. Aging influences the buildup of modified proteins subsequently due to AGEs being resistant to proteolysis (Snow et al. 2007), and particularly in proteins, such as myosin, with a low turnover (Ramamurthy and Larsson, 2013).

In conclusion, we have successfully set up methods to determine AGE levels in muscle, applying these techniques revealed that AGEs do indeed in increase with age in skeletal muscle. The age-related slowing may be attributed to this increased AGEs.
CHAPTER 6:

General discussion, Limitations,

Future work and Conclusion
6.1 General Discussion

Advanced glycation endproducts can be formed from a number of pathways involving interactions between proteins and sugars; this is known as the classical pathway, which results in a formation of an intermediate product. This process then leads to other pathways, including glucose autoxidation, Schiff base fragmentation and Amadori products glycoxidation, which then lead to the production of dicarbonyl intermediate compounds (Lapolla et al., 2003; Singh et al., 2014). These compounds can further react with protein to form AGE cross-links. Accumulation of AGEs are tissue specific (Snow et al., 2007) and are characterised by intra and inter protein crosslinking that alter the structure and function of proteins (Wu et al., 2009). AGE cross-links are found to be increased with increasing age (Nishigaki et al, 2008).

It has been reported that all pathways involving AGE formation generate oxygen free radicals (Gillery, 2001). Free radicals are atoms or molecules with unpaired electrons and oxygen radicals account for oxidative stress (Prasad et al, 2010). RAGE is recognized as an AGE receptor synthesized either in vitro or extracted from diabetic patients. The AGE-RAGE interactions result in activation of the mitogen-activated protein (MAP) kinase signalling and p21 ras pathways, which in turn activate nuclear factor-kB (NF-kB) and oxidative stress, resulting in the release of cytokines (Yan et al., 1994; Neumanna et al., 1999).

So far, it has been reported that the accumulation of AGEs can be caused by various factors, of which the most common is diabetes, obesity and lifestyle such as certain diets and smoking (Nedić et al., 2013), while exercise and a healthy diet attenuate the level of AGEs (Macías-Cervantes et al., 2014). Furthermore, smoking induces an increase in the levels of cytokines (Aula and Qadir, 2012).
Diabetes is a multifactorial disease, which is a major risk factor of micro- and macrovascular diseases affecting the heart, kidney, eye and nerves. Diabetes occurs from insulin resistance and/or impaired insulin production and is, if uncontrolled, characterised by hyperglycemia (Negre-Salvayre et al., 2009). Accumulation of AGEs in diabetes can result in protein modification and consequent protein dysfunction. An excessive accumulation of AGEs can lead to tissue damage (Negre-Salvayre et al., 2009).

This study provides better understandings of the levels of AGEs in healthy human participants, demonstrates that a number of conditions can have an impact on the level of AGEs, either being elevated or relegated. The conditions investigated in this study were 8 weeks aerobic exercise, smoking and 2 weeks of smoking cessation. Additionally, the levels of AGEs in the muscles from different aged rats were also studied. To do so, first methods to determine AGEs in muscles were validated. The muscle proteins were extracted and glycated for different periods and with two types of sugar (glucose and methylglyoxal) under non-physiological conditions. Surprisingly, each condition (8 weeks aerobic exercise smoking as well as 2 week smoking cessation and ageing) influenced the level of AGEs in a different manner.

Initially, the exercise study was carried out to investigate whether regular 8 weeks aerobic exercise influenced formation and/or accumulation of AGEs and oxidative stress. At first, it was essential to indicate whether biochemical parameters (cholesterol, triglycerides, HDL, LDL, serum glucose, and total protein) were influenced after training. Interestingly, there was no influence on the biochemical parameters; yet, Aguilo et al. (2003) reported that different intensity of exercise and different training status can influence the cholesterol profile. Total antioxidant status (TAS) and lipid peroxidation measurement were also not significantly affected maybe due to the
intensity of the exercise the subject were exposed to in this study. Therefore indicating that 8 weeks aerobic exercise had no influence on the level of AGEs in our study, as there was no detection of changes in the levels of AGEs after 8 weeks aerobic exercise.

On the other hand, it has been reported that smokers have higher serum levels of AGEs when compared to non-smokers (Cerami et al., 1997). Smoking tobacco results in generation of extremely reactive glycotoxins that invade the blood through lungs and speed up the generation of AGEs. Moreover, smoke produced from cigarettes is rich in methylglyoxal and glyoxal (Fujioka and Shibamoto, 2006; Talhout et al., 2006). In this study, we demonstrated that cigarette smoke and 2 weeks of smoking cessation influenced a number of factors. For example, albumin was reduced in smokers. Albumin functions as an important circulating antioxidant (Roche et al., 2008) and has ligand-binding capacities and free radical-trapping properties (Taverna et al, 2013).

Here we found that smokers had increased oxidative stress biomarkers (MDA and TAS). Free radical formation can accelerate the reaction of glycation (Lunenfeld et al., 2007). In addition, glycotoxins in cigarette smoke can rapidly react with proteins, such as serum proteins, to form AGEs (Cerami et al, 1997). In line with this, we demonstrated that smokers had higher levels of circulating AGEs.

Free radicals can damage unsaturated fatty acids, which subsequently leads to the increased MDA levels. Durak et al. (2002) and Lykkesfeldt et al. (2004) further demonstrated that lower antioxidants increased the levels of lipid peroxidation of smokers (Cobanoglu et al., 2011). Here we found that smokers had increased levels of MDA that did not return to normal levels after 2 weeks smoking cessation, it has been suggested that MDA has a long half-life (Naudí et al., 2013). The TAS levels are reduced in smokers, according to Mahmood et al, (2007) the reduction of TAS is due to
high amounts of free radicals presence in cigarette smoke that generate oxidative stress in the smoker’s body, which may in turn reduce the antioxidants. On the other hand, 2 weeks smoking cessation increased the level of TAS; this is due to an increase in serum antioxidant concentrations and consequently improves serum resistance towards oxidative stress (Polidori et al., 2003).

This study demonstrated that mild smoking alters the concentration of cytokines. Studies have shown that cigarette smoking may promote the release of different levels of cytokines even at lower levels of cigarette smoking (Hasnis et al., 2007), probably at least to some extent from leukocytes (Zhang and An., 2007). Here we demonstrated greater levels of cytokines (TNF-α and IL-2), whereas 2 weeks smoking cessation reduced the concentration of the cytokines to normal levels. The reduction of the cytokines level demonstrated in this study may have been a result of a reduction of immunosuppressive agents from the cigarettes smoking in the serum of the participants (Ouyang et al., 2000) and a reduced oxidative stress and exposure to radicals in cigarette smoke.

Certainly, reports have shown that reducing sugars other than glucose can speed up the glycation reaction where glucose is the least reactive of all sugars (Ahmed, 2005). As previously mentioned, methylglyoxal causes glycation by specifically targeting the arginine residue (Thornalley et al., 1999). The myosin has rich regions of lysine and arginine and is therefore susceptible to glycation, as demonstrated here by a significant increase of muscle protein glycation after methylglyoxal incubation. Furthermore, we demonstrated that the degree of crosslinking AGE formation was dependent on the duration of incubation with methylglyoxal and glucose. Finally, we showed an age-related increase in muscle AGE that may underlie the slowing of muscle in old age.
*In vivo* glycation of plantaris muscle is age-dependent. AGE formation is also dependent on the type of muscle (Snow *et al.*, 2007; Ramamurthy and Larsson, 2013) and whether or not one is diabetic (Snow *et al.*, 2007).

### 6.2 Limitations

The results of the current study may have been affected by a number of limitations described below.

- The human participants in this study were healthy young men with normal AGE levels to start with. Also different diets may have resulted in part of the variation in the level of AGEs observed in both smoking and exercise study. A more strict diet should be implemented in the future.

- *In vivo* glycation was carried out using rats muscle. Use of human muscle would have allowed for a better comparison with exercise study.

- The number of exercise testing group was rather small.
6.3 Future work

From the results obtained in this current study, future studies are suggested:

- To investigate the effect of AGEs on the cytotoxicity and cell viability of skeletal muscle cell line using cell culture and western blotting and AGEs antibody.
- To determine the changes in cell signalling after incubation with different AGE compounds.
- To identify whether an extended period of exercise exposure in old people will affect the levels of AGEs.
- Further experimental research, in order to establish whether 2 weeks smoking cessation in diabetic subjects will influence the levels of AGEs similarly to those in healthy subjects.

- The filament-sliding assay. An in vitro motility assay can provide important information about the function of motor proteins, such as myosin. For an in vitro motility assay, myosin is extracted from a muscle or muscle fibres and coated on a microscope slide (Hook et al., 2000). When the conditions are appropriate for myosin function the myosin molecules will propel actin filaments. This movement of the actin filaments can be visualised in fluorescent microscopy by labelling the filaments with rhodamine. In the absence of Mg-ATP, the actin filaments are tightly bound to myosin and no movement is visible, but when Mg-ATP is added the filaments will be propelled by the action of the myosin molecules. Sequential video images, captured by computer, allow filament positions to be tracked in time so that the speed and direction of filament sliding can be determined. The speed of the movement of the actin filaments gives an indication of the function of the myosin molecule. The technique has been applied to
show deterioration of myosin function with age (Hook et al., 2000) and after incubation of the myosin with glucose (Ramarthu et al., 2001), suggesting that glycation can contribute to the loss of myosin function in old age.

6.4 Conclusion
The present study shows that there is no significant change in serum AGE levels, total antioxidant status and lipid peroxidation after 8-weeks of aerobic exercise intervention in healthy young men. Thus, it may be concluded that aerobic exercise does not affect the levels of serum AGEs or oxidative stress in normal healthy subjects. Moreover, smoking was associated with increased levels of AGEs and reduced total antioxidant status increased lipid peroxidation as well as levels of certain types of cytokines. However, we show here, to our knowledge, for the first time, that 2 weeks of smoking cessation might be an easy and effective strategy to reduce the levels of AGEs as we also found that normalisation of cytokine levels, in particularly TNF-α, after 2 weeks smoking cessation.

Additionally, this study found that different types of reducing sugars can trigger glycation of extracted muscle protein; we demonstrated that methylglyoxal was much faster than glucose. Furthermore, our in vivo study concludes that muscle glycation is increased with age. As glycation impairs interaction between myosin and actin, it may contribute to the loss of muscle function in old age.

As smoking cessation did reduce AGEs levels we suggest that implementing a healthy lifestyle is an effective strategy for lowering the levels of AGE. Our current study has important implication for preventing diseases related with AGEs, such as diabetes and their complications.
7 References


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Appendix 1A

Materials
- 2-Mercaptoethanol (Sigma, UK).
- 4',6-diamidino-2-phenylindole (DAPI).
- 96-well plates (Scientific Laboratory Supplies, Nottingham, UK).
- Acrylamide/Bis solution 40% (Bio-Rad Laboratories, Germany).
- Acrylamide/bisacrylamide solution (Bio-Rad Laboratories, Hertfordshire, UK).
- Advanced glycation endproduct antibody; Rabbit polyclonal to AGE antibodies (Abcam, UK).
- Advanced glycation endproduct kit (Cell Biolabs').
- Albumin assay kit (Randox, UK).
- Ammonium persulphate (Sigma, UK).
- Bovine serum albumin (BSA) (Sigma, UK).
- Bromophenol blue (Serva, Germany).
- Capillary tube (Accu-Glass).
- Chloroform (Sigma, UK).
- ColorBurst electrophoresis marker for SDS-PAGE (Sigma-Aldrich.)
- Coomassie brilliant blue (Sigma, UK).
- Deionized water (Local store, Manchester, UK).
- D-glucose (BDH, UK).
- Eppendorf tubes, (Scientific Laboratory Supplies, Nottingham, UK).
- Gloves, sterile (Scientific Laboratory Supplies).
- Glucose assay kit (Randox, UK).
Glycerol (BDH, UK).
Glycine (BDH, UK).
Goat anti-rabbit IgG (Life technologies)
Hydrochloric acid (HCl) (BDH, UK).
Isopropanol (Sigma, UK).
Lipid peroxidation kit (Oxford biomedical research, UK).
Lysozyme (Sigma, UK).
Methanol (Fisher Scientific International, UK).
Methylglyoxal (Sigma, UK).
N, N, N', N'-tetramethylethylenediamine (TEMED) (Sigma, UK).
Normal goat serum (Vector).
Parafilm (Scientific Laboratory Supplier, UK).
Paraformaldehyde (Sigma).
Phosphate buffer saline (PBS) tablets (Oxoid limited, UK).
Plate sealers (R & D systems, USA).
PRO-PREP Protein Extraction Solution (European Biotech Network).
Quinine sulphate (Sigma- Aldrich, UK).
Silver stain (Sigma, UK).
Slides and coverslips, Thermanox, sterile (Nunc™, Fischer Scientific, Loughborough, UK).
Sodium azide (Sigma, UK).
Sodium dodecyl sulphate (SDS) (BDH, UK).
Sodium phosphate dibasic (Na2HPO4) (Sigma, UK).
Sodium phosphate monobasic (NaH2PO4) (Sigma, UK).
Sulphuric acid (H2SO4) (Sigma-Aldrich, UK)
Th1/Th2 11plex kit (eBioscience flow cytomix BMS810FF, Bender MedSystems).

Total antioxidant status kit (Randox, UK).

Trichloroacetic acid (Sigma, UK).

Tris (hydroxymethyl) methylamine (BDH, UK).

Tween 20 solution (Sigma, UK).

Universal tubes, sterile, 6 mL, 30 mL (Scientific Laboratory Supplies).

Vacutainer system (BD Biosciences).

**Equipments**

- Autovortex mixer SA1 (Stuart Scientific Co, UK).
- Analytical balance (Sartorius Machatronics Ltd, UK).
- Centrifuges (Eppendorf and Laborzentrifugen 3K10, Sigma, Hertfordshire, UK).
- Pipettes 0.5-10, 5-50, 50-200, 100-1000 µL and Multi-channel pipettes (Scientific laboratory supplies Ltd, Eppendorf, Germany).
- Cross power 500 for electrophoresis (Schleicher & Schuell, London, UK).
- Freezer (-80°C) (Juan Quality System, London, UK).
- Ice maker (Borolab Ltd, Abingdon, UK).
- Immunofluorescence microscope (Zeiss, Germany).
- Orbital shaker (Denley, London, UK).
- Grant-bio shaker POS-300 (Grant Instruments Ltd, UK).
- Laboratory freezer Bio cold (Scientific Laboratory Supplier, UK).
- Laboratory fridge (Scientific laboratory Supplies, UK).
- Laboratory pH/mV/temperature meter AGB-75 (Medical scientific instruments, England).
- LTE IP 30 incubator for protein glycation samples (Scientific Laboratory Supplier, UK).
- Magnetic stirrer hotplate (Stuart Scientific Co, UK).
- Microplate reader, 96-well (BioTek, USA).
- Sodium dodecyl sulphate-polyacrylamide gel electrophoresis tank (mini-Protean® 3 apparatus) (Bio-Rad Laboratories, Germany).
- Water bath (Grant Instruments Ltd, UK).
- Water de-ionizer (Millipore, UK).
- RandoxRX Daytona analyzer, (Randox Laboratories Ltd., Belfast, Ireland).
- Leica CM1950 cryostat.
- Haemocytometer.
- Digital dry bath (Labnet international).
- Biowave II spectrophotometer.
- Micro-haematocrit centrifuge (Howsely, England).
- HemoCue (HemoCue AB, Sweden).
- Height measurement instrument

## 2.1.3 Solutions

- Preparation of ammonium persulphate 10% for SDS-PAGE analysis: Ammonium persulphate (100 mg) was dissolved in 1ml of distilled water.
- Preparation of BSA (1 mg/ml) standard solution for the Bradford assay: Bovine serum albumin (100 mg) was dissolved in 100 ml of distilled water.
- Preparation of destaining solution for SDS-PAGE analysis: Methanol (250 ml) was mixed with 70 ml of acetic acid and the volume was made up to 1 L with distilled water.
- Preparation of 1X PBS: One tablet of PBS was dissolved in 100 ml of distilled water.
- Preparation of Sodium phosphate buffer (0.1 M): 3.1 g NaH2PO4 and 10.9 g Na2HPO4
were dissolved in 1 L of dH2O, pH adjusted to 7.4. To prevent any contamination the sodium azide (3 mM) was added. Then the buffer was stored at 4°C for up to one month.

❖ Preparation of running buffer for SDS-PAGE analysis: Tris-base (1.5 g), glycine (7.2 g) and 0.5g of SDS were dissolved in 500 ml of distilled water.

❖ Preparation of Tris-HCl buffer (0.5 M), pH 6.8 for SDS-PAGE analysis: Tris (Tris-hydroxymethyl aminomethane) 6.1 g, 0.4 g of SDS and 4.2 ml of HCl were dissolved in distilled water. The pH was adjusted to 6.8 and the solution was stored in a cold and dark place.

❖ Preparation of sample treatment buffer for SDS-PAGE analysis: Sodium dodecyl sulphate (0.1 g), and 0.1 ml of 2-mercaptoethanol were added to 1 ml of Tris-HCl buffer (0.5 M) and 2 ml of glycerine and the volume was made up to 10 ml with distilled water.

❖ Preparation of Tris-HCl buffer (1.5 M), pH 8.8 for SDS-PAGE analysis: Tris (Tris-hydroxymethyl aminomethane) (18.2 g), 0.4 g of SDS were dissolved in distilled water. The pH was adjusted to 8.8 and the solution was stored in a cold and dark place.

❖ Acrylamide/bisacrylamide solution (6 ml) and 4.5 ml of Tris-HCl buffer (1.5 M) were added to 7.5 ml of distilled water and mixed. Then, 80 µL of ammonium persulphate was added to the mixture. Then the 10 µL of TEMED was added to initiate the gel polymerization.

❖ Preparation of tracking dye solution for SDS-PAGE analysis: Bromophenol blue (1 mg) was added to 0.1 ml of glycerine and mixed with 0.9 ml of distilled water.

❖ Preparation of stacking gel (4.5%) for SDS-PAGE analysis: Acrylamide/bisacrylamide solution (0.9 ml) and 1.5 ml of Tris-HCl buffer (0.5 M) were added to 3.6 ml of distilled
water and mixed. Then, 20 μL of ammonium persulphate was added to the mixture. Polymerization was initiated by adding 10 μL of TEMED.

- Preparation of staining solution for SDS-PAGE analysis: Coomassie brilliant blue (2.5 g) was dissolved in 500 ml of methanol and 100ml of acetic acid. The volume was made up to 1 L with distilled water and then filtered.

- **Software**

  - Syngene G Box (Bio-imaging system) (Syngene, UK).
  - Microsoft office 2007 (Microsoft, USA).
  - Statistical Package for the Social Sciences 16.0 for Windows (SPSS) (SPSS Inc., Chicago, USA).
  - GraphPad Prism 6 software programme (GraphPad Software. Inc, 2012).
Appendix 1B

Faculty of Science & Engineering
School of Biology, Chemistry and Health Science
Manchester Institute of Healthcare Science

Dr Gethin Evans
School of Biology, Chemistry and Health Science
Manchester Metropolitan University
Chester St
Manchester M1 5GD

3rd February 2010

Dear Dr Evans,

The effect of an 8 week aerobic exercise intervention on endothelial progenitor cells, immune function, inflammation, advanced glycation end-products and metabolic characteristics – FAETC/09-10/17

I am pleased to inform you that the above project has been approved by Chairs Action. You and your co-applicants may now proceed with the Study,

Yours sincerely,

Signature

Professor Val Edwards-Jones
Director of Research
On behalf of the Chair of the Faculty of Science and Engineering
Ethics Committee

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Dierexperimentencommissie KUN

ons kenmerk 7520/GE/jv telefonnummer (024) 3615342 e-mail p.eggerink@azi.umcn.nl datum 6-11-2003

bericht
Archiefnr : KUNDEC 2002-117
Project : De rol van VEGF in hypertrofie op hoge leeftijd


De Commissie heeft u bij brief d.d. 21-11-2002 bericht gegeven bezwaren te hebben tegen de uitvoering van de dierexperimenten zoals voorgesteld in de projectaanvraag.

Gezien de ernst van het ongerief voor de dieren bij de door u beschreven dierexperimenten (mate van ongerief: matig/ernstig; code 4) verzoekt de Commissie u om haar, tijdens deze fase van het onderzoek, nader te informeren over de volgende punten:
1. Is de destijds ingeschatte mate van ongerief nog steeds van toepassing?
2. Zijn het aantal en de soort te gebruiken proefdieren nog conform de opgave in het aanmeldingsformulier?
3. Worden de dierproeven en ingrepen nog volgens de in het aanmeldingsformulier beschreven proefopzet uitgevoerd?
4. WORDEN, OP BASIS VAN DE RESULTATEN TOT NU TOE, ANPASSINGEN IN DE PROEFOPZET NOODZAKELIJK GECHT?
5. Is het wenselijk om, op basis van de resultaten tot nu toe, de doelstelling van het onderzoek aan te passen?

De Commissie ziet uw antwoord graag binnen twee weken tegemoet.

Dierexperimentencommissie KUN,

Prof. dr. P. Zwart
Voorzitter

Mw. dr. G.J.M. Eggerink
Secretaris

cc: Dr. J. Koopman, Directeur CDL

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151
Appendix 1C

Table 1: Average absorbance of fluorescence AGEs after period of incubation with methylglyoxal and glucose

<table>
<thead>
<tr>
<th>Day</th>
<th>Average absorbance of AGE (AU) after incubated with methylglyoxal (0.1 M)</th>
<th>Average absorbance of AGE (AU) after incubated with Glucose (0.5M)</th>
<th>P-values for methylglyoxal (compared to control)</th>
<th>P-values for glucose (compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average absorbance of AGE (AU) after incubated with methylglyoxal (0.1 M)</td>
<td>Average absorbance of AGE (AU) after incubated with Glucose (0.5M)</td>
<td>P-values for methylglyoxal (compared to control)</td>
<td>P-values for glucose (compared to control)</td>
</tr>
<tr>
<td>control</td>
<td>2.50±0.70</td>
<td>2.04±0.07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day0</td>
<td>8.54±0.70</td>
<td>3.05±0.21</td>
<td>0.013606</td>
<td>0.024352</td>
</tr>
<tr>
<td>Day1</td>
<td>20.50 ±0.70</td>
<td>11.52±0.72</td>
<td>0.00154</td>
<td>0.002972</td>
</tr>
<tr>
<td>Day3</td>
<td>36.02±2.82</td>
<td>24.08±2.29</td>
<td>0.003766</td>
<td>0.005368</td>
</tr>
<tr>
<td>Day5</td>
<td>58.53±3.54</td>
<td>33.31±0.19</td>
<td>0.002066</td>
<td>2.31E-05</td>
</tr>
<tr>
<td>Day7</td>
<td>97.51±3.54</td>
<td>52.39±0.83</td>
<td>0.000719</td>
<td>0.000139</td>
</tr>
</tbody>
</table>

Table 2: Average absorbance of fluorescence AGEs after period of incubation with methylglyoxal and glucose measured by ELISA

<table>
<thead>
<tr>
<th>Day</th>
<th>Average absorbance of AGE (nm) after incubated with methylglyoxal (0.1 M)</th>
<th>Average absorbance of AGE (nm) after incubated with Glucose (0.5M)</th>
<th>P values for methylglyoxal (compared to control)</th>
<th>P value for glucose (compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average absorbance of AGE (nm) after incubated with methylglyoxal (0.1 M)</td>
<td>Average absorbance of AGE (nm) after incubated with Glucose (0.5M)</td>
<td>P values for methylglyoxal (compared to control)</td>
<td>P value for glucose (compared to control)</td>
</tr>
<tr>
<td>control</td>
<td>0.036±0.02</td>
<td>0.02±0.007</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day0</td>
<td>0.09±0.010</td>
<td>0.09±0.001</td>
<td>0.061117</td>
<td>0.005494</td>
</tr>
<tr>
<td>Day1</td>
<td>0.15±0.004</td>
<td>0.10±0.014</td>
<td>0.01741</td>
<td>0.017705</td>
</tr>
<tr>
<td>Day3</td>
<td>0.20±0.004</td>
<td>0.19±0.001</td>
<td>0.007356</td>
<td>0.000985</td>
</tr>
<tr>
<td>Day5</td>
<td>0.46±0.03</td>
<td>0.21±0.016</td>
<td>0.003854</td>
<td>0.004426</td>
</tr>
<tr>
<td>Day6</td>
<td>0.57±0.07</td>
<td>0.36±0.01</td>
<td>0.011205</td>
<td>0.000518</td>
</tr>
<tr>
<td>Day7</td>
<td>1.12±0.01</td>
<td>0.40±0.01</td>
<td>0.000326</td>
<td>0.000362</td>
</tr>
</tbody>
</table>
Table 3: Intensity of AGEs staining in rat plantaris muscle in vivo (5, 25 and 32 months)

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Intensity of AGE staining (AU)</th>
<th>P value (compared to young rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 months (Young rat)</td>
<td>4.833135±0.063954</td>
<td>-</td>
</tr>
<tr>
<td>25-month-old rat</td>
<td>5.143513±0.109577</td>
<td>0.011314</td>
</tr>
<tr>
<td>32 month (very old rat)</td>
<td>5.592091±0.032026</td>
<td>1.35E-05</td>
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</tbody>
</table>