Advanced glycation end products as a biomarker for accelerated ocular ageing and glaucoma

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ADVANCED GLYCATION END PRODUCTS AS A BIOMARKER FOR ACCELERATED OCULAR AGEING AND GLAUCOMA

by

LEANNE SMEWING

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

DOCTOR OF PHILOSOHPHY

School of Health Professions

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee. Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Abstract

Advanced glycation end products as a biomarker for accelerated ocular ageing and glaucoma

Leanne Smewing

Advanced glycation end products (AGEs) have a large impact on the healthy ageing population and those diagnosed with pathology. Studies have linked AGEs to glaucomatous optic neuropathy, however there is little consensus on the role AGEs play in glaucoma development. Furthermore, it is known that diet is an exogenous source of AGEs, however it is not clear how dietary AGE (dAGE) influences tissue-bound levels in the body. The overarching theme of this thesis was to assess the impact of AGE level, measured both through diet and tissue-bound levels in the skin (skin autofluorescence, SAF), on retinal vessels and the cornea in healthy participants and patients diagnosed with ocular hypertension (OHT), early stage normal-tension glaucoma (NTG) and early stage primary open angle glaucoma (POAG).

A UK-specific food frequency questionnaire (FFQ) was developed and found to reliable and valid. This newly designed FFQ was subsequently used throughout the thesis to measure dAGE. In healthy controls, the contribution of dAGE to tissue-bound AGE levels appeared to be minimal. The level of AGE taken in via diet was similar between healthy, OHT, NTG and POAG participants.

Interestingly, tissue-bound AGE level (SAF) was found to be 16% higher in NTG and 14% higher in POAG than healthy control participants. Adding to the
evidence that SAF, as an accessible measure, may be a suitable long-term biomarker of glaucoma.

Higher SAF was associated with narrower retinal arteries (CRAE) in a healthy population, adding to the evidence that AGEs may be an accessible marker of vascular health. The NTG group had a significantly narrower CRAE than healthy controls as well as the highest SAF level. Increased SAF was also associated with a less viscoelastic, stiffer cornea in the NTG group only. These findings pose interesting questions about the possible association of SAF with ocular rigidity and subsequent increased susceptibility to IOP or arterial blood supply related injury, however larger scale studies are needed before any conclusions could be drawn.
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Table 9.8: Mean, SD and Pearson's correlations between vein parameters and corneal viscoelasticity parameters in OAG patients
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>AVR</td>
<td>Arterio-venous ratio</td>
</tr>
<tr>
<td>A1</td>
<td>Applanation one</td>
</tr>
<tr>
<td>A2</td>
<td>Applanation two</td>
</tr>
<tr>
<td>BDF</td>
<td>Baseline diameter fluctuation</td>
</tr>
<tr>
<td>BDR</td>
<td>Baseline diameter fluctuation</td>
</tr>
<tr>
<td>BFR</td>
<td>Baseline corrected flicker response</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BMR</td>
<td>Basal metabolic rate</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged coupling device</td>
</tr>
<tr>
<td>CCT</td>
<td>Central corneal thickness</td>
</tr>
<tr>
<td>CEE</td>
<td>Centre for eyecare excellence</td>
</tr>
<tr>
<td>CEL</td>
<td>N-Carboxyethyl-lysine</td>
</tr>
<tr>
<td>CML</td>
<td>N-Carboxymethyl-lysine</td>
</tr>
<tr>
<td>COAG</td>
<td>Chronic open angle glaucoma</td>
</tr>
<tr>
<td>COSH</td>
<td>Control of Substances Hazardous to Health</td>
</tr>
<tr>
<td>CRAE</td>
<td>Central retinal artery equivalent</td>
</tr>
<tr>
<td>CRVE</td>
<td>Central retinal vein equivalent</td>
</tr>
<tr>
<td>CT</td>
<td>Constriction time</td>
</tr>
<tr>
<td>dAGE</td>
<td>Dietary advanced glycation end products</td>
</tr>
<tr>
<td>DA</td>
<td>Dilation amplitude</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DD</td>
<td>Disc diameter</td>
</tr>
<tr>
<td>dmaPM</td>
<td>6-dimethylaminopyridoxamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>DVA</td>
<td>Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
</tr>
<tr>
<td>FR</td>
<td>Food record</td>
</tr>
<tr>
<td>GCP</td>
<td>Good clinical practice</td>
</tr>
<tr>
<td>GON</td>
<td>Glaucomatous Optic Neuropathy</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced)</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>8-OH-G</td>
<td>8-hydroxy-20-deoxyguanosine</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy control</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRA</td>
<td>Health Research Authority</td>
</tr>
<tr>
<td>hsCRP</td>
<td>High sensitivity C-Reactive Protein</td>
</tr>
<tr>
<td>ICC</td>
<td>Intraclass correlation coefficient</td>
</tr>
</tbody>
</table>
IL-1α  Interleukin 1α
IL-6  Interleukin 6
IMT  Intima-media thickness
IOP  Intraocular pressure
LOCS III  Lens Opacities Classification System III
MABP  Mean arterial blood pressure
MAPKs  Mitogen-activated protein kinases
MC%  Minimum percentage constriction
MD%  Maximum percentage dilation
MDA  Malondialdehyde
MD  Mean defect / Mean deviation
MG-H1  5-hydro-5-methyl-4-imidazolon-2-yl-ornithine
NF-κB  Transcription factor kappa B
NICE  National institute for health and care excellence
NO  Nitric oxide
NTG  Normal tension glaucoma
OAG  Open angle glaucoma
OBF  Ocular blood flow
OCT  Optical coherence tomography
OHT  Ocular hypertension
OHTS  Ocular hypertension treatment study
ONH  Optic nerve head
OPP  Ocular perfusion pressure
ORA  Ocular response analyser
POAG  Primary open angle glaucoma
PI3-K  Phosphatidylinositol-3 kinase
RAGE  Receptor for advanced glycation end products
REI  Royal eye infirmary
RGC  Retinal ganglion cell
RNFL  Retinal nerve fibre layer
RNS  Reactive nitrogen species
ROS  Reactive oxygen species
RPE  Retinal pigment epithelium
RT  Reaction time
RVP  Retinal venous pressure
SAF  Skin auto fluorescence
SBP  Systolic blood pressure
SDRA  Sequential and diameter response analysis
SOD  Superoxide dismutase
sRAGE  Soluble RAGE
TF  Transferrin
TM  Trabecular meshwork
TNF-α  Tumour necrosis factor α
UPLC–MS/MS  Ultra-performance liquid chromatography tandem mass-spectrometry
VEGF  Vascular endothelial growth factor
VCAM1  Vascular cell adhesion molecule 1
Advanced glycation end products (AGEs) as a biomarker for accelerated ocular ageing and glaucoma

1. Introduction

Globally, life expectancy has been rising and over the last two centuries it has more than doubled (1). This rise however, is due to global improvement of health rather than an increase in understanding or slowing of the ageing process (1-3). Increased age is a significant risk factor for both ocular and systemic disease (4, 5). Advanced glycation end products (AGEs) are a group of reactive compounds which accumulate gradually with age in cells, tissues and blood vessels throughout the body where they adversely affect structure and function. A sustained exposure to high levels of AGEs has been associated with the development of a number of chronic age-related systemic and ocular disorders. This body of research will utilise recent advances in technology to explore the role that AGEs may play in the acceleration of ocular ageing, as well as in the pathogenesis of neurodegenerative disease in the form of glaucomatous optic neuropathy (GON).

1.1 Accelerated ageing

The rate of ageing is not the same in all age matched individuals. The concept of accelerated ageing suggests that the presence of a number of specific genetic, environmental or systemic risk factors may cumulate to accelerate the ageing process in some individuals and lead to the development of age-related disease (5-7). Understanding the factors that influence accelerated ageing is vital for both
patient care and healthcare systems. As AGEs accumulate with age, the relationship between AGEs and accelerated ageing is an interesting one.

1.2 Advanced glycation end products (AGEs)

AGEs are a diverse group of reactive compounds produced naturally when a reducing sugar reacts with proteins, lipids or nucleic acids in a non-enzymatic way (8, 9). This reaction can occur endogenously within the body (8), and exogenously within food and tobacco (8, 10).

It is the tobacco curing process that promotes the formation of AGEs, consequently when smoking tobacco, glycotoxins are directly inhaled into the lungs where they then form AGEs (10). Smoking also causes increased oxidative stress levels (11), which encourages AGE formation. The relationship between AGEs and oxidative stress will be discussed further in Section 1.6.

Foods prepared by high heat cooking methods can promote deterioration reactions within the food products, including protein degradation, which can subsequently lead to AGE formation (12). The amount of AGE formed is dependent on a foods nutrient content and method of cooking; with formation of AGEs accelerating with exposure to heat (12-14). The relationship between AGEs and foods are discussed further in the ‘Dietary AGE’ Section 1.4.

AGEs accumulate gradually with age in cells, tissues and blood vessels throughout the body, where over time they can adversely affect structure and function by; binding to specific cell surface receptors, forming covalent cross links
on proteins such as collagen, creating reactive oxygen species (ROS), and oxidative stress (15-19). Each form of AGE has a complex structure with some displaying fluorescent properties (20). Due to AGEs accumulation in all tissues, including the skin, levels of AGE can be detected by skin biopsy and through measuring skin auto fluorescence (SAF) (21, 22). Skin biopsy is an invasive procedure that allows the collagen to be examined in the laboratory. Recent technology advances have produced a non-invasive means to measure AGEs by utilising SAF properties of tissue bound AGEs in the skin, examples of this include; the AGE Reader (DiagnOptics B.V., Groningen, Netherlands) and the TrūAge scanner (Morinda, Long Island City, NY) (21-24).

1.3 Formation of AGEs

AGE formation within the body is influenced by multiple factors such as levels of oxidative stress, blood glucose concentration and conversion and concentration of proteins (9). There are multiple mechanisms for the formation of AGEs and there are three that have been most studied (25). Firstly, the Maillard reaction has been studied since the 1900’s particularly by those interested in food, as the products from the Maillard reaction add a desired taste to foods along with a brownish colour (26). The two other most researched mechanisms include; the polyol pathway (27) and the autoxidation of glucose and peroxidation of lipids into dicarbonyls derivatives, by increase in oxidative stress (28). All three mechanisms are shown in Figure 1.1.
Figure 1.1: Formation of AGEs. Green pathway shows the Maillard reaction, blue pathway shows the Polyol pathway and purple pathway shows autoxidation of glucose and peroxidation of lipids into dicarbonyls derivatives.

The Maillard reaction is shown in green in Figure 1.1. The first step of the reaction is the formation of a Schiff base which has the general structure shown in Figure 1.2; it is formed by glucose reacting with a free amino acid. This occurs through the nucleophilic addition of a free amino acid from a protein or DNA to glucose (8). This reaction takes place over hours and is concentration dependent; a decrease in the concentration of glucose would enable the Schiff base reaction to be reversed (15). The formation of more stable Amadori products, also known as early glycation products, takes a number of days (29, 30). Although more stable than a Schiff base the reaction creating Amadori products is still a reversible one (8). Amadori products continue to accumulate over a number of
weeks where they undergo intra and intermolecular rearrangements via oxidative or non-oxidative pathways to create AGEs. This final stage in the Maillard reaction is irreversible (15, 31, 32).

![Figure 1.2 – General structure of a Schiff base](image)

The alternative pathways to the Maillard reaction are shown in purple and blue in Figure 1.1, these pathways occur at a faster rate than the Maillard reaction (33). The purple pathway illustrates the autoxidation of glucose and peroxidation of lipids, by increase in oxidative stress, into α-oxaldehydes (glyoxal, methylglyoxal, and 3-deoxyglucosone) also known as dicarbonyl derivatives. The reaction of the α-oxaldehydes with monoacids then produces AGEs (34). The blue pathway is known as the polyol pathway. Here glucose is reduced into sorbitol, which in turn converts to fructose which then converts to α-oxaldehydes. In the same mechanism as previously the α-oxaldehydes react with monoacids to produce AGEs (27).

It can be seen from Figure 1.1 that, as a result of the differing pathways of production, there is more than one type of AGE, each with different structure and properties. Ahmed (2005) categorised AGEs based on their chemical properties,
creating three subgroups; fluorescent cross-linking AGEs, non-fluorescent cross-linking AGEs and non-cross-linking AGEs (30). N-Carboxymethyl-lysine (CML), N-Carboxyethyl-lysine (CEL), pentosidine, Methylglyoxal-derived hydroimidazolone 1 (MG-H1) and pyrraline are some of the most studied AGEs and have been previously used as biomarkers for in vivo formation of AGEs (30, 35-37). The AGE glucosepane, a lysine-arginine product, as shown in Table 1.1 is a non-fluorescent cross-linking AGE. Although glucosepane previously has had less use as a biomarker compared to other AGEs, it is now thought to be one of the most abundant AGEs in human tissues (38, 39) and is becoming more widely studied. Table 1.1 shows examples of the AGE types mentioned applied to each category.

<table>
<thead>
<tr>
<th>Category</th>
<th>AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent cross-linking AGEs</td>
<td>Pentosidine</td>
</tr>
<tr>
<td></td>
<td>Crossline</td>
</tr>
<tr>
<td>Non-fluorescent cross-linking AGEs</td>
<td>Imadazolium dilysine</td>
</tr>
<tr>
<td></td>
<td>Alkyl formyl glycosyl pyrrole</td>
</tr>
<tr>
<td></td>
<td>Arginine–lysine imidazole</td>
</tr>
<tr>
<td></td>
<td>Glucosepane</td>
</tr>
<tr>
<td>Non-cross-linking AGEs</td>
<td>Pyrraline</td>
</tr>
<tr>
<td></td>
<td>N-Carboxymethyl-lysine (CML)</td>
</tr>
<tr>
<td></td>
<td>N-Carboxyethyl-lysine (CEL)</td>
</tr>
<tr>
<td></td>
<td>Methylglyoxal-derived hydroimidazolone 1 (MG-H1)</td>
</tr>
</tbody>
</table>

Table 1.1: Table categorising well-known AGEs
Meerwalt (2004) used the AGEs pentosidine, carboxymethyl-lysine (CML), and carboxyethyl-lysine (CEL) to look at AGE levels within the skin using skin biopsy. He showed that the fluorescent AGE pentosidine correlated with the non-fluorescent skin AGE levels of CML and CEL. From this Meerwalt went on to validate skin auto fluorescence as a technique for measuring AGEs (21). The use of skin auto fluorescence as a measure of AGE levels is discussed further in the methods Section 3.3.2.4.

1.4 Dietary AGE

Foods are a source of exogenous AGE; AGEs are formed during the heating process of food (40, 41). In the past, AGEs derived from diet (dAGEs) were disregarded as a contributor to circulating AGE levels as it was thought that the absorption into the gastrointestinal system was insignificant. It has been shown however in multiple studies that dAGEs could have a substantial influence on the body’s AGE level and contribute to pathology (42-48). Diabetes is an example of this, higher consumption of dAGE is thought to have a pathogenic significance by contributing to insulin resistance (49). Although there is a body of literature suggesting dAGEs are a risk factor for increasing circulatory AGE levels, a pilot study by Piroddi et al. (2011) contradicts this, having looked at the dAGE CML and found it did not contribute to circulating levels of AGE in chronic kidney disease patients (50). Further research is needed to precisely define the dAGE AGE relationship.

The process of heating foods, comprising of sugars and/or lipids and proteins, triggers the Maillard reaction, as described in Section 1.3. There are certain
influencing factors that determine the level of AGE within food namely; nutrient composition, humidity, pH, temperature, and length of time cooked (40). Low pH values are thought to slow the Maillard reaction (40), high AGE values are seen for foods cooked at high temperatures as well as by certain cooking methods; barbequing, grilling, frying and roasting (dry heat methods) (51). The length of time a food is cooked for is relevant too, however the temperature and type of cooking method is more significant to the AGE value (14, 36).

The AGE carboxymethyl-lysine (CML) is a key type of dAGE and is fairly stable and inert, as such it is commonly used as a dAGE marker (14, 40). Goldberg et al. (2004) were the first to produce a table showing AGE content from various food groups, with 250 foods measured for CML content (40). Uribarri et al. (2010) developed upon this database and measured the CML present in 549 foods (14). Since then other dAGE databases have been produced (52), with Scheijen et al. (2016) measuring other AGEs in addition to CML (53). Dresden University of Technology are developing a large dAGE database which different authors can contribute to, it currently has measured values for a wide variety of AGE types including: CML, CEL, methylglyoxal, Amadori-Product, pentosidine, pyrraline and furosine (45).

Foods such as fruits and vegetables in all of the databases have low AGE values recorded. The Goldberg et al. (2004) and Uribarri et al. (2010) databases found that foods with a high protein and lipid content have the highest AGE value, as illustrated by the fat and meat categories having much higher AGE values than other foods. Fats such as cheese have the highest dAGE although generally
these are eaten in smaller portions than meats (14, 40). The later developed Hull et al. (2012) and Scheijen et al. (2016) databases found that foods with a high sugar content such as chocolate, sweets and biscuits had the highest values, with these food categories having higher values than meats and cheeses (52, 53). The differences in CML values for similar foods between the databases is thought to be due to a different in analytical measurement methods used (48). Table 1.2 is a summary of the published AGE databases.

<table>
<thead>
<tr>
<th>AGE database</th>
<th>Type of AGE measured</th>
<th>Method of measurement</th>
<th>Units</th>
<th>Number of foods measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldberg et al. (2004) (40).</td>
<td>CML</td>
<td>ELISA</td>
<td>CML kU/100 g</td>
<td>249</td>
</tr>
<tr>
<td>Uribarri et al. (2010) (14).</td>
<td>CML</td>
<td>ELISA</td>
<td>CML kU/100 g</td>
<td>549</td>
</tr>
<tr>
<td>Hull et al. (2012) (52).</td>
<td>CML</td>
<td>UPLC–MS/MS</td>
<td>mg CML/100 g</td>
<td>257</td>
</tr>
<tr>
<td>Scheijen et al. (2016) (53).</td>
<td>CML CEL MG-H1</td>
<td>UPLC–MS/MS</td>
<td>mg CML/100g mg CEL/100g mg MG-H1/100g</td>
<td>190</td>
</tr>
</tbody>
</table>

Table 1.2: Summary of published AGE databases. CML: carboxymethyl-lysine, CEL: carboxyethyl-lysine, MG-H1: 5-hydro-5-methyl-4-imidazolon-2-yl-ornithine, ELISA: Enzyme-linked immunosorbent assay, UPLC–MS/MS: Ultra-performance liquid chromatography tandem mass-spectrometry

A food frequency questionnaire (FFQ) is considered one of the most suitable methods to measure the intake of a specific nutrient, such as dAGE. FFQ’s are discussed further in Section 3.3.2.5 and Section 4.3.2. There are currently very
few FFQs specifically designed to target dietary intake of AGEs and there are no AGE specific FFQs that have been designed for a UK based population. Indeed, the only existing validated AGE specific FFQ is that produced by Luevano-Contreras et al. (2013); which used the database produced by Uribarri et al. (2010) for AGE values, to measure dAGE in diabetic patients (54, 55). The Uribarri et al. (2010) database is the database most commonly used in the literature and has been used for both FFQ’s and food records (49, 54, 56-58). Hull et al. (2012) has been used by a 3-day food record (59), but no FFQ. Scheijen et al. (2016) database was used by the Scheijen et al. (2018) study which used the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) non-AGE specific FFQ.

When measuring the dAGE intake of two ethnicity groups with type 2 diabetes it was found that those with higher dietary intake of saturated fats had a higher dAGE level and those with a higher dAGE were more likely to have a moderate-high cardiovascular disease risk (55). Animal (rodent) studies have shown that a diet high in AGE is associated with increased circulating AGE levels as well as; atherosclerosis, weight gain, diabetes progression, lower plasma insulin and intolerance to glucose (60-62). Uribarri et al. (2003) found that if human participants with renal failure were given a low dAGE diet the circulating AGE levels decreased (44). Similarly, Vlassara et al. (2002) found that if participants diagnosed with type two diabetes were on a reduced dAGE diet for six weeks there would be a reduction of both circulating AGE level and reduction in the inflammatory markers tumour necrosis factor $\alpha$ (TNF-$\alpha$) and vascular cell adhesion molecule 1 (VCAM1) (43). In addition, it has been shown that giving a
single dietary oral AGE-rich liquid beverage (free from lipids) results in a rise in AGE serum levels. This serum AGE was measured 90 minutes after ingestion of the AGE beverage. Endothelial function was measured using flow-mediated arterial vasodilation, it was found that the AGE-rich beverage caused impairment of endothelial function (63). These studies suggest that restriction of dAGE may be a favourable approach for reducing the levels of AGE circulating within the body. Additionally they demonstrate the association between dAGE and circulating AGE level. Despite there being a number of dAGE studies the evidence has been questioned due to a lack of high quality randomised trials (64).

1.5 Effect of AGEs on cells and tissues

The accumulation of AGEs is important for the pathogenesis of different pathologies of the body (30). Endogenously AGEs are formed and accumulate where glycation reactions occur; in both tissues and bodily fluids. The damaging effects that AGEs have on tissues are primarily caused by their interference with protein function. There are two ways in which these damaging effects occur; one involves the cell surface receptor for AGE known as RAGE, the other is independent of RAGE, with AGEs causing direct damage to protein structure (30).

1.5.1 AGE – RAGE binding mechanism

AGE-binding receptors (RAGE) are scavenger receptors that mediate intracellular signalling (30, 65). They are expressed on a diverse range of cells; monocytes, macrophages, smooth muscle cells, vascular endothelial cells,
pericytes, podocytes, astrocytes, microglia, Müller, glia and retinal pigmented epithelial cells (65, 66). RAGE has the ability to bind non-AGE ligands, causing inflammation independent of AGE (67), making RAGE complex in itself.

As shown in Figure 1.3, when an AGE binds to RAGE it causes cellular signalling reactions; mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3 kinase (PI3-K) are activated, which in turn activate transcription factor kappa B (NF-κB) (60, 61). NF-κB is also activated due to the sudden increase in intracellular oxidative stress caused by NAD(P)H oxidase, created in response to the binding of AGE to RAGE (62, 63). Once NF-κB is activated it triggers the nucleus to begin to transcribe genes for; tissue growth factor, thrombomodulin, endothelin-1 and VCAM1, as well as cytokines; interleukin 1α (IL-1α), interleukin 6 (IL-6), TNF-α (60, 64). RAGE expression is increased by activation of NF-κB; hence a positive feedback cycle is also present here. Essentially, the AGE-RAGE binding mechanism initiates a state of inflammation and oxidative stress. It can also disturb the functioning of the vascular endothelium, which normally acts to regulate blood flow to ensure it always meets the demand of the tissues, through its action on endothelin-1. Oxidative stress and endothelial function are discussed further in Sections 1.6 and 1.8.
1.5.2 RAGE independent mechanism

The second mechanism involves AGEs causing direct damage to structure, properties and function independent of RAGE mediated events. AGEs can alter molecular recognition and protein binding (30, 38). In addition, as mentioned in Section 1.3, the AGEs pentosidine, crossline, imidazolium dilylsine, alkyl formyl glycosyl pyrrole, arginine–lysine imidazole and glucosepane form covalent cross-links (30, 68). Cross-linking can occur with short-lived molecules, however since time is a factor in cross-link formation it is thought that crosslinking occurs more often in long-lived proteins (69, 70), such as extracellular proteins collagen and
elastin. The AGE induced cross-link alters the mechanical properties of the protein, in collagen these cross-links result in increased stiffness of tissues and reduction in elasticity (38, 71, 72). Since normal collagen function is essential for organs and tissues throughout the body (19, 73) collagen cross-linking can have an adverse effect on physiology. In blood vessels for example, cross-linking causes increased stiffness of the protein matrix and decrease elasticity/flexibility, which affects cell interactions, as well as cell function and integrity (32, 72-75).

Recent research has found that as a result of their chemical, pro-inflammatory and pro-oxidant actions, sustained exposure to high levels of circulating AGEs could be a major factor in the development of a number of chronic degenerative age-related systemic disorders; diabetes (23), Alzheimer’s disease (76), renal disease (77), osteoarthritis (78), atherosclerosis (79, 80), as well as the ocular disorders; age-related macular degeneration (AMD) (22, 81), diabetic retinopathy (DR) (82, 83), cataract formation (81, 84-86), primary open angle glaucoma (POAG) (20) and other neurodegenerative diseases (76). These links with age-related disorders make AGEs and their mechanisms of damage an area of interest.

1.5.3 Soluble RAGE

Soluble RAGE (sRAGE) is different to the cell-surface RAGE described in 1.5.1, and is considered beneficial. Soluble RAGE contributes to the detoxification of AGEs (87). It does this by acting as a ‘decoy’ and competitor to RAGE, blocking RAGE-dependent responses (87, 88). It has been shown in a study investigating patients with diabetic retinopathy that sRAGE levels were significantly lower in
patients non-proliferative and proliferative diabetes compared to healthy controls and diabetic patients without retinopathy (88). In addition it has also been shown that levels of sRAGE are reduced in patients diagnosed with hypertension (89).

1.6 Oxidative stress

Oxidative stress is “an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage” (90). Oxidation is the loss of electrons during a reaction, carried out by oxidants. Oxidants are products of normal cellular metabolism, though in pathological conditions involving inflammation and infection oxidants tend to be found in increased amounts. Oxidants can be referred to as ‘reactive species’, for example; reactive oxygen and nitrogen species (ROS/RNS) (91, 92). They come in two forms, free radical and non-radical species and both can be reactive (93). Free radicals are unstable molecules which are reactive due to the unpaired electron in their outer orbit (94).

Free radicals derived from glycation cause oxidation of lipids and DNA as well as breaking down proteins (25, 30). Due to the glycation reactions involved in the formation of AGEs free radicals are often referred to in AGE literature.

An antioxidant is “any substance that, when present at low concentrations compared with that of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate” (91). Antioxidants are essentially able to ‘neutralise’ free radicals and reactive species as part of the protection mechanism for tissues of the body from the damage reactive oxidative species could cause (95, 96). Compounds of both oxidant and antioxidant form can be found in human diet (97). Vitamins A, C, and E are examples of anti-oxidants derived from foods that
are able to neutralise free radicals directly (98). However current clinical research on the effectiveness of dietary anti-oxidants at protecting the body is controversial as research is contradictory with no clear associations established (99). It has however been shown that a balanced diet is healthier than antioxidant supplementation (100, 101).

Oxidative stress can cause damage to DNA, proteins, and lipids. It is understood that oxidative stress contributes to; atherosclerosis, cardiovascular diseases, stroke, diabetes, cancer, rheumatoid arthritis, post-ischaemic perfusion injury, age-related neurodegenerative diseases and ageing (93, 96, 102, 103). Oxidative stress contributes to neurodegenerative damage via the mechanism of free radicals attacking the neural cells (96). The harmful effect the reactive species has on cells leads to tissue damage, oxidative injury and ultimately apoptosis (96, 104).

In more recent years it has been shown that reactive species such as ROS and RNS are linked to more than just pathology. They can also act as part of redox signalling throughout the body (100). Although generally reactive species are thought of as harmful it is not fully understood how damaging they are, as it seems to depend upon the circumstances (105). The understanding of oxidative stress has principally come through standard laboratory conditions. Laboratory conditions are limited in that they may not be related to free radical biology within the body (105).
Since reactive species are particularly unstable they are challenging to measure directly. Oxidative stress biomarkers give an indication of the level of oxidative stress present in the body. These biomarkers are molecules that are involved in the reactions with reactive oxygen species (106). There are a variety of biomarkers that exist, a summary of some commonly used biomarkers are listed in Table 1.3. The molecules; α- tocopherol (vitamin E), ascorbic acid (vitamin C) and malondialdehyde each have mechanisms relating to oxidative stress and will be used as biomarkers for oxidative stress in this thesis. Each biomarker is explained in Section 3.3.2.6. These are recognised biomarkers of oxidative stress in ageing and POAG (107-109) and have been shown to be sensitive to variations in AGE levels (110).

<table>
<thead>
<tr>
<th>Oxidants</th>
<th>Descriptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde (MDA)</td>
<td>Product of lipid peroxidation</td>
</tr>
<tr>
<td>4-hydroxynonenal (4-HNE)</td>
<td>Product of lipid peroxidation</td>
</tr>
<tr>
<td>Acrolein</td>
<td>Product of lipid peroxidation</td>
</tr>
<tr>
<td>8-isoprostane</td>
<td>Product of lipid peroxidation</td>
</tr>
<tr>
<td>8-hydroxy-20-deoxyguanosine (8-OH-G)</td>
<td>DNA lesion</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (vitamin C)</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>α-tocopherol (vitamin E)</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>Transferrin (TF)</td>
<td>Non-enzymatic antioxidant</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>Enzymatic antioxidant</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>Enzymatic antioxidant</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>Enzymatic antioxidant</td>
</tr>
</tbody>
</table>
1.7 AGE and Oxidative stress relationship

The relationship between AGE levels and oxidative stress is complex, with an increase in AGE levels having been shown to be both a cause and an effect of elevated oxidative stress (15). Oxidative stress contributes and accelerates the process of AGE formation (111). It is produced as a by-product of AGE formation and by the AGE-RAGE binding interaction; the interaction causes an intracellular generation of free radicals and oxidative stress, as shown in Figure 1.3 (112, 113). There is a positive feedback cycle between AGES and oxidative stress; hence AGE levels can be influenced by oxidative stress levels and vice versa. For this reason it is important to consider the AGE oxidative stress relationship when examining the effects of AGES.

Both AGES and oxidative stress levels increase with age and are both able to cause pathological consequence (8, 45, 114). The majority of the literature differentiates between AGES and oxidative stress, measuring each individually while discussing the known connection (8, 45, 115-117). However, there are examples in literature which use the terms interchangeably, with some using AGES as biomarkers of oxidative stress (92, 118, 119). Further investigation of the AGE oxidative stress relationship may help to clarify if it is acceptable to interchange between these terms.
1.8 Endothelial function

The vascular endothelium is a monolayer of cells which lines the inner surface of all blood vessel surfaces, acting as the barrier to blood and vessel interactions (120-122) see Figure 1.4. It is a multifaceted tissue that maintains vascular homeostasis in response to mechanical, chemical and biological stimuli (121, 123), through regulation of vascular tone, adhesion of cells, prevention of vessel wall inflammation, resistance of thrombus formation and resistance to smooth muscle cell proliferation (124). One of the fundamental roles of the microvascular endothelium is to regulate blood flow to ensure blood supply meets the demand of tissues, this is known as autoregulation. The endothelium controls this through the balanced secretion of endothelial derived vasoactive factors, the most potent of which are endothelin-1 (ET-1) and nitric oxide (NO) (125). ET-1 is a vasoconstriction agent and NO a vasodilation agent (126), they act on the underlying vascular smooth muscle to regulate blood vessel diameter, hence enabling regulation of the amount of blood that reaches a region to ensure supply meets demand.
Endothelial dysfunction refers to a failure in the ability of the endothelium to regulate blood vessel diameter and hence blood flow when the metabolic demand of the tissues is high. This is usually due to either decreased NO or increased ET-1 production (127). Unable to maintain vascular homeostasis, a pro-inflammatory and pro-thrombotic state is more likely, as well as the formation of atherosclerotic plaques (123, 128). A failure to increase blood supply to meet metabolic demand can leave the tissues ischaemic and repeated ischaemic episodes can cause damage through recurrent ischaemic reperfusion injury. Recurrent ischaemic reperfusion injury will be discussed in relation to GON in Section 1.13.2.2.

Endothelial function is essential to vascular biology. Endothelial dysfunction has been associated with: ageing, smoking, obesity, diabetes, coronary heart disease, chronic renal failure, hypercholesterolemia, hypertension, peripheral vascular disease, stroke and severe infectious diseases (129-131). Oxidative stress, as well as other factors, has also been linked to the cellular imbalance which causes endothelial dysfunction (127).

1.9 Relationship between AGEs and endothelial function

AGEs have an influence on endothelial function. The AGE-RAGE interaction produces NFκB which is the transcription factor that causes an increase in ET-1 levels (132). In addition, AGEs reduce availability and activity of NO (133). Hence
AGE-RAGE interaction has the overall effect of vasoconstriction, because ET-1 levels are increased and NO levels, as a ‘relaxing factor’, are reduced, resulting in a dysfunctional endothelium.

In addition to disturbing vascular endothelial function via the AGE-RAGE interaction, AGEs directly alter the functional properties of vessels due to their accumulation in vessel tissue (80, 134). Vessel walls are primarily made of collagen and elastin (135), as mentioned previously these long-lived proteins are susceptible to AGE cross-linking. This cross-linking results in a reduction in blood vessel elasticity and increased vascular stiffness (74, 80, 136). Animal studies have shown AGE cross-linking to be present on type I collagen, and found that increased vasculature stiffness occurs due to cross-links increasing the area of extra-cellular matrix (13, 137). In human studies aortic pulse wave velocity has been used as a method of measuring vessel stiffness. Semba et al. (2009) found a positive correlation between serum levels of CML and vessel stiffness (138), and Ueno et al. (2008) found a positive correlation between SAF and vessel stiffness (139). Reduced elasticity and increased vascular stiffness can also interfere with the ability of the blood vessels to regulate blood supply to meet demand, as blood vessel diameter cannot be so easily altered (74).

The apparent associations of AGEs with endothelial dysfunction, oxidative stress and vascular stiffness, and the known associations of endothelial dysfunction with age-related ocular and systemic disease, raises the question as to whether increased AGE levels could be an underlying causative factor for such disease states. Exploring this association will be a focus of this PhD research.
Indeed, the effect of AGEs on blood vessel function occurs throughout the body, including in the ocular vasculature. Reduced function of the ocular blood vessels is important as it affects the regulation of blood flow to and within the eye. Altered blood flow is a known risk factor for ocular pathology (140) and can also be a marker for systemic vascular disease. Hence, increasing our understanding of the role that AGEs play in microvascular dysfunction at the ocular level may increase our understanding of ocular pathology.

Measurement of microvascular function at the retinal level and examination of retinal vascular structure is possible with the Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena). As will be explained in Section 3.3.2.2 in a normal healthy person heightened metabolic demand triggers the retinal endothelium to release additional NO, resulting in vasodilation and consequently increased blood flow. The DVA can analyse vessel structure and measure altered retinal vessel response to flicker light which is thought to indicate any impaired endothelial function (141). Using the non-invasive AGE Reader alongside this will allow the comparison of AGE level and retinal endothelial function. This is something which has not previously been investigated and may provide insight as to AGEs effect on retinal vascular function in healthy ageing and ocular disease.

1.10 Advancing age as a risk factor for ocular disease

A significant risk factor for both ocular and systemic disease is increased age (4, 5). The ageing process is multifaceted, usually defined as deterioration of structure and function of cells and tissues throughout the body (142). The
cumulation of this deterioration causes increased risk of pathology, disease and death (143). The ocular cells and tissues are equally as affected by ageing as other cells and tissues in the body. Advancing age is a significant risk factor for a number of ocular diseases such as cataract (144), AMD (145) and GON (146). Of interest to this body of research is the role that ageing plays in the pathogenesis of the neurodegenerative disease glaucomatous optic neuropathy. Ageing is a risk factor for all neurodegenerative diseases and it is thought to exert its effects through mechanisms such as increased oxidative stress, mitochondrial dysfunction, impaired ability to self-repair; reduced DNA repair and decreased tissue regeneration (147). AGEs are associated with oxidative stress and their accumulation increases with age; hence AGEs may also be part of a neurodegenerative disease mechanism (148, 149).

1.11 The effect of advancing age and AGEs on ocular structures

The negative effect that physiological ageing has on structures of the eye will be discussed individually in this section, although it is important to remember that ageing of ocular components is not something that occurs in isolation, changes in certain structures has impact on others as a whole system. The structures in which AGEs accumulate within the eye will also be discussed.

1.11.1 Sclera

In spite of it having reduced collagen content, there is increased rigidity and stiffness of the sclera with age; it is thought that this rigidity is due to the accumulation of non-enzymatic cross-links. Girard et al. (2009) found that monkeys of increased age had a significantly stiffer posterior sclera. It is
postulated that this increased stiffness may cause overall globe stiffness and contribute to optic nerve head damage, contributing to glaucomatous changes (150). AGEs accumulate in the sclera (151) and could contribute to the increased collagen crosslinking that occurs with age.

1.11.2 Cornea

With physiological ageing the cornea becomes flatter, there is an increase in thickness of Descemet’s membrane, decrease in stromal density and a decrease in the density of endothelial cells (152-154). In the stroma there is an increase in collagen fibrils and collagen cross-linking (155) but no change in the thickness of Bowman’s layer with age, however calcific deposits at the periphery of the layer become more likely (7). Consequently there is a change in corneal curvature and a variation in thickness of structures which results in the cornea becoming stiffer (156). Stiffening as a result of collagen cross-linking has been shown to be beneficial in the treatment of keratoconus and is thought to be beneficial in treating other corneal conditions (157).

It has also been shown that there is an increase in AGEs in the stroma with increased age (158). It is thought that collagen modified by AGEs may contribute to the reduced corneal flexibility seen with ageing due to encouraging an increase in collagen fibrils (159, 160). Hence AGEs may be a contributing factor to the reduced corneal viscoelasticity seen with age. There is currently no research directly comparing tissue bound AGE levels to corneal viscoelastic properties. Corneal viscoelastic properties in relation to GON will be discussed in more detail
in Section 1.12 as it is relevant in understanding the mechanisms of glaucomatous damage.

1.11.3 Trabecular meshwork

The shape of the trabecular meshwork changes with physiological ageing; becoming shorter with the scleral spur more prominent, the uveal meshwork compacted, narrowing of the canal of Schlemm and increased thickening of trabeculae (161). There is a decrease in cellularity and absolute cell number with age (162). One of the postulated contributors to increased intraocular pressure (IOP) with age is that aqueous outflow is reduced and outflow resistance is increased (163). This is thought to be due to the changes in structure as well as reduction in giant vacuoles and intracellular pores in the canal of Schlemm through which aqueous humour passes (164, 165) and an accumulation of extracellular materials and changes to the collagen (163, 166, 167). In Section 1.13.2.2.3 endothelial dysfunction is discussed in relation to GON as an imbalance in NO and ET-1 can affect outflow of aqueous humour in the trabecular meshwork, any dysfunction located here would ultimately affect IOP. It is suggested that AGEs accumulate in the trabecular meshwork (116) and that AGEs may induce the oxidative stress thought to contribute to dysfunction.

1.11.4 Ciliary body
With increased age the ciliary stroma has an increase in collagen with some tissues replaced by collagen and the ciliary processes become shorter and less vascular. Similar to the trabecular meshwork, with age the ciliary body loses cellularity (7). The ciliary muscle appears to change with a displacement of muscle mass (168). As the ciliary body produces aqueous humour which ultimately affects IOP its ageing process is important.

1.11.5 Intraocular Lens

Lens shape and weight alters with physiological age; in adults the lens is more oval and heavier than at birth (169). Age related colour changes, yellowing, of the lens is a characteristic of cataract. It occurs with age due to lens proteins becoming oxidised (7). AGEs accumulate in lens (85) and are linked with the formation of cataract (81, 84, 170). The long-lived lens proteins crystallins are a site of glycation and cross-linking (170, 171), the modification to crystallin results in reduced transparency, and is thought to affect chaperoning and anti-apoptosis, resulting in cataract formation (170, 172, 173). The specific AGEs; CML and pentosidine have been found at increased levels in lenses with cataract compared to those without, with the intensity of brown cataract colour increasing with increased AGE level (86). In addition the AGE MG-H1 has been identified as a major AGE in human lens proteins (174). The lens capsule has more recently been identified as a glycation location, with different AGEs found at higher levels in capsules lenses with cataract than those without (171).

1.11.6 Vitreous
Ageing causes structural changes in the vitreous gel, including liquefaction and posterior vitreous detachment (175). The vitreous gel is comprised of a transparent extracellular matrix. Through studying diabetic patients vitreous it has been suggested that AGEs have a pathologic role in the degeneration of the vitreous (176).

1.11.7 Retina

Ageing occurs throughout the layers of the retina, Figure 1.5 shows a cross section of the retinal cells. The RPE will be discussed in the next Section with Bruch’s membrane. As the retina ages the internal limiting membrane thickens and gliosis occurs in the peripheral retina (7). The nuclei in the outer nuclear layer become displaced, move towards the photoreceptor layer, where their cell structure changes, eventually ending in cell death (177). Reduction of rods occurs before cones at the macula with age (178). The retina has physiological age related peripheral degenerations; typical and reticular peripheral cystoid degeneration (179), peripheral chorioretinal atrophy (paving stone degeneration) (180), and lattice degeneration (181). Additionally, small masses of degenerate cells named Corpora amylacea have been found to be present in autopsied eyes in the; retinal nerve fibre layer and optic nerve, optic nerve head, inner plexiform layer and inner nuclear layer with increased age (182-184). Corpora amylacea in the central nervous system are associated with ageing and neurodegeneration (185). AGEs are present in the retina (81), the presence of AGEs in retinal blood vessels as will be discussed in Section 1.11.11.
1.11.8 RPE & Bruch’s membrane

With increased age the retinal pigment epithelium (RPE) begins to change shape, this occurs in two distinct regions of the RPE (186). The macula RPE alters by narrowing with an increased height, while the peripheral RPE becomes shorter and wider (187). With age, the RPE macula cells have increased pigment density and irregular pigmentation and in the ageing peripheral RPE vacuoles form (187, 188). There is also an increase in lipofuscin accumulation in the cytoplasm of RPE cells with age (189). It is thought that lipofuscin accumulation in the RPE cell may contribute to the mechanism of macular degeneration (190). Drusen are found on the inner side of Bruch’s membrane, their accumulation come in different forms; hard, soft and confluent. With increased age there is an increase in presence of drusen. Soft, confluent and large drusen are seen in greater amounts in age-related macular degeneration (191). Bruch’s membrane thickens with age (192) and also has an accumulation of lipids (such as cholesterol) and can also become calcified (193, 194). It is these accumulates that form a barrier that is linked with age-related macular degeneration, photoreceptor dysfunction and pigment epithelial detachment (193).

AGEs accumulate in increased amounts in the RPE and basement membrane with age (81, 195-199), specifically the AGEs CML and pentosidine have been found in both the basement membrane and choroid with age (196). AGEs are thought to accumulate in the extracellular matrix of Bruch’s altering its physical
properties (200). This accumulation of AGEs induces VEGF, inflammation, and encourages neovascular AMD (22, 201).

1.11.9 Choroid
With age the thickness of the choroid does not change (192) but there is a decrease in choriocapillary density (192). With advancements in OCT the volume of choriocapillaries can now be measured non-invasively. OCT data has also confirmed that choriocapillary density decreases with advancing age (202).

1.11.10 ONH
With physiological ageing there is an increase in the amount of connective tissue present in the fibrovascular pial septae of the ONH. This increased thickness could impair transmission of essential nutrients and metabolic substances between nerve fibres and capillaries (7). Another consequence of physiological ageing on the optic nerve head is that cellular and extracellular material can collect in optic nerve fibre bundles and meninges (7).

It is the cribriform plates within the lamina cribrosa where AGEs accumulate within the ONH (203). It is thought that accumulation in this location may contribute to the mechanism of optic neuropathy (204). With age increased amounts of collagen cause the structure of the lamina cribrosa to alter, becoming stiffer and less resilient (203); when pressure is applied to the lamina cribrosa the ability to return to its original shape decreases (205). These age-related collagen changes are thought to be linked with age related glaucomatous optic
neuropathy. Along with an increase in the amount of collagen present in the lamina cribrosa, with age the AGE pentosidine has been found in increased amounts (203). The AGE pyrraline has also been located in the; sclera, pia matter, cribriform plates, optic nerve connective tissues and around the vessels in the optic nerve in donor eyes (204). The potential relationship between AGEs and GON is discussed in Section 1.11.4.1.

1.11.11. The retinal vasculature and the influence of AGEs on vascular function
With physiological ageing the body’s peripheral capillaries have a reduction in cellularity (206), hence the retinal microvasculature, as part of this peripheral network, also has a decrease in cellularity. Atherosclerotic and hypertensive vessel wall changes such as increased vessel wall thickness, are more likely to occur with ageing and as such ageing is a risk factor for both hypertension and atherosclerosis (7).

Research into the effect of AGE accumulation within blood vessels and tissues in cardiovascular disease is increasing as it appears that AGEs are part of the disease mechanism (13, 138, 142, 207) and play an important role in atherosclerosis (208). With ageing the vessels within the body experience; endothelial dysfunction, reduced elasticity, accumulation of collagen and increase in intimal medial thickness (thickening of the innermost two layers of artery wall) (209, 210). Elasticity of the blood vessels is dependent on the level of collagen fibres and collagen cross-links (211). An increase in covalent crosslinks, as a result of AGE accumulation causes a reduction in blood vessel elasticity (136), as was discussed in Section 1.8. Reddy (2004) using diabetic rats, demonstrated
this mechanism (212) and other studies have shown that increased vascular stiffness correlates with increased AGE accumulation (13, 138). Furthermore, SAF measured by the AGE Reader has been shown to be an independent predictor of cardiovascular disease in diabetic, dialysis and coronary heart disease participants (139, 213-216). SAF has also been shown to be a predictor for vessel AGE modifications in patients with coronary heart disease (216). Specific mechanisms linking AGEs and vascular endothelial function as well as vascular stiffness were discussed in Section 1.8.

1.12 Current knowledge of AGEs and Ocular disease

As explained throughout Section 1.11 AGEs accumulate throughout the structures of the eye and it is thought that they may be linked with both physiological and pathological age-related changes. There are certain specific conditions and structures that have been researched in more detail such as diabetic retinopathy, macular degeneration and diabetic keratopathy. The link between AGEs and GON is explained in detail in Section 1.13.4. A table summary of the AGEs and ocular disease literature can be found in Appendix Section 8.

1.12.1 AGEs and Diabetic Retinopathy

It has been estimated that there are approximately 93 million people worldwide with diabetic retinopathy, 17 million with proliferative diabetic retinopathy (217). Diabetic retinopathy is characterised by the development of blood vessels which are abnormally permeable, have impaired blood flow, basement membrane thickening, and ischaemia (218). Diabetic retinopathy is a complicated disease
with multiple pathological mechanisms, AGEs and AGE-RAGE interactions are thought to be one of these mechanisms (219). The link between hyperglycaemia, AGEs and diabetic retinopathy is becoming better understood, it has been shown that increased AGE level is correlated with increased diabetic retinopathy injury (220), with higher AGE levels found in those with proliferative retinopathy (221-223). There is also a relationship between increased levels of the AGE CML and increased expression of vascular endothelial growth factor (VEGF) (134). In post-mortem examination of retinas with background diabetic retinopathy and proliferative retinopathy both the AGE CML and VEGF were distributed around retinal blood vessels (134). The accumulation of AGEs on the endothelial membrane causes damage to the structure and function of the retinal vessel wall by increasing the permeability leading to vascular leakage (15). In addition, the thickening of vessel walls causes ischaemia and occlusion while increase in VEGF causes angiogenesis (224, 225).

It has been shown that AGE levels within the skin, particularly CML, are a good predictor for diabetic retinopathy (226). It is thought that skin levels of AGEs are superior predictors of diabetic retinopathy than HbA1c as they reflect hyperglycaemia over a longer time course (83, 227). The advancements in skin auto fluorescent technology is important as this may be a future method for predicting diabetic retinopathy.

1.12.2 AGEs and Age-related macular degeneration (AMD)

AMD is a complicated disease thought to occur via multiple pathology mechanisms and not only due to ‘normal’ physiologic ageing. The exact aetiology
of AMD remains largely unknown, however the degeneration of the macula is caused by changes in the retina and choroid due to atrophy, detachment and neovascularisation (145).

As mentioned in Section 1.11.8, AGEs accumulate in the RPE and basement membrane with age (195-199). AGEs are also found to accumulate within drusen (228). The alterations AGEs make to the basement membrane causes changes to the RPE proteome; with many proteins losing their physiological structure (229). The specific AGEs CML and pentosidine located in the basement membrane and choroid are thought to contribute to RPE-basement membrane-choroid dysfunction (196), as mentioned, we know that Bruch’s membrane thickens with age becoming less permeable (192). It is thought that AGE cross-link accumulation may be the cause of this thickening and decrease in permeability (201). As well as AGE contributing to AMD, RAGE accumulates in the RPE and photoreceptors of eyes with early AMD and geographic atrophy (199). It is thought that the AGE-RAGE interaction is related to photoreceptor loss, with NFkB, apoptosis and upregulation of RAGE having been observed in RPE cell cultures (199). Lipofuscin seen within drusen reduces the effectiveness of lysosomal antioxidant systems (230). The dysfunction of lysosomes has also been linked with reactive oxygen species and AGEs (173, 230, 231). Hence AGEs are not only located within drusen, but also encourage lysosome dysfunction, hence promoting drusen formation. As AGEs accumulate they induce inflammation via VEGF, promoting neovascular AMD (201). Mulder et al. (2010) found that there was an increased SAF measured using the AGE Reader in Caucasian subjects with neovascular AMD (22). It is thought that AGEs are
both formed and deposited with increased AMD (22). It appears that AGEs affect the progression of both dry and wet AMD.

1.12.3 AGEs and diabetic keratopathy

Collagen is the principal structural component in the cornea and as mentioned previously in Section 1.4, AGEs are found to accumulate during normal ageing in long-lived proteins such as collagen (19). This accumulation of AGEs occurs at an accelerated rate in those with diabetes (73, 232). Diabetic corneas have been well researched in this area due to this. Sady et al. (1995) showed that the specific AGE pentosidine and increased collagen are found in higher amounts in diabetic corneas, compared to age-matched healthy controls (232). It has also been shown that there is an increase in AGEs in the stroma with increased age (158). It is thought that collagen modified by AGEs may contribute to the reduced corneal flexibility seen with ageing due to encouraging an increase in collagen fibrils (159). Measurement of the AGE CML in diabetic compared to non-diabetic corneas, has shown that CML accumulates in the epithelial basement membrane of diabetic cornea’s (233). A large accumulation of CML was present in the laminin, in the basal lamina (233). As the function of the laminin is to aid adhesion and migration it is thought CML reduces adhesion and dispersion of corneal epithelial cells and may be relevant in the mechanism of diabetic corneal epithliopathy. Kaji et al. (2003) found that accumulation of AGEs and reactive oxygen species, products from AGE formation, cause endothelial cell death (234). It has also been shown that patients with proliferative diabetes have an increased corneal AGE level compared to healthy age-matched controls (82). It is
hypothesised that increased corneal AGE level may contribute to the mechanism for diabetic keratopathy.

1.13 Glaucoma and the influence of AGEs

1.13.1 Glaucoma background

Glaucoma is a degenerative optic neuropathy which is characterised by structural damage to the ONH and progressive visual field loss (235). Damage to the ONH causes retinal ganglion cell (RGC) death which can result in vision loss and visual field loss (236). Globally POAG affects over 60 million people, by 2020 it is expected that this will have increased to 80 million (237). It is estimated that the number of people living with glaucoma in the UK will rise in the coming years. Table 1.4 shows UK glaucoma epidemiology.

<table>
<thead>
<tr>
<th>Estimated number of people living with glaucoma</th>
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<td><img src="#" alt="Table 1.4" /></td>
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<table>
<thead>
<tr>
<th>UNITED KINGDOM</th>
<th>2015 (Population estimate 65.1 million)</th>
<th>2020</th>
<th>2025</th>
<th>2030</th>
<th>Percentage change from 2015 to 2030</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>598,560</td>
<td>626,300</td>
<td>655,080</td>
<td>668,400</td>
<td>12%</td>
</tr>
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Table 1.4: RNIB; key information and statistics (238).

GON is commonly classified by: age of onset (acquired/congenital) then further classified by aetiology (239, 240). In the early 20th century an advancement in methods to view the anterior drainage angle (gonioscopy) enabled a differentiation in aetiology; open angle and closed angle glaucoma, each with different cause of damage (235, 240). Figure 1.6 shows the classification of glaucoma. This study is interested in acquired POAG.
Further clinical separation of POAG patients into ‘high’ IOP and normal tension sub-categories has been common practice for many years; however NICE guidelines no longer subdivide open angle glaucoma based on IOP. The diagnosis ‘chronic open angle glaucoma’ (COAG) is used to describe primary open angle glaucoma with both normal and high IOP (241) as shown in Figure 1.7. This links with recent research which has indicated that glaucoma may be a disease continuum, whereby the balance of mechanical factors (IOP) and vascular factors vary on an individual basis and therefore suggest that the sub divisions; POAG, normal tension glaucoma (NTG) and ocular hypertension (OHT), based on the Bedford glaucoma survey in the 1970’s are now outdated to describe GON and should be abolished (242-244).

As shown in Figure 1.7, OHT is the diagnosis given when there is a repeatable high IOP measured with applanation tonometry, with no presence of visual field defect or glaucomatous ONH signs (241). The subdivision OHT enables
practitioners to distinguish between patients with simply increased IOP from those with both increased IOP and glaucomatous changes (245). Although NICE guidelines no longer subdivide COAG based on IOP it is important for this body of research to distinguish between participants to allow comparison with OHT participants. When recruitment for this study opened the NICE guidelines defined high IOP as 21mmHg or above, the NICE guidelines changed increasing the IOP threshold in November 2017. Current NICE guidelines recommend IOP treatment to occur if a patient has an “IOP of 24 mmHg or more” (246). Section 3.1.3 describes the sub-divisions used throughout this research.

Figure 1.7: NICE guideline pathway for OHT and COAG (241).

IOP is the most important risk factor for GON however it is not always associated with the disease. Knowing that glaucomatous changes can occur in those with
normal IOP indicates that more than one mechanism of damage can take place. Research into differences between NTG and POAG began in the 1980’s with some finding no significant difference between NTG and POAG (247, 248), and others finding that although total field loss between POAG and NTG were the same, NTG visual field defects were deeper, steeper, more localised and closer to fixation than in POAG (249-251). Hence there is still a disagreement in the literature as to if visual function differs between NTG and POAG.

1.13.2 Glaucoma pathogenesis
The exact pathogenesis of POAG is poorly understood, with two main theories, namely the mechanical theory and the vascular theory, having been proposed and a possible link to accelerated ageing having been made (20). High and fluctuating IOP linked with the mechanical theory are known factors affecting pathogenesis, it’s thought that changes in vascular function also play a significant role (120, 244).

1.13.2.1 Mechanical theory
Increased IOP has been identified as a cause of retinal ganglion cell death (236, 252) and the role that elevated IOP may play in the development of GON is the central focus of the ‘mechanical theory’; which suggests that high IOP causes mechanical strain/pressure on the ONH (253). This strain on the ONH causes deformation of the cribriform plates of the lamina cribrosa, activation of glial cells, possible compromise of the vasculature and compression of optic nerve fibre bundles which ultimately leads to nerve fibre damage (253-255). IOP is a clinically modifiable factor and its reduction slows the progression of glaucoma.
Due to our incomplete understanding of the mechanism of glaucoma, current treatment is focused on the reduction of IOP by medical and surgical treatment (246, 257, 258). Increased understanding of the other mechanisms in glaucoma would potentially enable expansion and improvement of glaucoma treatment.

1.13.2.2 Vascular theory

The vascular theory proposes that a compromised supply of blood to the optic nerve head causes retinal ganglion cell death (255, 259). Blood flow in POAG has been widely researched (260-263) and has been linked to increased severity of the disease (260, 261, 264). In the retina, reduced retinal arteriolar diameter has been found in glaucomatous eyes, compared to healthy controls and OHT patients (265). In addition, constriction of retinal arteries located near to the optic disc has been shown in POAG and NTG, which also correlated with extent of optic nerve head damage (266). The reduced blood flow observed in glaucomatous eyes was previously thought to be a consequence of GON, as ganglion cell loss causes a reduction in number of blood vessels (120). However, research has shown that reduced blood flow occurs before optic nerve damage develops (267-269). In addition, reduced blood flow is linked with disease progression (270), leading to the conclusion that reduced blood flow could be a mechanism of damage in GON. Over recent years it has emerged that unstable blood flow, in the form of altered regulation of blood supply to meet demand, is more relevant to GON pathogenesis than a steady, constant reduced blood flow.
An example which illustrates this well is multiple sclerosis (MS). MS causes a constant reduced blood flow which can cause atrophy, however glaucomatous optic nerve head signs such as excavation are not seen (273). Unstable blood flow in glaucoma has been hypothesised to result from the presence of disturbed autoregulation in these patients, as a result of endothelial dysfunction (274) and/or structural vessel wall changes (275).

1.13.2.2.1 Autoregulation

Ocular perfusion pressure (OPP) refers to the force of the blood flow through the intraocular vessels (120, 276), calculation shown in Chapter 3, equation 3.2. Under normal circumstances, if OPP falls autoregulation commences to make sure that adequate ocular perfusion is maintained (276, 277). This regulation occurs through automatic modification of vascular resistance i.e. vessel diameter, to ensure blood supply meets metabolic demand (276). Autoregulation occurs in the retina and ONH and is a neurovascular coupling response. This means that when the neural activity of the retina increases, it triggers the retinal vasculature to dilate to ensure that blood supply is increased, via the blood-retinal barrier, to meet the increased metabolic demand of the neurons. The endothelium plays an important role in the autoregulation of blood vessel diameter (120). Dysfunctional regulation is linked with endothelial dysfunction which has been shown to be associated with systemic factors such as systemic hypertension (278), cardiovascular disease (279), plus ocular vascular factors such as reduced OPP (279) and vasospasm (280).
Autoregulation of the retinal and ONH blood flow is only able to maintain blood flow within a certain range of OPPs. If OPP reduces to a level outside the capability of autoregulation, blood supply may not be able to be increased to meet demand, resulting in damage through ischaemia (276). Alternatively, if OPP is reduced to a level within the normal range for autoregulation but the mechanism is not functioning correctly, there is also the risk that ischaemic damage may occur (276). If blood flow regulation is dysfunctional the ONH is vulnerable to periods of ischaemia when demand is high, followed by periods of adequate perfusion when demand is low. This instability in blood flow can result in ischaemia-reperfusion injury and potentially GON as a consequence. Dysfunctional autoregulation is referred to in the literature as vascular dysregulation (275) and has been evidenced in glaucoma patients, both systemic (281) and ocular (282-285) with increased disturbance in those with NTG (286) and progressive GON (287).

1.13.2.2.2 Primary Vascular dysregulation
Primary vascular dysregulation describes an inherent inappropriate constriction (vasospasm) or inadequate dilation response of the microcirculation when stimulated which is present in some individuals (273). Within the eye the presence of primary vascular dysregulation could affect autoregulation, causing unstable blood flow and under/over perfusion to the retina and ONH hence increasing the likelihood of damage (273). It has been shown that vasospasm decreases OPP through increasing vascular resistance in the central retinal artery of primary vascular dysregulation patients compared to healthy controls (288). Primary vascular dysregulation syndrome has been linked with the
development of GON (289), with vasospasm shown to alter ocular blood circulation in NTG and POAG patients (290, 291). This provides support for the role that altered vascular regulation appears to play in the pathogenesis of GON.

1.13.2.2.3 Endothelial dysfunction

Endothelial dysfunction is introduced and explained in Section 1.8 along with the molecules ET-1 and NO. The endothelium is important in the regulation of systemic and ocular blood supply. A decrease in the availability of NO and increase in ET-1 results in excessive vasoconstriction and as a result, blood flow dysregulation (292, 293). As explained above, the endothelium plays an important role in the autoregulation of blood supply to meet demand, through the release of the vasoactive agents NO and ET-1 that act to increase and decrease the diameter of the blood vessels according to metabolic demand. A dysfunction of the endothelium and hence a failure in this autoregulation process can cause unstable blood flow to the ONH during periods of high neural activity, increasing the likelihood of glaucomatous damage through ischaemia-reperfusion injury (273). In NTG and POAG patients, levels of NO in the plasma have been found to be at a reduced level compared to healthy controls (294-296). ET-1 in the plasma has also been shown to be at higher levels in NTG (297, 298) and POAG patients (299, 300). In addition to ET-1 constricting vessels it has also been shown to facilitate an increase retinal venous pressure (RVP) (301). High RVP reduces perfusion pressure and hence reduces blood flow to ONH; which causes glaucomatous damage (301). In addition to indicating dysfunction by measuring plasma levels the clinical sign of ONH haemorrhages can be observed by viewing
the retina, ONH haemorrhages are associated with autoregulation and instability of ONH blood flow (302).

Endothelial dysfunction has been observed in the systemic macro-circulation of patients with NTG (256). Although this is important as it supports the vascular theory, endothelial dysfunction of the microcirculation is thought to be affected prior to macro-circulation (303). As mentioned in Section 1.9, the DVA can analyse microvasculature structure and measure altered retinal vessel response to flicker light which is thought to indicate any impaired endothelial function (302, 304). Garhofer et al. (2004) identified that POAG patients have a reduced venous dilation response compared to healthy controls (305). Further studies have shown that NTG patients have a reduced baseline-corrected flicker response (304) and found evidence of vascular dysfunction in both early-stage POAG and NTG compared to healthy controls (243). These associations infer that patients with GON have impaired autoregulation due to endothelial dysfunction.

As well as affecting the vasculature, endothelial dysfunction can affect the channels in the trabecular meshwork and canal of Schlemm. Endothelial cells line the trabecular meshwork channels, as outflow of aqueous humour occurs in the trabecular meshwork, any dysfunction located here would ultimately affect IOP (255, 293). Interestingly, levels of NO in the aqueous humour have been found to be significantly decreased in POAG patients (296) and in a separate study endothelin levels were found to be significantly higher than healthy control participants (299).
1.13.3 Oxidative stress and glaucoma

As described throughout this glaucoma section, glaucoma is a multifactorial disease. Oxidative stress is one factor that has been linked with glaucomatous RGC death (236, 306-311). Oxidative stress contributes to neurodegenerative damage via the mechanism of free radicals attacking the neural cells (96). The harmful effect the reactive species has on cells leads to tissue damage, oxidative injury and ultimately apoptosis (96, 104). It is thought that RGCs are susceptible to oxidative stress due to their high oxygen consumption (312) and light exposure (313).

In addition to affecting RGCs, oxidative stress is also thought to affect IOP. It has been shown that glaucoma patients have reduced antioxidant activity in the aqueous humour, suggesting increased oxidative stress is present (307, 314, 315). Increased levels of oxidative stress leads to inflammation, cellular loss and impairment and damage to trabecular endothelial cells (306, 316), affecting aqueous outflow and ultimately increasing IOP (317-319). As discussed in Section 1.13.2.1, increased IOP has been identified as a cause of retinal ganglion cell death.

Oxidative stress is also related to the vascular mechanisms of damage linked with glaucoma. As explained in 1.13.2.2 recurrent reperfusion injury refers to the damage caused to tissue when blood supply returns after a period of ischemia. During ischaemia, the absence of oxygen impairs electron transport, resulting in unused electrons. When the tissue returns to normal the additional electrons react with oxygen which leads to the formation of ROS (320). Astrocytes in the
ONH are susceptible to ROS. When activated, the astrocytes produce abnormal molecules such as ET-1 and NO which alter the cell environment (321). The activation of astrocytes is thought to be one of the mechanisms initiating inappropriate apoptosis of RGC’s in glaucoma. In addition, ROS also impact the vascular endothelium by promoting endothelial dysfunction through reduction in the bioavailability of the vasodilator NO (322).

The antioxidant glutathione (GSH) and total glutathione (t-GSH) in the plasma have been shown in glaucoma participants in be lower compared to healthy controls, suggesting a depleted antioxidant status, indicative of increased oxidative stress (323). A number of other oxidative stress biomarkers have also shown depleted antioxidant levels and increased pro-oxidants (107). Levels of the biomarker MDA has been found to be significantly correlated with glaucoma severity (109, 315). Recent studies have investigated reduction of oxidative stress as a possible treatment for glaucoma. The antioxidants alpha-lipoic acid delivered through the diet was found to decrease oxidative stress and increase protection of RGCs in mice (324). Similarly the antioxidant tempol was found to be beneficial in mice, the antioxidant caused a decrease in pro-inflammatory cytokines and decrease in decrease in NF-κB activation (325). The results of these animal studies suggest that antioxidant treatment may be a strategy for glaucoma prevention, although further research is required.
1.13.4 AGEs and glaucoma

The possibility that GON may develop as a manifestation of accelerated ageing has been suggested by previous studies (20, 326), and due to the known associations between accelerated ageing and AGE accumulation (142, 327), it is logical to hypothesise that increased AGE levels may contribute to the pathogenesis of glaucoma. To date, there have been few clinical studies that have been able to directly explore the relationship between AGEs and the development and progression of GON due to methodology restrictions, however as previously discussed, a number of the known associates of elevated AGE levels, namely increased oxidative stress, vascular dysfunction and altered ocular biomechanics, have been previously linked to the pathogenesis of GON. These associations and their potential relationship with AGE levels in glaucoma will be discussed further in these sections with relevance to the mechanical and vascular theories of glaucoma to show how AGEs could be hypothesised to be related throughout the glaucoma disease continuum.

1.13.4.1 AGEs in relation to the mechanical theory of GON

1.13.4.1.1 Accumulation of AGEs in the ONH

As discussed in Section 1.11.10 with age increased amounts of collagen cause the structure of the lamina cribrosa to alter, becoming stiffer and less resilient (203). These age-related collagen changes are thought to be linked with age related GON. As well as increased collagen present in the lamina cribrosa with age the AGE pentosidine has been found in increased amounts (203). Additionally Amano et al. (2001) investigated optic nerve specimens from diabetic
participants and the AGE pyrraline was located in the sclera, pia matter, cribriform plates, optic nerve connective tissues and around the vessels in the optic nerve (204). Tezel et al. (2007) measured the accumulation of AGEs (CML) in glaucomatous and age-matched control donor eyes and found that there was increased AGE and RAGE presence in those with more advanced age and those with glaucoma (20). The AGEs were primarily detected in the cribriform plates of optic nerve head, while RAGE was seen on the glial cells, mainly the Müller cells (20). It is thought that accumulation of AGEs in this region may have a role in the reduced ability of the cribriform plates to withstand strain and the increased rigidity of lamina cribrosa (20), leading to susceptibility to mechanical IOP induced damage (328).

1.13.4.1.2 Accumulation of AGEs in the trabecular meshwork

As previously explained in Section 1.5, AGEs accumulate in the extracellular matrix of tissues. In the trabecular meshwork the age related changes of the extracellular matrix is thought to contribute to increased outflow resistance (329), which in turn may contribute to elevated IOP in POAG (328). The decreased cellularity and structure changes of the trabecular meshwork may be attributed to AGE accumulation(116).

The increased presence of AGEs in glaucomatous tissues and the fact that AGEs accumulate with age supports the accelerated ageing theory that GON and the ageing process is related (20).
Corneal biomechanics is important as it is thought to be one of the hypotheses for why variations in IOP have a damaging effect on some optic nerve heads and not others (156). Central corneal thickness (CCT) and the curvature of the cornea are measurements known to influence measured IOP; eyes with increased CCT or steep corneas have an overestimated IOP, with the opposite true for decreased CCT or flat corneas (330). In addition, reduced CCT has been identified as an independent risk factor for GON (331, 332). The mechanism that causes this increased risk is not understood, although it is thought that improving the understanding of corneal biomechanics in eyes with reduced CCT may reveal a structural characteristic which makes the ONH more susceptible to damage (333).

CCT and corneal curvature are important biomechanical properties however they do not entirely explain IOP variations. It’s thought that corneal hysteresis is a biomechanical contributor to this variance (156). Corneal hysteresis is an assessment of the cornea’s ability to absorb and disperse energy, defined as; the difference between inward force to flatten the cornea and outward recovery (334). The resistance calculated is in theory related to viscoelastic corneal properties (334). The cornea’s viscoelastic behaviour is primarily derived from the stroma, and the stiffness that is known to increase with age is due to changes in the cornea’s extracellular matrix (328, 335, 336).

Corneal hysteresis in particular is of interest as lower hysteresis has been found in patients diagnosed with glaucoma (337), in addition corneal hysteresis has
been linked with progressive visual field worsening (337). Prata et al. (2012) similarly found that low corneal hysteresis is linked with glaucomatous nerve head signs; larger cup to disc ratio and increased cup depth (338), and in those with bilateral glaucoma, 75% of participants with lower corneal hysteresis had a larger cup to disc ratio (338). As explained in Section 1.11.2, AGEs accumulate in the collagen of the stroma with increased age (158) and encourage an increase in collagen fibrils (159). Hence it could be hypothesised that AGEs may be a contributing factor to the reduced corneal viscoelasticity seen with age and it could be further hypothesised that in individuals with elevated AGE levels, this age-related reduction in viscoelasticity could occur at an accelerated rate. There are no previous studies that have directly assessed the relationship between corneal hysteresis or viscoelasticity and tissue-bound AGE levels in healthy participants, or those with GON. It is possible that an increased rigidity of the extracellular matrix, not only in the cornea, but also throughout the structures of the eye, could contribute to overall ocular mechanical rigidity, including lamina cribrosa rigidity (338, 339).

The inclusion of OHT participants enabled the relationship of the structural properties between POAG, NTG and OHT to be examined. OHT patients have been shown to have a higher corneal hysteresis compared to patients with POAG and NTG (340). It is hypothesised that this relates to overall rigidity. Corneal biomechanic properties measured between groups as well as SAF will allow examination between altered rigidity and AGE level.
1.13.4.2 AGEs in relation to the vascular theory of GON

1.13.4.2.1 AGEs and endothelial dysfunction
The relationship between AGEs and endothelial dysfunction is explained previously in Section 1.8 with regard to specifically how AGEs create an imbalance in ET-1 and NO. In glaucoma research it has been reported there is an imbalance of ET-1 and NO (294, 296, 300). The associations between endothelial dysfunction and glaucoma were explained in more detail in Section 1.13.2.2.3. The exact relationship between AGE levels and microvascular endothelial function in healthy ageing and GON has however not previously been explored. It is possible that AGEs could potentially represent an underlying causative factor for retinal vascular dysfunction and hence could be suggested to contribute to the development of GON. The measurements taken by the DVA will help to indicate any impaired vascular endothelial function.

1.13.4.2.2 AGEs and structural alterations in the vasculature
As explained in Section 1.11.11 as part of the normal ageing process the vessels within the body experience; endothelial dysfunction, reduced elasticity, accumulation of collagen and increase in intimal medial thickness (209, 210). Elasticity of the blood vessels is dependent on the level of collagen fibres and collagen cross-links (211). AGE accumulation causes an increase in collagen covalent crosslinks (341), as a result there is an increase in stiffness of arteries (342). AGE accumulation also causes an increase in cross-links within elastin which also causes a reduction in blood vessel elasticity (136, 342). As discussed in Section 1.8 Reddy (2004) using diabetic rats, demonstrated this mechanism
and other studies have shown that increased vascular stiffness correlates with increased AGE accumulation (13, 138). Stiffness of arteries is a recognised risk factor for cardiovascular diseases (343). Increased systemic arterial stiffness has been shown in diabetic patients with glaucoma (344) and Mroczkowska et al. (2013) found evidence of vascular dysfunction in both POAG and NTG (243). However the relationship between increased vascular stiffness and glaucoma remains controversial as other studies have found relationships but none of which are statistically significant (345, 346). Altered dynamic retinal vessel function has been hypothesised to be linked to increased arterial stiffness and altered retinal vessel calibres infer an alteration in stiffness (304). Structural alterations in the retinal vessels will be analysed using the StaticVesselMap2 software (see Section 3.2.2.2.2).

1.13.5 AGEs measured with SAF in glaucoma

Only a few studies have used the AGE Reader (DiagnOptics B.V., Groningen, The Netherlands) and SAF in relation to glaucoma. The Schweitzer et al. (2018) study recruited 424 healthy controls and 31 POAG participants and found that high SAF was independently associated with an greater risk of glaucoma (115), they used SAF as a measurement of AGE level. A research group in Japan is also using SAF in glaucoma research (118, 119), however they use SAF as a biomarker of oxidative stress and not AGEs.
1.13.6 Summary of the potential mechanisms by which AGEs may link to glaucoma pathogenesis

The relationship of AGEs with the tissues associated with glaucoma and evidence from oxidative stress suggest the association between AGEs and glaucoma require further investigation. The accumulation of AGEs and RAGE detected in glaucomatous retina and ONH’s as well as AGEs accumulation in the collagen of the cornea presents the theory that AGE accumulation in these structures may be linked with the mechanical theory of GON. AGE accumulation in blood vessels as well as their negative effect on vascular function links AGE accumulation with the vascular theory of GON. No one has yet directly explored retinal vascular structure and function relationship with AGEs. The DVA and StaticVesselMap2 will help to build a picture of how AGEs may be linked with the vascular theory of GON. As the degree of mechanical and vascular involvement is thought to differ between POAG, NTG and OHT patients, evaluation of AGE levels between groups may improve understanding of the role AGEs play in GON. Any role AGEs do have could potentially be altered by advice about reduction of dietary AGEs if these are also found to be associated.

1.14 Strategies to reduce AGE level

It has been evidenced that AGEs have a strong pathological significance. As such research has been conducted with the aim of modifying/reducing AGE level, both exogenous and endogenous.
1.14.1 Restriction of dietary AGEs

As shown in Table 1.2 there are databases which have measured levels of AGEs within foods. Using these databases multiple studies have investigated the effect of AGEs on diet, predominantly using ‘high’ and ‘low’ AGE diets then measuring the resultant circulating AGE level, oxidative stress biomarkers and inflammation biomarkers. These studies are discussed in Section 1.4 and also listed in a Table summary in Appendix 9. It is generally considered that a reduction in dietary AGE level is beneficial to circulatory levels, however how this translates to tissue bound levels is not known.

1.14.2 Exercise and AGEs

Initial exercise AGE studies used animals, predominantly exercise trained rats, and with increased exercise there was decreased advanced glycation (347, 348). There are a few exercise AGE studies in humans. A study by Goon et al. (2009) found that participants performing tai chi twice a week had reduced plasma AGE levels and reduced levels of plasma MDA compared to the matched sedentary participants (349). In a different study the AGE CML was measured in relation to activity measured by a pedometer. It was found that serum levels of CML decreased with increased number of steps, this correlation was significant (350). The results of a study by Habacuc Macías-Cervantes et al. (2014) however found that exercise alone did not reduce serum AGE level, restriction of dAGE did reduce serum levels of AGE and when this was combined with exercise there was an improvement lipid profile (351).
1.14.3 Pharmacological Interventions and AGEs

Pharmacological interventions to reduce AGEs have been studied, they include agents that target AGE formation and reduce crosslinks as well as agents that block AGE-RAGE reactions (8). A few specific drugs have been studied in relation to AGEs. The drug metformin reduced serum levels of AGE in women diagnosed with polycystic ovary disease (352) and that the lipase inhibitor Orlistat also reduced serum AGE levels in women with polycystic ovary disease (353). In a group of diabetic patients with kidney disease it was found that the drug candesartan reduced CML levels measured from patient’s urine (354).

As explained in Section 1.5.3, soluble RAGE (sRAGE) is different to the cell-surface RAGE and is considered beneficial. Pharmacological agents have been studied in relation to regulating sRAGE. In a study using rats inhibiting the angiotensin-converting enzyme (ACE) resulted in increased expression of sRAGE (355). A relationship has been found between plasma levels of sRAGE and coronary artery calcium, with increased sRAGE inversely associated with coronary atherosclerosis (356). An atherosclerosis study found that the insulin sensitising thiazolidinedione medications increased levels of sRAGE (357). It is thought therefore that thiazolidinedione medications may be promising for regulating sRAGE (355). The pharmacological agents being researched are in the early stages of being trialled (358, 359), therefore effective safe treatments are not yet available to prescribe to patients.
2. Research rationale

AGEs are complex compounds with a potentially large impact both on the healthy ageing population and those diagnosed with pathology. Enhancing our understanding of the role that AGEs may play in accelerating the development of GON has the potential to offer additional insight into GON pathogenesis.

Total tissue-bound AGE level in the body includes AGEs produced endogenously, via normal glycation reactions occurring within the body, and the AGEs that are taken in exogenously, mainly through diet or smoking (as discussed in Sections 1.2 and 1.4). Measurement of tissue-bound AGE levels via skin auto-fluorescence does not allow the relative contribution of exogenous (dietary) and endogenous sources of AGE to be determined, which can make targeted treatment difficult. Dietary AGE intake represents a potentially modifiable target (360), however there is currently no UK specific AGE FFQ to measure dietary AGE intake (14, 56). Through the design of a specific and targeted FFQ, this research will investigate the contribution of dietary AGE to tissue-bound AGE levels with the aim of evaluating the viability of dietary treatment as an option to reduce the overall levels of AGE in the body and potentially reduce the development of age-related disease such as glaucoma.

When considering the influence that AGEs may have on the development of age-related disease, it is important to also take into consideration oxidative stress. Evidence suggests that oxidative stress is also part of the normal ageing process (114), in addition to this there is a complex relationship between AGE levels and
oxidative stress levels in the body. Indeed, an increase in AGE levels is both a cause and an effect of elevated oxidative stress (9), with oxidative stress accelerating AGE formation and the production of free radicals, as well as being produced as a by-product of AGE formation and AGE-RAGE binding interaction (see Section 1.7) (112, 113). AGEs are considered to be pro-oxidant substances, due to their formation increasing the cellular level of oxidative stress (361, 362).

To date, few studies have explored the possibility that AGEs may be an underlying causative factor for GON (20, 115, 204). It could be hypothesised that increased accumulation of AGEs could be the underlying causative factor for a number of the known pathological associations of GON and ultimately contribute towards its development in some individuals through acceleration of the ageing process. Establishing an association between AGE levels and these parameters in POAG, NTG and OHT patients therefore could increase our understanding of the pathogenesis of GON and potentially allow a new biomarker for accelerated ocular ageing, in the form of tissue-bound AGE levels to be realised. This evaluation is interesting as POAG, NTG and OHT are thought to have differing levels of vascular and mechanical involvement.
2.1 Research aims

- To develop and validate an AGE specific semi-quantitative FFQ.

- To determine whether dietary AGE intake and circulating oxidative stress levels are associated with tissue-bound AGE levels (SAF) in a healthy ageing population as well as to explore the influence of BMI, smoking history, alcohol consumption, body fat percentage and blood pressure on oxidative stress, SAF and dAGE, in the same population.

- To determine whether tissue bound and dietary AGE levels are elevated in POAG and NTG in comparison to age-matched OHT patients and healthy controls. Oxidative stress levels will be compared as a possible influencing factor.

- To determine how tissue-bound AGE levels are associated with static and dynamic retinal vessel parameters and corneal viscoelasticity in a healthy ageing population.

- To evaluate whether level of tissue bound AGEs (SAF) relates to static and dynamic retinal vessel structure and function and corneal viscoelasticity in POAG, NTG and OHT patients.

- To explore whether dynamic retinal vascular parameters are related to corneal viscoelastic parameters in HC, OHT, NTG and POAG patients.
3. Participants and Methods

3.1 Recruitment

All participants for the studies that make up this programme of work were recruited using convenience sampling. The study’s inclusion and exclusion criteria indicated which potential participants to approach. Written informed consent was received from all participants prior to study enrolment, and all study procedures were designed and conducted in accordance with the tenets of the Declaration of Helsinki. Four different participant groups were recruited for this study; healthy control, early POAG, early NTG and OHT.

The study design ensured that the recruiter did not apply undue pressure. In particular, it was ensured participants were fully aware that there was no personal benefit in terms of specialist care. As a registered health professional, the ability to judge capacity to provide valid consent is a familiar challenge, as the ethical principles of giving informed consent are core to professional optometric practice.

3.1.1 Participant recruitment

3.1.1.1 Recruitment of participants with early stage Primary Open Angle Glaucoma, early stage Normal Tension Glaucoma and Ocular Hypertension POAG, NTG and OHT participants were identified through their attendance at their routine outpatient’s appointment at Derriford Royal Eye Infirmary (REI). Participants that fit the study inclusion/exclusion criteria were identified by Mr Adam Booth FRCOphth PhD (Consultant Ophthalmic and Glaucoma Surgeon at the REI, Derriford Hospital, Plymouth) or a member of his team, and provided
with information about the study. If willing, suitable participants were either referred directly for further information and given a copy of the patient information sheet (Appendix 2.3 and 2.5), or asked whether they were happy for their contact details to be passed on and to be contacted at a later date.

**3.1.1.2 Recruitment of healthy control participants**

Some healthy control participants self-presented for involvement in the study following advertisement and promotion of the study within and around Plymouth University and through our Centre for Eyecare Excellence (CEE). In addition some suitable friends and relatives of the glaucoma or OHT participants also self-presented for involvement. After confirmation that the participant met the inclusion/exclusion criteria they were provided with the information sheet (Appendix 2.1).

Active recruitment of healthy control participants was also carried out through CEE at Plymouth University. Any patient who appeared to fit the inclusion/exclusion criteria for the study was given the opportunity to participate. They were provided with verbal information about the study and given the participant information sheet to review after their routine eye examinations. In addition, CEE patients who had consented to being contacted for research purposes were contacted if they fit the study criteria. These participants were informed about the study and if interested, provided with an information sheet by post inviting them to participate. The participants were given time to consider their involvement in the study.
3.1.2 Consent

Fully informed written consent was obtained at the outset of the study, in accordance with the principles of the Declaration of Helsinki (363) and Good Clinical Practice (GCP) (364). Persons who were deemed vulnerable or unable to give fully informed consent were not invited to take part. Participants were informed of their right to withdraw at any time, and no coercion or undue influence was used to encourage participation. A copy of the consent form can be found in Appendix 2.

3.1.3 Inclusion criteria for all study groups

• All participants had to be 50 years or older and have completed a comprehensive eye exam in the last two years.
• Informed and written content was required for participation in the study and all participants had to have the capacity to give informed consent themselves.
• All participants had to have adequate understanding of English language to be able to comprehend the oral and written instructions.
• All participants had to be able to complete a 12-hour overnight fast, which includes no alcohol or caffeine.

3.1.3.1 Definition of Ocular Hypertension (OHT)

For the purposes of this study OHT was defined as intraocular pressure (IOP) above 21 mmHg with no significant visual field defect and no signs of optic nerve head damage. Central corneal thickness was required to be less than or equal to 600 μm. Only reliable visual field results, with <20% fixation losses and <33% false positive and false negative responses were considered. The diagnosis of
OHT according to these criteria was made by Mr Adam Booth or one of his ophthalmology team.

3.1.3.2 Definition of early stage Normal Tension Glaucoma (NTG):
NTG patients were required to be in the early stage of their disease process and were only included if they met the definition outlined; for the purposes of this study early stage NTG was defined as patients with IOP equal to or below 21 mmHg, with a mild glaucomatous visual field defect and/or optic nerve heads changes indicative of glaucoma. A mild glaucomatous visual field defect was defined as a Mean Deviation (MD) between 0 and -6.00 dB along with either a Glaucoma Hemifield Test “outside normal limits” and/or a pattern standard deviation with p-value <0.05. Only reliable visual field plots, with <20% fixation losses and <33% false positive and false negative responses were considered. The diagnosis of NTG according to these criteria was made by Mr Adam Booth or one of his ophthalmology team.

3.1.3.3 Definition of early stage Primary Open Angle Glaucoma (POAG)
POAG patients were required to be in the early stage of their disease process and were only be included if they met the definition outlined; for the purposes of this study early stage POAG was defined as intraocular pressure above 21 mmHg with a mild glaucomatous visual field defect and/or optic nerve head changes indicative of glaucoma. A mild glaucomatous visual field defect was defined as detailed for NTG participants as above. The diagnosis of POAG according to these criteria was made by Mr Adam Booth or one of his ophthalmology team.
3.1.4 Exclusion criteria

Participants younger than 50 years were excluded, and there was no upper limit for age. This criterion was set since glaucoma is an age-related condition and has an older patient population (365).

Participants were excluded if they were smokers or had a positive diagnosis of severe cardio- or cerebro-vascular disease such as coronary artery disease, heart failure, arrhythmia, angina, stroke, transient ischaemic attacks, peripheral vascular disease, severe dyslipidaemia, diabetes, as well as other metabolic disorders. Any individuals suffering from chronic systemic diseases known to influence oxidative stress or taking cardiovascular preventative medication, hormone replacement therapy or antioxidant supplements were also not included. The previous lists of exclusions from the study are due to the possible effects on AGE level, oxidative stress and vascular function.

Participants with symptomatic cataract and/or cataract of grade 3.0 or above according to the Lens Opacities Classification System III (LOCS III) were excluded from the study because of the potential influences that cataract may have on the accurate conduction of DVA analysis. Due to the flicker light used in DVA testing any patients with photosensitive epilepsy were excluded from the examination.

All of the healthy control participants were screened for glaucoma and other ocular disease and were excluded if any signs consistent with glaucomatous optic neuropathy, ocular hypertension or retinal disease found. The glaucoma patient group were also be screened for ocular disease other than glaucoma and
excluded if any other ocular disease was revealed. The glaucoma patients were required to be in the early stage of their disease process as the DVA analyses the microvasculature and in vascular disease microcirculation is believed to be affected earlier than macro-circulation (303, 304). In addition it is known that ocular blood flow decreases in glaucoma with increased damage (120), hence investigation of the blood vessels prior to any damage is necessary to investigate any underlying causative factor. Recruitment of patients in the early stage of their disease process also enabled progression follow up to be possible.

A finger prick test was used prior to blood sampling to ensure the participant did not have high glucose levels. Normal blood sugar glucose levels after fasting were considered to be: 3.5–5.5 mmol/l, prediabetics/impaired glucose tolerance: 5.6–7.0 mmol/l and diagnosis of diabetes: more than 7.0 mmol/l. If the participant had a fasting glucose level of 7.0 mmol/l or above they were be excluded from the study due to the already established link between diabetes and AGE levels. In addition their GP was informed of their high fasting glucose level.

Potential participants incapable of giving informed consent due to reduced capacity, problems with communication or language that prevents that patient from fully understanding the procedure and nature of the study were excluded.
3.2 Ethical approval

Ethical approval was received from the Health Research Authority (HRA), Plymouth Hospitals R & D department and Plymouth University Ethics Committee (Appendix 1). Written informed consent was received from all participants before participating in the study and all procedures were designed and conducted in accordance with the tenets of the Declaration of Helsinki.

3.3 Methods

All techniques described in this chapter were performed by the author, including phlebotomy and blood sample analyses. Prior to commencing recruitment full training was received for all techniques to ensure reliability of data collection. Phlebotomy training took place at Derriford hospital and lab analysis training provided by Dr Desley White.

This programme of research will address six aims (Chapters four to nine). The methodology that will be implemented in this research is summarised in this section to provide an overview of techniques. All of the investigative techniques that form part of this research are detailed in Table 3.1.
### Table 3.1: Summary of the investigative techniques conducted in this research.

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Technique</th>
</tr>
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<tbody>
<tr>
<td><strong>Ocular</strong></td>
<td></td>
</tr>
<tr>
<td>Assessment of ocular vascular function and structure</td>
<td>Dynamic Retinal Vessel Analyser (DVA)</td>
</tr>
<tr>
<td>Assessment of corneal biomechanics</td>
<td>Corvis ST</td>
</tr>
<tr>
<td><strong>Systemic</strong></td>
<td></td>
</tr>
<tr>
<td>Measure height</td>
<td>Stadiometer</td>
</tr>
<tr>
<td>Measure weight and body fat percentage</td>
<td>Tanita body composition analyser</td>
</tr>
<tr>
<td>Measure blood pressure</td>
<td>Scian LD578</td>
</tr>
<tr>
<td>Measure blood glucose</td>
<td>Freestyle Optium Neo glucose monitor</td>
</tr>
<tr>
<td>Measurement of tissue-bound AGE</td>
<td>AGE Reader</td>
</tr>
<tr>
<td>Measure dietary AGE level</td>
<td>Food Frequency Questionnaire (FFQ)</td>
</tr>
<tr>
<td><strong>Plasma analysis – Fasted venous blood sample</strong></td>
<td></td>
</tr>
<tr>
<td>Biomarker for oxidative stress</td>
<td>Plasma levels of malondialdehyde (MDA)</td>
</tr>
<tr>
<td>Indicators of antioxidant status</td>
<td>Plasma levels of vitamin E</td>
</tr>
<tr>
<td>Indicators of antioxidant status</td>
<td>Plasma levels of ascorbate</td>
</tr>
</tbody>
</table>

3.3.1 Visit routine/procedures

The procedures and visit routine are detailed in each participant information sheet (Appendix 2). The different techniques used to address each aim are stated in each individual chapter. The recruitment and clinical tests for all groups were recruited and measured simultaneously with no one group set of data collected first.
3.3.2 Specific methods

3.3.2.1 General investigations

A demographic questionnaire was used for all participants to collect information such as: date of birth, gender, ethnicity, education, smoking history, alcohol consumption, general and ocular health history as well as current medication. As well as providing this data, the demographic questionnaire also served as a ‘check’ to ensure each participant met the inclusion/exclusion criteria. Data such as height, weight, body mass index (BMI) were collected and used to build a picture of overall health as well as aiding the match between healthy control participants and OHT, NTG and POAG participants.

Height was measured in meters using a stadiometer. Participants removed their shoes and socks and were asked to stand with their feet flat on the floor and to stand tall. Weight was measured in kg, patients were asked to remove heavy items. Weight, basal metabolic rate (BMR), and body fat percentage were measured using the Tanita body composition analyser (TBF-300 MA). BMI was calculated by; Dividing the participant’s weight in kilograms by their height in metres, and then dividing the answer by the participant height again.

Blood pressure was measured using a digital blood pressure monitor, Scian LD578 (GF Health Products., Atlanta, USA). The cuff size chosen for the measurement met the arm diameter requirements. A finger prick blood sugar test was performed prior to obtaining the blood sample to ensure normal fasting blood glucose levels. As mentioned previously (section 3.1.4), high glucose levels (7.0 mmol/l or more) resulted in exclusion. A fasting venous blood sample was
obtained to enable the measurement of oxidative stress biomarkers, venesection and sample handling is described in Section 3.3.2.6.1.

All healthy control participants and OHT, NTG and POAG participants had the health of their eyes examined and vision measured by the principal investigator (LS). Proxymetacaine 0.5% (Bausch & Lomb minims) drops were inserted and Goldmann tonometry performed. Tropicamide 0.5% (Bausch & Lomb minims) drops were inserted for pupil dilation which was required for dynamic and static vessel analysis with the DVA.

Results from these general and ocular health investigations were used for vascular calculations. Mean arterial blood pressure (MABP) was calculated using the blood pressure results, shown in equation 3.1. Ocular perfusion pressure (OPP) was calculated using MABP and IOP result, shown in equation 3.2.

\[
\text{MABP} = \frac{2}{3} \text{DBP} + \frac{1}{3} \text{SBP}
\]

Equation 3.1: MABP: mean arterial blood pressure, DBP: diastolic blood pressure, SBP: systolic blood pressure

\[
\text{OPP} = \frac{2}{3} (\text{MABP} - \text{IOP})
\]

Equation 3.2: OPP: Ocular perfusion pressure, MABP: mean arterial blood pressure, IOP: intraocular pressure
3.3.2.2 Dynamic retinal vascular function (DVA)

The Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) was used to assess both dynamic and static retinal vessel function. The DVA is a non-invasive device that evaluates the dynamic behaviour of retinal vessels through the continuous measurement of vessel diameter changes in response to flicker light provocation (366, 367). Dynamic retinal vessel parameters determined were baseline diameter fluctuation (BDF), baseline corrected flicker response (BFR), maximum vessel diameter, minimum vessel diameter, reaction time, percentage dilation and percentage constriction response. The DVA has been widely used as a measure of retinal microvascular function in both healthy ageing and ocular and systemic disease research (243, 368-370). Static retinal vessel parameters were measured using the VesselMap2 software on the DVA device. Measured parameters included central retinal artery equivalent (CRAE), central retinal vein equivalent (CRVE) diameters and arterio-venous ratio (AV ratio) (371).

The DVA device comprises a fundus camera (FF450, Zeiss Jena, Germany), a digital high-resolution charged coupling device (CCD) camera, CCD video camera, a computer with analysis software and a printer (Figure 3.1) (366, 372). An additional green (red-free) filter is inserted for dynamic retinal vessel analysis as this provides contrast for vessel visualisation (366). This filter is removed for static vessel analysis.
3.3.2.2.1 Technical specifications

The DVA and static VesselMap2 technical specifications are summarised in Table 3.2. As well as being non-invasive the DVA has high reproducibility (373), low variability (374), while allowing different vessel segments of artery and vein to be measured simultaneously. However, the DVA has shortcomings; it relies on the participant being able to hold stable fixation for 350 seconds, clear media to ensure sufficient image quality. Dynamic vessel analysis requires full pupil dilation, and the calculations performed by the software assume that the eye is emmetropic. The VesselMap2 software is non-invasive and requires the participant to fixate for a short period of time (approximately 1 minute). The vessel diameter for Vessel Map2 is measured in units (MU) (372), in Gullstrand’s ‘normal eye’ 1 MU is also defined as 1 μm (375).
### Table 3.2: Technical specifications of the DVA and Static VesselMap2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DVA value</th>
<th>Static VesselMap2 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spatial resolution</td>
<td>10 μm</td>
<td>10 μm</td>
</tr>
<tr>
<td>Scaling factor</td>
<td>12.3 μm/Pixel * 7 &gt;= ~ 85 μm</td>
<td>5.35 μm/Pixel * 7 &gt;= ~ 40 μm</td>
</tr>
<tr>
<td>Image field angle</td>
<td>30 degrees</td>
<td>50 degrees</td>
</tr>
<tr>
<td>Measuring time</td>
<td>350 seconds (although can be up to 10 min)</td>
<td>≤ 2 min</td>
</tr>
</tbody>
</table>

Table 3.2: Technical specifications of the DVA and Static VesselMap2 (372).

#### 3.3.2.2.2 Measurement of dynamic retinal vessel function

The DVA uses flicker light to instigate a vessel response and assess dynamic retinal vessel function (376). Flicker light increases the neural activity of the retina, which in turn increases the metabolic demand of the retina (377, 378). In a healthy person this heightened metabolic demand triggers the retinal endothelium to release more nitric oxide (NO) which causes vasodilation and consequently increases blood flow to meet the increased demand (379), as Figure 3.2 illustrates.
Increased metabolic demand of the photoreceptors

Retinal endothelium triggers increase in NO

Vasodilation

Increased blood flow

Figure 3.2: Pathway response to flicker light

This process is referred to as neurovascular coupling, see section 1.13.2.2.1. Flicker light as a stimulus is useful as it has the ability to stimulate the retina without involving any other vascular/microvascular bed. When normally functioning, both arteries and veins respond to flicker light with dilation. The arterial response is typically more marked than veins. Furthermore, whilst the arteries react almost instantly, the venous response is delayed several seconds (380, 381).
The DVA uses a flicker frequency of 12.5 Hz; this is within the maximum sensitivity (10-20 Hz) for the human visual system (380) and has been shown to be a suitable frequency for stimulation of retinal vessels (371, 376, 380, 381).

The duration of exposure to flicker is 20 seconds as the maximum vessel reaction has been shown to occur during this time (380). After the 20 second flicker ends vasodilation ceases and vasoconstriction begins. During the baseline that follows the end of the flicker there has been shown to be an overshoot period that lasts 6-10 seconds. Between 10-40 seconds after flicker has ceased the vessels will be at their minimum diameter, after which the vessel diameter returns to baseline (376, 380).

![Figure 3.3: Dynamic profile of an individual’s artery and vein response.](image)

The camera is positioned to provide the optimal illumination and the participant is directed to fixate on a fixation needle. This needle can be moved to enable a centre view of the area of interest. The real time video monitor allows vessel selection where the practitioner deems most appropriate, see Figure 3.4. From this area a section of retinal artery and vein approximately 1 to 1.5 disc diameters
away from the ONH and approximately half a disc diameter in length was selected, tortuous vessels, vessels with crossings and vessels very close to one another were avoided as per the recommendation outlined by Imedos (372, 382, 383).

The diameter of the vessel is calculated by an algorithm within the system which detects differences in reflectance. The selected portions of artery and vein automatically begin to be measured once the vessel selection is completed. Each selected vessel's diameter is continuously monitored during the procedure (total of 350 seconds). The first 50 seconds of baseline measurement are conducted in apparent constant illumination (25 Hz). This is followed by three flicker cycles (12.5 Hz) with 80 seconds of recovery (25 Hz) between each cycle, as shown in

Figure 3.4: Vessel selection for DVA measurement.
Figure 3.5. This length of test and inclusion of three flicker cycles was designed to enable the calculation of an average vessel response within an acceptable testing time length (376).

The procedure outlined above is in accordance with established protocols for carrying out assessments with the DVA (382, 384). Before testing began systemic blood pressure and pulse rate was measured. All participants were instructed to avoid alcohol, tobacco, nicotine, extreme exercise, unnecessary sleep deprivation, caffeine containing products or any dietary supplements/vitamins 12 hours prior to the study session due to their possible effect on retinal blood vessel function (382). However, they were asked to maintain normal eating habits as fasting can cause abnormal vascular activity (385). As mentioned previously in the exclusion criteria 3.1.4, participants taking vasoactive systemic drugs were excluded.

3.3.2.2.3 DVA dynamic output and parameters
The DVA algorithm calculates a baseline measurement, the results are based on this and expressed as percentage to baseline (141). The DVA software can analyse the dynamic response profile, and this output has been used by multiple
studies (386-389). However the DVA software averages all three cycles in combination such that responses from individual cycles cannot be distinguished. It also makes its calculation of maximum vessel diameter using an average of the vessel diameter in the last three seconds of flicker and first three seconds post flicker (17-23 seconds) Therefore using this method alone individuals who reach maximum dilation outside of this specified time window would have their dilatory response underestimated (390). In addition, the DVA software does not consider the baseline fluctuation of vessels diameter (BDF) that occurs in a normal resting state. This fluctuation is physiological and occurs due to arterial pulsation and vascular tone (384). Baseline corrected flicker response (BFR) is a calculation that accounts for BDF, it uses dilation amplitude (DA) and subtracts BFR to give BDF. This equation was established by Nagel et al. (2004) and has been used in other research using the DVA (243, 384, 390). For this reason, this thesis has not used the DVA software calculations.

Sequential and diameter response analysis (SDRA) was developed by Heitmar et al. (2010), it uses raw data from the DVA, which allows BDF, DA and BFR to be calculated in addition to percentage dilation and constriction (141, 390, 391). In a similar manner to SDRA, this thesis uses the raw data from the DVA to calculate the relevant parameters, see Table 3.3
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline diameter fluctuation (BDF)</td>
<td>The maximum range in vessel diameter during first 30 seconds of baseline readings (difference between max diameter and min diameter at baseline)</td>
</tr>
<tr>
<td>Dilation amplitude (DA)</td>
<td>Amplitude of the dilation response; difference between the maximum and minimum diameter (DA = MD – MC).</td>
</tr>
<tr>
<td>Baseline corrected flicker response (BFR)</td>
<td>Change in vessel diameter after considering the baseline diameter fluctuation (BFR = DA – BDF).</td>
</tr>
<tr>
<td>Maximum percentage dilation (MD%)</td>
<td>The percentage change in vessel diameter from baseline to maximum following flicker onset</td>
</tr>
<tr>
<td>Reaction time (RT)</td>
<td>Time taken to reach maximum diameter after flicker onset.</td>
</tr>
<tr>
<td>Minimum percentage constriction (MC%)</td>
<td>The smallest vessel diameter below baseline following cessation of flicker</td>
</tr>
<tr>
<td>Constriction time (CT)</td>
<td>Time taken to reach the point of maximum constriction following flicker cessation.</td>
</tr>
</tbody>
</table>

Table 3.3: Parameters calculated from raw data used for analysis of DVA response used in this thesis

The window of measurement for maximum percentage dilation was chosen to begin at 30 seconds, as the flicker commenced, and ended 10 seconds after flicker cessation to allow delayed dilation responses to be measured. Figure 3.3 is an example of a participant’s dynamic profile, the maximum dilation for the vein response is during flicker, however the artery maximum dilation response is delayed. Minimum percentage constriction was measured from flicker cessation at 50 seconds, until 90 seconds. Again, setting a wider window allowed any delayed response to be recorded.
Figure 3.6: Dynamic retinal vessel analysis profile with parameters used labelled.
The three measurement cycles outputted from the DVA raw data were assessed for quality, the cycles were reviewed individually and graded for inclusion/exclusion. Firstly, the raw data was checked and any cycle with a large number of missing values was excluded, this was checked both by looking at the list of data and visually at the cycles produced. Figure 3.7 is an example of three reviewed cycles; cycle 1 and 3 would be included, however cycle 2 has missing data at 30, 45 and 65 seconds. Due to the number of missing values the second cycle would therefore be excluded.

Figure 3.7: Shows the three stimulation cycles for the selected artery and vein. In this example based on missing readings cycle 2 would be excluded and cycle 1 and 3 included.
During the dynamic retinal vessel analysis trace artefacts can occur due to effects such as image noise, blinking, poor image quality, head or eye movement, change in illumination, and the measurement ‘jumping’ to a neighbouring vessel (392). Examples of common artefacts are shown in Figures 3.8 and 3.9. Cycles with a large number of artefacts were excluded.

![Image noise artefact](image1.png)

![Measurement ‘jump’ to neighbouring vessel](image2.png)

Figure 3.8: Illustration of common artefacts seen in the dynamic ‘real time’ trace

![Image noise](image3.png)

![Gaps in data due to blinking](image4.png)

Figure 3.9: The dynamic vessel trace of a participant in the first 14 seconds of measurement and the artefacts present.

In addition to checking for missing values and artefacts in the ‘real time’ trace the data was smoothed over the whole 350 seconds to check once more for any artefacts or errors. Figure 3.10 and 3.11 show examples of a trace that would and would not be included.
3.3.2.2.4 Measurement of static retinal vessel parameters

The VesselMap2 software (ImedosSystems, Jena, Germany) enables the retinal vasculature structure to be examined and evaluated (393). Static vessel analysis requires a disc centred red free fundus image. Insufficient image quality can result in errors when analysing, hence the device was set up to ensure optimal illumination and focused to produce the best possible images. Once the participant and equipment were in position 3-5 monochromatic disc centred fundus images were taken, the three best images were chosen to be analysed using the VesselMap2 software. Three images were taken as eye movements can cause vessel diameter variation as can pulse waves, the images taken were analysed and the average used to allow for this (375, 384).
Once the image was selected the ONH was manually marked with a superimposed ring. From this ring two additional superimposed rings were automatically generated by the software, with the first concentric ring 0.5 disc diameters (DD) away from the edge of the ONH, and the second 1DD away (394). The grader was then required to manually select all arteries and veins segments within the outer ring section, see Figure 3.12. The software automatically prevents selecting vessels 45 μm and smaller as vessels this size can be neglected (395), this is due to vessels this size having insufficient vessel edge resolution to be measured accurately (396). In accordance with the guidelines set out by the software manufacturers, the grader, who was the primary investigator, manually checked the vessel selections, ensuring that all branched vessels had the ‘branches’ selected and not the ‘trunk’ as well as ensuring each vessel has only been selected once. Once the vessels had been
selected the programme automatically included each vessel’s diameter to its Parr-Hubbard calculation and output CRAE, CRVE and AVR (393, 394), discussed further in section 3.3.2.2.5. As mentioned, the vessel selection was completed on each of the 3 images and the 3 sets of data output were averaged.

Subjective assessment of AVR has traditionally been used by practitioners to assess static retinal vessel parameters, however semi-automated analysis of AVR has been shown to be more reproducible, sensitive, specific and less observer-dependent (397). The parameters CRAE and CRVE are being increasingly used in research. Narrowing of CRAE is anticipated as part of the normal ageing process, as narrowing of arteries with age occurs throughout the body. However reduced CRAE has also been found to be associated with systemic hypertension (396, 398, 399) as well as kidney disease and heart disease (400, 401). Furthermore, an increase in CRVE has been found to be associated with obesity, dyslipidaemia, markers of vascular endothelial dysfunction (398, 402) and also associated with vascular dementia (403), diabetes (398, 404), cardiovascular disease and stroke (401, 405).

CRAE and CRVE have also been linked with ocular diseases. An increase in CRVE has been linked with incidence of proliferative retinopathy, and an increase of both CRAE and CRVE linked with progression of retinopathy in type one diabetics (406). Age-related macular degeneration is associated with increased CRVE (407) and narrowing of CRAE is associated with POAG, however it is not known if this narrowing is a cause or result of neurone loss (265). As such, participants recruited for this research were required to be in the early stage of
their disease process. Although AVR is more familiar in optometric practice, it is ambiguous in that an alteration in the diameter of the artery and or an alteration in the diameter of the vein can each cause an altered AVR. CRAE and CRVE provide much more information on the state of the retinal vasculature and have therefore been shown to be more profound biomarkers for systemic and ocular disease.

3.3.2.2.5 DVA static output and parameters
Semi-automated measurement of retinal artery and vein diameters is being increasingly used in vascular research. The static VesselMap2 software calculates AVR, CRAE and CRVE using the disc centred red-free fundus photograph and manually selected vessels, described above, with the Parr-Hubbard formula (396).

The Parr-Hubbard formula combines the vessel diameter measurements taken from the concentric ring to create an estimate of the larger trunk diameters. These trunks are then combined to give a single estimation for artery and vein; CRAE and CRVE (393, 396, 397). The Knudtson formula is the alternative method used by some semi-automated software (408). Unlike the Parr-Hubbard formula the Knudtson formula is based upon selection of the 6 largest arteries and veins within the concentric measurement ring. Both of these formulas are effective hence choice between the two is not imperative so long as the same formula is applied throughout (394). The VesselMap2 software uses the Parr-Hubbard formula hence this formula was used throughout this thesis.
The static output from the Vesselmap2 software is shown in Figure 3.13. It shows in the bottom left table the AVR, CRAE and CRVE for each image analysed. The three images for CRAE and CRVE are plotted on a graph, red cross for CRAE and blue cross for CRVE. In this example the three measurements are close to one another and this is reflected in the graph.

Figure 3.13: Static vessel analysis results sheet from Vesselmap2 software

Within this research CRAE and CRVE was used to assess vascular diameter changes in healthy ageing population as well as examining the narrowing or widening that takes place in the NTG, POAG and OHT groups.
3.3.2.3 Measurement of corneal parameters

The viscoelastic properties of the cornea was assessed using the Corvis ST (Oculus; Optikgeräte GmbH, Wetzlar, Germany) (Figure 3.14). The Corvis ST is a noncontact tonometry system integrated with an ultra-high speed Scheimpflug camera which allows dynamic real-time inspection of the corneal deformation process; speed of deformation and corneal deformation amplitude as well as measuring IOP and central corneal thickness (CCT) (409, 410). Full list of parameters measured by the Corvis ST is listed in Appendix 7. The parameters measured by the Corvis ST are linked with corneal biomechanics, an area of interest in relation to both AGES and glaucomatous optic neuropathy.

Figure 3.14: Corvis ST (Oculus; Optikgerate GmbH, Wetzlar)

The ultra-high speed Scheimpflug camera has a resolution of 640 x 480 pixels and frame rate of 4,330 images per second. The corneal deformation recorded with this camera is over 8.5mm horizontal coverage of the central cornea. The pachymeter used for measuring CCT has a measurement range of 200-1,200 μm.
and uses corneal section images taken (without ‘air-pulse’ effect) (410, 411). The non-contact tonometer has a measurement range 6-60 mmHg (411). The tonometer air puff is applied to the corneal apex while the camera records the air-pulse reaction of the cornea i.e. the cornea moving inwards and recovery outwards (409, 411). The Corvis-ST’s accuracy has been shown to be comparable with the gold standard equivalent techniques for IOP and CCT measurement (410).

The Ocular response analyser (ORA) is also a non-contact tonometer and it measures two corneal biomechanical parameters. The parameter corneal hysteresis is used to represent corneal viscoelasticity and the parameter corneal resistance factor to indicate the degree of elastic resistance (334). Unlike the ORA the Corvis ST measures the deflection behaviour of the cornea as it deforms rather than describe a specific biomechanical characteristic (412). A previous study by Matsuura et al. (2016) found statistically significant correlations between Corvis ST parameters and ORA measurements, however these correlations were not strong (413). It is thought that this may be due to the Corvis ST measuring further parameters which may reflect features of corneal biomechanics not measured by the two ORA biomechanical measurements (413).
3.3.2.3.1 Corvis ST output and parameters

The Corvis ST uses the captured sequence of images of the corneal deformation to calculate a range of parameters, the parameters used in this thesis are listed in Table 3.4 and Figure 3.15 shows the corneal deformation during the Corvis ST measurement.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time A1 (ms)</td>
<td>Measures the time from the initiation of the air puff to the first inward corneal movement</td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>Measures the time to second applanation / outward corneal movement</td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>Velocity of corneal surface movement at the first applanation</td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>Velocity of corneal surface movement at the second applanation</td>
</tr>
<tr>
<td>Deformation amplitude A1 (mm)</td>
<td>The magnitude of the movement of the corneal surface at the first applanation</td>
</tr>
<tr>
<td>Deformation amplitude A2 (mm)</td>
<td>The magnitude of the movement of the corneal apex at the second applanation</td>
</tr>
<tr>
<td>Highest deformation amplitude (mm)</td>
<td>The magnitude of the movement of the corneal surface at the highest concavity</td>
</tr>
<tr>
<td>Highest concavity time (ms)</td>
<td>Time from the initiation of the air puff to the highest concavity of the deformation of cornea</td>
</tr>
</tbody>
</table>

Table 3.4: Corvis ST parameters with descriptions. A1: first applanation 1, A2: second applanation.
Figure 3.15: shows the corneal deformation during the Corvis ST measurement. (A) shows an image prior to air puff applanation, (B) shows an image in the first applanation, (C) shows an image at the highest concavity, and (D) shows an image in the second applanation (414).
3.3.2.4 Measurement of AGE levels

As explained in Section 1.2 due to AGEs accumulation in the skin, tissue-bound AGE levels can be detected by skin biopsy. Although skin biopsy has been shown to be an accurate means of determining AGE levels, it is an invasive technique. The AGE Reader (DiagnOptics B.V., Groningen, The Netherlands) is a device which utilises the fluorescent properties of tissue-bound AGES in the skin to provide a measurement of skin autofluorescence (SAF), which has been proposed as a marker for tissue-bound AGE levels (23). The AGE Reader is a non-invasive device, which has been suggested to provide a low cost, fast, and reproducible means of determining tissue-bound AGE levels in the body (21-23). In addition the AGE Reader has been validated and is comparable to skin biopsies (21).

The protocol used for the AGE Reader is based upon the manufacturer’s instructions and previous research using the AGE Reader (21, 23). Each participant had the inside of their forearm measured for the assessment of SAF. The reader was set to take three measurements with the forearm repositioned for each repeated measurement. The average of the three SAF readings was recorded as well as average skin reflectance value. Self-tanning agents, sun cream and body lotion/moisturising cream were not permitted as they affect the SAF reading (415). As part of the appointment reminder participants were told to avoid all creams and lotions on their forearms. Noordzij et al. (2011) found that sun cream and self-tanning agents gave an SAF increase of >100% and body lotion increased SAF by 18% (415). They established that the hydration state of the skin did not influence SAF (415).
The AGE Reader as seen in Figure 3.16 is 280 mm wide, 150 mm deep with a height of 115 mm. At the top of the machine there is a 4 cm² window in the silicone mat through which the UV-A light is transmitted to enable direct illumination of the participant’s skin once the participants arm is resting on the silicone mat. The AGE reader contains an excitation light source which emits UV-A light with a peak wavelength of 375 nm which excites the fluorescent AGEs in the skin, these AGEs have a fluorescence range of 300–420 nm. The autofluorescent light from the skin is then measured by the integrated spectrometer which has a detection range of 300–600 nm (416). The AGE Reader acquires 50 scans, each scan approximately 200 ms, dependent on skin reflectance, is used to create each measurement. Each SAF measure takes
approximately 30 seconds, as mentioned this process is repeated three times as part of the software's triple measurement programme, with the participant’s arm being repositioned, hence measuring a different section of skin after each measurement. The results are automatically displayed once the measurement is completed (see Figure 3.17 for patient measurement set up and Figure 3.18 for an example output).
The automatic calculation for SAF by the software corrects for light absorption, the result is a ratio between emitted light intensity (per nm) and excitation light intensity (per nm) multiplied by 100, with the result in arbitrary units (22, 23).

Meerwaldt et al. (2004) validated the AGE reader by comparing SAF to skin biopsy assays in non-pigmented skin, SAF was found to be related to skin AGE accumulation (21, 418). Skin with darker pigmentation gives a lower SAF result. This has in the past resulted in studies excluding participants with dark skin.

Figure 3.18: AGE Reader measurement report (417)
pigmentation from SAF studies (22, 419). Koetsier et al. (2010) validated a new algorithm which enabled SAF to be assessed in pigmented skin (Fitzpatrick skin colour classes 1-4) (419). Skin reflectance <6% (Fitzpatrick skin colour classes 5 and 6) do not give the required reflectance, lower reflectance leads to a warning that the signals are too small to permit a valid analysis (416, 417, 419).

Repeated SAF readings on the same day showed the AGE reader to have an error of approximately 5% (21). McIntyre et al. (2011) when repeating 10 SAF measures using the AGE reader that there was a variation of 7%. This result was obtained on a single patient by a single operator (420). Ideally there should be no variation of the equipment used on the same day with the same method of use; however a small degree of error such as this has been deemed acceptable.

### 3.3.2.5 Measurement of dietary AGEs

In this thesis, an assessment of dietary AGE intake over the previous 12 months was made using a FFQ that was designed specifically to assess dietary AGE levels in a UK population for the purposes of this research. This questionnaire was administered to all participants with the aim of providing an estimate of AGE consumption through diet (see Figure 3.19, full FFQ in Appendix 4.1).

These is no single method to collect a participants nutritional status accurately as all measurements of food intake have an element of error (421). All methods are equally limited in that participants tend to under-report actual consumption and some also report what they consider as ‘healthier food’ rather than their true food consumption (34). FFQ was the method chosen for evaluating dietary AGE intake
in this research as it is suitable for large sample sizes, straightforward for participants to complete and is inexpensive (34, 54, 422). Additionally, FFQ’s are beneficial when aiming to measure a specific nutritional intake (423, 424), hence making FFQ a good choice for measuring dietary AGE.

<table>
<thead>
<tr>
<th>Dietary assessment method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Retrospective methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hour recall</td>
<td>Long-term memory not required, small respondent burden, actual intake over the last 24 hours, relatively detailed intake data.</td>
<td>Requires accurate memory of last 24 hours, if only 24 hours would not be representative – often multiple days required, time-consuming, moderate respondent burden.</td>
</tr>
<tr>
<td>Food frequency questionnaire (FFQ)</td>
<td>Useful for large sample sizes, cost effective, simple to complete/ small respondent burden, portion size can estimated to make the FFQ semi quantitative.</td>
<td>Long-term memory required, requires accurate interpretation - ideally with computer software, requires accurate long-term recall, and is reliant on the participant having literacy and numeric ability.</td>
</tr>
<tr>
<td><strong>Non retrospective methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weighed food records</td>
<td>Actual intake recorded, detailed dietary intake data, no recall bias/no long-term memory required.</td>
<td>Not suitable for large-scale studies, large respondent burden, time-consuming, multiple days required to assess usual intake</td>
</tr>
<tr>
<td>Food record with estimated weights</td>
<td>Actual intake recorded, relatively detailed dietary intake data, no recall bias/no long-term memory required.</td>
<td>Not suitable for large-scale studies, relatively large respondent burden, time-consuming, multiple days required to assess usual intake</td>
</tr>
</tbody>
</table>

Table 3.5 Advantages and disadvantages of different dietary assessment methods
The only existing validated AGE specific FFQ is that produced by Luevano-Contreras et al. (2013), the FFQ however was designed for use on diabetic patients in the USA. Luevano-Contreras describes in her validation paper that before being used in another population the FFQ requires further validation, due to the subjects having diabetes and there being a mean age of 56.6 years. Unfortunately further validation has not taken place (54). In its current format this FFQ does not therefore transfer well to UK based, non-diabetic populations, consequently the Luevano-Contreras FFQ was not appropriate to the population in this study and a new, targeted FFQ was developed.

Validation of a newly designed FFQ is important to ensure that the nutrient, dietary AGES, is measured correctly (425) as incorrect information could result in false associations (426). The design and validation of this FFQ is discussed in full in Chapter 4.
3.3.2.6 Measurement of oxidative stress

Plasma levels of malondialdehyde (MDA), alpha tocopherol (vitamin E) and ascorbate (vitamin C) were measured as biomarkers of oxidative stress. As explained in Section 1.6, these are recognised biomarkers of oxidative stress in ageing and POAG (107-109) and have been shown to be sensitive to variations in AGE levels (110). The relationship between AGE levels and oxidative stress is complex, with an increase in AGE levels having been shown to be both a cause and an effect of elevated oxidative stress (15).

3.3.2.6.1 Venesection and sample handling

The laboratory used for blood sampling and analysis is a Containment Level 2 biosafety level environment, which follows University of Plymouth Codes of Practice for Control of Hazardous Substances, in line with the Control of Substances Hazardous to Health (COSHH) Regulations 2002. Blood and plasma processing and sample preparation were carried out in a level 2 biosafety cabinet which is serviced annually. COSHH regulations were followed for the disposal of potentially infectious waste. Blood cells were not used or stored, they were destroyed in line with COSHH regulations. Plasma samples were stored in a -80 freezer until analysis took place. The plasma samples remaining after the end of processing were destroyed in line with COSHH regulations. All relevant material was disposed of with respect for the donor of the sample.

Three 3 mL lithium heparin vacutainers, green top tubes as shown in Figure 3.20, were used to collect the blood samples. These were spun in a centrifuge at 20G for 20 minutes at 4°C. The separated plasma was then pipetted into serum tubes,
see Figure 3.21, ready for freezing. Two batches were created, with each batch stored in a different freezer at -80°C. Storing the samples in parallel follows the Human Tissues Act licence. Once samples from all participant groups were collected the analysis began.

![3 mL blood collection](image1)

![Serum tubes](image2)

**Figure 3.20 - 3 mL blood collection**

**Figure 3.21 - Serum tubes**

**lithium heparin tubes**

### 3.3.2.6.2 Biochemical analyses

Biochemical analysis of oxidative stress biomarkers was carried out by the primary investigator (LS). Samples from each group of participants were analysed once all data collection had ended.

Ascorbic acid (vitamin C) is an essential nutrient. It is able to protect plasma lipids against oxidative damage from free radicals (427). The water soluble antioxidant ascorbate was measured by validated high performance liquid chromatography (HPLC) methods routinely used in the laboratory. Ascorbate was measured by the HPLC electrochemical method of Mitton and Trevithick (1994) (428), see Appendix 5.1 for method detail. The method allows the measurement of both
reduced and total ascorbate, from which the amount of oxidised ascorbate was calculated.

Figure 3.22 HPLC machine used to measure ascorbate (vitamin C).

MDA was measured as an index of free radical activity and free radical damage. The rationale and methodology for this is that free radicals may oxidatively damage protein, phospholipid and DNA forming MDA. Hence MDA is a sensitive and robust marker of lipid peroxidation (429). For more than 30 years MDA has been used as a biomarker for lipid oxidation (430). MDA has been used as an oxidative stress biomarker in previous studies investigating glaucoma (109, 315) and it has also been shown to be sensitive to variations in AGE levels (110).

MDA is routinely measured by reacting it with thiobarbituric acid which, under appropriate conditions, converts MDA to products which can be measured by HPLC. MDA was measured in the laboratory by the validated HPLC with

The phospholipid bilayer molecules that make up the cell membrane are vulnerable to free radicals as they have an unpaired electron. The free radical will attempt to take an electron from the cell membrane molecule in the process of oxidation. Vitamin E is a lipid soluble antioxidant, its role is to scavenge free radicals and donate an electron. This process protects the cell from any damage the free radical would have caused (432). Vitamin E occurs in eight forms, alpha tocopherol is the main lipid soluble antioxidant found in the plasma (433). For plasma vitamin E (α-tocopherol), the method is based on Julianto et al (1999) (434), using UV/vis HPLC detection, see Appendix 5.3 for method detail. Vitamin E plasma levels indicate the levels in cell membranes, where it protects against oxidative damage (433).
Figure 3.24. HPLC machine used to measure alpha-tocopherol (vitamin E)

The methods described throughout this chapter have each been chosen to meet the five aims (Chapters four to nine). Each method used will be listed in the Chapters which follow.
4. Validation of a specifically designed semi-quantitative food frequency questionnaire (FFQ)

4.1 Abstract

**Purpose:** To design and validate a UK-specific food frequency questionnaire (FFQ) for use in advanced glycation end product (AGE) research and assess its validity and reliability. In addition this study aims to compare the different databases available to calculate dietary AGE.

**Methods:** The newly designed FFQ was completed by 70 healthy participants and compared to plasma carboxymethyl-lysine (CML) levels and the outcome from a 4-day weighed food record (4-day FR). Reliability of the FFQ was assessed in a sub-sample of the participants through the completion of a second FFQ one month after the first.

**Results:** A relationship was found between dietary AGE (dAGE) values measured with the FFQ and that of the 4-day FR \( (n = 10, r = 0.894, p < 0.001, 95\% \text{ bootstrap confidence interval 0.669 to 0.996}) \). No significant relationship was found between dietary CML levels calculated from the FFQ and the plasma CML levels for 2 of the 3 dAGE databases used, a weak negative correlation was found between plasma CML level and dietary CML calculated with the Uribarri et al. (2010) database \( (n = 70, r = -0.256, p = 0.038, 95\% \text{ bootstrap confidence interval -0.014 to -0.477}) \). There was no significant difference in the dAGE values calculated across the two repeated FFQs \( (n = 20, p = 0.544) \).
Conclusion: The UK-specific semi-quantitative FFQ developed to measure dAGE was found to produce valid results when compared to a 4-day FR and to be reliable across completions. We were unable to validate the FFQ against the objective plasma CML-AGE measurement, however this could be due to the methodology used. The comparisons between databases suggests that they are more in line with each another than previously thought.
4.2 Introduction

AGEs are a group of reactive compounds with chemical, pro-inflammatory and pro-oxidant actions. They accumulate with age in cells and tissues throughout the body where they contribute to an acceleration of the ageing process by adversely affecting structure and function. AGEs exert their effects through binding to specific cell surface receptors, forming covalent cross links on proteins such as collagen and generating reactive oxygen species (ROS) and oxidative stress (15-18).

Strong associations have been demonstrated between elevated AGE levels and diabetes. Formation of AGEs is accelerated in hyperglycaemic conditions, and increased accumulation of AGEs has been associated with the occurrence and severity of diabetic complications (15, 23, 435), including diabetic retinopathy (82, 83). Independent of diabetes, it is also thought that sustained exposure to a high levels of circulating AGEs could be a major factor in the development of age-related degenerative disorders such as Alzheimer’s disease (76), renal disease (77), osteoarthritis (78), atherosclerosis (79), as well as a number of ocular age-related disorders such as age-related macular degeneration (22, 81), cataract (81, 84-86) and primary open angle glaucoma (20).

Foods are an exogenous source of AGE, (40) and it has been shown that dietary AGEs (dAGEs) can influence the body’s total AGE level and contribute to pathology in a similar manner to endogenous AGEs (42-47, 64). A high dAGE intake has been shown to increase oxidative stress, inflammation and endothelial dysfunction (64, 436, 437). It has been well evidenced in diabetes that dAGEs
have a pathogenic significance. Cardiovascular risk factors have been shown to be increased in the presence of high intake of dAGE in patients with diabetes (43, 438), and dAGEs are thought to contribute to insulin resistance (49). In addition it has been shown that patients with type 2 diabetes who have restricted dAGE consumption have lower levels of oxidative stress and inflammation, suggesting dAGE intake could act as a modifiable target (439). The evidence in this regard has been questioned due to a lack of high quality randomised trials (64).

However, a meta-analysis of randomised controlled trials investigating a diet low in dAGE on cardiometabolic parameters had similar results to previous dAGE research, in that a lower dAGE intake was found to be beneficial (437).

There is no gold standard method to determine a participants dAGE status, as all measures of dietary intake are known to carry an element of error (421, 440). Previous studies measuring dAGE have primarily used food frequency questionnaires (FFQs) and food records (FRs) (14, 54, 56). FRs have the advantage of not being retrospective and allowing portion size to be measured precisely (441, 442). In contrast, FFQs are suitable for large samples, straightforward to complete, and inexpensive (34, 54, 422, 442, 443). For measurement of a specific nutrient intake, such as dAGE, FFQs are considered to be most beneficial (423, 424, 441). To ensure that FFQ estimates of dAGE are representative of the population being investigated, the FFQ needs to be targeted to the food culture and preparation methods of the specific country or region (56). There is currently no UK specific FFQ to measure dAGE intake.
The AGE carboxymethyl-lysine (CML) has previously been used as a marker of dAGE (14, 40). The choice of CML as a dAGE biomarker is likely to be due to it being one of the wider studied AGEs as well as it being fairly stable and inert (14). However, it is known that other types of AGEs are present in foods (53, 444). Additionally the published associations between elevated AGE levels and pathology incorporate multiple types of AGEs and not one individual type (23, 76, 78, 435). In order to determine dAGE intake from the responses to a FFQ or FR, one needs to have knowledge of the relative AGE content of the listed foods.

There are three different databases available that have calculated and listed the level of CML in foods (Uribarri et al. (2010) (14), Hull et al. (2012) (52) and Scheijen et al. (2016) (53)), with one of these databases (Scheijen) also listing the levels of two additional methylglyoxal AGE derivatives (carboxyethyl-lysine (CEL) and 5-hydro-5-methyl-4-imidazolon-2-yl-ornithine (MG-H1)). Interestingly, the CML results of similar foods from each database differs from one another, this is thought to be due to a difference in the analytical methods used (53). The Uribarri et al. (2010) database uses an enzyme-linked immunosorbent assay (ELISA) method using a monoclonal anti-CML antibody, and Hull et al. (2012) and Scheijen et al. (2016) use ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). This inconsistency makes the decision of which database to use for determining dAGE intake problematic, as outlined in detail in Section 1.4.
4.2.1 Aim

The aim of this study was to design and validate a UK-specific FFQ for use in AGE research. In addition, this study aims to assess the reliability of the newly developed FFQ and to compare the dAGE outputs calculated using the three different databases.

4.2.2 Hypothesis

The newly designed FFQ will be related to plasma-CML level and 4-day food records and will be reliable. Dietary CML levels will be comparable between the Hull and Scheijen databases which use the same analytic method, but will not be comparable to the Uribarri database which uses a different analytical method.

4.2.3 Validity, repeatability and reliability

In this study, validity will be the term used to describe the probability that the method (the semi-quantitative FFQ) accurately measures dAGE (445). Repeatability is an evaluation of the variation in repeated measurements taken under the same conditions (446). Reliability is how consistent a result is when measured under the same conditions. Repeatability and reliability are often interchanged in the literature due to their similar definition (54, 447, 448). The test re-test of the FFQ in this study will be referred to as reliability, referring to the consistency of the FFQ to produce repeatable estimates of dAGE intake.
4.3 Methods

4.3.1 Participants

Convenience sampling was used to recruit participants who were eligible according to the inclusion and exclusion criteria for healthy ageing participants as detailed in Section 3.1.3. and Section 3.1.4. Due to the established link between diabetes and AGEs (449, 450), participants diagnosed with diabetes or participants found to have a high fasting glucose level were excluded from the study, as were current smokers, and previous smokers who quit less than 15 years ago due to the link between AGEs and tobacco (10). Participants who reported a major change to their diet over the last 12 months were excluded.

4.3.2 Development of the semi-quantitative FFQ

A total of 178 foods and drink choices were included in the semi-quantitative FFQ that was designed for use in this research (Appendix 4.1). Although this may seem lengthy compared to an average FFQ (79 foods) (443), an increased number of food choices was also used by Luevano-Contreras et al. (2013) for an AGE specific FFQ (130 foods used) (54). The foods were listed individually, rather than in groups, as AGE values can differ greatly with different temperatures and cooking methods, this therefore was reflected in the number of food choices within the FFQ. For example rather than ‘beef’ listed once there was a recorded section for: roast beef, pan fried steak, grilled steak, stewed beef, stir fried steak strips, meatballs, fried burger, fast food burger, beef mince browned and other beef to reflect the relative difference in AGE levels. Using the Uribarri database for example, a pan fried steak would have a value of 22,630 CML kU/portion and stewed beef only 2391 CML kU/portion. This method of listing
foods rather than grouping is known to possibly cause participants to overestimate intake due to some people unintentionally ‘double counting’ certain foods (451).

Careful consideration was given to the order in which the food and drink categories where inserted into the FFQ, as it is well known that errors are often made at the beginning of a FFQ and the format can initially be confusing to participants new to FFQs (443). The first section of the newly designed FFQ therefore focused on drinks, as drinks are known to have low AGE values (14, 52, 53) and any errors would have a low impact on the dAGE result calculated from the FFQ. Dairy and meat sections followed this, as they are thought to have high AGE content and are therefore of most interest (14, 52, 53). According to Cade et al (2001), placing food groups of high interest in the middle to early sections of the FFQ allows the important information to be collected at a point where the participant understands the FFQ format, but before any fatigue or boredom sets in (443).

Participants were required to indicate the frequencies at which they consume the various listed foods. The frequency options were based on the frequencies used in the European Prospective Investigation into Cancer (EPIC) FFQ (452). An example of the frequencies is shown in Figure 4.1. The full FFQ can be found in Appendix Section 4.
The FFQ required participants to also consider portion size, to allow the most accurate AGE calculation, making this a semi-quantitative FFQ (453). The average portion size stated next to each food was based on the food standards agency portion sizes (454). Portion pictures were included at the beginning of the questionnaire, and the plate and cup used for these photographs was shown to the participants to ensure the most accurate recalled portion reporting possible (455-457). It has been suggested that photographs improve reliability of portion estimates (458). The pictures of the portion sizes concentrated on foods with a high protein and lipid content, as these are the foods which are thought to have the highest AGE value and therefore required the most accurate responses (14).

All of the available databases that list the AGE content of foods were developed in different countries, the lists of foods measured and cooking methods reflect these cultural variations (14, 52, 53). It was therefore not possible to directly insert these listed foods and cooking methods into this new FFQ as they would not easily be translated by a UK population. In the development of this new FFQ therefore, all foods and cooking methods were ‘translated’ to traditional cooking method descriptions for the UK. Certain foods not eaten in the UK were omitted,
for example pop tarts. In addition, some foods commonly eaten in the UK were added, for example battered cod. All adjustments to the list of foods excluded and included in the FFQ were approved by a registered dietitian. In addition to adjusting foods, space in the FFQ was added to allow participants to list any foods they eat which had not already been named. The addition of space to record other foods allowed a complete report of all foods eaten by participants.

4.3.2.1 Procedure for completion of the FFQ

Prior to data collection, the FFQ was piloted with 12 people to ensure it was easy to fill out and follow. The pilot study raised the issue that the columns showing foods eaten per month, week and day were difficult to separate from each other. Colour was added to help address this difficulty. There was also a query about the portion size of cheese, e.g. “how big is 30 g of cheese?” An additional portion page was added at the end of the questionnaire to illustrate visually some other general portion sizes, such as 250 ml displayed in a mug and cup, 30 g cornflakes, 30 g of cheddar cheese as well as some other foods (see Appendix Section 4). These photos were taken on the same plate as previous portion photos. No queries about the names of foods or cooking methods were raised from these pilot questionnaires. All adjustments to the FFQ design were approved by a registered dietitian.

Every study participant completed the FFQ, it was interview-administered with questions and prompts made by the primary-investigator throughout the questionnaire. This was appropriate as there were some open questions throughout the questionnaire; it also allowed the participant to raise any queries
about nutrient details and for these to be addressed, as well as helping to ensure accurate portion size was recorded where possible to add to the reliability of the resulting data (425, 459). A flow chart of food prompts used is detailed in Appendix 4.2.

4.3.2.2 Calculation of dAGE from the FFQ

It is standard practice when investigating diet to use a FFQ in conjunction with analysis software which can calculate nutrient content (460, 461). For example, a study that was part of EPIC-Norfolk study used a 130 item semi quantitative FFQ and the dietary assessment software FETA (FFQ EPIC Tool for Analysis) (462). There is currently no software which can calculate or analyse dAGE values. The FFQ responses were recorded and calculated using Microsoft Excel (Seattle, WA, USA).

The dAGE consumption value was calculated by totalling the dAGE value for each food and portion selected in the FFQ and scaled up to its relative frequency eaten over the year. For example (using the Uribarri database) a 30 g portion of cheddar cheese has a dAGE value of 1657 CML kU; if a participant reported eating cheese twice a week then 1657 x 104 = 172328 CML kU is the dAGE value for cheese eaten in a year. The dAGE value was assigned using the three databases which have measured AGE content in foods (and drinks). These databases were introduced in Section 1.4 and the three that were used in this study to calculate dAGE were the Uribarri database, which measures CML in 549 foods (14), Hull database, which measures CML in 257 foods (52), and the
Scheijen database which measures the AGEs; CML, CEL and MG-H1 in 190 foods (53), (see Table 2 in Section 1.4)

As mentioned in Section 1.4 Dresden University of Technology are developing a large dAGE database which different authors can contribute to (444). It includes a wide variety of AGEs including; CML, CEL, methylglyoxal, amadori-product, pentosidine, pyrraline, furosine, however this database is not complete and has limited AGEs measured for certain food groups compared to others, for example the ‘milk and milk products’ section has values for seven types of AGEs, whereas ‘meats’ only have one type of AGE measured (444). For this reason this database was not included in this study.

Of the three databases, the Uribarri database lists the most foods and is the database most commonly used in the literature (49, 54, 56-58). Hence, to ensure findings were comparable to previous literature, the Uribarri database was the principal database used in this study to calculate dAGE. Despite the large number of FFQ studies using the Uribarri database however, its calculations are not without criticism. As shown in Table 2 in Section 1.4, ELISA is the method used by Uribarri to measure AGE in foods. Hull and Scheijen use the validated method UPLC MS/MS, which has increased sensitivity (463). The immunoassay ELISA method used by Uribarri has not been validated and as such, it has been suggested that its analysis may be misleading (51, 53, 463, 464). As previously described by Poulsen et al. (2013) the Uribarri database uses different units to the UPLC MS/MS databases, making comparison between databases particularly difficult (51). Scheijen compared its newly developed database to Uribarri by
comparing which food groups were generally considered to have ‘high’ AGE levels (53), however they were unable to make any calculations or analysis from this. In this study, participants’ FFQ responses were used with each database individually, allowing dAGE calculated from each database to be directly compared, despite unit differences.

The dAGE value of the foods listed in the newly designed FFQ was assigned using the AGE values calculated from each database (14, 52, 53). All foods without a value listed had their dAGE value assigned by using the next most similar food, for example, apple crumble was assigned with the dAGE value of apple pie. The databases considered agree with one another in that fruits, vegetables and drinks have low or negligible values. However, the CML results of similar foods from each database differ; cheese, oil, butter and meats have a high dAGE value with Uribarri and low with Hull and Scheijen. Biscuits, chocolate and cereals had a high dAGE value with Hull and Scheijen, and low with Uribarri. As stated previously, these discrepancies are thought to be due to the different measurement methods used (53). There is currently no guidance as to which of the available databases should be used for calculation of dAGE.

4.3.3 Validation

The newly designed semi-quantitative FFQ was validated against plasma CML levels and results from a 4-day food record (FR). Using FRs is an accepted method for validating a FFQ (443, 465) and ELISA is a method widely used to measure AGEs from serum and plasma samples (43, 466-469).
4.3.3.1 Enzyme-linked immunosorbent assay (ELISA)

A 3 mL lithium heparin vacutainer was used to collect a blood sample; this was then spun in a centrifuge at 20 G for 10 minutes at 4°C. The separated plasma was pipetted into serum tubes and stored in a freezer at -80°C. Analysis of the samples began once all samples were obtained. ELISA analysis was carried out by Dr Desley White.

The plasma samples were measured by a direct double antibody (monoclonal CML antibody and polyclonal biotin-labelled secondary antibody) sandwich ELISA kit which analysed the presence of the CML-AGE (469). The human CML-AGE ELISA commercial kit was from MyBioSource (MBS263691, San Diego, CA, USA) and was used according to the manufacturer’s instructions.

4.3.3.2 4-day food records (FR)

Using weighed food records in a sub-sample of the main study participants is an accepted method for validation of a FFQ (54, 465). The length of days used for a FR can range from 24 hours to 16 days, the number of days chosen should be sufficient to represent average nutrient intake (470). It is considered that 3 days is the minimum number of days to be representative (191). Multiple consecutive days, more than four, can result in poor compliance (471) and it has been shown that in 7-day weighed FRs the later days are less likely to comply, instead recording foods retrospectively (472). For this reason it was decided that a 4-day food record would be used and that the days did not have to be consecutive.
Prior to completing the 4-day FR, training was given to each participant in the form of written and verbal instructions. The food diary was given to the participants approximately 1 month after the FFQ was administered (Appendix 5). The 4-day records consisted of three weekdays and one weekend day to obtain an accurate reflection of participants’ diet over the week (473, 474). Participants were instructed that food/drink items and portions should be recorded in a real time and that they should not modify their dietary habits and the food choices just because they are being recorded. Participants were asked to avoid recording at times of any unusual fasting or feasting, for example birthdays, as any highly atypical intake would affect the mean total of the 4-day FR (475).

Digital kitchen scales (Colour match digital scale, Milton Keynes, UK) were loaned to participants to allow all food and drink to be measured accurately. The dAGE intake was calculated using the same database values used to calculate dAGE for the FFQ. The dAGE results from the 4-day FR were averaged to give the average daily dAGE value.

4.3.4 Reliability

To assess the reliability of the FFQ (FFQ1) a second FFQ (FFQ2) was administered approximately 1 month after the first. The one month interval made it unlikely that initial responses could be remembered, but also made it unlikely that substantive changes in diet had taken place (443, 476).
4.3.5 Database Comparison

Annual dAGE intake was calculated from the FFQ responses using the corresponding food values from each of the three databases (Uribari CML, Hull CML, Scheijen CML, Scheijen CEL and Scheijen MG-H1) in order to determine if the relative calculations were comparable.

4.3.6 Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM). The normality of data was assessed by the Shapiro–Wilk test and through inspection of quantile-quantile (QQ) plots. The Mann-Whitney U test was used to test for differences in the demographic data.

P values of less than 0.05 were considered statistically significant. There was a mix of parametric and non-parametric distributions in the different groups of participants. For non-parametric data median and interquartile range should be reported (477), however, for consistency in the tables in this Chapter and throughout this thesis mean and standard deviation have been reported. Tables reporting the median and interquartile range for all non-parametric data can be found in Appendix 10.1.

4.3.6.1 Validity

Due to nonparametric distribution Spearman’s rank correlation was used to analyse the association between FFQ1 and plasma CML levels in order to evaluate its validity. This analysis was carried out separately for each dAGE database under consideration, namely: Uribari, Hull and Scheijen.
Pearson’s correlation was used to analyse the association between FFQ1 and the 4-day FR, in order to evaluate its validity. This analysis was carried out using the principal database (Uribarri). The use of one database was chosen as the FFQ validation was centred upon participants’ dietary responses, and hence the relative associations would be the same with whichever database values were used. In order to visualise the differences between dAGE calculated using the FFQ and that calculated from the 4-day FR, a Bland-Altman plot with 95% limits of agreement was also constructed (478).

4.3.6.2 Reliability
Reliability of the FFQ using the principal database (Uribarri) was analysed using Pearson’s correlation and intraclass correlation coefficient (ICC). An ICC of 0.60 or below would be considered low, and 0.8 or above would be considered to have good reliability (446). To visualise the difference between FFQ1 and FFQ2, a Bland-Altman plot with 95% limits of agreement was constructed.

4.3.6.3 Database Comparison
Spearman’s rank correlation was used to analyse the association between each dAGE value calculated by the databases under consideration, namely: Uribari, Hull and Scheijen.

4.3.6.4 Power Calculation
Sample size for FFQ validation using CML-plasma levels was determined using G*Power 3 programme (Heinrich-Heine, Universität Düsseldorf). In order to achieve 80% power at the 5% alpha level with a medium effect size of 0.3, 64
participants would be required. To account for unusable data an allowance was made and a sample size of 71 participants was chosen.

Sample size for FFQ reliability and validation using the weighed FR was determined using G*Power 3 programme (Heinrich-Heine, Universität Düsseldorf) using data from previous research which also assessed FFQ reliability and validated a FFQ using FR to measure AGE in the diet (54). In order to achieve 80% power at the 5% alpha level a sample size of 14 was required. To account for unusable data an allowance was made and a sample size of 20 participants was chosen.

4.4 Results
A total of 70 participants were recruited for this study. All 70 participants completed the newly designed FFQ and had their plasma CML levels measured. 10 of the 70 participants additionally completed the 4-day weighed food diary. 20 out of the 70 participants were asked to complete a second FFQ for evaluation of reliability. The characteristics of the participant group with mean and standard deviation are detailed in Table 4.1.
Table 4.1: Mean and standard deviation of participant group characteristics (n = 70) * P < 0.05, ** P < 0.001 difference between M: males and F: females.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
</tr>
<tr>
<td>Female</td>
<td>47</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>60.84 ± 9.54</td>
</tr>
<tr>
<td><strong>Ethnicity (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>64</td>
</tr>
<tr>
<td>White other</td>
<td>2</td>
</tr>
<tr>
<td>Mixed white/Asian</td>
<td>1</td>
</tr>
<tr>
<td>Mixed white/black Caribbean</td>
<td>2</td>
</tr>
<tr>
<td>Indian</td>
<td>1</td>
</tr>
<tr>
<td><strong>Body mass index (BMI)</strong></td>
<td>26.11 ± 4.56</td>
</tr>
<tr>
<td><strong>Basal metabolic rate (BMR) (kcal)</strong></td>
<td>All: 1445 ± 230</td>
</tr>
<tr>
<td></td>
<td>M: 1666 ± 212, F: 1337 ± 149 **</td>
</tr>
<tr>
<td><strong>Body fat percentage (%)</strong></td>
<td>31.74 ± 8.77</td>
</tr>
<tr>
<td></td>
<td>M: 23.32 ± 6.34, F: 35.86 ± 6.56 **</td>
</tr>
<tr>
<td><strong>Fat mass (Kg)</strong></td>
<td>23.69 ± 9.84</td>
</tr>
<tr>
<td></td>
<td>M: 19.74 ± 8.28, F: 25.62 ± 10.04 *</td>
</tr>
<tr>
<td><strong>SBP</strong></td>
<td>129.24 ± 17.25</td>
</tr>
<tr>
<td><strong>DBP</strong></td>
<td>81.16 ± 10.20</td>
</tr>
<tr>
<td><strong>Alcohol units per week</strong></td>
<td>6.21 ± 6.56</td>
</tr>
<tr>
<td><strong>Smoking history (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>44</td>
</tr>
<tr>
<td>Previous smoker, quit &gt;20yrs ago</td>
<td>25</td>
</tr>
<tr>
<td>Previous smoker, quit &gt;15yrs ago</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.1: Mean and standard deviation of participant group characteristics (n = 70) * P < 0.05, ** P < 0.001 difference between M: males and F: females.
4.4.1 Validity of the FFQ

4.4.1.1 FFQ vs. Plasma CML levels

No significant correlations existed between the dAGE values calculated from the FFQ and plasma CML for two out of the three databases (Hull and Scheijen). Spearman’s correlation did however show a weak negative relationship between the average CML kU/day calculated from the FFQ responses using the Uribarri database and plasma CML (n = 70, r = -0.256, p = 0.038, 95% bootstrap confidence interval -0.014 to -0.477) (Figure 4.2) (Table 4.2).

<table>
<thead>
<tr>
<th>Mean</th>
<th>SD</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uribarri (average CML kU/day) Hull CML (average mg CML/day) Scheijen CML (average mg CML/day) Scheijen CEL (average mg CEL/day) Scheijen MG-H1 (average mg MG-H1/day)</td>
</tr>
<tr>
<td>Plasma CML (ng/ml)</td>
<td>101.91 ± 28.81</td>
<td>-0.256</td>
</tr>
<tr>
<td></td>
<td>p = 0.038*</td>
<td>p = 0.526</td>
</tr>
</tbody>
</table>

Table 4.2: Mean, standard deviation and Spearman’s correlations between plasma CML and dAGEs calculated with each database. *p < 0.05
Figure 4.2: Negative relationship between the average CML kU/day calculated from the FFQ responses and plasma CML ng/ml (n = 70, r = -0.256, p = 0.038, 95% CI -0.014 to -0.477). The dashed grey line represents the line of best fit.

4.4.1.2 FFQ vs. 4-day food record

Pearson’s correlation showed a strong relationship (n = 10, r = 0.894, p = < 0.001, 95% bootstrap confidence interval 0.669 to 0.996) between the average CML kU/day calculated from the FFQ using the Uribarri database and that calculated from the 4-day food record (Figure 4.3).
Figure 4.3: Relationship between FFQ1 and 4-day FR using the Uribarri database to calculate dAGE (n = 10, r = 0.894, p = < 0.001, 95% CI 0.669 to 0.996). The dashed grey line represents the line of equality (1:1).

The Bland–Altman plot in Figure 4.4 shows the level of agreement between the dietary CML level calculated with the FFQ and that calculated with the 4-day FR, with the 95% limits of agreement calculated. There was good agreement between the two measures and no evidence of proportional bias, however this is based upon a small number of participants (n=10).
Figure 4.4: Agreement between FFQ1 and 4-day FR using the principal database. The red line shows the mean difference and green lines the 95% (1.96 ± SD) limits of agreement between and 4-day FR for dAGE (n=10).

4.4.2 Reliability of the FFQ

Pearson’s correlation showed a strong association between dAGE value calculated with FFQ1 and FFQ2, (n = 20, r = 0.934, p = < 0.001, 95% bootstrap confidence interval 0.75 to 0.973), (Figure 4.5). There was no significant difference between the mean and standard deviations of the calculated dAGE values for FFQ1 and FFQ2 (Table 4.3, t-test; p = 0.544). In addition the FFQ had
an ICC (2 way mixed) of 0.965 with 95% a confidence interval of 0.913 to 0.986.

The Bland–Altman plot in Figure 4.6 shows the mean difference between FFQ1 and FFQ2 with the 95% limits of agreement calculated. There was good agreement and no evidence of proportional bias.

<table>
<thead>
<tr>
<th></th>
<th>Mean (average CML kU/day)</th>
<th>Standard deviation</th>
<th>Pearson’s correlation (R values)</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFQ1</td>
<td>23011 ± 8115</td>
<td></td>
<td>0.934</td>
<td>0.965</td>
</tr>
<tr>
<td>FFQ2</td>
<td>22758 ± 9881</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3: Means, standard deviation, Pearson’s correlation and ICC between FFQ1 and FFQ2.
Figure 4.5: Relationship between FFQ1 and FFQ2 using the Uribarri database to calculate dAGE (n = 20, r = 0.934, p = < 0.001, 95% CI 0.75 to 0.973). The dashed grey line represents the line of equality (1:1).
Figure 4.6: Agreement between FFQ1 and FFQ2 using the principal database. The red line shows the mean difference and green lines the 95% (1.96 ± SD) limits of agreement between FFQ1 and FFQ2 for dAGE (n = 20).

In Figure 4.6 the Bland-Altman plot indicates heteroscedasticity. For this reason, the Bland–Altman plot was repeated using dAGE data which had been log transformed. This repeat log transformed plot is shown in Figure 4.7. There was no evidence of proportional bias.
Figure 4.7: Agreement between LogFFQ1 and LogFFQ2 using the principal database. The red line shows the mean difference and green lines the 95% (1.96 ± SD) limits of agreement between LogFFQ1 and LogFFQ2 for dAGE (n = 20).

4.4.3 Database comparison

Strong/moderate correlations were found between each of the FFQ calculated dAGEs from each database, with one exception. No correlation was found between the dAGE calculated from the FFQ using the Uribarri CML database and that calculated with the Scheijen MG-H1 database. The mean and standard deviations of the calculated dAGE values, as well as the correlations between the databases are shown in Table 4.4.
4.5 Discussion

The aim of this study was to design and validate a new UK-specific FFQ for use in research. It is thought that the FFQ designed in this study is the first UK specific FFQ to measure dAGE intake and the first to directly compare dAGE values calculated from a range of databases.

Careful consideration was given to the design of this FFQ to ensure it targeted food products known to have high AGE values and to ensure maximum reliability of responses. It is important that any newly designed FFQ undergoes validation against other known measures of the dietary target and that its measures are reliable.

4.5.1 Validity of the FFQ

Strong agreement was found between the dietary CML levels calculated using the FFQ (Uribarri database) and those calculated from the 4-day FR. Using food records is an established method for validating FFQs (425) and has been used

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**Table 4.4:** Means, standard deviation and Spearman’s correlations between dAGEs for each database measured with FFQ1 (n = 70). *p < 0.05. **p < 0.01.

<table>
<thead>
<tr>
<th>Database</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Uribarri (average CML kU/day)</td>
<td>20534.94</td>
<td>± 8981.31</td>
<td>0.274*</td>
<td>0.337**</td>
<td>0.525**</td>
<td>0.176</td>
<td></td>
</tr>
<tr>
<td>2. Hull CML (average mg CML/day)</td>
<td>5.99</td>
<td>± 2.25</td>
<td>0.745**</td>
<td>0.705**</td>
<td>0.771**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Scheijen-CML (average mg CML/day)</td>
<td>3.98</td>
<td>± 1.59</td>
<td>0.740**</td>
<td>0.731**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Scheijen-CEL (average mg CEL/day)</td>
<td>3.07</td>
<td>± 1.04</td>
<td>0.764**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 5. Scheijen-MGH1 (average mg MG-H1/day) | 30.20 | ± 9.93 | ** | ** | ** | ** | ** | ** | ** | **
by multiple studies as a means of validating newly designed FFQs (54, 425, 479, 480). This finding suggests that the FFQ developed in this study is valid in its measurement of dAGE. Looking at the data, there was one extreme value where the 4-day FR result was much lower than the FFQ result, which goes against the trend. Interestingly this participant’s FFQ showed good agreement on repeat, suggesting perhaps the participant modified their eating habits for the 4-day FR, or that the four days they recorded were over a period of irregularly low dAGE consumption. This sort of variation is recognised in regard to weighed food records, since it is known that participants tend to underestimate food intake in this style of reporting (481, 482). Macdiarmid and Blundell (1998) found that underestimating food intake is most prevalent in overweight and obese participants, with women more likely to underestimate intake than men (482). Interestingly the individual with this extreme value was a woman and had a BMI of 41, classed as very obese.

Consideration should be given to the fact that the comparison of the dietary CML levels calculated from the newly designed FFQ and the 4-day FR in this study was based on the data obtained from 10 participants. Weighed food records and diaries place a high level of burden on the individual and FRs are known to only be suitable for small sample studies (471). Sample sizes of the order used in this study are considered acceptable for FFQ validation and these numbers are comparable to other validation FFQ studies (54, 443), the EPIC study for example, which had a cohort of 25,000 had 179 participants complete a 7-day food diary (483).
Interestingly, despite good agreement between the FFQ and 4-day FR output, no strong relationships were found between dietary CML calculated with the FFQ and CML-AGE plasma level. In fact, a weak negative relationship was found to exist between CML-AGE plasma level (ng/ml) and CML calculated from the newly designed FFQ (Uribarri database). Two previous studies have found no association between dietary and circulating CML-AGE levels (50, 484). However, the majority of the literature suggests that there is an association between dAGE intake and circulating AGE levels (45, 437, 485-488). There are no previous studies which report a negative correlation between CML-AGE plasma level and dAGE. The ELISA kit used for this study had unfortunately not been validated, and the standards provided with the kit were not in the same range as the results. If pre-tests had been carried out this may have been able to be accounted for, as plasma samples could have been diluted to be in the same range as the standards. Due to the limited number of pre-set wells in the kit this was not possible. It has to be considered that this could have impacted the results and may explain why the CML-AGE plasma level in this study is not consistent with the previous literature. This finding therefore needs to be considered with caution.

4.5.2 Reliability
Assessing the reliability of any newly designed FFQ is considered to be a standard requirement (443, 448, 476, 489). Strong agreement was demonstrated between the first completion of the newly designed FFQ (FFQ1) and the second completion, one month later (FFQ2), suggesting that the newly designed FFQ is reliable. Review of the Bland-Altman plots indicates there was no proportional
bias and no consistent trend that one FFQ measured dAGE levels that were higher than the other, with there being an equal number of points above and below the mean. There is an indication on the Bland-Altman plot, Figure 4.6, that the data is skewed, showing heteroscedasticity, in that higher values calculated by the FFQ have slightly increased variability. This would suggest that the FFQ is less reliable when measuring high dAGE values. It is common for nutritional data in Bland-Altman plots to be skewed (490) since intake will be above zero. In addition it is common that those with higher intakes will appear relatively more variable. The non-uniform variability in Figure 4.6 can be overcome by using logarithms of the data for each method (447, 491). Figure 4.7 shows the Bland-Altman plot using the log-transformed data, the heteroscedasticity is no longer present. FFQ1 and FFQ2 agree with each other and as shown in Figure 4.7 and are approximately +/- 0.3 log units, which corresponds to be within a factor of 2, approximately.

4.5.3 Database Comparison

There is currently no guidance or gold standard on which of the available databases should be used as a reference for calculation of dAGE levels, furthermore the databases are known to have dAGE measurement differences. As described in Section 4.3.4 the calculation of dAGE from the FFQ responses, using the values provided within each individual database in this study has allowed the first direct comparison of the database outputs, despite there being unit differences between them. Perhaps, unexpectedly, poor agreement was found between the CML levels calculated from the FFQ using all three of the available dAGE databases (Uribarri, Hull and Scheijen). These correlations are
likely to exist in part due to the fact they are measuring the same type of AGE, despite the measuring techniques having been different. This suggests that the databases may be more in line with each another when measuring the same AGE than previously thought by Poulsen et al. (2013) and Scheijen et al. (2016) (51, 53).

The Scheijen database, alongside CML, also provides values for the AGEs CEL and MG-H1. Interestingly, the CEL levels calculated from the FFQ with the Scheijen database, showed good agreement with the CML levels for each of the other databases. It is known that CML and CEL are similar to one another (492), this can be evidenced when comparing the chemical structures of CML and CEL, since they only differ by a methyl group (see Figure 4.8). The strong correlations between CEL and CML values from each database suggests that these AGEs are found in close association in foods.

![Chemical structure of AGEs: N-carboxymethyllysine: CML, N-carboxyethyllysine: CEL.](image)

**CML: R = H**

**CEL: R = CH₃**

Figure 4.8: Chemical structure of AGEs: N-carboxymethyllysine: CML, N-carboxyethyllysine: CEL.
MG-H1 has a different chemical structure to CEL and CML, as shown in Figure 4.9. MG-H1 levels calculated from the FFQ using the Scheijen database, were found to have good agreement with the CEL and CML levels calculated from the same Scheijen database and that calculated from the Hull database, however no relationship was found between MG-H1 and CML calculated using the Uribarri database. It may be that this reflects the difference in measurement techniques since MG-H1 levels correlated with all AGEs measured by UPLC MS/MS but not ELISA.

The three types of AGEs measured by Scheijen: CML, CEL and MG-H1 all had different values for foods within the database, for example, Kellogs rice crispies, CML = 0.59, CEL = 0.47, MG-H1 = 14.70. Despite the within database differences in food values, strong correlations existed between each of the different annual AGE calculated intakes. This emphasises the importance of considering the diet as a whole.
4.6 Conclusion:

The UK-specific semi-quantitative FFQ developed to measure dAGE was found to produce valid results when compared to a 4-day FR and to be reliable on two completions; this indicates that it is a useful tool for estimating dAGE in a UK population. We were however unable to validate the FFQ against the objective plasma CML-AGE measurement and the reasons behind this require further research, but could relate to the measurement technique used. The comparisons between databases suggests that they are more in line with each another than previously thought. The choice of database should therefore be based upon which is most relevant to the population, and has enough foods measured to ensure minimal instances of assigning ‘assumed values’ to foods with no assigned measured value. The strong correlations found between the three different types of AGEs measured by Scheijen (CML, CEL and MG-H1) suggests that when considering diet as a whole, an individual dAGE may be sufficient to give a representation as to if a person has a high or low AGE diet.

The UK-specific semi-quantitative FFQ developed to measure dAGE was found to produce valid results when compared to a 4-day FR and to be reliable across completions. The comparisons between databases suggests that they are more in line with each another than previously thought.
4.7 Limitations:

FFQs and FRs are limited in that one cannot be certain that participants have not misreported their diet. It is possible that both methods of collecting a participant’s dietary status could be equally flawed.

The Plasma AGE-CML ELISA kit used was not validated and produced results which did not agree with the previous research. The standards given with this kit were not in the same range as the measurements found, this will have impacted the results. In future research plasma AGE-CML will be carried out using the UPLC–MS/MS method.

Although this study identifies relationships between variables it cannot identify causative factors.

4.8 Future work:

Further validation of the FFQ by using UPLC-MS/MS to measure CML in the plasma.

This is the first UK-specific semi-quantitative FFQ for the measurement of dAGEs, with increased use further validation and review should take place.
5. The relationship between dietary AGE intake, skin autofluorescence and circulating oxidative stress levels in a healthy ageing population.

5.1 Abstract

Purpose: To determine how dAGE intake influences tissue-bound AGE level (SAF) in a healthy ageing population, as well as to determine how circulating oxidative stress levels relate to SAF and dAGE. The relative influence that a variety of demographic parameters may have on these measures were also assessed.

Methods: Skin autofluorescence (SAF) as a measure of tissue-bound AGE level was measured in 68 healthy participants and compared to dAGE measured by FFQ. Fasting venous blood samples were obtained to enable the measurement of oxidative stress biomarkers.

Results: A positive relationship between SAF and dAGE (MG-H1) was calculated from the Scheijen database (n = 68, r = 0.319, p = 0.008, 95% CI 0.099 to 0.511). Spearman’s correlation showed a positive relationship between SAF and the oxidative stress biomarker alpha tocopherol (n = 68, r = 0.252, p = 0.044, 95% CI 0.007 to 0.463). When MG-H1 and alpha-tocopherol were included in a multiple linear regression along with relevant demographic variables they were not found to be significant predictors of SAF; age was the single best predictor (p < 0.001), followed by BMI (p = 0.008).
Conclusion: The contribution of dietary AGE to tissue-bound AGE levels appeared to be minimal. This unexpected finding requires further investigation, which will be aided by the development of new AGE databases. No statistically significant associations between oxidative stress biomarkers and dAGE were found. AGEs measured by SAF and the circulating oxidative stress biomarkers measured in this study were not closely related. This suggests that the two measures should be considered alongside each other, rather than interchangeably and that the output from the AGE Reader, SAF, should not simply be considered as a marker of oxidative stress.
5.2 Background

Foods are a known exogenous source of AGEs (40), and dAGEs have been shown to influence the body’s total AGE level (see Section 4.2). It is understood that approximately a third of dietary AGEs consumed are absorbed through the digestive tract, then circulate in the body (493). It is not currently known if dAGEs accumulate in tissues or if they cause biological consequence (51). It has however been suggested that after urinary excretion a proportion of dAGEs remain in the body (494) and rodent studies have evidenced that CML-rich diets result in accumulation in tissues (495-497). Dietary AGE intake represents a potentially modifiable target (360), hence this is an area of interest.

AGEs are known to accumulate in cells and tissues throughout the body, however the amount of AGEs that are contributed exogenously from the diet versus those that produced endogenously is unclear. Measuring tissue-bound AGE levels is thought to give an indication of the total AGE levels in the body. The contribution of dAGE to tissue-bound AGE levels has not been widely researched due to difficulties in assessing human tissue (493). As explained in Section 3.3.2.4., the AGE reader provides a measurement of skin autofluorescence (SAF) which has been proposed as a marker for tissue-bound AGE levels (21, 23).

Using the AGE-reader, Klenovics et al. (2014) found that infants had a higher SAF if they were formula fed compared to breast fed infants (498), Nongnuch et al. (2015) measured SAF levels in haemodialysis patients and found those with a vegetarian diet had a lower SAF level (499), Botros et al. (2017) found that SAF
and coffee intake positively correlated with one another (500), and Kellow et al. (2017) measured SAF in a group of healthy adults and found those with a higher intake of meat products had a higher SAF level. None of these studies used an AGE specific FFQ or AGE database to calculate dAGE consumption. A recent study by Hörner et al. (2018) used a FFQ to measure dAGE in a group of hemodialysis patients and found that SAF did not correlate with dAGE intake (501).

SAF, by its nature, can only measure the level of fluorescent AGEs in the skin; examples of fluorescent AGEs include pentosidine and crossline, the previously discussed AGEs CML, CEL and MG-H1 do not fluoresce. The lack of fluorescence of all types of AGEs is therefore considered a limitation of SAF and consequently of the AGE-reader (37, 502). However, Meerwaldt et al. (2004) did show that levels of fluorescent and non-fluorescent AGEs do correlate with one another, suggesting that SAF could in fact be a marker of total AGE in the skin (21). Another consideration with SAF readings is that other molecules in the skin fluoresce as well as AGEs, examples include porphyrins (organic compounds) and redox regulated fluorophores such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide (502, 503). While this is important to consider, Smit et al. (2010) found that approximately 75% of the SAF variance was due to variability of the AGE pentosidine, confirmed by skin biopsies (33).

In a similar manner to AGEs, oxidative stress develops as part of the body’s normal metabolism and is linked with ageing (114). This supports the notion that ageing is multifactorial (114, 504). When considering the influence that AGEs
may have on the development of age-related disease, it is important to take into consideration the complex relationship between AGE levels and oxidative stress levels in the body. Indeed, endogenous AGE formation is accelerated by increased levels of oxidative stress and blood glucose (9). Increase in AGE levels is both a cause and an effect of elevated oxidative stress (11), with oxidative stress accelerating AGE formation and the production of free radicals, as well as being produced as a by-product of AGE formation and AGE-RAGE binding interaction (see Section 1.7) (112, 113). AGES are considered to be pro-oxidant substances, because their formation increases the cellular level of oxidative stress (361, 362). It has been found that serum CML levels correlate with the ELISA measured oxidative stress biomarker 8-isoprostane (45). It has been suggested that dAGEs are directly associated with oxidative stress as a low-AGE diet resulted in a significant reduction in 8-isoprostanes and vascular cell adhesion molecule one (VCAM-1) (505). In addition it has been found that dAGE intake weakly positively correlates with the inflammation marker high sensitivity C-Reactive Protein (hsCRP) (45). Although statistically significant results have been reported the proposed risk of dAGEs to health has been questioned, since the results of these studies have been described as low grade evidence (493).

As described in Section 1.7, the vast majority of the literature differentiates between AGES and oxidative stress, measuring each individually while discussing the known complex relationship (8, 45, 115-117). However, there are examples in the literature which use the terms interchangeably, with some using AGES as biomarkers of oxidative stress (92, 118, 119), suggesting that the two are perhaps measurements of the same thing. Understanding the relationship
between circulating oxidative stress markers and AGE levels could help increase understanding of which may be the primary influence and whether they are measures of the same thing, or should be considered, and potentially targeted, individually.

Increased body mass index (BMI) is known to result in increased oxidative stress (506) as well as smoking (507) and alcohol (508). Kellow et al. (2017) found that increased age, smoking and BMI correlated with increased SAF (494). These demographic, general health parameters are known associates of the AGEs and oxidative stress and therefore need to be considered. Understanding which parameters influence AGE and oxidative stress levels in healthy participants could help increase understanding as to the demographic factors that accelerate the physiological ageing process. It is also important to identify possible confounders in this healthy population before examining participants with pathology.

5.2.1 Aims
To determine whether dietary AGE intake is associated with tissue-bound AGE levels (SAF) in a healthy ageing population, to determine whether circulating oxidative stress levels relate to tissue-bound AGE levels (SAF) and dAGE in a healthy ageing population and to explore the influence of BMI, smoking history, alcohol consumption, body fat percentage and blood pressure on oxidative stress, SAF and dAGE, in the same population.
5.2.2 Hypothesis
Dietary AGE intake will be associated with SAF in healthy controls, and both dAGE and SAF will relate to the measured oxidative stress biomarkers.

5.3 Methods
The methodology that will be used in this study is summarised here; more detailed information on the specifics of each technique can be found in Chapter 3.

5.3.1 Participants
Convenience sampling was used to recruit participants who were eligible according to the inclusion and exclusion criteria for healthy ageing participants as detailed in Section 3.1.3. and Section 3.1.4. Due to the established link between diabetes and AGEs (449, 450), participants diagnosed with diabetes, or participants found to have a high fasting glucose level were excluded from the study, as were current smokers, and previous smokers who quit less than 15 years ago due to the link between AGEs and tobacco (10).

5.3.2 General health investigations
As described in 3.3.2.1, height was measured in meters using a stadiometer, weight was measured in kg and from these measurements BMI was calculated. Weight, basal metabolic rate (BMR), and body fat percentage were measured using the Tanita body composition analyser (TBF-300 MA). Blood pressure was measured and a fasting venous blood sample was obtained to enable the measurement of oxidative stress biomarkers in plasma. Three oxidative stress
biomarkers were used, the non-enzymatic antioxidants ascorbate (vitamin C) and alpha tocopherol (a form of vitamin E) and the product of lipid peroxidation malondialdehyde (MDA). Each biomarker is described in Section 3.3.2.6. A normal range for ascorbate levels would be 30 to 150 µM (509) and MDA range 0.36 and 1.24 µM, and (429). There is currently no established normal range for plasma alpha tocopherol concentration, however the mean plasma alpha tocopherol concentration is 32.5 µM for adults aged 19-64, in the UK (454).

5.3.3 Skin autofluorescence (SAF)

The measurement of tissue-bound AGE levels was obtained using the AGE Reader (DiagnOptics B.V., Groningen, The Netherlands). The AGE Reader is a non-invasive device, which provides a low cost, fast, and reproducible means of determining AGE levels in the body through utilisation and measurement of the fluorescent properties of tissue-bound AGEs in the skin (21-23). Specific detail on the AGE reader and its protocol can be found in Chapter 3, Section 3.3.2.4.

5.3.4 Dietary AGE, semi-quantitative FFQ

An assessment of dietary AGE intake over the last year was made using a semi-quantitative FFQ that was designed specifically to assess dietary AGE levels in a UK population for the purposes of this research (Chapter 4, Appendix 4.1). The FFQ was administered to all participants. The results from the FFQ were calculated with each of the databases described in Chapter 4. As explained previously in Section 4.3.1, use of all of the different AGE databases available in this study enabled the measurement of dietary intake of CEL and MG-H1 in addition to CML from the FFQ responses. It is important to
note that CML, CEL and MG-H1 do not fluoresce (510), hence the FFQ measures different types of AGEs to the AGE-reader. Furthermore, it is thought that the fluorescent AGE pentosidine may have only a minor role in the Maillard reaction within foods (511).

5.3.5 Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM). The normality of data was assessed by the Shapiro–Wilk test and visual inspection of the QQ plots. P values of less than 0.05 were considered significant.

5.3.5.1 SAF and dAGE

Spearman’s rank correlation was used to analyse the association between SAF and dAGE. This analysis was carried out separately for each dAGE database under consideration, namely: Uribari et al. (2010), Hull et al. (2012) and Scheijen et al. (2016).

5.3.5.2 Relationship between oxidative stress, SAF and dAGE

Spearman’s rank correlation was used to analyse the association between oxidative stress and SAF. This analysis was carried out separately for each oxidative stress biomarker, namely: ascorbate (vitamin C), malondialdehyde (MDA) and alpha tocopherol (form of vitamin E).

Spearman’s rank correlation was used to analyse the association between oxidative stress and dAGE. This analysis was carried out for each oxidative stress biomarker and each dAGE database.
5.3.5.3 Relationship between demographic variables, SAF, dAGE and oxidative stress

Mann-Whitney U test and Kruskal-Wallis test were used to analyse the demographic data. Spearman’s rank correlation was used to assess the relationship between SAF and each clinical measure namely: blood pressure, BMI, BMR, body fat percentage, fat mass as well as dietary AGE, and oxidative stress biomarkers. The same statistical analyses were repeated for dAGE database and each oxidative stress parameter. Any significant correlations were entered into a stepwise forward and backward multiple linear regression to further assess the influence of different variables on SAF.

5.3.5.4 Power calculation

Sample size was determined using G*Power 3 programme (Heinrich-Heine, Universität Düsseldorf). In order to achieve 80% power at the 5% alpha level with a medium effect size of 0.3, 64 participants would be required. To account for unusable data an allowance was made and a sample size of 71 participants was chosen.

5.4 Results

A total of 70 participants were recruited, 68 of which were eligible for the study. The 68 eligible participants were free of acute or chronic diseases, including diabetes, with the exception of controlled hypertension. Participants who reported a major change to their diet over the last 12 months were excluded.
characteristics of the participant group with mean and standard deviation are
detailed in Table 5.1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong> (n)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
</tr>
<tr>
<td>Female</td>
<td>46</td>
</tr>
<tr>
<td><strong>Age</strong> (years)</td>
<td>60.84 ± 9.67</td>
</tr>
<tr>
<td><strong>Ethnicity</strong> (n)</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>62</td>
</tr>
<tr>
<td>White other</td>
<td>2</td>
</tr>
<tr>
<td>Mixed white/Asian</td>
<td>1</td>
</tr>
<tr>
<td>Mixed white/black Caribbean</td>
<td>2</td>
</tr>
<tr>
<td>Indian</td>
<td>1</td>
</tr>
<tr>
<td><strong>Body mass index</strong> (BMI)</td>
<td>26.12 ± 4.62</td>
</tr>
<tr>
<td><strong>Basal metabolic rate</strong> (BMR) (kcal)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1444 ± 232</td>
</tr>
<tr>
<td>M</td>
<td>1667 ± 217, <strong>F: 1338 ± 150</strong></td>
</tr>
<tr>
<td><strong>Body fat percentage</strong> (%)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>31.75 ± 8.73</td>
</tr>
<tr>
<td>M</td>
<td>23.41 ± 6.51, <strong>F: 35.74 ± 6.59</strong></td>
</tr>
<tr>
<td><strong>Fat mass</strong> (kg)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>23.73 ± 9.94</td>
</tr>
<tr>
<td>M</td>
<td>19.88 ± 8.44, <strong>F: 25.57 ± 10.15</strong></td>
</tr>
<tr>
<td><strong>Systolic blood pressure</strong> (SBP) (mmHg)</td>
<td>129.54 ± 17.36</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure</strong> (DBP) (mmHg)</td>
<td>81.18 ± 10.29</td>
</tr>
<tr>
<td><strong>Alcohol units per week</strong></td>
<td>6.07 ± 6.59</td>
</tr>
<tr>
<td><strong>Smoking history</strong> (n)</td>
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</tr>
<tr>
<td>Non-smoker</td>
<td>43</td>
</tr>
<tr>
<td>Previous smoker, quit &gt;20yrs ago</td>
<td>24</td>
</tr>
<tr>
<td>Previous smoker, quit &gt;15yrs ago</td>
<td>1</td>
</tr>
<tr>
<td><strong>Skin autofluorescence</strong> (SAF) (AU)</td>
<td>2.24 ± 0.39</td>
</tr>
</tbody>
</table>

Table 5.1: Mean and standard deviation of participant group characteristics. AU: arbitrary units.

* P < 0.05, ** P < 0.001 difference between M: males and F: females.
The characteristic BMR (kcal) was significantly higher in males, both body fat percentage and fat mass measurements were significantly higher in females. All other characteristics were not statistically different between genders. Figure 3.17, Chapter 3 shows the AGE Reader measurement report displayed after an AGE reading and visually displays what a 'normal' SAF reading would be depending on age of the participant. The reference values used in the graph are from mean SAF data for age from Koetsier et al. (2010) (512). The average SAF value for the average age of 61 would be 2.25 AU.

5.4.1 SAF and dAGE

Spearman’s correlation showed a positive relationship between SAF and dAGE (MG-H1) calculated from the Scheijen database (n = 68, r = 0.319, p = 0.008, 95% bootstrap confidence interval 0.099 to 0.511) (Figure 5.1). No significant correlations existed between the other dAGE values calculated from the FFQ responses (Uribarri CML, Hull CML, Scheijen CML and CEL) and SAF (Table 5.2).
Figure 5.1: Positive relationship between SAF and dAGE MG-H1 calculated from the FFQ responses using the Scheijen et al. (2016) database (n = 68, r = 0.319, p = 0.008, 95% CI 0.099 to 0.511).

Table 5.2: Mean, standard deviation and Spearman’s correlations between SAF (AU) and dAGEs calculated with each database.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAF</td>
<td></td>
<td>Uribarri Hull CML Scheijen CML Scheijen CEL Scheijen MG-H1</td>
</tr>
<tr>
<td></td>
<td>2.24 ± 0.39</td>
<td>0.075</td>
<td>0.210 0.190 0.175 0.319</td>
</tr>
<tr>
<td></td>
<td>p = 0.545</td>
<td>p = 0.085</td>
<td>p = 0.120 p = 0.153 p = 0.008*</td>
</tr>
</tbody>
</table>

Table 5.2: Mean, standard deviation and Spearman’s correlations between SAF (AU) and dAGEs calculated with each database. *p<0.05
5.4.2. Oxidative stress, SAF and dAGE

5.4.2.1 Relationship between oxidative stress and SAF

Spearman’s correlation showed a positive relationship between SAF and the oxidative stress biomarker alpha tocopherol \((n = 68, r = 0.252, p = 0.044, 95\% \text{ bootstrap confidence interval 0.007 to 0.463})\) (Figure 5.2). No significant correlations existed between MDA and SAF or ascorbate and SAF (Table 5.3).

<table>
<thead>
<tr>
<th>Oxidative Stress Biomarker</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>Spearman’s Correlation Coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate (µM)</td>
<td>50.41</td>
<td>± 18.52</td>
<td>-0.207, ( p = 0.093 )</td>
</tr>
<tr>
<td>MDA (µM)</td>
<td>0.61</td>
<td>± 0.19</td>
<td>0.072, ( p = 0.570 )</td>
</tr>
<tr>
<td>Alpha tocopherol (µM)</td>
<td>43.68</td>
<td>± 16.01</td>
<td>0.252, ( p = 0.044^* )</td>
</tr>
</tbody>
</table>

\( ^* \text{p}<0.05 \)

Table 5.3: Mean, standard deviation and Spearman’s correlations between SAF (AU) and each oxidative stress biomarker: ascorbate (vitamin C), malondialdehyde (MDA) and alpha tocopherol (form of vitamin E). 

\( ^* \text{p}<0.05 \)
Figure 5.2: Weak positive relationship between alpha tocopherol (µM) and SAF (AU) \( (n = 68, r = 0.252, p = 0.044, 95\% \text{ CI} 0.007 \text{ to } 0.463) \).

5.4.2.2 Relationship between oxidative stress and dAGE

No statistically significant correlations existed between the oxidative stress biomarkers (ascorbate, MDA and alpha tocopherol) and dAGEs derived from any of the databases.
5.4.3. Relationship between demographic variables, SAF, dAGE and oxidative stress

5.4.3.1 Relationship between demographic variables and SAF

Spearman’s correlation showed a strong positive relationship between increased age and SAF \((n = 68, r = 0.420, p < 0.001, 95\% \text{ bootstrap confidence interval } 0.182 \text{ to } 0.613)\) (Figure 5.3). No statistically significant correlations existed between any other demographic variables and SAF.

Figure 5.3: Positive relationship between age (years) and SAF (AU) \((n = 68, r = 0.420, p < 0.001, 95\% \text{ CI } 0.182 \text{ to } 0.613)\).
The demographic history of previous smokers who had quit more than 15 years ago, and participants who had ‘never’ smoked, were analysed to detect if there was a difference related to SAF. There was no significant difference (p = 0.788).

In a similar manner alcohol units consumed per week were assessed. Nine participants did not drink alcohol, 53 drank a low to moderate amount of units (1-14 units per week) and 6 participants drank 15 or more units, classified as high consumption due to it being above the UK recommended units consumed per week (513). There was no significant difference in SAF level between the different alcohol consumption groups (Kruskal-Wallis; p = 0.616).

An incidental finding regarding reflectance, a measurement obtained from the AGE-reader when measuring SAF, was discovered during data analysis. Reflectance was found to correlate with BMI (R = 0.244, p = 0.044, 95% bootstrap confidence interval -0.001 to 0.473), body fat percentage (R = 0.311, p = 0.01, 95% bootstrap confidence interval 0.057 to 0.530), and fat mass (R = 0.268, p = 0.027, 95% bootstrap confidence interval 0.020 to 0.502).

5.4.3.2 Relationship between demographic variables and dAGE

No statistically significant correlations existed between the demographic variables and different measured dAGEs.

5.4.3.3 Relationship between demographic variables and oxidative stress

Spearman’s correlation showed a negative relationship between ascorbate and BMI (n = 68, r = -0.345, p = 0.004, 95% bootstrap confidence interval -0.115 to -0.535) (Figure 5.4) and negative relationship between ascorbate and BMR (n =
68, r = -0.328, p = 0.007, 95% bootstrap confidence interval -0.108 to -0.517)

(Figure 5.5). No statistically significant correlations were found between any other demographic variables and oxidative stress biomarkers.

Figure 5.4: Negative relationship between ascorbate (µM) and BMI (n = 68, r = -0.345, p = 0.004, 95% bootstrap confidence interval -0.115 to -0.535).
5.4.4. Multiple linear regression

Considering all findings together, within the variables considered (dAGE, oxidative stress markers and demographic parameters), there were three variables which correlated with SAF, the demographic parameter age as well as the dAGE MG-H1 and oxidative stress biomarker alpha tocopherol. Table 5.4 summarises all of the variables which correlated with SAF.
Forwards stepwise and backwards multiple linear regression analysis was performed to determine the factors that had the most influence on SAF. The independent variables included were those which had previously correlated with SAF: age, dAGE MG-H1 and alpha tocopherol. BMI did not correlate with SAF \((n = 68, r = 0.173, p = 0.159, 95\% \text{ bootstrap confidence interval } -0.065 \text{ to } 0.381)\), however BMI was included since the literature suggests that it is a confounding variable (494).

Age was the single best predictor \((p < 0.001)\), followed by BMI \((p = 0.008)\). These findings were also found to be true in the backwards model.
5.5 Discussion

The aim of this study was to determine how dAGE intake relates to SAF in a healthy ageing population, as well as to determine whether circulating oxidative stress biomarker levels relate to SAF and dAGE and the relative influence that a variety of demographic parameters may have on these measures. We believe that this is the first study to examine the influence of dAGE on tissue-bound AGE (SAF) levels using a specifically designed FFQ for measuring dAGE with different databases.

5.5.1 SAF and dAGE

A positive relationship was found in this study between the dAGE MG-H1 and SAF, however no relationship was found between SAF and dietary intake of CML and CEL. As explained in Section 5.2 only a few studies have previously assessed the relationship between dAGE and SAF. Only one of these studies used an AGE specific FFQ to calculate dAGE consumption, Hörner et al. (2018) in a group of haemodialysis patients found that SAF did not correlate with dAGE intake. The FFQ used by Hörner et al. (2018) only used the Uribarri database, hence could only measure the dAGE CML (501). Lack of correlation between the dAGE CML and the results of this study agree with one another. No previous studies have examined the relationship between CEL and MG-H1 and SAF.

MG-H1 is known to be a major type of AGE (514), although CML is currently the favoured dAGE marker, the result from this study supports the notion that databases should expand and examine multiple types AGES in the diet. The weak correlation between MG-H1 and SAF suggests that MG-H1 absorbed from
the diet may be accumulating in the tissues. The lack of relationship between dietary CML and CEL and SAF suggests that these dAGEs may not contribute, or contribute only to a low extent, to tissue-bound AGE levels. The difference between the types of dAGEs may be due to reactivity or structural differences (see Figure 4.8 and Figure 4.9). CML and CEL can bind to RAGE when in an embedded peptidic structure (515), however when considering free CML and CEL it has been suggested that they do not readily bind to RAGE, whereas free MG-H1 does (492, 516). These differences in reactivity with RAGE suggests that circulating levels of MG-H1 may be more likely to react and therefore be less likely to be excreted from the body and potentially more likely to accumulate in the tissues. This difference in reactivity between types of dAGEs could indicate possible differing pathological effects which would be important to consider.

Important to note is that whilst the correlation between MG-H1 and SAF was moderate, when MG-H1 was included in the multiple linear regression it was not a significant predictor of SAF. This supports the theory that AGEs formed endogenously create the largest burden on the tissues of the body, and that diet makes only a minor contribution to overall tissue-bound levels. Further research is required to assess if dAGE restriction is a viable treatment option to reduce tissue-bound AGE levels.

5.5.2 Relationship between oxidative stress, SAF and dAGE
As explained in Section 5.2, when considering AGEs, it is important to take into consideration the complex relationship between AGE and oxidative stress levels
in the body. Increased understanding is needed to establish if they are measures of the same thing, or should be considered, and potentially targeted, individually.

In an environment of increased oxidative stress it would be expected that the oxidant MDA would be increased and the antioxidants ascorbate and alpha tocopherol decreased (103). There are only a few studies directly comparing oxidative stress biomarkers and SAF. Hartog et al. (2006) measured vitamin C (ascorbate) and vitamin E plasma levels in renal transplant recipients and found that increased SAF was related to decreased plasma vitamin C levels and no association was found between SAF and vitamin E (517). Himori et al. (2016) found that the oxidative stress biomarker 8-hydroxy-2’-deoxyguanosine did not correlate with SAF (119). The oxidative stress biomarker alpha tocopherol in this study was found to weakly positively correlate with SAF and borderline significant (*p = 0.044*). Both ascorbate and MDA were found not to correlate with SAF. The lack of correlation of MDA and ascorbate with SAF may be attributed to the participants being healthy and not being in a state of oxidative stress. Indeed, MDA and ascorbate levels were within a healthy normal range, and the antioxidant alpha tocopherol levels were above normal (429, 454, 509). In addition oxidative stress biomarkers are short-term measures, providing only a measurement for that moment in time whereas the tissue-bound SAF measure is thought to indicate longer-term tissue damage (418).

Both ascorbate and alpha tocopherol are thought to be appropriate oxidative stress biomarkers (518). Frei (1989) proposed that ascorbate is the most effective antioxidant present in the plasma and is vital in the protection against
oxidative stress (427). Low levels of ascorbate have been linked with AGEs and AGE inhibitors such as aminoguanidine (519) as well as being linked to endothelial dysfunction (520, 521). Alpha tocopherol has also been shown to regulate inflammation related to neurodegeneration in Alzheimer's disease (522, 523).

As explained in 3.3.2.6.2, alpha tocopherol is one of the eight forms of vitamin E and is the main lipid soluble antioxidant found in the plasma (433). The mean UK plasma alpha tocopherol concentration is 32.5 µmol/L for adults aged 19-64 (454), as shown in Table 5.1 the adults in this study (mean age 61) had mean alpha tocopherol of 43.68 ± 16.01 µmol/L. This higher mean may be in part due to a 'healthy volunteer effect', in that the inclusion/exclusion criteria and protocol of this study is likely to have attracted a healthier volunteer sample (524, 525). Nevertheless, the finding of a positive correlation between alpha tocopherol and SAF in this study was not expected. Indeed, as alpha tocopherol is a lipid soluble antioxidant, higher levels are considered to be beneficial, as alpha tocopherol has been shown to inhibit low density lipoprotein oxidation and inhibit glycation (526-528). Culbertson et al. (2003) used a novel AGE inhibitor, 6-dimethylaminopyridoxamine (dmaPM) which was pharmacologically designed to scavenge free radicals in a similar manner to non-enzymatic antioxidants, such as alpha tocopherol. Interestingly, they similarly found an unexpected result, as dmaPM (antioxidant) increased the levels of pentosidine (fluorescent AGE) (529). The similarities in unexpected results could however be coincidence, as the theory of an antioxidant encouraging AGE formation opposes the previous literature. Indeed, it should also be considered that the positive correlation
between alpha tocopherol and SAF found in this study was only weak (R = 0.252, p = 0.044) and when put through multiple regression analysis alpha tocopherol was not found to be a predictor of SAF.

Previous research has used the AGE reader’s SAF measure as a biomarker for oxidative stress (118, 119). The AGE reader has not been validated or developed to measure oxidative stress. Oxidative stress is particularly challenging to measure as reactive species are unstable, oxidative stress biomarkers are used to give an indication of the level within the body. As mentioned above the recognised oxidative stress markers ascorbate and MDA did not correlate with SAF and alpha tocopherol had a weak positive correlation. These results suggest that SAF may not be a suitable biomarker of oxidative stress and helps to clarify that it is not acceptable to interchange between oxidative stress and AGE (SAF) level, since although related, they appear to measure different things.

With regard to dAGE, none of the oxidative stress biomarkers tested in the study related to dAGE however previous studies have suggested that increased intake of dAGEs is directly linked to oxidative stress in mice and humans (45, 57, 505, 530). The markers of oxidative stress used in these studies however were: CML, MG derivatives, hsCRP, VCAM-1, plasma 8-isoprostane and fibrinogen. Although relevant, CML is a type of AGE, MG a precursor to AGE formation, hsCRP and VCAM-1 are considered inflammation markers, and fibrinogen is a coagulation factor. The only specific oxidative stress biomarker measured in previous studies was plasma level of 8-isoprostane, which is a product of lipid peroxidation (531). MDA was chosen as a biomarker in this thesis as it has been linked with AGEs
as well as glaucoma, with increased levels of MDA having been associated with glaucoma (108) and glaucoma severity (107, 109, 315). Since both MDA and 8-isoprostane are products of lipid peroxidation it was expected that MDA would also correlate with dAGE, however this was not the case. This difference in findings may in part be related to the way in which dAGE was measured between the studies however, rather than oxidative stress biomarker differences. Methodology differences can make study findings more difficult to compare.

5.5.3 Demographic variables with SAF, dAGE and oxidative stress

No meaningful relationships were found in this study between dAGEs and any of the different demographic variables measured. Hörner et al. (2018) presented similar results suggesting the lack of a meaningful association between measurements such as BMI and dAGE (501).

Both age and BMI were found to be statistically significant predictors of SAF in this study. The strong correlation between age and SAF was an expected finding as it is known that AGEs accumulate with age (494, 500, 532). There are conflicting conclusions in previous research regarding the correlation between SAF and BMI. Some studies have found that in healthy adults BMI and SAF do not correlate (498, 500, 533), and others have found statistically significant correlations (494, 532, 534). However Corstjens et al. (2008) found that BMI was only significantly related to SAF in healthy adults under the age of 40 (532). Indeed, this may explain the lack of a correlation found in this study as all recruited participants were aged 50 or older. Higher BMI and BMR in this study were also related to lower ascorbate levels and in an environment of increased
oxidative stress it would be expected that ascorbate, as an antioxidant would be at a decreased level (103). These results support the already established relationship between oxidative stress and increased BMI and obesity (506, 535, 536), with Kimmons et al. (2006) similarly finding that ascorbate levels were lower obese participants (537).

No relationship was found between SAF, smoking history and alcohol intake in this study, or between circulating oxidative stress markers and these parameters. Tobacco smoke can increase oxidative stress and is a known exogenous source of AGEs (10, 507, 538). It has previously been shown that smoking and smoking history are positively correlated with increased SAF (512, 533, 534). However, the finding that SAF was not at a higher level in the previous smoker participants compared to ‘never’ smoked participants in this study is consistent with previous research by van Waateringe et al. (2017) who found that smoking cessation had a reversible effect on SAF level. After approximately 15 years of smoking cessation SAF levels are thought to be comparable to ‘never’ smokers (539). All of the participants who were ‘previous smokers’ in this study had been non-smokers for a minimum of 15 years, with the majority having not smoked for 20 or more years (see Table 5.1). In a similar manner the finding that there was no significant correlation between circulating oxidative stress biomarkers and smoking history is consistent, since it had been such a long period of smoking cessation (540). The lack of relationship between alcohol consumption and SAF is also in accordance with the previous literature; Jochemsen et al. (2009) found alcohol consumption not to be correlated with SAF (541). In addition the different
AGE databases agree with one another, estimating alcohol intake to be of either little or no value (14, 53).

As previously stated in Section 3.3.2.4 the AGE reader is able to measure pigmented skin ranging from 1 to 4 in the Fitzpatrick skin colour classification. It is unable to read darker skin pigment classifications 5 and 6. As shown in Table 5.1 the majority of participants in this study were Caucasian or ‘white other’. This reflects the population in the South West of England where 95% of the population describe themselves as ‘White British’ or ‘White other’ (542). All participants who volunteered for this study had their AGE reading successfully measured, this included participants who described their ethnicity as ‘Indian’ and ‘Mixed white/black Caribbean’ whose characteristics would usually be linked to Fitzpatrick class 5 (416, 543). This suggests reflectance thought to be associated with certain ethnicities and the capabilities of the AGE reader should be reviewed. Due to the low number of participants with darker skin pigmentation in this study the relationship between ethnicity, skin reflectance and SAF could not be determined.

An incidental finding regarding reflectance, an additional output from the AGE-reader when measuring SAF, was revealed during data analysis. Reflectance positively correlated with body fat percentage, fat mass and BMI. Fat cells (adipocytes) aggregate to form adipose tissue. Bashkatov et al. (2005) measured human subcutaneous adipose tissue and found it to have a spectral range from 400 to 1500 nm (544). As explained in Section 3.3.2.4 the AGE reader emits UV-A light with a peak wavelength of 375 nm to excite the fluorescent AGEs in the
skin. Since these wavelengths do not overlap the adipocytes should not be excited. However results from this study show a correlation between the demographic variables body fat percentage, fat mass and BMI with skin reflectance. Reflectance is not a parameter usually reported by studies using the AGE reader, hence this correlation has not previously been reported. This relationship requires further investigation since reflectance is part of the SAF calculation. A possible relationship could therefore impact and influence SAF readings.

5.6 Conclusion

This study suggests that the contribution of dietary AGE to tissue-bound AGE levels may be minimal. This however requires further investigation, this will be aided by the development of new AGE databases, expanding with more types of AGEs measured than in the past (444).

The relationship between oxidative stress and AGES was investigated. No associations between oxidative stress biomarkers and dAGE were found. The oxidative stress biomarker alpha tocopherol weakly correlated with SAF, this was however in the opposite way than was hypothesised and therefore requires further investigation before the relevance and insight that could be gained from this finding can be determined. The results suggest that tissue-bound AGES measured by SAF and the circulating oxidative stress biomarkers measured in this study are not closely related. For this reason the AGE reader, SAF, should not simply be used as a marker for oxidative stress and instead the two
measures should be used alongside each other, rather than interchangeably in place of each other.

5.7 Limitations

Plasma CML (ng/ml) was not included in this chapter. Statistical analyses were performed to investigate the relationships between plasma CML and SAF, demographic parameters, dAGE and oxidative stress biomarkers. No relationships were found between plasma CML and any of these listed parameters. We believe that this may be due to the standards given with the ELISA kit not being in the same range as the measurements found which impacted the results.

As mentioned in Section 5.2 the AGE reader is only able to measure fluorescent AGEs. None of the dAGEs calculated from the databases available have fluorescent properties. It has however been shown that fluorescent AGEs positively correlate with other non-fluorescent AGEs (21), as part of validation of the AGE reader. In addition, other molecules in the skin are known to fluoresce and influence SAF measurement.

FFQs are heavily reliant on accurate responses from the participant. This is a limitation of all FFQ based studies.

This study did not measure the levels of AGEs excreted by the body in the urine. This data would have enabled a calculation of the amount of dAGEs absorbed in
the body. It would be interesting to know if amount of dAGE absorbed relates to SAF.

5.8 Future work

To measure dAGE intake, dAGE excretion, and SAF to assess if absorption is associated with tissue-bound AGE levels. Since the AGE reader was able to successfully measure SAF readings in participants whose characteristics would be linked to Fitzpatrick class 5 further research is required to better understand the capabilities of the AGE reader and establish which ethnicity groups can be measured accurately. Further investigation into how body fat percentage and fat mass impact SAF measurements is also required.
6.0 AGE levels in early stage POAG, NTG and OHT patients

6.1 Abstract

Purpose: To determine whether tissue bound and dietary AGE levels are elevated in POAG and NTG in comparison to age-matched OHT patients and healthy controls. Oxidative stress levels were considered as a possible influencing factor.

Methods: Skin autofluorescence (SAF) as a measurement of tissue-bound AGE level was measured in 33 healthy control participants, 16 OHT, 12 NTG and 32 POAG patients. A fasting venous blood sample was obtained to enable the measurement of oxidative stress biomarkers.

Results: SAF was significantly higher in NTG and POAG compared to healthy controls (p = 0.005). Furthermore, POAG patients had a lower ascorbate (p = <0.001) and alpha tocopherol (p = <0.001) level than healthy controls. There was no statistically significant difference in dAGE value between the groups nor between contributing factors such as BMI and blood pressure.

Conclusion: AGE (SAF) level was significantly higher in NTG and POAG compared to healthy controls. This finding adds to the evidence that SAF, as an accessible measure, may be a suitable long-term biomarker of glaucoma. Patients with POAG exhibited lower levels of the antioxidants ascorbate and alpha tocopherol compared to OHT and healthy controls, suggesting oxidation inhibition. Dietary AGE intake was similar between healthy controls, OHT, NTG and POAG patients.
6.2 Background

AGEs are known to accumulate in and around the ONH with the specific AGE pentosidine having been found in increased amounts in cribiform plates of the lamina cribrosa (203) and the specific AGE pyrraline being found in the sclera, pia matter, cribiform plates, optic nerve connective tissues and around the vessels in the optic nerve (204). It is hypothesised that AGE accumulation in and around the ONH may contribute to the mechanism of GON (204). Indeed, Tezel et al. (2007) measured the accumulation of the specific AGE CML in glaucomatous and age-matched control donor eyes, and found an increase in AGE and RAGE in those with glaucoma (20). The AGEs were primarily detected in the cribiform plates of the optic nerve head, while RAGE was seen on glial cells, mainly the Müller cells (20). It is thought that AGEs may be responsible for the reduced ability of the cribiform plates to withstand strain, increased rigidity of lamina cribrosa (20) and may also impair the ONH vasculature. The increased presence of AGEs in glaucomatous tissues and AGEs accumulation with age supports the accelerated ageing theory of glaucomatous optic neuropathy development (20).

As well as accumulating in the ONH AGEs affect vascular function in the eye by creating crosslinks in the vessel wall collagen causing stiffening (136, 341, 342) and by creating an imbalance in ET-1 and NO (132, 133) promoting endothelial dysfunction. The cumulative effect is that accumulation of AGEs in blood vessels negatively affects vessel structure and function. This is relevant as compromised or variable supply of blood to the optic nerve head causes retinal ganglion cell death (255, 259). Blood flow regulation in GON has been widely researched
(260-263) and has been linked to increased severity of the disease (260, 261, 264).

To date, only a few studies have been able to explore the possibility that AGEs may be an underlying causative factor for GON. In addition to the study by Tezel et al. (2007), which used healthy control and glaucoma donor eyes (20), Moschos et al. (2017) measured two RAGE gene polymorphisms in POAG patients and healthy controls. The results showed that the gene and allele frequencies were not significantly different between the groups, suggesting that RAGE polymorphisms are not associated with POAG susceptibility (545). A study by Hondur et al. (2017) examined AGE levels in the serum of glaucoma patients and found AGE levels were higher than in control patients, however this study did not state the type of AGE measured by ELISA, hence this result should be viewed with caution (546). The other three studies examining AGE in glaucoma have used the AGE-reader (DiagnOptics B.V., Groningen, The Netherlands) to measure AGE level (SAF). A recent study by Schweitzer et al. (2018) measured SAF in 31 open angle glaucoma patients, and found that SAF level was higher in glaucoma patients than healthy controls (115). The other two studies were conducted by the same research group; the first examined patients with NTG and found that there was no significant difference in SAF levels between NTG and healthy controls, but did report that SAF was correlated with circumpapillary retinal nerve fibre layer thickness and visual field mean deviation (119). The second study similarly found no significant difference in SAF level between OAG patients and healthy controls (118).
It is known that a proportion of OHT participants are likely to develop POAG. The Ocular Hypertension Treatment Study (OHTS) recruited 1,636 participants and over a 5 year follow up found that 4.4% of the treated group and 9.5% of the observation group went on to develop POAG (547). Age is an independent risk factor for OHT participants developing glaucoma (548, 549). It is this link with ageing as well as the known links with glaucoma that makes AGE levels in OHT patients an area of interest. Indeed, in addition to this, OHT patients are interesting as a large proportion of them do not develop POAG. Understanding if AGEs are at similar levels or not to POAG may aid understanding as to why many OHT do not develop POAG.

6.2.1 Aims

To determine whether tissue bound and dietary AGE levels are elevated in early stage POAG and NTG in comparison to age-matched OHT patients and healthy controls. Oxidative stress levels will be compared as a possible influencing factor.

6.2.2 Hypothesis

Tissue bound AGE (SAF) level and dAGE intake will be higher in the glaucoma groups than OHT and control groups. Oxidative stress marker MDA will be higher, and antioxidants ascorbate and alpha tocopherol lower in glaucoma groups compared to OHT and control groups.
6.3 Methods

The methodology used in this study is summarised here; more detailed information on the specifics of each technique can be found in Chapter 3.

6.3.1 Participants

Convenience sampling was used to recruit participants who were eligible according to the study’s inclusion and exclusion criteria, the inclusion criteria for participants are detailed in section 3.1.3, exclusion detailed in section 3.1.4. Due to the established link between diabetes and AGEs (449, 450) participants diagnosed with diabetes, and participants found to have a high fasting glucose level, were excluded from the study, as were current smokers, and previous smokers who quit less than 15 years ago due to the link between AGEs and tobacco (10). POAG, NTG and OHT participants were identified through their attendance at routine outpatient appointments at Derriford REI.

6.3.2 General investigations

As described in 3.3.2.1, height was measured in meters using a stadiometer, weight was measured in kg and from these measurements BMI was calculated. Weight, basal metabolic rate (BMR), and body fat percentage were measured using the Tanita body composition analyser (TBF-300 MA). Blood pressure was measured and a fasting venous blood sample was obtained to enable the measurement of oxidative stress biomarkers. Three oxidative stress biomarkers were used, the non-enzymatic antioxidants ascorbate (vitamin C) and alpha tocopherol (form of vitamin E) and the product of lipid peroxidation malondialdehyde (MDA). Each biomarker is described in Section 3.3.2.6.
Oxidative stress is linked with glaucoma and is thought to be a contributing factor to glaucomatous RGC death (306-309). RGCs are understood to be susceptible to oxidative stress due to their high oxygen consumption (312) and light exposure (313). MDA has been identified as one of the best circulating oxidative stress biomarkers and is commonly used in glaucoma research (550). Oxidative stress and AGEs accompany one another, AGE levels can be influenced by oxidative stress levels and vice versa. Previous chapters have assessed the AGE oxidative stress relationship in healthy controls, the results suggest that AGES measured by SAF and the circulating oxidative stress biomarkers MDA, ascorbate and alpha tocopherol are not closely related. Although oxidative stress is not thought to be the primary influence on AGEs its contribution still needs to be considered. Each biomarker is described in Section 3.3.2.6 and method for analysis described in Appendix 6.

6.3.3 Skin autofluorescence (SAF)

The measurement of tissue-bound AGE levels was obtained using the AGE Reader (DiagnOptics B.V., Groningen, The Netherlands). The AGE Reader is a non-invasive device, which provides a low cost, fast, and reproducible means of determining AGE levels in the body through utilisation and measurement of the fluorescent properties of tissue-bound AGEs in the skin (21-23). Specific detail on the AGE reader and its protocol can be found in Chapter 3, section 3.3.2.4.

6.3.4 Dietary AGE, semi-quantitative FFQ

An assessment of dietary AGE intake over the last year was made using a semi-quantitative FFQ that was designed specifically to assess dietary AGE levels in a
UK population for the purposes of this research. The FFQ design and delivery is discussed in Chapter 4. The FFQ was administered to all participants. The results from the FFQ were calculated with each of the databases described in Chapter 4. As explained previously in Section 4.3.1, use of all of the different AGE databases available in this study enabled the measurement of dietary intake of CEL and MG-H1 in addition to CML from the FFQ responses. It is important to note that CML, CEL and MG-H1 do not fluoresce (510), hence the FFQ measures different types of AGEs to the AGE-reader.

6.3.5 Statistical analysis
Statistical analysis was performed using SPSS version 24 (IBM). The normality of data was assessed by the Shapiro–Wilk test and visual inspection of the QQ plots. P values of less than 0.05 were considered significant.

6.3.5.1 General characteristics between groups
Mann-Whitney U test was used to analyse the demographic data. An ANOVA was used to compare the results of general investigations, namely: gender, age, BMI, SBP, DBP, IOP, MABP and OPP between the four groups (POAG, NTG, OHT and HC).

6.3.5.2 SAF, dAGE and oxidative stress between groups
An ANOVA was used to compare the SAF level between groups, followed by the post-hoc analysis Scheffe. An ANOVA was then used to compare dAGE between groups and oxidative stress between groups.
Parameters identified as different between groups were entered into a correlation with SAF, to examine any possible relationship between SAF and oxidative stress.

6.3.5.3 Power calculation

The sample size was determined using data from previous research which also measured tissue-bound AGE level using AGE Reader between healthy and disease groups (83). In order to achieve 80% power at the 5% alpha level a sample size of 37 per group was required (NTG, POAG, OHT and controls). To account for unusable data an allowance was made and a sample size of 40 participants was chosen per group.

6.4 Results

A total of 32 POAG, 12 NTG, 16 OHT and 33 healthy control participants were recruited and eligible for the study. These recruitment numbers are below intended target; this was due to recruitment difficulties of finding participants that met the strict inclusion criteria. Post-hoc power calculation using G*Power 3 programme (Heinrich-Heine, Universität Düsseldorf) revealed a 66% power at a 5% alpha level. The number of participants recruited were sufficient to obtain significant results.
6.4.1 Group characteristics

There were no significant differences in age, BMI, systemic blood pressure, IOP, MABP and OPP between the four groups (p > 0.05). The characteristics of the participant group with mean and standard deviation are detailed in Table 6.1. It was expected that there would not be a significant difference in IOP between the groups since the OHT, POAG and NTG patients were all taking their prescribed IOP lowering treatment as normal. There was no significant difference between the NTG and POAG group’s visual field mean deviation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HC</th>
<th>OHT</th>
<th>NTG</th>
<th>POAG</th>
<th>ANOVA p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>33</td>
<td>16</td>
<td>12</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.97 ±9.43</td>
<td>65.94 ±8.10</td>
<td>72.17 ±7.16</td>
<td>67.72 ±7.398</td>
<td>0.146</td>
</tr>
<tr>
<td>BMI</td>
<td>25.62 ±3.36</td>
<td>26.48 ±4.58</td>
<td>26.60 ±4.39</td>
<td>26.26 ±4.32</td>
<td>0.844</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>134.82 ±16.93</td>
<td>139.56 ±16.84</td>
<td>140.17 ±17.35</td>
<td>140.00 ±19.16</td>
<td>0.625</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.67 ±10.17</td>
<td>85.38 ±9.28</td>
<td>81.33 ±9.56</td>
<td>84.91 ±9.56</td>
<td>0.252</td>
</tr>
<tr>
<td>IOP (mmHg)</td>
<td>16.39 ±2.72</td>
<td>18.00 ±3.16</td>
<td>14.83 ±3.16</td>
<td>16.71 ±3.93</td>
<td>0.099</td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>98.72 ±11.11</td>
<td>103.44 ±10.68</td>
<td>100.94 ±10.79</td>
<td>103.27 ±11.38</td>
<td>0.340</td>
</tr>
<tr>
<td>OPP</td>
<td>49.63 ±7.91</td>
<td>50.96 ±9.16</td>
<td>52.46 ±7.07</td>
<td>52.17 ±9.36</td>
<td>0.623</td>
</tr>
<tr>
<td>MD (dB)</td>
<td>-0.19 ±0.95</td>
<td>-3.62 ±1.32</td>
<td>-3.95 ±1.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1: Summary of mean and standard deviation of participant characteristics for the study groups. P<0.05 is considered a significant difference. BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, IOP: intraocular pressure, MABP: mean arterial blood pressure, OPP: ocular perfusion pressure, was subsequently calculated using MABP and IOP values, MD: mean deviation from visual field data collected from REI appointment, OHT MD added for reference in brackets.
6.4.2 SAF

A significant difference was found between groups with regard to SAF (p = 0.005, Table 6.2). Post-hoc testing found SAF to be significantly higher in the POAG and NTG patients than in healthy controls (POAG p = 0.018, NTG p = 0.05). SAF level in the OHT patients was not significantly different to healthy controls or to the glaucoma groups (p>0.05).

<table>
<thead>
<tr>
<th></th>
<th>HC (1)</th>
<th>OHT (2)</th>
<th>NTG (3)</th>
<th>POAG (4)</th>
<th>ANOVA p value</th>
<th>Post-hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAF (AU)</td>
<td>2.19 ±0.39</td>
<td>2.44 ±0.42</td>
<td>2.6 ±0.39</td>
<td>2.54 ±0.45</td>
<td>0.005</td>
<td>3, 4 &gt; 1</td>
</tr>
</tbody>
</table>

Table 6.2: Difference in mean SAF between groups. SAF: Skin autofluorescence, AU: arbitrary units. P<0.05 is considered significant.

6.4.3 Dietary AGE

No significant differences were found between groups with regard to any of the dAGE values calculated using FFQ responses (p > 0.05, Table 6.3). In addition, no significant relationships were present between the dAGE calculated for each database with SAF for each group (p = >0.05)
Table 6.3: Difference in mean dAGE between groups (± standard deviation). dAGE: dietary advanced glycation end-products. P<0.05 is considered a significant difference. Uribarri CML (average CML kU/day), Hull CML (average mg CML/day), Scheijen CML (average mg CML/day), Scheijen CEL (average mg CEL/day), Scheijen MG-H1 (average mg MG-H1/day).

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>OHT</th>
<th>NTG</th>
<th>POAG</th>
<th>ANOVA p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uribarri CML</strong></td>
<td>18560 ±6672</td>
<td>19485 ±5963</td>
<td>19899 ±6849</td>
<td>22837 ±15733</td>
<td>0.430</td>
</tr>
<tr>
<td><strong>Hull CML</strong></td>
<td>5.66 ±1.61</td>
<td>6.72 ±2.05</td>
<td>6.13 ±1.83</td>
<td>6.45 ±1.99</td>
<td>0.213</td>
</tr>
<tr>
<td><strong>Scheijen CML</strong></td>
<td>4.03 ±1.53</td>
<td>4.07 ±2.05</td>
<td>3.23 ±0.65</td>
<td>3.69 ±1.17</td>
<td>0.330</td>
</tr>
<tr>
<td><strong>Scheijen CEL</strong></td>
<td>3.03 ±0.89</td>
<td>2.99 ±1.04</td>
<td>2.98 ±0.83</td>
<td>3.12 ±0.91</td>
<td>0.954</td>
</tr>
<tr>
<td><strong>Scheijen MG-H1</strong></td>
<td>30.52 ±9.45</td>
<td>30.86 ±12.91</td>
<td>27.72 ±6.40</td>
<td>32.83 ±10.24</td>
<td>0.497</td>
</tr>
</tbody>
</table>

6.4.4 Oxidative stress biomarkers

A significant difference was found between groups with each of the oxidative stress biomarkers measured (see Table 6.4). Post-hoc testing found the antioxidant ascorbate to be significantly lower in POAG compared to all other groups. In addition, post-hoc testing found that alpha tocopherol was lower in the POAG group compared to HC and OHT groups, and not significantly different to NTG. The ANOVA showed a significant difference in MDA between the groups, however the post-hoc tests did not confirm this, with the tests between groups not significant (p > 0.05).
Table 6.4: Difference in mean oxidative stress biomarker levels between groups (± standard deviation). P<0.05 is considered a significant difference. Ascorbate (uM), MDA (uM), Alpha tocopherol (umol/L).

<table>
<thead>
<tr>
<th></th>
<th>HC (1)</th>
<th>OHT (2)</th>
<th>NTG (3)</th>
<th>POAG (4)</th>
<th>ANOVA p value</th>
<th>Post-hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate</td>
<td>54.14 ±18.44</td>
<td>62.69 ±11.37</td>
<td>52.81 ±11.85</td>
<td>30.83 ±10.85</td>
<td>&lt;0.001</td>
<td>4 &lt; 1,2,3</td>
</tr>
<tr>
<td>MDA</td>
<td>6.43 ±1.85</td>
<td>7.88 ±2.75</td>
<td>5.36 ±1.16</td>
<td>6.09 ±2.73</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>Alpha tocopherol</td>
<td>42.31 ±15.98</td>
<td>41.66 ±6.46</td>
<td>37.85 ±5.36</td>
<td>29.44 ±8.91</td>
<td>&lt;0.001</td>
<td>4 &lt; 1,2</td>
</tr>
</tbody>
</table>

Pearson’s correlation between SAF and ascorbate in the POAG group ($r = -0.130$, $p = 0.226$, 95% bootstrap confidence interval -0.336 to 0.087), and between SAF and alpha tocopherol in the POAG and NTG groups ($r = 0.149$, $p = 0.163$, 95% bootstrap confidence interval -0.064 to 0.351) were not statistically significant. Hence, there was no significant relationship between reduced levels of antioxidants and SAF.

6.5 Discussion

The aim of this study was to determine if both tissue-bound AGE levels (SAF) and dAGE were different in POAG, NTG, OHT and healthy control participants. Oxidative stress was also assessed due to the known links with AGEs and glaucoma. It is thought that this is the first study to examine dAGE and tissue-bound AGE (SAF) levels in OHT, NTG, POAG patients in conjunction with each other.
6.5.1 SAF

POAG and NTG patients were found to have a significantly higher SAF level compared to healthy controls. These results are in agreement with the previously published study by Schweitzer et al. (2018) which found SAF was higher in OAG patients that controls (115), and also agrees with the Tezel et al. (2007) which found accumulation of the AGE CML and RAGE in glaucomatous compared to control donor eyes (20). The result of the study does differ to the two studies by Himori et al., in which SAF was not significantly different between OAG patients and healthy controls (118, 119). The results of the Himori et al. studies may have been impacted by the inclusion of participants with diabetes in the healthy control group.

This study recruited OHT patients in addition to glaucoma patients. Interestingly the mean SAF level for OHT patients was above healthy control levels and below both glaucoma groups. This result does to some extent match what is known about OHT patients in that they do not have a signs of pathology, yet are at increased risk of ONH damage compared to healthy individuals. A long-term follow-up study would aid understanding as to if SAF is linked with OHT patients progression to glaucoma.

Although there are many similarities between NTG and POAG it is thought that there are differing levels of vascular and mechanical involvement (551). Although vascular dysregulation has been identified in individuals with POAG, it is not traditionally considered a primary cause, as opposed to in NTG, where it is thought to be a more significant part of glaucomatous development and
progression (552, 553). On the other hand, IOP, is not considered a primary causative factor in NTG (554), but is strongly linked with the mechanical theory of POAG development, with higher IOP associated with POAG development and progression (552, 555, 556). In more recent years, rather than segregating POAG and NTG, it has been proposed that open angle glaucoma should be thought of as a disease continuum (242, 244), with the relative influences of mechanical and vascular factors varying on an individual basis. Indeed, it has previously been shown that both early stage POAG and early stage NTG patients can have similar ocular and systemic circulation alterations (243). The results of this study found that both NTG and POAG patients had a significantly higher SAF compared to healthy controls. In addition the SAF result between NTG and POAG was not statistically different to one another.

In a similar manner to OAG disease continuum theory it may be that the extent to which AGEs affect the different structures related to mechanical involvement and vascular function differs in different patients. It may also be that AGEs equally affect mechanical and vascular function and are hence a factor in both POAG and NTG pathogenesis. Since SAF is raised in both POAG and NTG a reduction in AGE level would likely be beneficial for both forms of OAG.

6.5.2 Dietary AGE

There was no difference in dAGE intake between the groups. This was the case for each of the dAGEs calculated using FFQ responses with each of the different AGE databases available. This result indicates that the patients diagnosed with glaucoma recruited for this study had a similar dAGE intake to healthy controls,
suggesting that dAGE intake is not a causative factor in glaucoma onset. It should however be noted that this estimation of dAGE is a short-term indicator as the FFQ estimates intake over the previous 12 months. Current dAGE research is focused on only short-term effects (54) as this is what is feasible to measure with dietary recall methods (425). It could however be that a sustained high diet of dAGE over many years could have an influence, or could contribute in part to elevated tissue bound AGE levels. Further research is required to understand if long-term restriction of dAGE is in anyway beneficial.

6.5.3 Oxidative stress
The antioxidant alpha tocopherol was found to be at reduced levels in POAG patients compared to HC and OHT in this study. Furthermore, levels of the antioxidant ascorbate were found to be reduced in POAG compared to all other groups. This reduced antioxidant level agrees with the findings of Gherghel et al. (2005) who found that the antioxidant glutathione was reduced in POAG compared to healthy controls (323). The reduced levels of circulating ascorbate and alpha tocopherol may be due to a higher rate of oxidative reactions, however if this was the case it would be expected that levels of the oxidant MDA would be increased. MDA has been used in multiple glaucoma studies to measure oxidative stress (550). It was expected that MDA levels would be significantly higher in the glaucoma groups compared to OHT and healthy controls based on previous literature, however this was not the case. The lack of result with MDA could be related to a difference in glaucoma severity between this study and previous POAG MDA studies, as this study only included patients with early stage glaucoma. This notion could not be verified as the previous studies
measuring circulating levels of MDA in POAG have not specified how advanced/the progression of POAG in their participants. Each did however state that having glaucomatous visual field loss was part of the inclusion criteria (108, 557-559).

The lack of correlation between reduced antioxidant levels and SAF supports the conclusion of Chapter 5 that AGEs measured by SAF are not closely related to circulating oxidative stress biomarkers. This suggests that a higher SAF in glaucoma patients may be independent of oxidative stress levels.

6.6 Conclusion

SAF was significantly higher in patients with NTG and POAG compared to healthy controls. This finding adds to the evidence that SAF, as an accessible measure, may be a suitable long-term biomarker of glaucoma. Understanding if AGEs influence vascular parameters more than structural parameters of tissues is not yet established. Dietary AGE intake was similar between healthy controls, OHT, NTG and POAG patients. Patients with POAG exhibited lower levels of the antioxidants ascorbate and alpha tocopherol compared to OHT and healthy controls, suggesting oxidation inhibition. There was however no increased level of MDA.

6.7 Limitations

The sample size recruited was lower than the target sample size, this potentially limits the conclusions drawn from this study. The challenge with recruitment of early stage glaucoma participants and OHT participants was due to the strict
inclusion/exclusion criteria. These criteria however were necessary to avoid any other influences on AGE level, e.g. if smokers were included this may have masked any other relationships. In addition the recruitment between glaucoma groups was not even, with fewer NTG patients recruited. The number recruited participants however was able to provide statistically significant results.

6.8 Future work

To assess if baseline AGE (SAF) levels are associated with an increased rate of progression in NTG and POAG patients. In addition, to determine whether SAF levels are associated with OHT progression to develop POAG.

To assess if long-term restriction of dAGE is beneficial in terms of glaucoma progression.
7. The relationship between tissue-bound AGE levels (SAF), retinal vessel structure and function and corneal viscoelastic properties in a healthy ageing population

7.1 Abstract

Purpose: To determine how SAF is associated with static and dynamic retinal vessel parameters as well as corneal viscoelasticity, in a healthy ageing population.

Methods: Skin autofluorescence (SAF) as a measurement of tissue-bound AGE level was measured in 60 healthy participants and compared to dynamic and static vessel parameters measured by the Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) and corneal viscoelasticity measured by the Corvis ST (Oculus; Optikgeräte GmbH, Wetzlar, Germany). A fasting venous blood sample was obtained to enable the measurement of oxidative stress biomarkers.

Results: Central retinal artery equivalent diameter (CRAE) was found to be negatively associated with SAF ($r = -0.313$, $p = 0.015$, 95% CI -0.077 to -0.512) and positively associated with circulating levels of the antioxidant ascorbate ($r = 0.258$, $p = 0.048$, 95% CI 0.004 to 0.473). This association was further assessed with forwards and backwards multiple regression analysis, the independent variables included were CRAE, age, ascorbate, BMI, BMR and MG-H1. The model revealed that age ($p = <0.001$), BMI ($p = 0.010$) and CRAE ($p = 0.015$) were significantly associated with SAF. These findings were also found to be true
in the backwards model. No statistically significant relationships existed between SAF and dynamic retinal parameters or corneal viscoelastic parameters.

**Conclusion:** Higher levels of tissue-bound AGE (SAF) are associated with narrower retinal arteries in a healthy population. This finding adds to the evidence that AGEs are an accessible marker of vascular health. Increased levels of ascorbate were associated with wider retinal arteries, this supports the notion that diet derived antioxidants may have a protective effect. In this healthy population SAF was not found to be associated with dynamic vessel parameters or corneal viscoelastic parameters.
7.2 Background

In the eye the earliest signs of age-related change are often alterations in structure and function of the transparent structures, such as the lens and cornea (158, 560-562) and the microvascular blood supply to the retina and optic nerve (563-566). These age-related changes have been linked to a number of disease states (562, 565, 566). Determining whether AGE levels are influencing the structure and function of these components of the eye will be a focus of this study. The relationship between ageing, AGES and cataract formation in the lens is well established (see Section 1.11.5), and will not be discussed in this Chapter (7, 81, 84, 170, 171). In the cornea it is known that there is an increase AGES in the stroma with increased age (158), and it is thought that the modification to collagen contributes to the reduced flexibility seen with age (159, 160).

Accumulation of AGES has been shown to disturb the structure of blood vessels by altering the extra-cellular matrix and blood vessel function via the AGE-RAGE reaction (13, 138, 142, 207, 342). Altered retinal vessel structure and function has previously been linked to a number of ocular and systemic disease states, including cardiovascular disease, Alzheimer’s disease, diabetes, obesity, hypertension, hyperlipidaemia, AMD and glaucoma (243, 368-370).

There are no previous studies that have explored whether tissue-bound AGE levels are directly associated with retinal vessel structure and function. Establishing this relationship across a healthy ageing population is the first step to increasing our understanding of the impact that AGE accumulation in tissues may be having on the microvasculature and hence the potential contribution it
could make to the pathogenesis of diseases with a vascular origin. The potential association between AGEs and vascular function is important as altered retinal vascular function has been linked with ocular pathology (140, 281).

The Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) has been used as a measure of retinal microvascular function in both healthy ageing and ocular and systemic disease research (243, 368-370). The DVA uses luminance flicker light to instigate vessel response and assess dynamic retinal vessel function (376). In a normal healthy person this heightened metabolic demand causes the retinal endothelium to release additional NO, resulting in vasodilation and consequently increased blood flow (379) (see Section 3.3.2.2). A failure to observe this vasodilation response has been linked to the presence of vascular endothelial dysfunction (304) or perhaps to an increase in vascular stiffness (342).

Although the association between AGE level and retinal vessel structure and function has not previously been explored there are a number of studies that have linked AGEs to alterations in blood vessel structure and function at the systemic level. AGEs and the AGE-RAGE interaction for example has been shown to cause an increase in ET-1 levels (132) and reduced bioavailability and activity of NO (133), with the overall effect of vasoconstriction and a disturbance in the regulatory function of the endothelium. Furthermore, the AGE-RAGE interaction has been linked to endothelial cell hyperpermeability which can eventually lead to impairment of the blood-retinal barrier (567). In addition, as explained in Section 1.5, AGEs have also been shown to cause direct damage to
the systemic vasculature by forming covalent cross-links in the long-lived proteins of extracellular matrix, such as collagen and elastin in blood vessels (74, 568). These AGE induced cross-links alter the mechanical properties of the protein, resulting in increased vessel stiffness, increased vessel wall thickness, increased rigidity and reduced elasticity (38, 71, 72, 75, 136). Finally, a study by Yoshioka (2018) examined the maximum intima–media thickness (IMT) of the carotid artery in diabetic participants and found that increased SAF was associated with increased IMT (569). All of these findings indicate that AGE accumulation has the potential to influence blood vessel structure and the ability of blood vessels to regulate blood flow to ensure supply meets demand, with the potential effect of putting tissues at risk of ischaemic-hypoxic damage (570, 571).

As previously mentioned it has been shown that there is an increase in AGEs in the corneal stroma with increased age (2). There are however no previous studies that have explored whether AGE levels are directly associated with corneal viscoelasticity. A recent study by Bekmez and Kocaturk (2018) used the ORA to measure corneal biomechanics in diabetic patients and healthy controls and found that mean corneal hysteresis was lower in diabetic patients than healthy controls. They go on to explain that “We do not know ‘how and why’ diabetes affects corneal biomechanical measurements. It may be related to glucose level or something else possibly affecting the elasticity or viscosity of the cornea.” (572). Since AGE levels are known to be increased in diabetic patients (69, 573) it may be that increased an AGE level is altering corneal mechanical properties.
We hypothesise that AGEs may be a contributing factor to the reduced corneal viscoelasticity seen with age and that in individuals with elevated AGE levels, this age-related reduction in viscoelasticity could occur at an accelerated rate. Establishing this relationship across a healthy ageing population is the first step to increasing our understanding of the impact that AGE accumulation may be having and the potential contribution to the pathogenesis of diseases. The viscoelastic biomechanics of the cornea has been linked with glaucoma (337, 574). In addition, it has previously been suggested by Kotecha et al. (2007) that the biomechanical properties of the cornea may indicate overall globe mechanics, indicating stiffness of structures that cannot easily be measured (156).

7.2.1 Aims

To determine how tissue-bound AGE levels are associated with static and dynamic retinal vessel parameters and corneal viscoelasticity in a healthy ageing population.

7.2.2 Hypothesis

Retinal microvasculature structure and function will be associated with tissue-bound AGE levels (SAF), with increased SAF having a negative impact on the microvasculature. Corneal viscoelasticity will be related to AGEs, with an increased AGE level resulting in reduced elasticity.
7.3 Methods

The methodology that will be used in this study is summarised here; more detailed information on the specifics of each technique can be found in Chapter 3.

7.3.1 Participants

Convenience sampling was used to recruit participants who were eligible according to the inclusion and exclusion criteria for healthy ageing participants as detailed in section 3.1.3. and section 3.1.4. Due to the established link between diabetes and AGEs (449, 450) participants diagnosed with diabetes or participants found to have a high fasting glucose level were excluded from the study, as were current smokers, and previous smokers who quit less than 15 years ago due to the link between AGEs and tobacco (10).

A total of 71 participants were recruited, 60 of which were eligible for the study. One participant was excluded due to a diagnosis of arrhythmia between recruitment and study appointment. A second was excluded due to them revealing that although they quit smoking tobacco 15 years ago they continue to smoke non-tobacco products. A third participant was excluded due to use of fake tanning agent. As explained in section 3.3.2.2.3, the DVA raw data was assessed for quality. A further eight participants were excluded due to DVA cycles not meeting the quality criteria.
7.3.2 General investigations

Participant’s eyes were examined with visual acuity recorded as well as a slit lamp exam: anterior eye health examined, angles graded with Van Herick technique and posterior pole assessed. Proxymetacaine 0.5% drops were inserted, Goldmann tonometry performed and tropicamide 0.5% drops inserted for pupil dilation.

As described in 3.3.2.1, height was measured in meters using a stadiometer, weight was measured in kg and from these measurements BMI was calculated. Weight, basal metabolic rate (BMR), and body fat percentage were measured using the Tanita body composition analyser (TBF-300 MA). Blood pressure was measured and a fasting venous blood sample was obtained to enable the measurement of oxidative stress biomarkers.

In Chapter 5 the relationship between tissue-bound AGEs and oxidative stress was examined. The results suggested that tissue-bound AGEs and the circulating oxidative stress biomarkers used in this study cannot be used interchangeably, although there is clear evidence from the literature that AGEs and oxidative stress have a complex relationship and therefore it was decided that for this study oxidative stress markers would also be considered. Three oxidative stress biomarkers were used: ascorbate, MDA and alpha tocopherol. Each biomarker is described in section 3.3.2.6. Oxidative stress markers were measured due to their known associations with AGEs.
7.3.3 Dynamic Retinal Vessel Analyser (DVA)

The Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) was used to assess both dynamic and static retinal vessel function. It is a non-invasive device, with high reproducibility (373) and low variability (374). Specific detail on the DVA and its protocol for both dynamic and static measurements can be found in Section 3.3.2.2.

The dynamic parameters calculated from the raw data included: baseline diameter fluctuation (BDF), dilation amplitude (DA), baseline corrected flicker response (BFR), maximum percentage dilation (MD%), reaction time (RT), minimum percentage constriction (MC%), constriction time (CT) for artery and vein. For full list of descriptions of these parameters see Table 3.4 in Section 3.3.2.2. Participants were asked to maintain normal eating habits before DVA measurement as fasting can cause abnormal vascular activity (385).

The VesselMap2 software (ImedosSystems, Jena, Germany) was used to enable the retinal vasculature structure to be examined and evaluated (393). The static VesselMap2 software calculates central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) using the disc centred red-free fundus photograph and manually selected vessels, described in Section 3.3.2.2.

7.3.4 Corvis ST

The viscoelastic properties of the cornea were assessed using the Corvis ST (Oculus; Optikgeräte GmbH, Wetzlar, Germany). The Corvis ST is a noncontact tonometry system, for specific detail see Section 3.3.2.3. The parameters used:
first applanation time, second applanation time, velocity of corneal surface movement at the first applanation, velocity of corneal surface movement at second applanation, deformation amplitude at first applanation, deformation amplitude at second applanation, highest deformation amplitude, corneal highest concavity and highest concavity time. Corvis measurements were taken for each eye of each participant.

7.3.5 Skin autofluorescence (SAF)

The measurement of tissue-bound AGE levels was obtained using the AGE Reader (DiagnOptics B.V., Groningen, The Netherlands). The AGE Reader is a non-invasive device, which provides a low cost, fast, and reproducible means of determining AGE levels in the body through utilisation and measurement of the fluorescent properties of tissue-bound AGEs in the skin (21-23). Specific detail on the AGE reader and its protocol can be found in Section 3.3.2.4.

7.3.6 Dietary AGE, semi-quantitative FFQ

An assessment of dietary AGE intake over the last year was made using a semi-quantitative FFQ that was designed specifically to assess dietary AGE levels in a UK population for the purposes of this research. The FFQ design and delivery is discussed in Chapter 4. The FFQ was administered to all participants. The results from the FFQ were calculated with each of the databases described in Chapter 4. The results of Chapter 5 suggested that the contribution of dietary AGE to tissue-bound AGE levels is minimal, and that further investigation is required to understand which, if any, types of AGEs are impacting tissue-bound levels. MG-
H1 appears to be the dAGE most likely to impact tissue-bound AGE levels, for this reason MG-H1 was the dAGE included in this study.

7.3.7 Statistical analysis
Statistical analysis was performed using SPSS version 24 (IBM). The normality of data was assessed by the Shapiro–Wilk test and visual inspection of the QQ plots. P values of less than 0.05 were considered significant.
Mann-Whitney U test was used to check for differences in categorical data.

7.3.7.1 Relationship between retinal vessel structure and function parameters and SAF
Spearman’s rank correlation was used to analyse the association between each dynamic retinal vessel parameter, namely: BDF, DA, BFR, MD%, RT, MC% and CT for artery and vein with SAF.
Spearman’s rank correlation was used to analyse the association between each structural static retinal vessel parameter, namely: CRAE and CRVE with SAF.

Parameters found to correlate with SAF were entered into a stepwise forward and backward multiple linear regression was used to further assess the influence of different variables on SAF. Age and BMI were included in the multiple regression since in Chapter 5 it was found they influence SAF, the dAGE MG-H1 as a possible relevant form of dAGE. Due to oxidative stress having previously been shown to be both a cause and an effect of increased AGE formation (see Section 1.7) oxidative stress biomarkers were also included in the multiple linear regression.
7.3.7.2 Relationship between corneal viscoelasticity and SAF

Spearman’s rank correlation was used to analyse the association between each corneal parameter, namely: first and second applanation time, velocity of corneal surface movement at the first and second applanation, deformation amplitude at first and second applanation, highest deformation amplitude and time of highest concavity with SAF.

7.3.7.3 Power Calculation

Sample size was determined using G*Power 3 programme (Heinrich-Heine, Universität Düsseldorf). In order to achieve 80% power at the 5% alpha level with a medium effect size of 0.3, 64 participants would be required. To account for unusable data an allowance was made and a sample size of 71 participants was chosen.
7.4 Results

All 60 eligible participants were free of acute or chronic diseases, including diabetes, with the exception of controlled hypertension. The characteristics of the participant group with mean and standard deviation are detailed in Table 7.1. Collectively the participants blood pressure fell within normal limits, as described by the World Health Organisation & International Society of Hypertension guidelines (SBP: 120-129, DBP: 80-84) (575). The group were overweight, with a mean BMI of 26 ± 4 kg/m².

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
</tr>
<tr>
<td>Female</td>
<td>43</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.02 ± 8.86</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>26.29 ± 4.84</td>
</tr>
<tr>
<td>Systolic blood pressure (SBP) (mmHg)</td>
<td>129.12 ± 17.23</td>
</tr>
<tr>
<td>Diastolic blood pressure (DBP) (mmHg)</td>
<td>80.57 ± 10.28</td>
</tr>
<tr>
<td>Mean arterial blood pressure (MABP)</td>
<td>96.75 ± 11.27</td>
</tr>
<tr>
<td>Intraocular pressure (IOP) (mmHg)</td>
<td>15.70 ± 3.21</td>
</tr>
<tr>
<td>Ocular perfusion pressure (OPP)</td>
<td>48.79 ± 7.79</td>
</tr>
<tr>
<td>Skin autofluorescence (SAF) (AU)</td>
<td>2.22 ± 0.36</td>
</tr>
<tr>
<td>Ascorbate (µM)</td>
<td>51.41 ± 18.31</td>
</tr>
<tr>
<td>Malondialdehyde (MDA) (µM)</td>
<td>6.23 ± 1.91</td>
</tr>
<tr>
<td>Alpha tocopherol (µM)</td>
<td>43.12 ± 16.10</td>
</tr>
</tbody>
</table>

Table 7.1: Mean and standard deviation of participant group characteristics. AU: arbitrary units.
7.4.1 Dynamic retinal vessel analysis

No statistically significant correlations were found between any of the dynamic retinal vessel parameters (artery and vein) and SAF as shown in Table’s 7.2 and 7.3.

<table>
<thead>
<tr>
<th>Artery</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SAF (AU)</td>
</tr>
<tr>
<td>BDF</td>
<td>2.32</td>
<td>± 1.21</td>
<td>0.007, p = 0.956</td>
</tr>
<tr>
<td>DA</td>
<td>4.51</td>
<td>± 3.20</td>
<td>0.144, p = 0.272</td>
</tr>
<tr>
<td>BFR</td>
<td>2.24</td>
<td>± 2.50</td>
<td>0.165, p = 0.207</td>
</tr>
<tr>
<td>MD %</td>
<td>2.89</td>
<td>± 2.27</td>
<td>0.131, p = 0.319</td>
</tr>
<tr>
<td>RT (secs)</td>
<td>15.31</td>
<td>± 5.41</td>
<td>-0.033, p = 0.803</td>
</tr>
<tr>
<td>MC %</td>
<td>1.61</td>
<td>± 1.63</td>
<td>0.123, p = 0.349</td>
</tr>
<tr>
<td>CT (secs)</td>
<td>22.85</td>
<td>± 7.82</td>
<td>0.215, p = 0.098</td>
</tr>
</tbody>
</table>

Table 7.2: Artery dynamic parameters, mean, standard deviation and spearman’s correlation with SAF. AU: arbitrary units, BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.
Table 7.3: Vein dynamic parameters, mean, standard deviation and spearman’s correlation with SAF. AU: arbitrary units, BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.

<table>
<thead>
<tr>
<th>Vein</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAF (AU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDF</td>
<td>2.34 ± 1.51</td>
<td>-0.140, p = 0.287</td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>5.55 ± 2.81</td>
<td>-0.075, p = 0.570</td>
<td></td>
</tr>
<tr>
<td>BFR</td>
<td>3.21 ± 1.87</td>
<td>0.008, p = 0.950</td>
<td></td>
</tr>
<tr>
<td>MD %</td>
<td>4.57 ± 2.11</td>
<td>-0.098, p = 0.458</td>
<td></td>
</tr>
<tr>
<td>RT (secs)</td>
<td>18.47 ± 3.07</td>
<td>-0.004, p = 0.978</td>
<td></td>
</tr>
<tr>
<td>MC %</td>
<td>0.991 ± 1.26</td>
<td>0.015, p = 0.912</td>
<td></td>
</tr>
<tr>
<td>CT (secs)</td>
<td>31.07 ± 7.31</td>
<td>-0.147, p = 0.262</td>
<td></td>
</tr>
</tbody>
</table>

7.4.2 Static retinal vessel analysis

Table 7.4 shows the mean, standard deviation of the static retinal parameters. Spearman’s rank correlation showed a negative relationship between SAF and CRAE \( r = -0.313, \ p = 0.015, \) 95% bootstrap confidence interval -0.077 to -0.512, Figure 7.1. There was no statistically significant association between CRVE and SAF \( r = 0.061, \ p = 0.641, \) 95% bootstrap confidence interval -0.204 to 0.302.
Table 7.4: Static vessel parameters, mean, standard deviation and Spearman’s correlation with SAF. AU: arbitrary units, CRAE: central retinal artery equivalent, CRVE: central retinal vein equivalent.

<table>
<thead>
<tr>
<th>Static</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAF (AU)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRAE</td>
<td>180.95</td>
<td>± 18.36</td>
<td>-0.313, p = 0.015</td>
</tr>
<tr>
<td>CRVE</td>
<td>210.80</td>
<td>± 17.57</td>
<td>0.061, p = 0.641</td>
</tr>
</tbody>
</table>

Figure 7.1: Relationship between Skin autofluorescence (SAF) (AU) and central retinal artery equivalent (CRAE) (MU) (n = 60, r = -0.313, p = 0.015, 95% CI -0.077 to -0.512).
Spearman’s rank correlation also showed a positive relationship between CRAE and ascorbate ($r = 0.258$, $p = 0.048$, 95% bootstrap confidence interval 0.004 to 0.473), Figure 7.2, there was no statistically significant association between ascorbate and CRVE ($r = 0.123$, $p = 0.354$, 95% bootstrap confidence interval -0.145 to 0.364). There and no association between static vascular parameters and MDA or alpha tocopherol.

Figure 7.2: Relationship between Ascorbate ($\mu$M) and central retinal artery equivalent (CRAE) (MU) ($n = 60$, $r = 0.123$, $p = 0.354$, 95% CI -0.145 to 0.364).
The association between SAF and CRAE was further assessed with forwards and backwards multiple regression analysis, the independent variables included were CRAE, age, ascorbate, BMI, BMR and MG-H1. The model revealed that age ($p = <0.001$), BMI ($p = 0.010$) and CRAE ($p = 0.015$) were significantly associated to SAF. These findings were also found to be true in the backwards model.

7.4.3 Corvis ST analysis

Spearman’s Rank revealed no statistically significant correlations between any of the corneal parameters and SAF, as shown in Table 7.5.
<table>
<thead>
<tr>
<th>Corneal parameters</th>
<th>Mean ± Standard deviation</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SAF (AU)</td>
</tr>
<tr>
<td><strong>Time A1 (ms)</strong></td>
<td>7.33 ± 0.31</td>
<td>0.140, p = 0.394</td>
</tr>
<tr>
<td><strong>Time A2 (ms)</strong></td>
<td>21.83 ± 0.45</td>
<td>-0.105, p = 0.526</td>
</tr>
<tr>
<td><strong>Velocity A1 (m/s)</strong></td>
<td>0.15 ± 0.02</td>
<td>-0.227, p = 0.165</td>
</tr>
<tr>
<td><strong>Velocity A2 (m/s)</strong></td>
<td>-0.27 ± 0.03</td>
<td>0.290, p = 0.074</td>
</tr>
<tr>
<td><strong>Deformation amp. A1 (mm)</strong></td>
<td>0.13 ± 0.13</td>
<td>0.033, p = 0.840</td>
</tr>
<tr>
<td><strong>Deformation amp. A2 (mm)</strong></td>
<td>0.44 ± 0.44</td>
<td>0.113, p = 0.492</td>
</tr>
<tr>
<td><strong>Highest deformation amp. (mm)</strong></td>
<td>1.09 ± 0.10</td>
<td>-0.265, p = 0.103</td>
</tr>
<tr>
<td><strong>Highest concavity time (ms)</strong></td>
<td>16.47 ± 0.50</td>
<td>0.012, p = 0.942</td>
</tr>
</tbody>
</table>

7.5 Discussion

The aim of this study was to determine if SAF is associated with static and dynamic retinal vessel parameters and corneal viscoelasticity in a healthy ageing population. It is thought that this is the first study to compare SAF levels with these retinal vessel and corneal parameters in a healthy ageing population.

7.5.1 Relationship between retinal vessel structure and function and SAF

Higher SAF levels were found to be associated with narrower retinal arteries in this healthy ageing population. Narrowing of microvascular arteries is part of the normal ageing process (576), but the influence of tissue-bound AGE (SAF) levels on this finding has not been previously explored. At the macrovascular level however, increased SAF has been associated with increased maximum IMT of the carotid artery in diabetic patients (569). It is thought that the microvasculature is affected earlier than the macrovasculature (304, 577), hence SAF may be associated with IMT of retinal vessels. AGEs are known to increase cross-links in the proteins of the extra-cellular matrix of blood vessels (74, 136, 568). This accumulation may contribute to thickening of the inner layers of the artery wall and could be a possible explanation for why a narrower CRAE was associated with SAF. It is worth noting that changes to retinal vessels are likely to be multifactorial, and although this study has identified a relationship between SAF and CRAE it cannot ascertain if AGEs are indeed a causative factor in retinal artery narrowing.

An increase in CRVE has been associated with obesity, dyslipidaemia and markers of vascular endothelial dysfunction (398, 402). As the AGE-RAGE
interaction disrupts endothelial function (132, 133), it was hypothesised that increased SAF level may be related to increased CRVE. However in contrast to CRAE, no statistically significant relationship was found between CRVE and SAF. This could be related to structural component differences between arteries and veins, whereby arteries have a thicker tunica media and increased collagen and elastin in comparison to veins, since they have more extracellular matrix (578). As explained previously AGEs form covalent cross-links in the collagen and elastin of the extracellular matrix (74, 568). The structure of the arteries may therefore be more susceptible to AGE crosslinking than the vein (216).

A positive correlation was found in this study between the antioxidant ascorbate and CRAE, suggesting that lower antioxidant levels are associated with narrower retinal artery diameters. An increased ascorbate level suggests that ascorbate has not had to scavenge free radicals, hence there is not an environment of oxidative stress (103). Therefore the lack of relationship with MDA is consistent, as MDA is a lipid peroxidation product present in environments of increased oxidative stress. It has previously been proposed that ascorbate is the most effective non-enzymatic antioxidant present in the plasma (427), this may explain why only ascorbate and not alpha tocopherol correlated with CRAE. Ascorbate, as a diet derived antioxidant is considered to have a protective effect (579), this appears to be evidenced here with increased ascorbate correlating with a wider CRAE.

No statistically significant correlations existed between any of the dynamic vascular function parameters and SAF or oxidative stress. This suggests that the
AGE and oxidative stress levels are not affecting retinal microvascular function in the healthy population examined in this study. This however may be related to the fact this healthy ageing group do not have a high AGE level, hence the AGE related associated effects not present. Previously Seshadri et al. (2015) found that the non-enzymatic antioxidant GSH did correlate with retinal microvascular dynamic responses and suggested that this was due to increased levels of oxidative stress impairing endothelium-dependent dilation (370). Since ascorbate and alpha tocopherol are both non-enzymatic antioxidants (92), it was hypothesised that the GSH correlation would be replicated, however this was not the case. One possible explanation for the difference is that ascorbate and alpha tocopherol levels in the healthy participants in this study were above or within normal range (454, 509), indicating that oxidative stress was not present, hence may explain why oxidative stress associated effects were not present. In addition, it is worth noting that GSH has a large range in what is considered normal values in the literature (150-1500 µM). Rossi et al. (2002) described difficulties in the measurement of GSH and concluded that most published data may be affected by multiple artefacts (580), this may have impacted the Seshadri et al. (2015) results and may also add to why there is a difference between Seshadri et al. (2015) results and those from this study.

7.5.2 Relationship between corneal viscoelasticity and SAF

There were no statistically significant correlations between the corneal viscoelastic parameters measured and SAF. This suggests that the AGE levels may not be related to corneal viscoelasticity in the healthy population examined in this study. It may also be related to the fact this healthy ageing group do not
have a high AGE level, hence the AGE related associated changes may not be present. The recruited participants in this study were aged 50 to 90 years, it was noted that the older participants free from any general health conditions had a lower AGE level than expected for their age, for example a participant aged 90 had a SAF of 2.80 AU, which would be approximately normal for an 80 year old. It was anticipated that recruiting participants in the age range of 50 to 90 would give differing results, however this was not the case. Future studies examining SAF and corneal viscoelasticity would benefit from recruiting a larger age range, to enable a larger range of SAF and viscoelasticity results.

7.5.3 Possible healthy volunteer effect
The mean SAF of this healthy group of participants was 2.22 ± 0.36. This result is lower than expected, since a study with a healthy participant group of similar mean age had a mean SAF of 2.46 ± 0.57 (420). Indeed the mean SAF of a study by Kellow et al. (2017) was closer at 2.10 ± 0.60, however the average age of the group was considerably younger (47 years) (494). This suggests that the group recruited in this study has possibly been subject to a healthy volunteer effect. This may help to explain why there are no adverse associations with vascular or corneal parameters that would be expected to be affected by increased AGE level. In addition the standard deviation of SAF measures was low, this low variability of the data means that there are no particularly high or low AGE values in this data. Low variance is possibly problematic here as the lack of association may be due more to the fact participants have a normal AGE level and little AGE effect rather than there truly being no association between AGES and the retinal vasculature and cornea.
7.6 Conclusion

Higher levels of tissue-bound AGE (SAF) are associated with narrower retinal arteries in a healthy population. This finding adds to the evidence that AGEs are an accessible marker of vascular health. Increased levels of ascorbate were associated with wider retinal arteries, this supports previous literature that diet derived antioxidants may have a protective effect (579). In this healthy population SAF was not found to be associated with dynamic vessel parameters or corneal viscoelastic parameters. This suggests that AGE levels may not be related to dynamic vessel parameters or corneal viscoelasticity, although it should be considered that this recruited healthy group had a lower mean SAF than expected.

7.7 Limitations

By excluding all disease linked to AGEs the resultant group of participants were particularly healthy and as a result had a lower SAF level than was expected for mean age.

7.8 Future work

Having a wider age range would enable a better comparison of corneal elasticity. The relationship between corneal viscoelasticity and SAF should be further assessed with participants with a wider age range. In addition the use the ORA in conjunction with the Corvis ST and AGE-reader to enable examination of corneal hysteresis and corneal resistance factors relationship with SAF.
8.0 The relationship between tissue bound AGE levels, static and dynamic retinal vessel structure and function, and corneal viscoelasticity in POAG, NTG and OHT patients

8.1 Abstract

**Purpose:** To evaluate how tissue bound AGE level (SAF) relates to static and dynamic retinal vessel structure and function and corneal viscoelasticity in POAG, NTG and OHT patients.

**Methods:** 33 healthy control (HC) participants, 16 OHT, 12 NTG and 32 POAG patients had skin autofluorescence (SAF) measured using the AGE Reader (DiagnOptics B.V., Groningen, The Netherlands), static and dynamic retinal vessel structure and function evaluated using the Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) and corneal viscoelasticity measured using the Corvis ST (Oculus; Optikgeräte GmbH, Wetzlar, Germany).

**Results:** Increased SAF was found to correlate with a decrease in corneal viscoelasticity velocity A1 (m/s) \( r = -0.748, p = 0.033, 95\%\ CI -0.838\) to 0.102) and reduced deformation amplitude at A1 \( r = -0.714, p = 0.047, 95\%\ CI -0.115\) to -0.997) in NTG patients. There were no significant differences with OHT or POAG patients. No significant relationships were found between dynamic and static retinal vascular parameters and SAF for any group, however NTG patients were found to have a significantly smaller static CRAE diameter \( p=0.048\) than healthy controls.
**Conclusion:** Ageing is a multifactorial process and similarly glaucoma is thought to be influenced by multiple factors. In the NTG group a less viscoelastic, stiffer cornea was related to increased SAF level. There was no significant relationship in the OHT or POAG groups. There were no statistically significant relationships between SAF and dynamic and static vascular parameters in any groups, however CRAE was found to be narrowest in the NTG group.
8.2 Background

In Chapter 6 SAF was shown to be significantly higher in NTG and POAG patients compared to healthy controls. This finding adds to the evidence that SAF, as an accessible measure, may be a suitable long-term biomarker of glaucoma. To establish how AGEs may contribute to glaucoma pathogenesis it is important to establish the structures they may be affecting. This evaluation is particularly interesting as POAG, NTG and OHT are thought to have differing levels of vascular and mechanical involvement.

Studies investigating the vascular effects of AGEs have linked increased AGE levels to vascular stiffness and endothelial dysfunction, both of which have also been linked to glaucoma pathogenesis (243, 304, 328, 344, 345). AGEs are thought to cause increased vessel stiffness due to increased cross-linking (136), and to contribute to endothelial dysfunction by increasing ET-1 levels (132) and reducing availability and activity of NO (133). A decrease in the availability of NO and an increase in ET-1 can result in excessive vasoconstriction and subsequent blood flow dysregulation (292, 293). Interestingly, in NTG and POAG patients, levels of NO in the plasma have been found to be at a reduced level compared to healthy controls (294-296), and levels of ET-1 have been shown to be at higher levels in NTG (297, 298) and POAG patients (299, 300). It is therefore of interest to explore whether a relationship between vascular parameters and SAF is evident within these patient groups due to the known underlying AGE mechanism.
Hysteresis is a viscoelastic property of the cornea; it describes the difference between behaviour in ‘loading’ and ‘unloading’ conditions (574). A lower corneal hysteresis (less viscoelastic cornea) has been found in patients diagnosed with glaucoma; in addition, a lower corneal hysteresis has been linked with progressive visual field worsening (337, 581, 582). Prata et al. (2012) found that low corneal hysteresis is linked with glaucomatous nerve head signs such as larger cup to disc ratio and increased cup depth, and in those with bilateral glaucoma, 75% of participants with lower corneal hysteresis had a larger cup to disc ratio (338). Murphy et al. (2017) examined corneal hysteresis in patients with glaucoma, OHT and glaucoma-like optic discs and found corneal hysteresis to be higher (more viscoelastic) in OHT and glaucoma-like discs compared to glaucoma, suggesting that increased viscoelasticity of the cornea may have a beneficial, protective role against glaucoma (340).

As previously mentioned it has been shown that there is an increase in AGEs in the corneal stroma with increased age (2). Chapter 7 explored whether AGE levels were directly associated with corneal viscoelasticity in healthy control participants, and the results showed no significant relationship. This result however may have been influenced by a healthy volunteer effect. No previous studies have directly linked AGE (SAF) level and corneal viscoelasticity in OHT, NTG and POAG patients.
8.2.1 Aims
To evaluate whether level of tissue bound AGEs (SAF) relates to static and dynamic retinal vessel structure and function and corneal viscoelasticity in POAG, NTG and OHT patients.

8.2.2 Hypothesis
Tissue bound AGE (SAF) level will be associated with static and dynamic vessel parameters and corneal viscoelasticity parameters in NTG, POAG, OHT groups.

8.3 Methods
The methodology used in this study is summarised here; more detailed information on the specifics of each technique can be found in Chapter 3.

8.3.1 Participants
Convenience sampling was used to recruit participants who were eligible according to the study’s inclusion and exclusion criteria. The inclusion criteria for participants are detailed in section 3.1.3, exclusion detailed in section 3.1.4. Due to the established link between diabetes and AGEs (449, 450) participants diagnosed with diabetes or participants found to have a high fasting glucose level were excluded from the study, as were current smokers, and previous smokers who quit less than 15 years ago due to the link between AGEs and tobacco (10). POAG, NTG and OHT participants were identified through their attendance at routine outpatient appointments at Derriford REI.
IOP lowering medication was not an exclusion factor for this study as diagnosed early stage glaucoma or OHT patients were required. With this in mind, it should be noted that some participants were prescribed a beta-blocker or combination drop which contained a beta-blocker, this applied to 18 of the recruited participants (OHT: n = 2, NTG: n = 3, POAG: n = 13). It is possible that vasoactive eye-drops influence retinal blood vessel parameters (583-585), however AGE level would likely be unaffected. In addition participants were asked to refrain from using their drops on the morning of the study.

8.3.2 General investigations

As described in 3.3.2.1, height was measured in meters using a stadiometer, weight was measured in kg and from these measurements BMI was calculated. Weight, basal metabolic rate (BMR), and body fat percentage were measured using the Tanita body composition analyser (TBF-300 MA). Blood pressure was measured and a fasting venous blood sample was obtained to enable the measurement of oxidative stress biomarkers. Three oxidative stress biomarkers were used, the non-enzymatic antioxidants ascorbate (vitamin C) and alpha tocopherol (form of vitamin E) and the product of lipid peroxidation malondialdehyde (MDA). Each biomarker is described in Section 3.3.2.6.

8.3.3 Dynamic Retinal Vessel Analyser (DVA)

The Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) was used to assess both dynamic and static retinal vessel function. It is a non-invasive device, with high reproducibility (373) and low variability (374). Specific
detail on the DVA and its protocol for both dynamic and static measurements can be found in Section 3.3.2.2.

The dynamic parameters calculated from the raw data included: baseline diameter fluctuation (BDF), dilation amplitude (DA), baseline corrected flicker response (BFR), maximum percentage dilation (MD%), reaction time (RT), minimum percentage constriction (MC%), constriction time (CT) for artery and vein. For full list of descriptions of these parameters see Table 3.3 in Section 3.3.2.2. Participants were asked to maintain normal eating habits before DVA measurement as fasting can cause abnormal vascular activity (385).

The VesselMap2 software (ImedosSystems, Jena, Germany) was used to enable the retinal vasculature structure to be examined and evaluated (393). The static VesselMap2 software calculates central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) using the disc centred red-free fundus photograph and manually selected vessels, described in Section 3.3.2.2.

8.3.4 Corvis ST

The viscoelastic properties of the cornea were assessed using the Corvis ST (Oculus; Optikgeräte GmbH, Wetzlar, Germany). The Corvis ST is a noncontact tonometer system, for specific detail see Section 3.3.2.3. The parameters used: first applanation time (time A1), second applanation time (time A2), velocity of corneal surface movement at the first applanation (velocity A1), velocity of corneal surface movement at second applanation (velocity A2), deformation amplitude at first applanation (deformation amplitude A1), deformation amplitude
at second applanation (deformation amplitude A2), highest deformation amplitude, and highest concavity time. Corvis measurements were taken for each eye of each participant.

8.3.5 Skin autofluorescence (SAF)

The measurement of tissue-bound AGE levels was obtained using the AGE Reader (DiagnOptics B.V., Groningen, The Netherlands). The AGE Reader is a non-invasive device, which provides a low cost, fast, and reproducible means of determining AGE levels in the body through utilisation and measurement of the fluorescent properties of tissue-bound AGEs in the skin (21-23). Specific detail on the AGE reader and its protocol can be found in Section 3.3.2.4.

8.3.6 Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM). The normality of data was assessed by the Shapiro–Wilk test and visual inspection of the QQ plots. P values of less than 0.05 were considered significant.

8.3.6.1 General characteristics between groups

Mann-Whitney U test was used to analyse the demographic data. A one-way ANOVA was used to compare the results of general investigations between the groups.
8.3.6.2 SAF associated with vascular parameters

Vascular parameters, both from retinal artery and vein, were entered into a Pearson’s correlation with SAF, to examine any possible relationships.

8.3.6.3 Static vessel parameters between groups

A one-way ANOVA was used to compare the static retinal vessel parameters, namely: CRAE and CRVE followed by the post-hoc analysis Scheffe.

8.3.6.4 SAF associated with corneal parameters

Corneal parameters were entered into a Pearson’s correlation with SAF, to examine any possible relationships.

8.3.6.5 Relationship between corneal parameters in the groups

An ANOVA was used to compare the corneal parameters measured by the Corvis ST between the groups, followed by the post-hoc analysis Scheffe.

8.3.6.6 Power calculation

The sample size was determined using data from previous research which also measured tissue-bound AGE level using AGE Reader between healthy and disease groups (83). In order to achieve 80% power at the 5% alpha level a sample size of 37 per group was required (NTG, POAG, OHT and controls). To account for unusable data an allowance was made and a sample size of 40 participants was chosen per group.
8.4 Results

A total of 32 POAG, 12 NTG, 16 OHT and 33 healthy control participants were recruited and eligible for the study.

8.4.1 Group characteristics

There were no significant differences in age, BMI, systemic blood pressure, IOP, MABP and OPP between the four groups (p > 0.05). The characteristics of the participant group with mean and standard deviation are detailed in Table 8.1. It was expected that there would not be a significant difference in IOP between the groups since the OHT, POAG and NTG patients were all taking their prescribed IOP lowering treatment as normal.

Table 8.1: Summary of mean and standard deviation of participant characteristics for the study groups. P<0.05 is considered a significant difference. BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, IOP: intraocular pressure, CDR: cup to disc ratio, CCT: central corneal thickness, MABP: mean arterial blood pressure, OPP: ocular perfusion pressure, was subsequently calculated using MABP and IOP values. MD: mean deviation from visual field data collected from REI appointment, OHT MD added for reference in brackets.
As shown in Chapter 6 a significant difference was found between groups with regard to SAF ($p = 0.005$, Table 6.2). Post-hoc testing found SAF to be significantly higher in the POAG and NTG patients than in healthy controls (POAG $p = 0.018$, NTG $p = 0.05$). SAF level in the OHT patients was not significantly different to healthy controls or to the glaucoma groups ($p>0.05$).

<table>
<thead>
<tr>
<th></th>
<th>HC (1)</th>
<th>OHT (2)</th>
<th>NTG (3)</th>
<th>POAG (4)</th>
<th>ANOVA p value</th>
<th>Post-hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAF (AU)</td>
<td>2.19 ±0.39</td>
<td>2.44 ±0.42</td>
<td>2.6 ±0.39</td>
<td>2.54 ±0.45</td>
<td>0.005</td>
<td>3, 4 &gt; 1</td>
</tr>
</tbody>
</table>

Copy of Table 6.2 from Chapter 6: Difference in mean SAF between groups (± standard deviation). SAF: skin autofluorescence, AU: arbitrary units. $P<0.05$ is considered significant.

8.4.2 Dynamic retinal vessel analysis

8.4.2.1 Relationship between SAF and dynamic retinal artery parameters

In Chapter 7 Table 7.2 and 7.3 show that there were no statistically significant correlations between SAF and any of the dynamic retinal vessel parameters (artery and vein) in healthy control participants. Table 8.2 shows the mean and standard deviation for the retinal artery dynamic parameters of each group. Table 8.3 shows the correlations between SAF and each dynamic retinal artery parameter, no statistically significant relationships were detected (all $p > 0.05$).
### Table 8.2: Mean and SD of artery dynamic parameters, BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.

<table>
<thead>
<tr>
<th>Artery</th>
<th>OHT</th>
<th>NTG</th>
<th>POAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDF</td>
<td>2.42 ± 1.31</td>
<td>4.77 ± 4.04</td>
<td>3.11 ± 2.21</td>
</tr>
<tr>
<td>DA</td>
<td>5.53 ± 3.08</td>
<td>5.59 ± 3.59</td>
<td>4.96 ± 7.96</td>
</tr>
<tr>
<td>BFR</td>
<td>3.12 ± 2.55</td>
<td>1.46 ± 2.05</td>
<td>2.02 ± 2.05</td>
</tr>
<tr>
<td>MD%</td>
<td>3.27 ± 3.03</td>
<td>3.58 ± 4.30</td>
<td>2.87 ± 2.48</td>
</tr>
<tr>
<td>RT (secs)</td>
<td>15.22 ± 4.59</td>
<td>19.55 ± 5.67</td>
<td>14.69 ± 9.56</td>
</tr>
<tr>
<td>MC%</td>
<td>2.27 ± 2.02</td>
<td>2.16 ± 1.67</td>
<td>2.31 ± 2.90</td>
</tr>
<tr>
<td>CT (secs)</td>
<td>26.26 ± 8.55</td>
<td>21.09 ± 9.82</td>
<td>24.57 ± 7.49</td>
</tr>
</tbody>
</table>

### Table 8.3: Artery dynamic parameters Pearson’s correlation with SAF. AU: arbitrary units, BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.

<table>
<thead>
<tr>
<th>Artery</th>
<th>Pearson’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAF (AU)</td>
</tr>
<tr>
<td></td>
<td>OHT</td>
</tr>
<tr>
<td>BDF</td>
<td>0.386, p = 0.155</td>
</tr>
<tr>
<td>DA</td>
<td>-0.023, p = 0.935</td>
</tr>
<tr>
<td>BFR</td>
<td>-0.226, p = 0.418</td>
</tr>
<tr>
<td>MD%</td>
<td>-0.428, p = 0.112</td>
</tr>
<tr>
<td>RT (secs)</td>
<td>0.211, p = 0.450</td>
</tr>
<tr>
<td>MC%</td>
<td>0.306, p = 0.107</td>
</tr>
<tr>
<td>CT (secs)</td>
<td>0.048, p = 0.865</td>
</tr>
</tbody>
</table>

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8.4.2.2 Relationship between SAF and dynamic retinal vein parameters

Table 8.4 shows the mean and standard deviation for the retinal vein dynamic parameters of each group. Table 8.5 shows the correlations between SAF and each dynamic vein artery parameter, no significant relationships existed (all $p > 0.05$).

<table>
<thead>
<tr>
<th>Vein</th>
<th>OHT</th>
<th>NTG</th>
<th>POAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDF</td>
<td>2.66 ±1.92</td>
<td>3.83 ±2.29</td>
<td>3.11 ±2.21</td>
</tr>
<tr>
<td>DA</td>
<td>5.76 ±3.60</td>
<td>5.89 ±3.79</td>
<td>4.92 ±2.28</td>
</tr>
<tr>
<td>BFR</td>
<td>3.35 ±2.79</td>
<td>2.54 ±2.63</td>
<td>2.54 ±1.86</td>
</tr>
<tr>
<td>MD%</td>
<td>4.54 ±2.86</td>
<td>5.55 ±3.79</td>
<td>4.36 ±2.13</td>
</tr>
<tr>
<td>RT (secs)</td>
<td>16.80 ±3.91</td>
<td>19.73 ±2.80</td>
<td>19.53 ±3.21</td>
</tr>
<tr>
<td>MC%</td>
<td>1.22 ±1.42</td>
<td>0.65 ±1.43</td>
<td>0.97 ±1.47</td>
</tr>
<tr>
<td>CT (secs)</td>
<td>26.11 ±8.55</td>
<td>30.00 ±10.43</td>
<td>32.56 ±6.32</td>
</tr>
</tbody>
</table>

Table 8.4: Mean and SD of vein dynamic parameters, BFR: baseline diameter fluctuation, DA: dilation amplitude, BRF: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.
Table 8.5: Vein dynamic parameters Pearson’s correlation with SAF. AU: arbitrary units, BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.

<table>
<thead>
<tr>
<th>Vein</th>
<th>SAF (AU)</th>
<th>OHT</th>
<th>NTG</th>
<th>POAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDF</td>
<td>0.151, p = 0.592</td>
<td>-0.376, p = 0.254</td>
<td>0.068, p = 0.723</td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>-0.156, p = 0.578</td>
<td>-0.262, p = 0.437</td>
<td>-0.043, p = 0.820</td>
<td></td>
</tr>
<tr>
<td>BFR</td>
<td>-0.390, p = 0.151</td>
<td>-0.140, p = 0.682</td>
<td>-0.137, p = 0.470</td>
<td></td>
</tr>
<tr>
<td>MD%</td>
<td>-0.320, p = 0.245</td>
<td>-0.220, p = 0.516</td>
<td>-0.035, p = 0.854</td>
<td></td>
</tr>
<tr>
<td>RT (secs)</td>
<td>-0.366, p = 0.181</td>
<td>0.156, p = 0.647</td>
<td>0.060, p = 0.753</td>
<td></td>
</tr>
<tr>
<td>MC%</td>
<td>0.247, p = 0.374</td>
<td>-0.137, p = 0.687</td>
<td>-0.049, p = 0.798</td>
<td></td>
</tr>
<tr>
<td>CT (secs)</td>
<td>-0.293, p = 0.290</td>
<td>0.032, p = 0.926</td>
<td>-0.010, p = 0.957</td>
<td></td>
</tr>
</tbody>
</table>

8.4.3 Static retinal vessel analysis

Chapter 7 showed a negative relationship between SAF and CRAE (r = -0.313, p = 0.015, 95% bootstrap confidence interval -0.077 to -0.512), with no statistically significant association between CRVE and SAF (r = 0.061, p = 0.641, 95% bootstrap confidence interval -0.204 to 0.302) in healthy control participants. Table 8.7 shows the lack of significant correlation between SAF and static parameters in OHT, NTG and POAG patients. Figure 8.1 demonstrates the trend in association between CRAE and the groups when considered together.
Table 8.6: Central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) mean and standard deviation.

<table>
<thead>
<tr>
<th>Static</th>
<th>HC</th>
<th>OHT</th>
<th>NTG</th>
<th>POAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRAE</td>
<td>180.18 ±20.81</td>
<td>175.64 ±17.94</td>
<td>164.61 ±11.51</td>
<td>171.56 ±17.97</td>
</tr>
<tr>
<td>CRVE</td>
<td>210.67 ±19.27</td>
<td>204.22 ±20.84</td>
<td>206.83 ±18.86</td>
<td>214.59 ±2.28</td>
</tr>
</tbody>
</table>

When comparing CRAE and CRVE across the study groups the artery static parameter CRAE was significantly different between groups (ANOVA p = 0.024), post hoc testing revealed that NTG patients have a lower CRAE than HC (p = 0.048). No significant difference was found between CRVE in the study groups (ANOVA p>0.05).

Table 8.7: Central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) correlations with SAF. P<0.05 is considered a significant difference.

<table>
<thead>
<tr>
<th>Static</th>
<th>Pearson’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAF (AU)</td>
</tr>
<tr>
<td></td>
<td>OHT</td>
</tr>
<tr>
<td>CRAE</td>
<td>-0.161, p = 0.567</td>
</tr>
<tr>
<td>CRVE</td>
<td>0.018, p = 0.949</td>
</tr>
</tbody>
</table>
8.4.4 Corvis ST analysis

The corneal viscoelasticity velocity A1 (m/s) significantly correlated with SAF ($r = -0.748$, $p = 0.033$, 95% bootstrap confidence interval -0.838 to 0.102) in the NTG group. As SAF increased, the velocity at A1 was slower, however as shown by the confidence interval, due to the low participant number we cannot be confident in this result. Deformation amplitude at A1 was also found to correlate with SAF in the NTG group ($r = -0.714$, $p = 0.047$, 95% bootstrap confidence interval -0.115
to -0.997). With increased SAF there was a decreased deformation amplitude, with the cornea becoming less deformed on A1.

An ANOVA comparison of means revealed that there was no significant difference between the groups for velocity A1 and deformation amplitude A1 (P > 0.05). The parameter CCT was however found to be different between the groups (ANOVA p = 0.011), post hoc testing revealed that NTG (p = 0.046) and POAG (p = 0.022) patients have a lower CCT than HC.
<table>
<thead>
<tr>
<th>Corneal viscoelasticity</th>
<th>HC</th>
<th>OHT</th>
<th>NTG</th>
<th>POAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>33</td>
<td>13</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>Time A1 (ms)</td>
<td>7.33 ±0.37</td>
<td>7.45 ±0.30</td>
<td>7.18 ±0.18</td>
<td>7.34 ±0.25</td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>21.95 ±0.44</td>
<td>21.66 ±0.48</td>
<td>22.03 ±0.28</td>
<td>21.67 ±0.44</td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>0.13 ±0.02</td>
<td>0.13 ±0.03</td>
<td>0.16 ±0.01</td>
<td>0.15 ±0.02</td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>-0.26 ±0.03</td>
<td>-0.26 ±0.02</td>
<td>-0.28 ±0.02</td>
<td>-0.27 ±2.48</td>
</tr>
<tr>
<td>Deformation amp. A1 (mm)</td>
<td>0.13 ±0.01</td>
<td>0.13 ±0.01</td>
<td>0.13 ±0.01</td>
<td>0.13 ±0.01</td>
</tr>
<tr>
<td>Deformation amp. A2 (mm)</td>
<td>0.44 ±0.07</td>
<td>0.44 ±0.08</td>
<td>0.45 ±0.08</td>
<td>0.42 ±0.05</td>
</tr>
<tr>
<td>Highest deformation amp. (mm)</td>
<td>1.09 ±0.11</td>
<td>1.07 ±0.12</td>
<td>1.10 ±0.06</td>
<td>1.09 ±0.09</td>
</tr>
<tr>
<td>Highest concavity time (ms)</td>
<td>16.52 ±0.51</td>
<td>16.42 ±0.54</td>
<td>16.45 ±0.39</td>
<td>16.41 ±0.59</td>
</tr>
<tr>
<td>CCT (μm)</td>
<td>555 ±34.34</td>
<td>550 ±21.22</td>
<td>527 ±45.39</td>
<td>526 ±36.62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Corneal parameters</strong></th>
<th>Pearson’s correlation coefficients (R values)</th>
<th>SAF (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OHT</td>
</tr>
<tr>
<td><strong>Time A1 (ms)</strong></td>
<td>0.550, p = 0.053</td>
<td>0.000, p = 0.999</td>
</tr>
<tr>
<td><strong>Time A2 (ms)</strong></td>
<td>-0.484, p = 0.093</td>
<td>0.003, p = 0.993</td>
</tr>
<tr>
<td><strong>Velocity A1 (m/s)</strong></td>
<td>-0.414, p = 0.159</td>
<td>-0.748, p = 0.033*</td>
</tr>
<tr>
<td><strong>Velocity A2 (m/s)</strong></td>
<td>0.424, p = 0.148</td>
<td>0.334, p = 0.419</td>
</tr>
<tr>
<td><strong>Deformation amp. A1 (mm)</strong></td>
<td>0.115, p = 0.708</td>
<td>-0.714, p = 0.047*</td>
</tr>
<tr>
<td><strong>Deformation amp. A2 (mm)</strong></td>
<td>0.129, p = 0.675</td>
<td>-0.609, p = 0.109</td>
</tr>
<tr>
<td><strong>Highest deformation amp. (mm)</strong></td>
<td>-0.167, p = 0.587</td>
<td>-0.652, p = 0.080</td>
</tr>
<tr>
<td><strong>Highest concavity time (ms)</strong></td>
<td>0.090, p = 0.770</td>
<td>-0.183, p = 0.664</td>
</tr>
<tr>
<td><strong>CCT (μm)</strong></td>
<td>-0.086, p = 0.779</td>
<td>0.488, p = 0.220</td>
</tr>
</tbody>
</table>

Figure 8.2 demonstrates the lack of relationship between CCT and the groups when considered together. Pearson’s correlation, including all groups showed no significant relationship between SAF and CCT ($r = -0.005$, $p = 0.968$, 95% bootstrap confidence interval -0.252 to 0.239), Figure 8.2.

Figure 8.2: Relationship between Skin autofluorescence (SAF) (AU) and central corneal thickness (CCT) with all groups shown ($r = -0.005$, $p = 0.968$, 95% CI -0.252 to 0.239).
8.5 Discussion

The aim of this study was to determine how tissue-bound AGE levels (SAF) are related to retinal vascular parameters and corneal viscoelasticity parameters in POAG, NTG, OHT and healthy control participants. It is thought that this is the first study to examine SAF levels with retinal parameters and corneal parameters in these groups.

8.5.1 Retinal vessel structure and function

Early stage NTG patients were found to have significantly narrower CRAE in comparison to healthy controls. Early stage POAG participants also had a narrower CRAE than HC, however this difference was not statistically significant. The previous literature agrees that retinal arteriolar narrowing is associated with glaucoma (265, 586) and Kawasaki et al. (2013), in addition to concluding that a narrower CRAE was associated with higher risk of glaucoma incidence, also found that CRVE was not associated with glaucoma risk (587), which is consistent with the findings of this study.

The exact mechanism for CRAE narrowing in glaucoma is unknown. One hypothesis is that the narrowing may occur as a secondary event to retinal ganglion cell loss, as fewer retinal ganglion cells results in reduction in oxygen demand (587). An alternative hypothesis is that tissue-bound AGEs are causing changes to the retinal vasculature. SAF has been related to the macrovasculature, with increased SAF associated with increased maximum IMT of the carotid artery in diabetic patients (569). It is thought that the microvasculature is affected earlier than the macro-vasculature (304, 577); hence, the finding of a
narrower CRAE may be linked to thickening of the inner layers of the retinal microvascular wall.

NTG patients, alongside having a narrower CRAE, also had the highest mean value of SAF in this study. A significant correlation between CRAE and SAF, however was only evident in the healthy control participants and not in the NTG group. This makes it difficult to draw conclusions around whether tissue-bound AGEs are part of the mechanism causing retinal arteries to narrow in glaucoma patients from this study. It is possible that the lack of a significant relationship between SAF and CRAE found here may be a consequence of there being only a small number of NTG patients. Exploring CRAE and SAF levels with a larger group of NTG patients as well as assessing differing stages of glaucoma progression may help to aid understanding of whether this narrowing is indeed related to AGE levels within the tissues and whether this carries any pathogenic significance.

As well as increased vessel wall thickness it is thought that AGE induced cross-links alter the mechanical properties of the vessels resulting in increased vessel stiffness, and reduced elasticity (38, 71, 72, 75, 136). No relationship however was found between the dynamic artery or vein parameters and SAF in the POAG, NTG and OHT participants in this study. This result agrees and follows on from the dynamic vascular results in Chapter 7 where there was also no significant relationship between SAF and retinal dynamic vascular parameters in healthy control participants. AGEs are thought to affect systemic vascular function (216), and in type 2 diabetic patients SAF readings have been found to be an
independent predictor for the development of microvascular complications (588), these diabetic patients had a higher mean SAF level (2.74). It may be that since the patients in this study were systemically healthy and had a lower SAF level that AGE level had not yet influenced microvascular function. It must also be considered that since patients continued the use of their IOP lowering medication as normal this may have affected retinal vessel function. However the vascular effects of IOP lowering drops on retinal vascular calibre is not fully understood (265), a review by Newman et al. (2018) found there was no consistent effect on retinal vessel width with the use of IOP lowering drops (585).

8.5.2 Corneal viscoelasticity
A less viscoelastic cornea has been linked with glaucoma (337, 581, 582), and it has been suggested that an increased viscoelasticity of the cornea may have a beneficial, protective role against glaucoma (340). As shown in Table 8.8 CCT was significantly different between the groups, with NTG and POAG having a thinner CCT than HC. It has been previously been shown that a thinner cornea experiences increased corneal deformation (589), however there was no significant difference in deformation between the groups despite CCT difference. This suggests that in this group of patients CCT was not influencing corneal deformation. This may be due to there being only a 29 μm difference between thinnest and thickest CCT.

In a similar manner to the HC participants, the OHT and POAG patients had no significant correlations between SAF and the different corneal viscoelastic parameters. In NTG however, velocity and deformation amplitude at the first
applanation was found to be reduced with increased SAF. This suggests that in NTG patients, SAF is related to corneal viscoelasticity, with increased SAF linked to a less viscoelastic cornea. Ocular rigidity although thought to be relevant to pathology is not often considered clinically due to the difficulties in acquiring accurate measurements (590). The lamina cribrosa is an example of this, as rigidity cannot be easily measured. Although differing structures, the stiffening of the cornea may be an indicator of overall ocular mechanical rigidity, including lamina cribrosa rigidity (338, 339). This is relevant as a lamina cribrosa that is more rigid/less viscoelastic is thought to be less able react to changes in IOP levels as it is less able to elastically absorb IOP (591). Hence it has been hypothesised that a stiffer/less viscoelastic lamina cribrosa is more susceptible to damage by increased IOP as well as normal IOP levels (328, 591, 592). This links to the results of this study since the relationship with SAF and decreased viscoelasticity existed only in the NTG group. These corneal results within the NTG group should however be reviewed with caution due to the low participant numbers in this group. This relationship should be reviewed and replicated in a study with increased numbers of systemically healthy NTG patients.

8.6 Conclusion

Ageing is a multifactorial process and similarly glaucoma is thought to be influenced by multiple factors. The results from Chapter 6 suggest that AGEs may be one possible factor influencing glaucoma. The aim of this study was to evaluate whether tissue bound AGE level (SAF) related to static and dynamic retinal vessel structure and function and corneal viscoelasticity in POAG, NTG and OHT patients. The NTG group, who are known to have a higher SAF level in
comparison to healthy controls from Chapter 6, were shown in this study to also have a significantly narrower CRAE. A direct relationship between SAF and CRAE was however not found within this group and it is thought this could relate to the small overall number of NTG participants recruited for this study. Indeed, the negative relationship shown in the larger HC group could indicate that narrower CRAE is related to increased SAF levels in principle. Increased SAF levels were found to associate with a less viscoelastic, stiffer cornea in the NTG group only. It is possible that increased SAF levels contribute to increased rigidity of the ocular structures which leave them more susceptible to damage from more ‘normal’ IOP levels, or small reductions in arterial blood supply, however larger scale studies would be needed before any conclusions could be drawn from these findings. There was no significant relationship in the OHT or POAG groups. There were no statistically significant relationships between SAF and dynamic vascular parameters in any groups, suggesting at the early stages of the disease process tissue-bound AGE levels do not significantly influence the vasoactive response of the retinal microvasculature to increased metabolic demand from flicker light stimulation.

8.7 Limitations
Participants that were prescribed anti-glaucomatous drops were not excluded. It is known that drops can affect the ocular surface, which in turn may have affected the Corvis ST measurement (410, 593). In addition some anti-glaucomatous drops are known to be vasoactive, this may affect the retinal vasculature and hence affect the dynamic and static retinal vascular measurements.
The sample size recruited was lower than the target sample size, this potentially limits the conclusions drawn from this study. The challenge with recruitment of early stage glaucoma participants and OHT participants was due to the strict inclusion/exclusion criteria. These criteria however were necessary to avoid any other influences on AGE level, e.g. if smokers had been included then this may have masked any other relationships. In addition the recruitment between glaucoma groups was not even, with fewer NTG patients recruited. The number of recruited participants was able to provide statistically significant results, however analysis may have been aided by increased participant numbers.

8.8 Future work

To repeat this study with a larger group of participants and include sub groups of varying levels of glaucomatous damage.
9.0 The relationship between retinal vessel function and corneal viscoelasticity in open angle glaucoma (OAG), OHT and healthy patients

9.1 Abstract

Purpose: To explore whether dynamic retinal vascular parameters are related to corneal viscoelastic parameters in HC, OHT and OAG patients.

Methods: 33 healthy control participants, 13 OHT and 36 OAG patients had static and dynamic retinal vessel structure and function evaluated using the Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) and corneal viscoelasticity measured using the Corvis ST (Oculus; Optikgeräte GmbH, Wetzlar, Germany).

Results: On consideration of retinal artery response, in OAG patients a less viscoelastic cornea was related to increased arterial dilation response to flicker light (A1 velocity and artery BFR; \( r = -0.521, p = 0.001, 95\% \text{ CI } -0.236 \text{ to } -0.757 \)). No significant relationships were found between dynamic retinal artery parameters and corneal viscoelasticity parameters in HC and OHT groups.

On consideration of retinal venous response, in OAG participants, a less viscoelastic cornea was related to a faster venous response and reaction time (A2 velocity and vein RT \( r = 0.484, p = 0.003, 95\% \text{ CI } -0.055 \text{ to } 0.809 \); A2 velocity and vein CT \( r = 0.434, p = 0.009, 95\% \text{ CI } -0.070 \text{ to } 0.736 \)). Similarly, in HC participants, a less viscoelastic cornea was found to relate to increased venous dilation response (A1 time with venous BFR \( r = 0.533, p = 0.002, 95\% \text{ CI } 0.112 \text{ to } 0.821 \) and A1 time with vMD; \( r = 0.497, p = 0.005, 95\% \text{ CI } 0.006 \text{ to } 0.755 \)). In
OHT participants however, a less viscoelastic cornea was found to relate to a decreased venous dilation response (A1 time with vein BFR; \( r = -0.772, \ p = 0.002 \), 95% CI -0.529 to -0.929 and highest corneal deformation amplitude with vein BFR \( r = 0.693, \ p = 0.009 \), 95% CI 0.164 to 0.927).

**Conclusion:** Retinal artery and vein parameters of dynamic retinal function were found to be related to viscoelastic corneal properties. However, the results are not consistent between parameters, which causes some uncertainty to the relationship.
9.2 Background

AGE accumulation can induce direct structural change to cells and tissues, through the creation of cross-links with proteins such as extracellular proteins collagen and elastin (69, 70). The AGE induced cross-link alters the mechanical properties of the protein. In collagen, these cross-links result in increased stiffness of tissues and reduction in elasticity (38, 71, 72). Normal collagen and elastin function is important for organs, tissues and vascular function throughout the body (19, 73), hence modifications are thought to have an adverse effect on physiology.

A main site of RGC axonal injury in glaucoma is thought to be the lamina cribrosa (591, 594, 595). The lamina cribrosa is known to stiffen with age (592) and on becoming less elastic it also becomes less able to respond at high IOP levels and therefore more vulnerable to the effects of increased IOP. AGEs have been detected in the cribriform plates of optic nerve head (20). Age related stiffening of the lamina cribrosa is linked with AGEs and as such AGEs could be encouraging the RGC axonal injury seen in glaucoma. It is difficult to measure deformation of the lamina cribrosa in living patients (556).

The mechanical properties of the cornea can be indicated by viscoelasticity measurement and mechanical properties of retinal blood vessels by dynamic vascular measurements. Although differing structures, both are thought to be stiffened by AGE collagen cross-linking. This stiffening throughout the structures of the eye, could contribute to overall ocular mechanical rigidity, including lamina cribrosa rigidity (338, 339). It could be hypothesised therefore that rigidity and
dynamic response of the cornea and retinal vasculature may be able to infer if there is rigidity of the lamina cribrosa.

Deformation amplitude is considered to be one of the main corneal viscoelastic parameters. Deformation amplitude has been linked with glaucoma with Wang et al. (2015), Tian et al. (2015) and Jung et al. (2017) each finding that deformation amplitude is lower in POAG than HC (596-598), indicating a less viscoelastic, stiffer, cornea in these patients. Recent literature has also investigated the viscoelastic properties of myopic corneas, Chansangpetch et al. (2017) found that myopic participants both with and without glaucoma had an increased maximum deformation amplitude compared to emmetropes with and without glaucoma (599), similarly He et al. (2017) found that eyes with high myopia (-6.00D) had increased deformation amplitude compared to emmetropes (600). This suggests that myopic eyes have a more viscoelastic cornea. There is only one study which relates Corvis ST measures of deformation amplitude to the posterior pole. The results of this were inconsistent compared to other studies assessing deformation in glaucoma patients since the results showed that increased deformation amplitude significantly correlated with increased parapapillary atrophy area and optic disc tilt (601). These results however were not found to be significant after multiple regression analysis and should be reviewed with caution due to myopia possibly influencing the results (601). Exploring if retinal vessels are linked with deformation amplitude and other corneal viscoelastic parameters is interesting since both structures largely consist of extracellular matrix and both are linked with glaucoma.
There is evidence to suggest that AGEs induce structural changes to the extracellular matrix of the cornea, lamina cribrosa and the systemic blood vessel walls by increasing the stiffness. Exploring if there is any relationship between retinal and corneal parameters may reveal if there are links and a general trend towards stiffness in the eye at a similar rate, or if the structures are affected independently. The elements investigated in this chapter are explorative, based on AGE theory. No previous studies have examined the relationship between corneal viscoelasticity and vascular dynamic properties.

9.2.1 Aims
To explore whether dynamic retinal vascular parameters are related to corneal viscoelastic parameters in HC, OHT, NTG and POAG patients.

9.2.2 Hypothesis
A relationship will exist between retinal vascular and corneal parameters.

9.3 Methods
The methodology used in this study is summarised here; more detailed information on the specifics of each technique can be found in Chapter 3.

9.3.1 Participants
Convenience sampling was used to recruit participants who are eligible according to the study’s inclusion and exclusion criteria, the inclusion criteria for participants are detailed in section 3.1.3, exclusion detailed in section 3.1.4. Due to the established link between diabetes and AGEs (449, 450) participants diagnosed
with diabetes or participants found to have a high fasting glucose level were excluded from the study, as were current smokers, and previous smokers who quit less than 15 years ago due to the link between AGEs and tobacco (10). POAG, NTG and OHT participants were identified through their attendance at routine outpatient appointments at Derriford REI.

9.3.2 General investigations

As described in 3.3.2.1, height was measured in meters using a stadiometer, weight was measured in kg, and from these measurements BMI was calculated. Weight, basal metabolic rate (BMR), and body fat percentage were measured using the Tanita body composition analyser (TBF-300 MA). Blood pressure was measured, and a fasting venous blood sample was obtained to enable the measurement of oxidative stress biomarkers. Three oxidative stress biomarkers were used, the non-enzymatic antioxidants ascorbate (vitamin C) and alpha tocopherol (a form of vitamin E) and the product of lipid peroxidation malondialdehyde (MDA). Each biomarker is described in Section 3.3.2.6. As mentioned in Section 9.2 myopia has been linked with deformation amplitude. All participants met the inclusion criteria of having a refractive error below +/- 6.00D.

9.3.3 Dynamic Retinal Vessel Analyser (DVA)

The Dynamic Retinal Vessel Analyser (DVA; IMEDOS GmbH, Jena, Germany) was used to assess dynamic retinal vessel function. It is a non-invasive device, with high reproducibility (373) and low variability (374). Specific detail on the DVA and its protocol for dynamic measurements can be found in Section 3.3.2.2.
The dynamic parameters calculated from the raw data included: baseline diameter fluctuation (BDF), dilation amplitude (DA), baseline corrected flicker response (BFR), maximum percentage dilation (MD%), reaction time (RT), minimum percentage constriction (MC%), constriction time (CT) for artery and vein. For full list of descriptions of these parameters see Table 3.3 in Section 3.3.2.2. Participants were asked to maintain normal eating habits before DVA measurement as fasting can cause abnormal vascular activity (385).

9.3.4 Corvis ST

The viscoelastic properties of the cornea were assessed using the Corvis ST (Oculus; Optikgeräte GmbH, Wetzlar, Germany). The Corvis ST is a noncontact tonometry system, for specific detail see Section 3.3.2.3. The parameters used were: first applanation time (time A1), second applanation time (time A2), velocity of corneal surface movement at the first applanation (velocity A1), velocity of corneal surface movement at second applanation (velocity A2), deformation amplitude at first applanation (deformation amplitude A1), deformation amplitude at second applanation (deformation amplitude A2), highest deformation amplitude, and highest concavity time. Corvis measurements were taken for each eye of each participant.

Reduced CCT has been identified as an independent risk factor for GON (331, 332). Previous studies examining the link between CCT and corneal hysteresis have differing results as to if a relationship does or does not exist. Touboul et al. (2008) found no significant correlation between CCT and corneal hysteresis (602), however Shah et al. (2006) found a moderate positive correlation between
increasing CCT and increased corneal hysteresis, and a recent study by Murphy et al. (2017) found a strong positive correlation between CCT and corneal hysteresis (340). For this reason CCT was measured in addition to viscoelastic properties.

9.3.5 Selection of parameters

Static parameters were not included in the study since it is the responsiveness of the structures that is being compared. In addition BDF was not included since it describes the vessel in baseline conditions and is also incorporated into the BFR result. All other vascular parameters, listed above in Section 9.3.1.3 were included and all of Corvis ST viscoelastic properties listed in 9.3.1.4 were also included.

9.3.6 Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM). The normality of data was assessed by the Shapiro–Wilk test and visual inspection of the QQ plots.

P values of less than 0.01 were considered significant for correlations. This stricter p-value was adopted for this explorative Chapter in order to correct for multiple comparisons taking place, and to minimise any bias towards type II errors.

9.3.6.1 General characteristics between groups

An ANOVA was used to compare the results of general investigations between the groups followed by the post-hoc analysis Scheffe. P values of less than 0.05 in an ANOVA was considered significant.
9.3.6.2 Relationship between arterial retinal vessel and corneal parameters

Pearson’s correlation was used to analyse the association between the arterial parameters: DA, BFR, MD%, RT, MC%, CT with the corneal parameters: time A1, time A2, velocity A1, velocity A2, deformation A1, deformation A2, highest deformation and highest concavity time. Any significant correlations were entered into a stepwise forward and backward multiple linear regression to further assess the influence of different variables on one another.

9.3.6.3 Relationship between venous retinal vessel and corneal parameters

Pearson’s correlation was used to analyse the association between the venous parameters: DA, BFR, MD%, RT, MC%, CT with the corneal parameters: time A1, time A2, velocity A1, velocity A2, deformation A1, deformation A2, highest deformation and highest concavity time. Any significant correlations were entered into a stepwise forward and backward multiple linear regression to further assess the influence of different variables on one another.

9.3.6.4 Power calculation

The sample size was determined for Chapters 7 and 8, using data from previous research which also measured tissue-bound AGE level using AGE Reader between healthy and disease groups (83). In order to achieve 80% power at the 5% alpha level, a sample size of 37 per group was required (NTG, POAG, OHT and controls). To account for unusable data an allowance was made and a sample size of 40 participants was chosen per group.
9.4 Results

A total of 28 POAG, 8 NTG, 13 OHT and 33 healthy control participants participated in the study. Due to the NTG group having only eight participants the NTG and POAG groups were combined into an open angle glaucoma group.

9.4.1 Group characteristics

There were no significant differences in age, BMI, systemic blood pressure, IOP, MABP and OPP between the four groups (p > 0.05). The characteristics of the participant group with mean and standard deviation are detailed in Table 9.1. It was expected that there would not be a significant difference in IOP between the groups since the OHT, POAG and NTG patients were all taking their prescribed IOP lowering treatment as normal.
Table 9.1: Summary of mean and standard deviation of participant characteristics for the study groups. P<0.05 is considered a significant difference. BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, IOP: intraocular pressure, MABP: mean arterial blood pressure, OPP: ocular perfusion pressure, was subsequently calculated using MABP and IOP values.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HC (1)</th>
<th>OHT (2)</th>
<th>OAG (3)</th>
<th>ANOVA p value</th>
<th>Post hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>33</td>
<td>13</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>M:22</td>
<td>M:11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F:5</td>
<td>M:8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F:17</td>
<td>M:19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.97 ±9.43</td>
<td>67.23 ±8.10</td>
<td>68.92 ±7.06</td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>25.62 ±3.36</td>
<td>25.77 ±4.56</td>
<td>26.53 ±4.28</td>
<td>0.247</td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>134.82 ±16.93</td>
<td>141.15 ±17.60</td>
<td>138.25 ±19.18</td>
<td>0.146</td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.67 ±10.17</td>
<td>84.85 ±9.50</td>
<td>84.06 ±9.77</td>
<td>0.426</td>
<td></td>
</tr>
<tr>
<td>IOP (mmHg)</td>
<td>16.39 ±2.72</td>
<td>18.25 ±3.13</td>
<td>16.22 ±3.56</td>
<td>0.140</td>
<td></td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>98.72 ±11.11</td>
<td>103.62 ±11.13</td>
<td>102.12 ±11.22</td>
<td>0.189</td>
<td></td>
</tr>
<tr>
<td>OPP</td>
<td>49.63 ±7.91</td>
<td>51.08 ±9.87</td>
<td>52.08 ±8.36</td>
<td>0.414</td>
<td></td>
</tr>
<tr>
<td>SAF</td>
<td>2.19 ±0.39</td>
<td>2.39 ±0.44</td>
<td>2.52 ±0.40</td>
<td>0.001</td>
<td>1 &lt; 3</td>
</tr>
</tbody>
</table>

Corneal viscoelasticity parameters between groups

The corneal viscoelasticity parameter velocity A1 (m/s) was significantly different between groups (p = 0.042) on ANOVA comparison of the means, however on post hoc testing this significant difference between the groups was not held. In addition no significant difference was found between any of the other corneal viscoelasticity parameters between the study groups (p>0.05, Table 9.2). CCT however was found to be different between the glaucoma groups and HC, with HC having increased CCT.

<table>
<thead>
<tr>
<th>Corneal viscoelasticity</th>
<th>HC (1)</th>
<th>OHT (2)</th>
<th>OAG (3)</th>
<th>ANOVA p value</th>
<th>Post hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>33</td>
<td>13</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time A1 (ms)</td>
<td>7.33 ±0.37</td>
<td>7.45 ±0.30</td>
<td>7.30 ±0.24</td>
<td>0.225</td>
<td></td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>21.95 ±0.44</td>
<td>21.66 ±0.48</td>
<td>21.75 ±0.43</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>0.15 ±0.02</td>
<td>0.13 ±0.03</td>
<td>0.15 ±0.02</td>
<td>0.042 p&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>-0.26 ±0.03</td>
<td>-0.26 ±0.02</td>
<td>-0.27 ±0.03</td>
<td>0.156</td>
<td></td>
</tr>
<tr>
<td>Deformation amp. A1 (mm)</td>
<td>0.13 ±0.01</td>
<td>0.13 ±0.01</td>
<td>0.13 ±0.01</td>
<td>0.862</td>
<td></td>
</tr>
<tr>
<td>Deformation amp. A2 (mm)</td>
<td>0.44 ±0.07</td>
<td>0.44 ±0.08</td>
<td>0.43 ±0.05</td>
<td>0.699</td>
<td></td>
</tr>
<tr>
<td>Highest deformation amp. (mm)</td>
<td>1.09 ±0.11</td>
<td>1.07 ±0.12</td>
<td>1.09 ±0.09</td>
<td>0.530</td>
<td></td>
</tr>
<tr>
<td>Highest concavity time (ms)</td>
<td>16.48 ±0.44</td>
<td>16.42 ±0.54</td>
<td>16.43 ±0.54</td>
<td>0.874</td>
<td></td>
</tr>
<tr>
<td>CCT (μm)</td>
<td>555 ±34.34</td>
<td>550 ±21.22</td>
<td>526.94 ±38.08</td>
<td>0.004 1 &gt; 3</td>
<td></td>
</tr>
</tbody>
</table>
9.4.2 Relationship between arterial retinal vessel and corneal parameters

A more viscoelastic, deformable cornea is thought to have a shorter A1 time, faster velocity at A1, increased deformation amplitude at A1, increased maximum deformation amplitude and have a longer time A2, and slower velocity at A2 (410). Vasodilation in response to flicker stimulation relies on the dilation of blood vessel walls, which is driven by endothelial function (141, 379). Decreased DA, BFR and maximum percentage dilation, slower reaction time and constriction time are thought to infer vascular dysfunction.

As shown in Table 9.1 and Table 9.2 SAF and CCT were significantly different between the groups. Previous research has suggested that a thinner cornea experiences increased corneal deformation (589). For this reason, all correlations were run within each group rather than with all participants grouped together. In addition, any significant correlations between corneal and vascular parameters were entered into a stepwise forward and backward multiple linear regression with the variables CCT and SAF included.

9.4.2.1 Healthy controls

Table 9.3 shows the correlations between the six different retinal artery dynamic parameters and the eight different corneal viscoelasticity parameters in healthy control participants. No significant correlations existed.
### Healthy controls

<table>
<thead>
<tr>
<th>Artery</th>
<th>Mean ± SD</th>
<th>DA</th>
<th>BFR</th>
<th>MD%</th>
<th>RT (secs)</th>
<th>MC%</th>
<th>CT (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time A1 (ms)</td>
<td>7.281 ± 0.366</td>
<td>0.352</td>
<td>0.376</td>
<td>3.356</td>
<td>15.629</td>
<td>1.722</td>
<td>24.237</td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>22.019 ± 0.429</td>
<td>-0.187</td>
<td>-0.208</td>
<td>-0.160</td>
<td>-0.102</td>
<td>-0.145</td>
<td>-0.133</td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>0.152</td>
<td>-0.312</td>
<td>-0.299</td>
<td>-0.205</td>
<td>-0.066</td>
<td>-0.325</td>
<td>-0.217</td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>-0.263</td>
<td>0.422</td>
<td>0.413</td>
<td>0.341</td>
<td>0.141</td>
<td>0.354</td>
<td>0.313</td>
</tr>
<tr>
<td>Deformation amp. A1 (mm)</td>
<td>0.135</td>
<td>0.286</td>
<td>0.301</td>
<td>0.239</td>
<td>-0.408</td>
<td>0.229</td>
<td>0.136</td>
</tr>
<tr>
<td>Deformation amp. A2 (mm)</td>
<td>0.443</td>
<td>0.058</td>
<td>0.039</td>
<td>0.013</td>
<td>0.005</td>
<td>0.094</td>
<td>-0.130</td>
</tr>
<tr>
<td>Highest deformation amp. (mm)</td>
<td>1.092</td>
<td>-0.199</td>
<td>-0.214</td>
<td>-0.202</td>
<td>-0.035</td>
<td>-0.110</td>
<td>-0.302</td>
</tr>
</tbody>
</table>

Table 9.3: Mean, SD and Pearson’s correlations between the six artery parameters and eight corneal viscoelasticity parameters. P<0.01* only correlations of P<0.01 were considered significant. BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time, Time A1: first applanation time, Time A2: second applanation time, Velocity A1: velocity of corneal surface movement at the first applanation, Velocity A2: velocity of corneal surface movement at second applanation, Deformation amp. A1: deformation amplitude at first applanation, Deformation amp. A2: deformation amplitude at second applanation, Highest deformation amp: highest deformation amplitude.

Table 9.4 shows the correlations between the six different retinal vein dynamic parameters and the eight different corneal viscoelasticity parameters in healthy control participants. A significant correlation was found between A1 time and
venous BFR ($r = 0.533$, $p = 0.002$, 95% bootstrap confidence interval 0.112 to 0.821) as well as between A1 time and venous MD% ($r = 0.497$, $p = 0.005$, 95% bootstrap confidence interval 0.006 to 0.755). These correlations suggest that a less viscoelastic cornea is related to a greater venous dilation response.

**Healthy controls**

<table>
<thead>
<tr>
<th>Vein</th>
<th>Pearson's correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DA</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.624 ± 2.756</td>
</tr>
<tr>
<td>Time A1 (ms)</td>
<td>7.281 ± 0.366</td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>22.019 ± 0.429</td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>0.152 ± 0.018</td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>-0.263 ± 0.033</td>
</tr>
<tr>
<td>Deformation amp. A1 (mm)</td>
<td>0.135 ± 0.011</td>
</tr>
<tr>
<td>Deformation amp. A2 (mm)</td>
<td>0.443 ± 0.076</td>
</tr>
<tr>
<td>Highest deformation amp. (mm)</td>
<td>1.092 ± 0.113</td>
</tr>
<tr>
<td>Highest concavity time (ms)</td>
<td>16.480 ± 0.449</td>
</tr>
</tbody>
</table>

Table 9.4: Mean, SD and Pearson’s correlations between the six vein parameters and eight corneal viscoelasticity parameters. P<0.01*, only correlations of P<0.01 were considered significant. BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time, Time A1: first applanation time, Time A2: second applanation time, Velocity A1: velocity of corneal surface movement at the first applanation, Velocity A2: velocity of corneal surface movement at second applanation, Deformation amp. A1: deformation amplitude at first applanation, Deformation amp. A2: deformation amplitude at second applanation, Highest deformation amp: highest deformation amplitude.
Forwards stepwise and backwards multiple linear regression analysis was performed to determine the factors that had the most influence on corneal time A1. The independent variables included were those which had previously correlated, vein BFR and vein MD%. In addition, CCT, SAF and age were included due to the literature suggesting they influence corneal viscoelasticity.

The variable CCT was the single best predictor ($p = 0.005$), followed by vein MD% ($p = 0.020$). These findings were also found to be true in the backwards model.

### 9.4.2.2 Ocular hypertension

Table 9.5 shows the correlations between the six different retinal artery dynamic parameters and the eight different corneal viscoelasticity parameters in OHT patients. In a similar manner to the healthy control patients, no significant correlations existed.
**Table 9.5:** Mean, SD and Pearson’s correlations between the six artery parameters and eight corneal viscoelasticity parameters. P<0.01** only correlations of P<0.01 were considered significant. BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time, Time A1: first applanation time, Time A2: second applanation time, Velocity A1: velocity of corneal surface movement at the first applanation, Velocity A2: velocity of corneal surface movement at second applanation, Deformation amp. A1: deformation amplitude at first applanation, Deformation amp. A2: deformation amplitude at second applanation, Highest deformation amp: highest deformation amplitude.
Table 9.6 shows the correlations between the six different retinal vein dynamic parameters and the eight different corneal viscoelasticity parameters in OHT patients. A negative correlation between corneal A1 time and vein BFR existed ($r = -0.727, p = 0.002, 95\%$ bootstrap confidence interval $-0.529$ to $-0.929$), with a shorter A1 time related to increased vein BFR. A positive correlation between highest deformation amplitude and vein BFR existed ($r = 0.693, p = 0.009, 95\%$ bootstrap confidence interval $0.164$ to $0.927$), as there was increased maximum deformation there was increased vein BFR. The results of both of these correlations suggest that a less deformable viscoelastic cornea is related to reduced venous dilation response.
Table 9.6: Mean, SD and Pearson’s correlations between the six vein parameters and eight corneal viscoelasticity parameters. P<0.01**, only correlations of P<0.01 were considered significant.

- **BDF**: baseline diameter fluctuation, **DA**: dilation amplitude, **BFR**: baseline corrected flicker response, **MD%**: maximum percentage dilation, **RT**: reaction time, **MC%**: minimum percentage constriction, **CT**: constriction time, **Time A1**: first applanation time, **Time A2**: second applanation time, **Velocity A1**: velocity of corneal surface movement at the first applanation, **Velocity A2**: velocity of corneal surface movement at second applanation, **Deformation amp. A1**: deformation amplitude at first applanation, **Deformation amp. A2**: deformation amplitude at second applanation, **Highest deformation amp.**: highest deformation amplitude.

<table>
<thead>
<tr>
<th>Vein</th>
<th>Mean ± SD</th>
<th>DA</th>
<th>BFR</th>
<th>MD%</th>
<th>RT (secs)</th>
<th>MC%</th>
<th>CT (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4.613 ± 2.066</td>
<td>2.315 ± 2.516</td>
<td>3.761 ± 2.135</td>
<td>17.077 ± 4.141</td>
<td>0.853 ± 0.919</td>
<td>25.385 ± 9.301</td>
</tr>
<tr>
<td>Time A1 (ms)</td>
<td>7.45 ± 0.302</td>
<td>-0.502 p = 0.080</td>
<td>-0.772 * p = 0.002</td>
<td>-0.582 p = 0.037</td>
<td>-0.640 p = 0.019</td>
<td>0.224 p = 0.462</td>
<td>-0.524 p = 0.066</td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>21.656 ± 0.477</td>
<td>0.345 p = 0.248</td>
<td>0.599 p = 0.030</td>
<td>0.474 p = 0.101</td>
<td>0.526 p = 0.065</td>
<td>-0.326 p = 0.277</td>
<td>0.583 p = 0.036</td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>0.134 ± 0.027</td>
<td>0.356 p = 0.233</td>
<td>0.621 p = 0.023</td>
<td>0.430 p = 0.142</td>
<td>0.548 p = 0.052</td>
<td>-0.199 p = 0.513</td>
<td>0.628 p = 0.021</td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>-0.257 ± 0.024</td>
<td>-0.448 p = 0.125</td>
<td>-0.564 p = 0.044</td>
<td>-0.440 p = 0.132</td>
<td>-0.553 p = 0.050</td>
<td>0.017 p = 0.956</td>
<td>-0.611 p = 0.026</td>
</tr>
<tr>
<td>Deformation amp. A1 (mm)</td>
<td>0.133 ± 0.009</td>
<td>-0.111 p = 0.718</td>
<td>-0.040 p = 0.897</td>
<td>-0.085 p = 0.782</td>
<td>0.009 p = 0.977</td>
<td>-0.052 p = 0.866</td>
<td>-0.023 p = 0.939</td>
</tr>
<tr>
<td>Deformation amp. A2 (mm)</td>
<td>0.442 ± 0.077</td>
<td>0.369 p = 0.215</td>
<td>0.601 p = 0.030</td>
<td>0.419 p = 0.155</td>
<td>0.033 p = 0.914</td>
<td>-0.143 p = 0.640</td>
<td>0.355 p = 0.235</td>
</tr>
<tr>
<td>Highest deformation amp. (mm)</td>
<td>1.067 ± 0.118</td>
<td>0.399 p = 0.177</td>
<td>0.693 * p = 0.009</td>
<td>0.485 p = 0.093</td>
<td>0.264 p = 0.383</td>
<td>-0.229 p = 0.452</td>
<td>0.480 p = 0.097</td>
</tr>
<tr>
<td>Highest concavity time (ms)</td>
<td>16.419 ± 0.537</td>
<td>0.473 p = 0.103</td>
<td>0.595 p = 0.032</td>
<td>0.436 p = 0.137</td>
<td>0.045 p = 0.883</td>
<td>0.050 p = 0.870</td>
<td>0.598 p = 0.031</td>
</tr>
</tbody>
</table>
Forwards stepwise and backwards multiple linear regression analysis was performed to determine the factors that had the most influence on corneal time A1. The variable vein BFR was included due to the correlation. In addition, CCT, SAF and age were included due to the literature suggesting they influence corneal viscoelasticity. The variable vein BFR was the single best predictor (p = 0.001), followed by SAF (p = 0.009). These findings were also found to be true in the backwards model.

Forwards stepwise and backwards multiple linear regression analysis was also performed to determine the factors that had the most influence on the corneal viscoelastic parameter ‘highest deformation amplitude’. The variable vein BFR was included due to the correlation. In addition, CCT, SAF and age were included due to the literature suggesting they influence corneal viscoelasticity. The variable vein BFR was the single best predictor (p = 0.009), with no other significant variables. These findings were also found to be true in the backwards model.

9.4.2.3 Open angle glaucoma (NTG and POAG patients)

Table 9.7 shows the correlations between the six different retinal artery dynamic parameters and the eight different corneal viscoelasticity parameters in the open angle glaucoma patients. One significant correlation existed between the parameters, a faster velocity at A1 related to reduced artery BFR (r = -0.521, p = 0.001, 95% bootstrap confidence interval -0.236 to -0.757). This relationship suggests that in this group of open angle glaucoma patients a more viscoelastic cornea is related to a reduced arterial dilation response to flicker light.
<table>
<thead>
<tr>
<th>Artery</th>
<th>DA</th>
<th>BFR</th>
<th>MD%</th>
<th>RT (secs)</th>
<th>MC%</th>
<th>CT (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time A1 (ms)</td>
<td>7.299 ± 0.240</td>
<td>0.324 p = 0.057</td>
<td>0.324 p = 0.045</td>
<td>0.282 p = 0.101</td>
<td>-0.011 p = 0.948</td>
<td>0.134 p = 0.444</td>
</tr>
<tr>
<td></td>
<td>Time A2 (ms)</td>
<td>-0.144 p = 0.410</td>
<td>-0.322 p = 0.059</td>
<td>-0.150 p = 0.389</td>
<td>0.096 p = 0.582</td>
<td>-0.021 p = 0.905</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.749 ± 0.431</td>
<td></td>
<td></td>
<td>0.045 p = 0.799</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.134 ± 0.009</td>
<td>0.089 p = 0.613</td>
<td>-0.167 p = 0.337</td>
<td>0.074 p = 0.674</td>
<td>0.151 p = 0.385</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.429 ± 0.057</td>
<td>0.027 p = 0.877</td>
<td>-0.131 p = 0.454</td>
<td>0.193 p = 0.266</td>
<td>0.206 p = 0.236</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.106 ± 0.096</td>
<td>-0.317 p = 0.063</td>
<td>-0.410 p = 0.014</td>
<td>-0.200 p = 0.248</td>
<td>0.035 p = 0.842</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.421 ± 0.547</td>
<td>-0.068 p = 0.698</td>
<td>-0.172 p = 0.324</td>
<td>-0.020 p = 0.910</td>
<td>-0.100 p = 0.567</td>
</tr>
</tbody>
</table>

Forwards stepwise and backwards multiple linear regression analysis was performed to determine the factors that had the most influence on the corneal viscoelastic parameter velocity A1. The variable artery BFR was included due to the correlation. In addition, CCT, SAF and age were included due to the literature suggesting they influence corneal viscoelasticity. Artery BFR was the single best predictor ($p = 0.006$), followed by CCT ($p = 0.026$). These findings were also found to be true in the backwards model.

Table 9.8 shows the correlations between the six different retinal vein dynamic parameters and the eight different corneal viscoelasticity parameters in the open angle glaucoma patients. As previously mentioned a slower A2 velocity indicates a more viscoelastic cornea. A2 velocity was found to correlate with vein reaction time ($r = 0.484$, $p = 0.003$, 95% bootstrap confidence interval -0.055 to 0.809), with a faster A2 velocity related to faster vein reaction time. In a similar manner A2 velocity correlated with vein constriction time ($r = 0.434$, $p = 0.009$, 95% bootstrap confidence interval -0.070 to 0.736), with a faster A2 velocity related to a faster vein constriction time. Both of these results indicate that a less viscoelastic cornea is related to a faster venous response and recovery time.
<table>
<thead>
<tr>
<th>Glaucoma</th>
<th>Pearson's correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vein</strong></td>
<td><strong>DA</strong></td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>5.299 ± 2.997</td>
</tr>
<tr>
<td><strong>Time A1 (ms)</strong></td>
<td>7.299 ± 0.240</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Time A2 (ms)</strong></td>
<td>21.749 ± 0.431</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Velocity A1 (m/s)</strong></td>
<td>0.151 ± 0.020</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Velocity A2 (m/s)</strong></td>
<td>-0.276 ± 0.037</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Deformation amp. A1 (mm)</strong></td>
<td>0.134 ± 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Deformation amp. A2 (mm)</strong></td>
<td>0.429 ± 0.057</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Highest deformation amp. (mm)</strong></td>
<td>1.106 ± 0.096</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Highest concavity time (ms)</strong></td>
<td>16.421 ± 0.547</td>
</tr>
</tbody>
</table>

Forwards stepwise and backwards multiple linear regression analysis was performed to determine the factors that had the most influence on corneal velocity. The independent variables included were those which had previously correlated, vein RT and vein CT. In addition, CCT, SAF and age were included due to the literature suggesting they influence corneal viscoelasticity. The variable vein RT was the single best predictor ($p = 0.003$), followed by CCT ($p = 0.041$). These findings were also found to be true in the backwards model.

### 9.5 Discussion

The aim of this study was to explore if any relationship existed between the viscoelastic properties of the cornea and dynamic retinal vessel response to flicker stimulation. It is thought that this is the first study to compare these parameters. In the population examined, which included healthy, OHT, NTG and POAG participants, correlations were found between corneal viscoelasticity and retinal vessel responsiveness. There are no previous studies which relate corneal viscoelastic properties to the retina or retinal vascular properties. Hence, there is no research to directly compare the results of this study to.

A less viscoelastic cornea has been linked with glaucoma (337, 581, 582), and it has been suggested that an increased viscoelasticity of the cornea may have a protective role against glaucoma (340). In this study, no significant differences in corneal viscoelasticity were found between the different groups. The only parameter that was significantly different was CCT, with healthy participants having a thicker CCT than glaucoma patients. The other measurement different...
between groups was SAF, with the OAG group having a higher SAF level than healthy controls. As potential confounding variables, CCT and SAF were included in multiple linear regressions along with the relevant corneal and vascular parameters at each stage of the analysis. CCT and SAF were not found to be consistent predictors in the different regression analysis. This suggests that the relationships found between corneal and vascular parameters were not dependent on SAF or CCT levels.

In this study, a less viscoelastic cornea was found to relate to an increased arterial dilation response to flicker light in OAG patients only. Based on the findings of previous studies that have separately evaluated corneal viscoelasticity and retinal artery dilation response in glaucoma patients, this finding is perhaps contradictory, as a less viscoelastic cornea and a reduced, rather than increased, arterial dilation response has typically been linked to the disease. Previous studies using the DVA have however found differing results in relation to arterial dilation response to flicker. Garhöfer et al. (2004) found no significant difference in arterial flicker response between glaucoma and healthy control groups (305) where-as Gugleta et al. (2013) found that response to flicker light was decreased in POAG patients compared to controls (603). It is interesting that this relationship between arterial dilation response and corneal viscoelasticity was only present in the OAG patients and not the HC or OHT patients considering there were no significant differences in either corneal viscoelasticity or vascular parameters between groups. The result may have been influenced by there being a wider variation of results in the OAG group. Due to the small sample sizes, in this study, NTG and POAG patients have been grouped together. In Chapter 8
altered CRAE was found in our NTG patients, with this group having a significantly narrower artery equivalent compared to healthy controls. It may be that the relationship found in this study is being driven by a mechanism occurring in NTG patients, however more data is needed to better understand this.

With regard to retinal venous response to flicker light and corneal viscoelasticity, in this study, a less viscoelastic cornea was found to relate to a faster venous response and recovery time in OAG patients and to an increased venous dilation response in healthy participants. Somewhat contradictorily to this however, in the OHT group a less viscoelastic cornea was found to relate to a reduced venous dilation response to flicker light. Retinal venous dilation response following flicker light stimulation has been found to be reduced in NTG patients (304) and OAG patients (305, 603) compared to healthy controls. A reduced vascular response of the tissue is thought to indicate altered endothelial function in these patients.

There are fewer studies that have assessed retinal venous responses to flicker in OHT patients, with studies focusing on response of vessels after IOP rise (282). In a recent study the viscoelastic property, corneal hysteresis, was found to be higher, i.e. more viscoelastic, in OHT patients compared to glaucoma patients however, suggesting that increased viscoelasticity of the cornea may have a beneficial, having a protective role against glaucoma (340). This is of interest when considered in relation to the OHT results of this study, as a less viscoelastic cornea was related to reduced vein dilation response to flicker light. If reduced venous reactivity does indeed indicate endothelial function, this could suggest that endothelial dysfunction is related to a stiffer, less viscoelastic cornea in OHT patients and potentially an increased risk of glaucomatous damage. If this were
the case however, it would have been expected that the OAG patients would have demonstrated a similar relationship, however no such relationship was found. The OAG patients in this study were at the early stages of their disease process. There were no significant differences in the vascular parameters between OAG and OHT patients in this study, so although it appears viscoelasticity and dynamic vascular response of the vein are indeed related, in which way is not clear and larger scale studies would be needed to confirm this relationship.

It is not known whether the extracellular matrix of the cornea is affected by AGEs in a different way to the extracellular matrix in the vessels due to differing ratios of elastin and collagen within the structures, and differing types of collagen (604, 605). It is known that AGE crosslinks form in different types of collagen, however collagen type I is thought to be particularly prone to cross-linking (74, 137, 606). There is currently no evidence that vessels are more or less prone to AGE cross-linking than the cornea. This is relevant as differences may help to explain why the structures respond differently to one another.

An additional hypothesis important to consider is that the results of this study may be unusual and different to what was expected due to the difference in how the measurements are achieved. The Corvis ST uses a stimulus that forces the cornea to respond in a manner that is not ‘normal’ and would not occur under normal conditions. The DVA however relies upon the normal autoregulation mechanism to measure response to a stimulus. The response is one which would occur under normal conditions. One possible reason for the difference could
therefore be how the tissues respond differently when reacting in a recreation of ‘normal circumstances’, as opposed to those when a forced response is prompted. In addition the vascular parameters described here were the result of multiple reactions and responses including, myogenic factors, metabolic factors and endothelial agents such as NO (607). Whereas the reaction of the cornea is a structural response, the corneal deformation and recovery. Hence the vascular parameters has more possible factors that could affect the dilation response measurements. In addition, one possible factor that could have affected the results is that all glaucoma patients continued to use their glaucoma IOP-lowering drops as normal. It is known that drops can affect the ocular surface, which in turn may have affected the Corvis ST measurement (410, 593).

9.6 Conclusion

Retinal artery and vein parameters of dynamic retinal function were found to be related to viscoelastic corneal properties. In open angle glaucoma patients, a less viscoelastic cornea was found to be related to increased arterial dilation response to flicker light and a faster venous response and recovery time. The results however are not consistent between the groups, which causes some uncertainty to the relationships. It is not clear from these results if AGEs cause increased stiffness to one structure more than another.

9.7 Limitations

Participants that were prescribed anti-glaucomatous drops were not excluded. This may have impacted the results as these drops can affect the ocular surface, which could affect the Corvis ST measurement (410, 593). In addition some anti-
glaucomatous drops are known to be vasoactive, this may affect the retinal vasculature and hence affect the dynamic retinal vascular measurements. However the vascular effects of IOP lowering drops on retinal vascular calibre is not fully understood (265).

There was an upper limit exclusion criteria for CCT, however due to the lower participant numbers in the NTG and OHT groups CCT matching was not possible between the groups, in future studies increased numbers of participants would allow better matching of patients.

Some results in this study were close to significance. Any larger scale studies may help to confirm/deny the findings which are close to significance.
10. Summary & conclusions

10.1 Summary

AGEs have the potential to impact both the healthy ageing population and those diagnosed with pathology. Enhancing understanding of the role that AGEs may play in accelerating the development of age-related conditions, such as open angle glaucoma has the potential to offer additional insight into pathogenesis and act as a biomarker. Dietary AGE intake represents a potentially modifiable target, design of a UK specific AGE FFQ was an aim of this thesis to enable the measurement of dietary AGE intake in the UK population. Evaluating the impact of AGES and dietary AGES on a healthy ageing population as well as patients with OHT, NTG and POAG has been a focus of this thesis, as well investigating the relationships between AGES, the retinal vasculature and corneal viscoelasticity.

In summary the findings of this work were:

10.1.1 (4.0) Validation of a specifically designed semi-quantitative food frequency questionnaire

The known links between dAGE and pathology and the concept that they are a potentially modifiable factor makes assessment of dAGE intake an area of great interest. The aim of this study was to design and validate a UK-specific FFQ for use in AGE research. In addition, this study aimed to assess the reliability of the newly developed FFQ and to compare the dAGE outputs calculated using the three different databases.
The comparisons between databases suggested that they are more in line with each other than previously thought. The choice of database should therefore be based upon which is most relevant to the population, and has enough foods measured to ensure minimal instances of assigning ‘assumed values’ to foods with no assigned measured value. The strong correlations found between the three different types of AGEs measured by Scheijen (CML, CEL and MG-H1) suggests that when considering diet as a whole, an individual dAGE may be sufficient to give a representation as to if a person has a high or low AGE diet.

The UK-specific semi-quantitative FFQ developed to measure dAGE was found to produce valid results when compared to a 4-day FR and to be reliable across two completions; this indicates that it is a useful tool for estimating dAGE in a UK population. We were however unable to validate the FFQ against the objective plasma CML-AGE measurement and the reasons behind this require further research, but could relate to the measurement technique used.

10.1.2 (5.0) The relationship between skin autofluorescence, dietary AGE intake and circulating oxidative stress levels in a healthy ageing population

Questions remain as to if dAGEs accumulate in tissues or if they cause biological consequence. As such, the aim of this study was to determine whether dAGE intake is associated tissue-bound AGE levels (SAF) in a healthy ageing population. If addition, due to oxidative stress having strong links with AGEs the study aimed to determine whether circulating oxidative stress levels relate to tissue-bound AGE levels (SAF) and dAGE in this same population.
The results of this study suggest that the contribution of dAGE to tissue-bound AGE levels may be minimal. This however requires further investigation. New AGE databases, and different AGES are being measured which will aid further research. The relationship between oxidative stress and AGES was investigated, no significant associations between oxidative stress biomarkers and dAGE were found. The oxidative stress biomarker alpha tocopherol correlated with SAF, this was however in the opposite way to that hypothesised and therefore requires further investigation before the relevance and insight that could be gained from this finding can be determined. The results also suggest that tissue-bound AGES measured by SAF and the circulating oxidative stress biomarkers measured in this study are not closely related. For this reason the AGE reader, SAF, should not simply be used as a marker for oxidative stress and instead the two measures should be used alongside each other, rather than interchangeably in place of each other.

10.1.3 (6.0) AGE levels in early stage POAG, NTG and OHT patients
The results of Chapter 5 indicated that the contribution of dAGE to tissue-bound AGE levels is minimal, and requires further investigation to understand which, if any, types of AGES are impacting tissue-bound levels in a healthy ageing population. Previous research however has shown that diets higher in dAGE are linked with pathology, for this reason it was decided that dAGE would be included in this study to allow the assessment of dAGE intake between HC and pathology groups.
It has been hypothesised that increased accumulation of AGEs could be the underlying causative factor for a number of the known pathological associations of GON and ultimately contribute towards its development in some individuals through acceleration of the ageing process. The aim of this study was to determine whether tissue bound and dAGE levels are elevated in POAG and NTG in comparison to age-matched OHT patients and healthy controls. Oxidative stress levels were compared as a possible influencing factor. No previous studies had examined SAF in these three groups in conjunction with each other.

Tissue-bound AGE, SAF level, was significantly higher in NTG and POAG compared to healthy controls. This finding adds to the evidence that SAF, as an accessible measure, may be a suitable long-term biomarker of glaucoma. Understanding if AGEs influence vascular parameters more than structure of tissues is not yet established. Dietary AGE intake was found to be similar between healthy controls, OHT, NTG and POAG patients. Patients with POAG exhibited lower levels of the antioxidants ascorbate and alpha tocopherol compared to OHT and healthy controls, suggesting oxidation inhibition. There was however no increased level of MDA.

10.1.4 (7.0) The relationship between tissue-bound AGE levels (SAF), retinal vessel structure and function and corneal viscoelastic properties in a healthy ageing population

Age-related changes in the eye have been linked with pathology. There are no previous studies that have explored whether tissue-bound AGE levels are directly associated with retinal vessel structure and function, or corneal viscoelasticity,
hence the aim of this study was to determine how tissue-bound AGE levels are associated with static and dynamic retinal vessel parameters and corneal viscoelasticity in a healthy ageing population.

Higher levels of tissue-bound AGE (SAF), were associated with narrower retinal arteries in a healthy population. This finding adds to the evidence that AGEs are an accessible marker of vascular health. Increased levels of ascorbate were associated with wider retinal arteries, this supports previous literature that diet derived antioxidants may have a protective effect. In this healthy population SAF was not found to be associated with dynamic vessel parameters or corneal viscoelastic parameters, however this may be due to the healthy group recruited as mean SAF was lower than expected.

10.1.5 (8.0) The relationship between tissue bound AGE levels, static and dynamic retinal vessel structure and function, and corneal viscoelasticity in POAG, NTG and OHT patients

In Chapter 6 SAF was significantly higher in NTG and POAG compared to healthy controls. This finding adds to the evidence that SAF, as an accessible measure, may be a suitable long-term biomarker of glaucoma. Understanding if AGEs influence vascular parameters in glaucoma is not yet established. Therefore, the aim of this study was to evaluate whether level of tissue bound AGEs (SAF) relates to static and dynamic retinal vessel structure and function and corneal viscoelasticity in POAG, NTG and OHT patients. It was shown in Chapter 6 that dAGE level is not significantly different between HC, OHT, NTG and POAG groups. For this reason, dAGEs were not assessed in this study.
The NTG group, who were shown to have a higher SAF level in comparison to healthy controls in Chapter 6, were found to have a significantly narrower CRAE. A direct relationship between SAF and CRAE was however not found within this group and it is thought this could relate to the small overall number of NTG participants recruited for this study. Indeed, the negative relationship shown in the larger HC group in Chapter 7 could indicate that narrower CRAE is related to increased SAF levels in principle.

Increased SAF levels were found to associate with a less viscoelastic, stiffer cornea in the NTG group only. It is possible that increased SAF levels contribute to increased rigidity of the ocular structures which leave them more susceptible to damage from more ‘normal’ IOP levels, or small reductions in arterial blood supply, however larger scale studies would be needed before any conclusions could be drawn from these findings. There was no significant relationship in the OHT or POAG groups. The lack of statistically significant relationships between SAF and dynamic vascular parameters in any groups suggests that at the early stages of the disease process tissue-bound AGE levels do not significantly influence the vasoactive response of the retinal microvasculature to increased metabolic demand from flicker light stimulation.

10.1.6 (9.0) The relationship between retinal vessel function and corneal viscoelasticity in OAG, NTG, OHT and healthy patients

Although differing structures, both retinal vessels and the cornea are thought to be stiffened by AGE collagen cross-linking. This stiffening throughout the structures of the eye could contribute to overall ocular mechanical rigidity. The
aim of this study was to explore whether dynamic retinal vascular parameters are related to corneal viscoelastic parameters in HC, OHT, OAG patients.

Retinal artery and vein parameters of dynamic retinal function were found to be related to viscoelastic corneal properties. In open angle glaucoma patients, a less viscoelastic cornea was found to be related to increased arterial dilation response to flicker light and a faster venous response and recovery time. The results however are not consistent between the groups, which causes some uncertainty to the relationships. It is not clear from these results if mechanical rigidity occurs at an equal rate throughout the eye, or if structure is affected more than another. Larger scale studies may help to identify which way relationships exist in different patient groups.

10.2 Overall limitations

The studies outlined in this thesis are subject to potential limitations. The sample size recruited for OHT and NTG was lower than the target sample size, this potentially limits the conclusions drawn from this study. The challenge with recruitment of early stage glaucoma participants and OHT participants was due to the strict inclusion/exclusion criteria. These criteria however were necessary to avoid any other influences on AGE level, e.g. if smokers were included then this may have masked any other relationships. As a result of this strict criteria and exclusion of other disease linked to AGEs the resultant group of participants were particularly healthy and as a result had a lower SAF level than was expected for mean age.
The AGE-reader (DiagnOptics B.V., Groningen, The Netherlands) as explained in previous Chapters, is a device which utilises the fluorescent properties of tissue-bound AGEs in the skin to provide a measurement of skin autofluorescence (SAF). A limitation of the device is that it can only assess Fitzpatrick skin colour classes 1-4 (419). Fitzpatrick skin colour classes 5 and 6 (Skin reflectance <6%) do not give the required reflectance for a reliable measurement (416, 417, 419).

As described in Chapter 6, SAF has been found to be significantly higher in NTG and POAG compared to healthy controls. A problem arises here in that ethnicity is a risk factor for glaucoma, with people of African descent more at risk of developing glaucoma (608-611). It is this same ethnicity group that has the darkest skin and would not be able to have a reliable SAF measurement taken.

The Plasma AGE-CML ELISA kit used was not validated and produced results which did not agree with the previous research. The standards given with this kit were not in the same range as the measurements found, this will have impacted the results. In future research plasma AGE-CML will be carried out using the UPLC–MS/MS method.

Participants that were prescribed anti-glaucmatous drops were not excluded. This may have impacted the results as these drops can affect the ocular surface, which could affect the Corvis ST measurement (410, 593). In addition some anti-glaucmatous drops are known to be vasoactive, this may affect the retinal vasculature and hence affect the dynamic retinal vascular measurements. However the vascular effects of IOP lowering drops on retinal vascular calibre is not fully understood (265).
10.3 Clinical implications

10.3.1 Implications in regard to dAGE

The UK-specific semi-quantitative FFQ developed for this thesis offers a method of dAGE intake measurement that could be used in both future research and a clinical setting.

10.3.2 Implications in regard to open angle glaucoma patients

The results of this study found that SAF, as a measure of tissue-bound AGE level, may be a suitable long-term biomarker of glaucoma. SAF is an easily accessible fast measurement. The introduction of AGE readers to measure SAF could be something seen in a clinical setting either in a hospital or ‘high street’ setting and may be a useful tool as part of glaucoma screening.

10.4 Future directions

10.4.1 Further FFQ validation

Despite the results of this thesis suggesting that the newly developed UK specific AGE FFQ is repeatable and a useful tool for estimating dAGE in a UK population, further validation and review should take place. This validation should use UPLC-MS/MS to measure CML in the plasma.
10.4.2 Assessment of AGE-reader capabilities

Since the AGE reader was able to successfully measure SAF readings in participants whose characteristics would be linked to Fitzpatrick class 5 further research is required to better understand the capabilities of the AGE reader and establish which ethnicity groups can be measured accurately. In addition further investigation into how body fat percentage and fat mass impact SAF measurements is of interest.

10.4.3 Larger scale studies

Despite statistically significant results being demonstrated in this thesis any larger scale studies may help to confirm/deny the findings which are close to significance. In addition to this, including sub-groups with varying levels of glaucomatous damage would be of interest as to AGEs influence on glaucoma progression.

10.4.4 AGE levels influence on the rate of glaucoma progression

Assessing if baseline AGE (SAF) levels are associated with an increased rate of progression in NTG and POAG patients as well as determine whether SAF levels are associated with OHT progression to develop POAG. This would be a long-term follow up study assessing early stage glaucoma patients and following the changes in visual field mean deviation and OCT retinal nerve fibre layer. These parameters are measured routinely in the glaucoma follow-up clinics and would therefore be easily accessible. The literature suggests that a follow-up period of between 3-5 years is preferential for determining the potential role of influencing factors on rate of progression in glaucoma.
Appendices

1. REC, REC amendment & HRA approval letters

2. Participant information sheets and consent forms;
   2.1 Healthy control information sheet
   2.2 Healthy control consent form
   2.3 POAG and NTG information sheet
   2.4 POAG and NTG consent form
   2.5 OHT information sheet
   2.6 OHT consent form

3. Demographic questionnaire

4. Food Frequency Questionnaire (FFQ)
   4.1 FFQ
   4.2 FFQ prompt flow chart

5. 4 - Day Weighed Food Diary

6. Lab analysis methods:
   6.1 Measurement of ascorbate (vitamin C)
   6.2 Measurement of MDA
   6.3 Measurement of alpha-tocopherol (vitamin E)

7. Corvis full list of parameters

8. Table summary of AGEs and ocular disease literature

9. Table summary of dietary AGEs literature

10. Statistical tests
    10.1 Median and IQR for non-parametric data in Chapter 4
    10.2 Median and IQR for non-parametric data in Chapter 5
    10.3 Median and IQR for non-parametric data in Chapter 6

11. Gant chart

12. Research training activities
Appendix 1 – REC & HRA approval

Health Research Authority
North East - Tyne & Wear South Research Ethics Committee

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval.

12 July 2016

Miss Leanne Smewing
Post graduate researcher
Plymouth University
School of Health Professions, Plymouth University
Peninsula Allied Health Centre, Room FF01
Derriford Road
Plymouth
PL6 8BH

Dear Miss Smewing

Study title: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy

REC reference: 16/NE/0247
Protocol number: N/A
IRAS project ID: 204642

The Proportionate Review Sub-committee of the North East - Tyne & Wear South Research Ethics Committee reviewed the above application on 07 July 2016.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all

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14 December 2016

Miss Leanne Smewing
Post graduate researcher
Plymouth University
School of Health Professions, Plymouth University
Peninsula Allied Health Centre, Room FF01
Derriford Road, Plymouth
PL6 8BH

Dear Miss Smewing

Study title: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy

REC reference: 16/NE/0247

Protocol number: N/A

Amendment number: Substantial Amendment 1, 21/11/16

Amendment date: 21 November 2016

IRAS project ID: 204642

The above amendment was reviewed at the meeting of the Sub-Committee held by correspondence.

Summary of amendment

This amendment was submitted in order include an optional ‘session 3’ for hospital recruited participants.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.
Miss Leanne Smewin
Post graduate researcher
Plymouth University
School of Health Professions, Plymouth University
Peninsula Allied Health Centre, Room FF01
Derrford Road, Plymouth
PL5 6BQ

26 July 2016

Dear Miss Smewin,

Letter of HRA Approval

Study title: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy
IRAS project ID: 204642
Protocol number: N/A
REC reference: 16/NE/0247
Sponsor Plymouth University

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England
The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. Please read Appendix B carefully, in particular the following sections:

- Participating NHS organisations in England – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- Confirmation of capacity and capability – this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) – this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.
15/12/16

Dear Leanne,

Further to the below, I am pleased to confirm that HRA Approval has been issued for the referenced amendment, following assessment against the HRA criteria and standards.

The sponsor should now work collaboratively with participating NHS organisations in England to implement the amendment as per the below categorisation information. This email may be provided by the sponsor to participating organisations in England to evidence that the amendment has HRA Approval.

Please contact hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Yours sincerely,

Kind Regards
Steph Macpherson
HRA Senior Assessor

Health Research Authority
HRA, Ground Floor, Skipton House, 80 London Road, London, SE1 6LH
E: hra.approval@nhs.net
www.hra.nhs.uk
Participant information sheet

Project title: Advanced glycation end products as a biomarker for accelerated ageing

We would like to invite you to participate in a research study being conducted by Miss Leanne Smewing, Optometrist and PhD researcher, in conjunction with Dr Stephanie Mroczkowska, Dr Desley White and Professor Paul Artes from Plymouth University.

Before deciding if you would like to participate it is important for you to understand why the research is being undertaken and what it will involve. This information sheet explains the background and aims of the study. Please take time to read it carefully and discuss it with others if you wish. If anything is unclear, or if you would like more information, please ask us. Your participation in this study is entirely voluntary.

Information related to your participation

1. What is this study about?
Glaucoma is a disease of the eye which affects over 60 million people worldwide. The exact cause of glaucoma is still relatively poorly understood, however one of the most significant risk factors for its development is thought to be advancing age. This study aims to explore whether ageing products that accumulate naturally within the body over time (called advanced glycation end-products (AGEs)) influence the function of the blood vessels at the back of the eye in healthy individuals. It also aims to explore whether the accumulation of these ageing products occurs to a higher level in those with glaucoma and/or in those with high pressure in the eye.

2. Why have I been invited to participate?
To allow measurement of ‘normal ageing’ and to allow the comparison of ‘normal’ results to those of our participants diagnosed with glaucoma or high eye pressure, this study requires the participation of healthy volunteers, like yourself, who do not suffer from either eye condition.

3. What does the study involve?
Data collection for the study will be carried out across 2 sessions. Session 1 will be carried out at Peninsula Allied Health Centre (PAHC), Derriford, PL6 8BH or the Wellbeing Centre, Endsleigh Place, Plymouth University, Drake Circus, PL4 8AA.
Session 2 will be carried out at Human Metabolism laboratory, Food and Nutrition Unit, Link Building, Drake Campus, Plymouth University.

Session 1 procedures:
- Completion of a short questionnaire
- Measurement of vision
- Examination of the health of your eyes and insertion of drops
- Measurement of eye pressure
- Have a scan taken of the front of your eye (cornea)
- Have a scan taken of the back of your eye (retinal vessel function)

Total visit time approximately 2.5 hours

Session 2 procedures:
- Height and weight measured
- Measurement of blood pressure
- Measure AGE level with skin reader
- Finger prick blood sugar test
- Obtain a blood sample
- Complete a food questionnaire

Total visit time approximately 2 hours

4. What is expected of me at these sessions?

Session 1: Carried out at Peninsula Allied Health Centre (PAHC), Derriford, PL6 8BH or Wellbeing Centre, Endsleigh Place, Plymouth University, Drake Circus, PL4 8AA

Before session 1:
- Leanne Smewing (chief investigator) will call you and remind you of your appointment time and remind you that as drops will be inserted into one of your eyes at this visit you will not be able to drive for 4 hours after the appointment (alternative travel arrangements will need to be made).
- Due to their possible effect on your blood vessel measurement, you will be asked to avoid the following in the 12 hours before your appointment:
  - alcohol
  - tobacco/nicotine
  - extreme exercise
  - unnecessary sleep deprivation
- caffeine containing products (tea/coffee)
- taking any dietary supplements or vitamins (especially vitamin C)

However, you will be asked to maintain your normal eating habits on this day i.e. you are NOT required to fast.

On the day of session 1:

- If you decide to participate in this study you will be asked to sign a consent form. A copy of this consent form can be found at the end of this information pack.

- You will be asked to complete a quick questionnaire to allow us to collect information about your age, ethnicity and any medication you are taking etc. This will aid us with the analysis of our results and also ensure that there are no unforeseen medical factors that would prevent you being included in our study.

- You will have your vision measured and a short health exam to ensure your eyes won’t have any allergic reaction to the drops used.

- A machine will be used to take measurements of your cornea at the front of your eye. This procedure requires you to sit down with your head on a comfortable support.

- A drop and some fluorescein eye dye will be put into each of your eyes so that your eye pressure can be measured. This drop is an anaesthetic drop called proxymetacaine. The drops take about 60 seconds to work and the anaesthetic effect can last for up to 20 minutes. You will be advised not to rub your eyes and avoid getting dust or grit in your eye during this 20 minute period.

- So that good images of the back of your eye can be obtained with the retinal camera another drop will be put into one of your eyes only. This drop, called tropicamide, increases the size of your pupil so that we can get a full view of the back of your eye. Having big pupils can reduce your quality of vision temporarily and you may experience some dazzling in bright sunlight or artificial light for a few hours after you’ve had the drops in. You may therefore benefit from bringing some sunglasses with you and will be advised not to drive a motor vehicle, ride a bicycle or operate moving machinery for 4 hours after the appointment.

- The pupil dilating drops take approximately 20 minutes to work, while the drops are working we will decide a suitable date for part 2 and you will be provided with a campus map. You will also have the opportunity to ask any further questions.

- Once the drops have dilated your pupil the retinal camera will be used to take measurements of the blood vessels at the back of your eye. This procedure requires you to sit down with your head on a comfortable support. You will be required to look
straight ahead and you will see a flickering light for short periods of time. This will not harm your eyes but may leave you with an afterimage for a few seconds.

Due to the flickering light it is important that you tell us if you have a known history of epilepsy. Although the camera poses very little harm, there is a small risk of triggering an episode of epilepsy in those not diagnosed.

**At the end of session 1:**
- You will be advised **not** to drive a motor vehicle, ride a bicycle or operate moving machinery for **at least 4 hours** after the appointment due to the insertion of the eye drop.
- Your appointment for session 2 will be confirmed

**Session 2:**

**Before session 2:**
- You will be required to **fast** (i.e. not eat anything) for **12 hours before this appointment**. This is because eating food can affect the outcome of your blood test during the blood test.

During the 12 hours before your appointment you will **also be asked to avoid:**
  - alcohol
  - tobacco/nicotine
  - extreme exercise
  - unnecessary sleep deprivation
  - caffeine containing products
  - taking any dietary supplements or vitamins (especially vitamin C)

This is because these factors can also affect the outcome of the results.

- We recommend that you wear comfortable, loose fitting clothing on the day of this appointment. This is to allow us to easily access your upper arm for both blood pressure measurement and blood sample.

- Self-tanning agents must not have been used for at least 2 days before this appointment as they can affect the AGE reading taken from your skin.

**On the morning of session 2:**

At Plymouth University main campus Link building Leanne Smewing will meet you at the main entrance.

- Basic measurements will be taken first. These will include your; height, weight and blood pressure. Your BMI will be calculated from these measurements.
• If you have any sun blockers or skin creams on your forearm this will be removed as it can influence the AGE measurement.
• Next, your tissue bound AGE level will be measured using a non-invasive device called the AGE Reader. You will place your forearm on the machine a light from the machine will be used to take the AGE measurement. The measurement will take approximately 20-30 seconds and will be repeated 3 times.
• A finger prick blood sugar test will be taken prior to your blood sample.
• A blood sample will be taken by a researcher trained in phlebotomy in a specialist blood laboratory. This will be carried out with you sitting up and will involve taking blood in a similar way to that which you may have experienced at your GP’s surgery or at the hospital. This is a routinely performed procedure and the risks associated with this procedure are minimal.
• You will be asked to complete a questionnaire relating to your diet. The researcher will go through the questions with you. This allows us to collect information about the foods you eat and your methods of cooking.

After session 2:
• It is recommended that you **avoid alcohol for 4 hours after the study** and also avoid any unnecessary hazardous/strenuous/physical activity.
• As we require you to fast for 12 hours prior to the appointment a light snack will be provided after the study is completed. Alternatively you may wish to bring some refreshments with you

5. **What are the risks of taking part in this study?**
All the devices used in this study are commercially available and CE marked to ensure they comply with European Health and Safety requirements. We have worked hard to limit the risks associated with this study and all participants will be selected carefully. Despite this it is still important that you are aware of the possibility of adverse effects.

Leanne Smewing is a qualified optometrist and is fully trained in the techniques being performed. In addition first aiders will be present on both sites.

**Use of Proxymetacaine 0.5% and fluorescein**

This is a drop that is used when measuring eye pressure. It is a drop routinely used by Optometrists and Eye Hospitals so it possible you may have had it before. You may experience mild stinging when the drop is first put into your eyes, but this will subside very quickly. The drops are a type of local anaesthetic and take about 60 seconds to work and the effect may last for up to 20 minutes. You must not rub your eyes and try to avoid getting dust or grit in your eye as the anaesthetic would prevent you feeling any effect of this until after the drops wear off.

In the unlikely event that you experience any unusual symptoms such as pain and soreness in or around your eyes or your vision seems blurred after the appointment, contact a member of the research team, your optometrist or seek medical advice as you might be experiencing an adverse reaction to the drops.
Use of Tropicamide 0.5%

This is the drop that is used with the retinal camera to make your pupils larger. It takes approximately 15 to 30 minutes to work and up to 6 hours to wear off. Again, it is a drop routinely used by Optometrists and Eye Hospitals so it possible you may have had it before.

As mentioned previously, the enlargement of the pupil may cause a reduced quality of vision temporarily and you may experience some glare in bright sunlight or artificial light for a few hours afterwards. You are therefore advised not to drive a motor vehicle, ride a bicycle or operate moving machinery for at least 6 hours after the appointment. You may also wish to use sunglasses during this time to alleviate any increased light sensitivity. You may experience mild stinging when the drop is first put into your eyes, but this will subside very quickly.

There is a very small chance that using this drop can trigger unusual symptoms such as pain and redness around the eyes or 'misty' vision, usually immediately after drop insertion. This is very unlikely but if any of these symptoms do occur you are advised to speak to the research team and seek medical advice immediately as you may be experiencing an adverse reaction to the drops.

Use of the retinal camera

The retinal camera is a non-invasive device that poses very little harm. As it uses a flickering light it is important to be aware that there is a small risk of it triggering an episode of epilepsy in those not diagnosed.

Taking a blood sample

There can be a mild short irritation when taking a blood sample. Only a small amount of blood is taken during this study so you shouldn't feel any significant after-effects. However some people may feel dizzy and faint during and after the test. If this has happened to you in the past, or happens during the test please tell the person carrying out the test so they're aware and can help you feel more comfortable. After the test, you may have a small bruise where the needle went in. Bruises can be painful, but are usually harmless and fade over the next few days.

A small blood sample of 9mL will be taken. This blood sample will provide us with information about oxidative stress. The sample we store will not contain any of your DNA and will be stored and destroyed following the human tissue act procedures. The sample will be kept in the -80 degree C freezer in the locked laboratory in the Human Metabolism Laboratory at the Food and Nutrition Unit, Link Building, Plymouth University. They will be stored until all analysis is completed, stored for maximum of 2 years. Only the research team and technical staff will have access to the samples. Any abnormal findings found from this study would be sent in a letter to your GP and a copy would also be sent to you.

6. Are there any benefits of participating in this study?
This study is not likely to benefit you directly, but it will help researchers at Plymouth University evaluate whether AGE levels influence the function of the blood vessels in
your eye or contribute to the development of the eye disease glaucoma. In the future it may open up the possibility of new diagnostic and treatment options in the form of dietary, lifestyle or targeted therapeutic interventions.

7. Who has reviewed the study?
All research is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and approved by the Health Research Authority and Plymouth University’s Faculty Research Ethics Committee by the ‘Central Office of the Research Ethics Committee (REC) for the NHS and the Plymouth University Research Ethics Committee.

8. Do I have to take part?
No. You are free to decide whether or not to participate in this study. You should take time to consider your participation in this research and ask questions if there are aspects which you do not understand or if you need further information. If you decide to take part you will be asked to sign two copies of a consent form in the presence of an appropriate member of the research team. You will be given a signed copy of the consent form and an information sheet for your own records. You may decide not to participate or withdraw at any time without giving a reason or without it affecting your relationship with Plymouth University, or your current or future treatment in any way. If you decide to withdraw from the study all identifiable data and samples collected from you would be withdrawn. The samples would be destroyed as per human tissue authority guidelines. The lead investigator may also remove you from the study if they believe it to be in your best interest.

9. Is there remuneration for participation?
Unfortunately, you will not get paid to participate in this project.

10. Is taking part confidential?
If you decide to participate in the study we will inform your GP you are doing so.

All of the information you provide as a participant and any data collected as a result of your participation in this study will remain confidential. Your records may be looked at by the research team involved in this study and the monitoring or audit team approved by the university. All information will be stored electronically on a computer which is password protected, in a document file that is also password protected. All information will be handled in compliance with the Data Protection Act (1998).

Your name and address (which we need in order to contact you) will be stored separately from the other information you supply during the study so that you cannot be identified from your study records. After 10 years, all of your records will confidentially be disposed of in accordance with the guidelines laid out by Plymouth University.

Any data collected in this investigation may be submitted for publication or used in presentations. Neither your name nor information disclosing your identity will be released or published without your explicit consent to the disclosure.

11. What will happen to the results of the research study?
The results will be available through University of Plymouth, if you require a copy of the final report please write to the address given below.
12. What if I have any further questions or require further information?

If you have any questions about our project, either now or in the future, please feel free to contact:
Leanne Smewing;
Email: leanne.smewing@plymouth.ac.uk
Phone: (0)1752 587541

13. What if I have a complaint?

Should you have reason to complain about the way you have been treated at any stage during the study you can contact;

Dr Stephanie Mroczkowska;
Email: stephanie.mroczkowska@plymouth.ac.uk
Telephone: 01752 587549

Alternatively, you can make your complaint directly to Leanne Smewing, (contact details as above).

In the unlikely situation that you experience any unusual side effects to any of the procedures carried out you are advised to seek immediate medical advice. You will be issued with a list of any drugs administered to you and this should be shown to any medic that you consult.

If you have any concerns about the way in which the study has been conducted, please contact the Secretary of the Health & Human Sciences University Research Ethics Committee Sarah C Jones by email, or telephone (details below) as well as contacting the Patient Advice and Liaison Service (PALS) on 0800 328 3205.

Sarah C Jones;
Email: hhsethics@plymouth.ac.uk
Telephone: 01752 585339

If there is negligent harm during the clinical trial, Plymouth University owes a duty of care to the person harmed.

If you decide to participate in this study you will be asked to sign a consent form. A copy of this consent form can be found at the end of this information pack.

Thank you for taking the time to read this information sheet
Appendix 2.2 – Healthy control consent form

Declaration of informed consent

Title of Project: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy

Name of researcher: Leanne Smewing

(Please initial the boxes, if you agree)

I confirm that I have read and understood the patient information sheet for the above study and have had an opportunity to ask questions and have received acceptable answers.

I understand that the information collected during this study will remain strictly confidential and accessible only to appropriate members of the research team.

I understand that my participation in this study does not replace or constitute a complete eye examination in any way. During the study and after completion of the scheduled study visit I agree to continue eye care at my regular eye care practitioner.

I am aware that this study has been reviewed and approved by the Health Research Authority and Plymouth University's Faculty Research Ethics Committee, and that if I have any concerns or questions about my participation in this study I may contact Leanne Smewing, leanne.smewing@plymouth.ac.uk or at (0)1752675641.

I agree that the research team, auditors, monitors, regulatory authorities and ethics committees may have restricted access to my medical records.

I am aware that I may withdraw from the study at any time without giving any reason and without affecting my medical or care or legal rights. I understand that the investigator reserves the right to discontinue my participation from the study at any time, with regards to the research or the health of my eyes.

I am aware that my participation in this study is voluntary. I agree of my own free will, to participate in this study. I also consent to the release of information from the study to my eye care practitioner, where relevant.

Signature of participant: ___________________________ Date: ______________

Printed name of participant: ___________________________

As the chief investigator responsible for this research or a designated deputy, I confirm that the nature and purpose of this research have been explained to the participant named above.

Signature of person explaining consent: ___________________________ Date: ______________

Printed name of person explaining consent: ___________________________

(Two copies to be signed, one copy is to be kept by the participant and one copy is kept by the research team)
Participants information sheet

Project title: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy

We would like to invite you to participate in a research study being conducted by Miss Leanne Smewing, Optometrist and PhD researcher, in conjunction with Dr Stephanie Mroczkowska, Dr Desley White and Professor Paul Artes from Plymouth University.

Before deciding if you would like to participate it is important for you to understand why the research is being undertaken and what it will involve. This information sheet explains the background and aims of the study. Please take time to read it carefully and discuss it with others if you wish. If anything is unclear, or if you would like more information, please ask us. Your participation in this study is entirely voluntary.

Information related to your participation

1. What is this study about?

Glaucoma is a disease of the eye which affects over 60 million people worldwide. The exact cause of glaucoma is still relatively poorly understood, however one of the most significant risk factors for its development is known to be advancing age. With this in mind, this study aims to explore whether certain ageing products, (called advanced glycation end-products, (AGEs), that accumulate naturally within the body over time, are present in higher amounts in those with glaucoma and/or in those with high pressure in the eye compared to those without glaucoma. This study will also aim to explore whether the level of these ageing products in your body at the start relates to how much your glaucoma progresses over a 5 year period.

2. Why have I been invited to participate?

The study requires the participation of individuals who have been diagnosed with or are suspected of having early glaucoma and are otherwise healthy, like yourself.

3. What does the study involve?

Data collection for the study will be carried out across 2 sessions. Session 1 will be carried out at The Royal Eye Infirmary, level 3 Derriford Hospital, Derriford Road, Plymouth, PL6 8DH. Session 2 will be carried out at Human Metabolism laboratory, Food and Nutrition Unit, Link Building, Drake Campus, Plymouth University. There is an optional 3rd visit which will be carried out at Peninsula Allied Health Centre (PAHC),
Session 1 procedures:
- Normal NHS appointment procedures
- Given this information sheet and a short questionnaire

Session 2 procedures:
- Height and weight measured
- Measurement of blood pressure
- Measure AGE level with skin reader
- Finger prick blood sugar test
- Obtain a blood sample
- Complete a food frequency questionnaire

Total visit time approximately 2 hours

Optional session 3:
- Examination of the health of your eyes and insertion of drops
- Measurement of eye pressure
- Have a scan taken of the back of your eye (retinal vessel function)

After completion of session 1 and 2 and with your consent, your NHS eye records will be reviewed yearly for 5 years by a member of the research team. The results of your visual field test and optic nerve scan will be noted from your eye records and used as a way of monitoring the progression of your glaucoma. You will not need to be present for this section of data collection.

4. What is expected of me at these sessions?

**Session 1:**
If you decide to participate in this study you will be asked to sign a consent form. A copy of this consent form can be found at the end of this information pack.

Once you have given your consent you will be asked to complete a quick questionnaire which will allow us to collect information about your age, ethnicity and any medication you are taking. This will aid us with the analysis of the results and also ensure that there are no unforeseen medical factors that would prevent you being included in our study. Following this we will arrange a date that is suitable for you to visit Plymouth University main campus for your second data collection session. You will be provided with a campus map and given full instructions on how to locate the required building in preparation for this second session.

**Session 2:**
Before session 2:

- You will be required to fast (i.e. not eat anything) for **12 hours before this appointment**. This is because eating food can affect the outcome of your blood test.

During the 12 hours before your appointment you will also be asked to avoid:

- alcohol
- tobacco/nicotine
- extreme exercise
- unnecessary sleep deprivation
- caffeine containing products
- taking any dietary supplements or vitamins (especially vitamin C)

This is because these factors can also affect the outcome of the results.

- We recommend that you wear comfortable, loose fitting clothing on the day of this appointment. This is to allow us to easily access your upper arm for both blood pressure measurement and blood sample.
- Self-tanning agents must not have been used for at least 2 days before this appointment as they can affect the AGE reading taken from your skin.

On the morning of session 2:

At Plymouth University main campus Link building Leanne Smewing will meet you at the main entrance.

- Basic measurements will be taken first. These will include your; height, weight and blood pressure. Your BMI will be calculated from these measurements.
- If you have any sun blockers or skin creams on your forearm this will be removed as it can influence the AGE measurement.
- Next, your tissue bound AGE level will be measured using a non-invasive device called the AGE Reader. You will place your forearm on the machine and a light from the machine will be used to take the AGE measurement. The measurement will take approximately 20-30 seconds and will be repeated 3 times.
- A finger prick blood sugar test will be taken prior to your blood sample.
- A blood sample will be taken by a researcher trained in phlebotomy in a specialist blood laboratory. This will be carried out with you sitting up and will involve taking blood in a similar way to that which you may have experienced at your GP’s surgery or at the hospital. This is a routinely performed procedure and the risks associated with this procedure are minimal.
- You will be asked to complete a questionnaire relating to your diet. The researcher will go through the questions with you. This allows us to collect information about the foods you eat and your methods of cooking.
After session 2:

- It is recommended that you **avoid alcohol for 4 hours after the study** and also avoid any unnecessary hazardous/strenuous/physical activity.

- As we require you to fast for 12 hours prior to the appointment a light snack will be provided after the study is completed. Alternatively you may wish to bring some refreshments with you.

- Should you be interested, we can arrange an optional 3rd session where we can take measurements of the blood vessels at the back of your eye. The drops used at this 3rd session are the same as the drops used for your hospital visits.

Optional Session 3: Carried out at Peninsula Allied Health Centre (PAHC), Derriford, PL6 8BH or Wellbeing Centre, Endsleigh Place, Plymouth University, Drake Circus, PL4 8AA

Before session 3:

- Leanne Smewing (chief investigator) will call you and remind you of your appointment time and remind you that as drops will be inserted into one of your eyes at this visit you will not be able to drive for 4 hours after the appointment (alternative travel arrangements will need to be made).

- Due to their possible effect on your blood vessel measurement, you will be asked to avoid the following in the 12 hours before your appointment:
  - alcohol
  - tobacco/nicotine
  - extreme exercise
  - unnecessary sleep deprivation
  - caffeine containing products (tea/coffee)
  - taking any dietary supplements or vitamins (especially vitamin C)

However, you will be asked to maintain your normal eating habits on this day i.e. you are NOT required to fast.

On the day of session 3:

- You will have your vision measured and a short health exam to ensure your eyes won't have any allergic reaction to the drops used.

- A drop and some fluorescein eye dye will be put into each of your eyes so that your eye pressure can be measured. This drop is an anaesthetic drop called proxymetacaine. The drops take about 60 seconds to work and the anaesthetic effect can last for up to 20 minutes. You will be advised not to rub your eyes and avoid getting dust or grit in your eye during this 20 minute period.
So that good images of the back of your eye can be obtained with the retinal camera another drop will be put into one of your eyes only. This drop, called tropicamide, increases the size of your pupil so that we can get a full view of the back of your eye. Having big pupils can reduce your quality of vision temporarily and you may experience some dazzling in bright sunlight or artificial light for a few hours after you’ve had the drops in. You may therefore benefit from bringing some sunglasses with you and will be advised not to drive a motor vehicle, ride a bicycle or operate moving machinery for 4 hours after the appointment.

The pupil dilating drops take approximately 20 minutes to work.

Once the drops have dilated your pupil the retinal camera will be used to take measurements of the blood vessels at the back of your eye. This procedure requires you to sit down with your head on a comfortable support. You will be required to look straight ahead and you will see a flickering light for short periods of time. This will not harm your eyes but may leave you with an afterimage for a few seconds.

Due to the flickering light it is important that you tell us if you have a known history of epilepsy. Although the camera poses very little harm, there is a small risk of triggering an episode of epilepsy in those not diagnosed.

At the end of session 3:

- You will be advised not to drive a motor vehicle, ride a bicycle or operate moving machinery for at least 4 hours after the appointment due to the insertion of the eye drop.

5. Are any risks associated with this project?

All the devices used in this study are commercially available and CE marked to ensure they comply with European Health and Safety requirements. We have worked hard to limit the risks associated with this study and all participants will be selected carefully. Despite this it is still important that you are aware of the possibility of adverse effects.

Leanne Smewing is a qualified optometrist and is fully trained in the techniques being performed. In addition first aiders will be present on both sites.

Use of Proxymetacaine 0.5% and fluorescein

This is a drop that is used when measuring eye pressure. It is a drop routinely used by Optometrists and Eye Hospitals so it possible you may have had it before. You may experience mild stinging when the drop is first put into your eyes, but this will subside very quickly. The drops are a type of local anaesthetic and take about 60 seconds to work and the effect may last for up to 20 minutes. You must not rub your eyes and try to avoid getting dust or grit in your eye as the anaesthetic would prevent you feeling any effect of this until after the drops wear off.

In the unlikely event that you experience any unusual symptoms such as pain and soreness in or around your eyes or your vision seems blurred after the appointment,
contact a member of the research team, your optometrist or seek medical advice as you might be experiencing an adverse reaction to the drops.

Use of Tropicamide 0.5%

This is the drop that is used with the retinal camera to make your pupils larger. It takes approximately 15 to 30 minutes to work and up to 6 hours to wear off. Again, it is a drop routinely used by Optometrists and Eye Hospitals so it possible you may have had it before.

As mentioned previously, the enlargement of the pupil may cause a reduced quality of vision temporarily and you may experience some glare in bright sunlight or artificial light for a few hours afterwards. You are therefore advised not to drive a motor vehicle, ride a bicycle or operate moving machinery for at least 6 hours after the appointment. You may also wish to use sunglasses during this time to alleviate any increased light sensitivity. You may experience mild stinging when the drop is first put into your eyes, but this will subside very quickly.

There is a very small chance that using this drop can trigger unusual symptoms such as pain and redness around the eyes or ‘misty’ vision, usually immediately after drop insertion. This is very unlikely but if any of these symptoms do occur you are advised to speak to the research team and seek medical advice immediately as you may be experiencing an adverse reaction to the drops.

Use of the retinal camera

The retinal camera is a non-invasive device that poses very little harm. As it uses a flickering light it is important to be aware that there is a small risk of it triggering an episode of epilepsy in those not diagnosed.

Taking a blood sample

There can be a mild short irritation when taking a blood sample. Only a small amount of blood is taken during this study so you shouldn't feel any significant after-effects. However some people may feel dizzy and faint during and after the test. If this has happened to you in the past, or happens during the test please tell the person carrying out the test so they're aware and can help you feel more comfortable. After the test, you may have a small bruise where the needle went in. Bruises can be painful, but are usually harmless and fade over the next few days.

A small blood sample of 9mL will be taken. This blood sample will provide us with information about oxidative stress. The sample we store will not contain any of your DNA and will be stored and destroyed following the human tissue act procedures. The sample will be kept in the -80 degree C freezer in the locked laboratory in the Human Metabolism Laboratory at the Food and Nutrition Unit, Link Building, Plymouth University. They will be stored until all analysis is completed, stored for maximum of 2 years. Only the research team and technical staff will have access to the samples.

Any abnormal findings found from this study would be sent in a letter to your GP and a copy would also be sent to you.
6. What are the benefits of participation?
This study is not likely to benefit you directly, but it will help researchers at Plymouth University evaluate whether AGE levels influence the function of the blood vessels in your eye or contribute to the development of the eye disease glaucoma. In the future it may open up the possibility of new diagnostic and treatment options in the form of dietary, lifestyle or targeted therapeutic interventions.

7. Who has reviewed the study?
All research is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and approved by the Health Research Authority and Plymouth University’s Faculty Research Ethics Committee.

8. Do I have to take part?
No. You are free to decide whether or not to participate in this study. You should take time to consider your participation in this research and ask questions if there are aspects which you do not understand or if you need further information. If you decide to take part you will be asked to sign three copies of a consent form in the presence of an appropriate member of the research team. You will be given a signed copy of the consent form and an information sheet for your own records. You may decide not to participate or withdraw at any time without giving a reason or without it affecting your relationship with Plymouth University, or your current or future treatment in any way. If you decide to withdraw from the study all identifiable data and samples collected from you would be withdrawn. The samples would be destroyed as per human tissue authority guidelines. The lead investigator may also remove you from the study if they believe it to be in your best interest.

9. Is there remuneration for participation?
Unfortunately, you will not get paid to participate in this project.

10. Is taking part confidential?
If you decide to participate in the study we will inform your GP you are doing so.

All of the information you provide as a participant and any data collected as a result of your participation in this study will remain confidential. Your records may be looked at by the research team involved in this study and the monitoring or audit team approved by the university. All information will be stored electronically on a computer which is password protected, in a document file that is also password protected. All information will be handled in compliance with the Data Protection Act (1998).

Your name and address (which we need in order to contact you) will be stored separately from the other information you supply during the study so that you cannot be identified from your study records. After 10 years, all of your records will confidentially be disposed of in accordance with the guidelines laid out by Plymouth University.

Any data collected in this investigation may be submitted for publication or used in presentations. Neither your name nor information disclosing your identity will be released or published without your explicit consent to the disclosure.
11. What will happen to the results of the research study?
The results will be available through University of Plymouth, if you require a copy of the final report please write to the address given below.

12. What if I have any further questions or require further information?
If you have any questions about our project, either now or in the future, please feel free to contact:
Leanne Smewing:
Email: leanne.smewing@plymouth.ac.uk
Phone: (0)1752 587541

13. What if I have a complaint?
Should you have reason to complain about the way you have been treated at any stage during the study you can contact;

Dr Stephanie Mroczkowska;
Email: stephanie.mroczkowska@plymouth.ac.uk
Telephone: 01752 587549
Alternatively, you can make your complaint directly to Leanne Smewing, (contact details as above).

In the unlikely situation that you experience any unusual side effects to any of the procedures carried out you are advised to seek immediate medical advice. You will be issued with a list of any drugs administered to you and this should be shown to any medic that you consult.

If you have any concerns about the way in which the study has been conducted, please contact the Secretary of the Health & Human Sciences University Research Ethics Committee Sarah C Jones by email, or telephone (details below) as well as contacting the Patient Advice and Liaison Service (PALS) on 0800 328 3205.

Sarah C Jones;
Email: hhsethics@plymouth.ac.uk
Telephone: 01752 585339

If there is negligent harm during the clinical trial, Plymouth University owes a duty of care to the person harmed.

If you decide to participate in this study you will be asked to sign a consent form. A copy of this consent form can be found at the end of this information pack.

Thank you for taking the time to read this information sheet
Appendix 2.4 – POAG and NTG consent form

Declaration of informed consent

Title of Project: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy

Name of researcher: Leanne Smewing

(Please initial the boxes, if you agree)

I confirm that I have read and understood the patient information sheet for the above study and have had an opportunity to ask questions and have received acceptable answers.

I understand that the information collected during this study will remain strictly confidential and accessible only to appropriate members of the research team.

I understand that appropriate members of the research team will review my NHS records as part of a 5 year longitudinal aspect of this study.

I understand that my participation in this study does not replace or constitute a complete eye examination in any way. During the study and after completion of the scheduled study visit I agree to continue eye care at my regular eye care practitioner.

I am aware that this study has been reviewed and approved by the Health Research Authority and Plymouth University’s Faculty Research Ethics Committee, and that if I have any concerns or questions about my participation in this study I may contact Leanne Smewing, leanne.smewing@plymouth.ac.uk or at (0)175257541.

I agree that the research team, auditors, monitors, regulatory authorities and ethics committees may have restricted access to my medical records.

I am aware that I may withdraw from the study at any time without giving any reason and without affecting my medical or care or legal rights. I understand that the investigator reserves the right to discontinue my participation from the study at any time, with regards to the research or the health of my eyes.

I am aware that my participation in this study is voluntary. I agree of my own free will, to participate in this study. I also consent to the release of information from the study to my eye care practitioner, where relevant.

Signature of participant: …………………………………. Date: ………………….

Printed name of participant: ………………………………………………………………………

As the chief investigator responsible for this research or a designated deputy, I confirm that the nature and purpose of this research have been explained to the participant named above.

Signature of person explaining consent: ………………………………… Date: ………………….

Printed name of person explaining consent: ………………………………………………………

(Three copies to be signed, one copy is to be kept by the participant and one copy is kept by the research team)
Participant information sheet

Appendix 2.5 – OHT information sheet

Project title: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy

We would like to invite you to participate in a research study being conducted by Miss Leanne Smewing, Optometrist and PhD researcher, in conjunction with Dr Stephanie Mroczkowska, Dr Desley White and Professor Paul Artes from Plymouth University.

Before deciding if you would like to participate it is important for you to understand why the research is being undertaken and what it will involve. This information sheet explains the background and aims of the study. Please take time to read it carefully and discuss it with others if you wish. If anything is unclear, or if you would like more information, please ask us. Your participation in this study is entirely voluntary.

Information related to your participation

1. What is this study about?
Glaucoma is a disease of the eye which affects over 60 million people worldwide. The exact cause of glaucoma is still relatively poorly understood, however one of the most significant risk factors for its development is known to be advancing age. With this in mind, this study aims to explore whether certain ageing products (called advanced glycation end-products, AGEs), that accumulate naturally within the body over time, are present in higher amounts in those with glaucoma and/or in those with high pressure in the eye compared to those without glaucoma or high pressure. This study also aims to explore whether the level of these ageing products in your body at the start relates to the progression of your eye condition over a 5 year period.

2. Why have I been invited to participate?
The study requires the participation of those who have been diagnosed with or are suspected of having ocular hypertension and are otherwise healthy, like yourself.

3. What does the study involve?
Data collection for the study will be carried out across 2 sessions. Session 1 will be carried out at The Royal Eye Infirmary, level 3 Derriford Hospital, Derriford Road, Plymouth, PL6 8DH. Session 2 will be carried out at Human Metabolism laboratory, Food and Nutrition Unit, Link Building, Drake Campus, Plymouth University. There is an optional 3rd visit which will be carried out at Peninsula Allied Health Centre (PAHC), Derriford, PL6 8BH or Wellbeing Centre, Endsleigh Place, Plymouth University, Drake Circus, PL4 8AA.
Session 1 procedures:
- Normal NHS appointment procedures
- Given this information sheet and a short questionnaire

Session 2 procedures:
- Height and weight measured
- Measurement of blood pressure
- Measure AGE level with skin reader
- Finger prick blood sugar test
- Obtain a blood sample
- Complete a food frequency questionnaire

Total visit time approximately 2 hours

Optional session 3:
- Examination of the health of your eyes and insertion of drops
- Measurement of eye pressure
- Have a scan taken of the back of your eye (retinal vessel function)

After completion of session 1 and 2 and with your consent, your NHS eye records will be reviewed yearly for 5 years by a member of the research team. The results of your visual field test and optic nerve scan will be noted from your eye records and used as a way of monitoring the progression of your glaucoma. You will not need to be present for this section of data collection.

4. What is expected of me at these sessions?

Session 1:

If you decide to participate in this study you will be asked to sign a consent form. A copy of this consent form can be found at the end of this information pack.

Once you have given your consent you will be asked to complete a quick questionnaire which will allow us to collect information about your age, ethnicity and any medication you are taking. This will aid us with the analysis of the results and also ensure that there are no unforeseen medical factors that would prevent you being included in our study. Following this we will arrange a date that is suitable for you to visit Plymouth University main campus for your second data collection session. You will be provided with a campus map and given full instructions on how to locate the required building in preparation for this second session.

Session 2:

Before session 2:
• You will be required to **fast** (i.e. not eat anything) for **12 hours before this appointment**. This is because eating food can affect the outcome of your blood test.

During the 12 hours before your appointment you will also be asked to avoid:

- alcohol
- tobacco/nicotine
- extreme exercise
- unnecessary sleep deprivation
- caffeine containing products
- taking any dietary supplements or vitamins (especially vitamin C)

This is because these factors can also affect the outcome of the results.

• **We recommend that you wear comfortable, loose fitting clothing on the day of this appointment.** This is to allow us to easily access your upper arm for both blood pressure measurement and blood sample.

• Self-tanning agents must not have been used for at least 2 days before this appointment as they can affect the AGE reading taken from your skin.

**On the morning of session 2:**

At Plymouth University main campus Link building Leanne Smewing will meet you at the main entrance.

• Basic measurements will be taken first. These will include your; height, weight and blood pressure. Your BMI will be calculated from these measurements.
• If you have any sun blockers or skin creams on your forearm this will be removed as it can influence the AGE measurement.
• Next, your tissue bound AGE level will be measured using a non-invasive device called the AGE Reader. You will place your forearm on the machine and a light from the machine will be used to take the AGE measurement. The measurement will take approximately 20-30 seconds and will be repeated 3 times.
• A finger prick blood sugar test will be taken prior to your blood sample.
• A blood sample will be taken by a researcher trained in phlebotomy in a specialist blood laboratory. This will be carried out with you sitting up and will involve taking blood in a similar way to that which you may have experienced at your GP’s surgery or at the hospital. This is a routinely performed procedure and the risks associated with this procedure are minimal.
• You will be asked to complete a questionnaire relating to your diet. The researcher will go through the questions with you. This allows us to collect information about the foods you eat and your methods of cooking.

**After session 2:**
- It is recommended that you **avoid alcohol for 4 hours after the study** and also avoid any unnecessary hazardous/strenuous/physical activity.

- As we require you to fast for 12 hours prior to the appointment a light snack will be provided after the study is completed. Alternatively you may wish to bring some refreshments with you.

- Should you be interested, we can arrange an optional 3rd session where we can take measurements of the blood vessels at the back of your eye. The drops used at this 3rd session are the same as the drops used for your hospital visits.

**Optional Session 3:** Carried out at Peninsula Allied Health Centre (PAHC), Derriford, PL6 8BH or Wellbeing Centre, Endsleigh Place, Plymouth University, Drake Circus, PL4 8AA

**Before session 3:**
- Leanne Smewing (chief investigator) will call you and remind you of your appointment time and remind you that as drops will be inserted into one of your eyes at this visit you will not be able to drive for 4 hours after the appointment (alternative travel arrangements will need to be made).
- Due to their possible effect on your blood vessel measurement, you will be asked to avoid the following in the 12 hours before your appointment:
  - alcohol
  - tobacco/nicotine
  - extreme exercise
  - unnecessary sleep deprivation
  - caffeine containing products (tea/coffee)
  - taking any dietary supplements or vitamins (especially vitamin C)

However, you will be asked to maintain your normal eating habits on this day i.e. you are NOT required to fast.

**On the day of session 3:**
- You will have your vision measured and a short health exam to ensure your eyes won’t have any allergic reaction to the drops used.

- A drop and some fluorescein eye dye will be put into each of your eyes so that your eye pressure can be measured. This drop is an anaesthetic drop called proparacaine. The drops take about 60 seconds to work and the anaesthetic effect can last for up to 20 minutes. You will be advised not to rub your eyes and avoid getting dust or grit in your eye during this 20 minute period.
• So that good images of the back of your eye can be obtained with the retinal camera another drop will be put into one of your eyes only. This drop, called tropicamide, increases the size of your pupil so that we can get a full view of the back of your eye. Having big pupils can reduce your quality of vision temporarily and you may experience some dazzling in bright sunlight or artificial light for a few hours after you’ve had the drops in. You may therefore benefit from bringing some sunglasses with you and will be advised not to drive a motor vehicle, ride a bicycle or operate moving machinery for 4 hours after the appointment.

• The pupil dilating drops take approximately 20 minutes to work.

• Once the drops have dilated your pupil the retinal camera will be used to take measurements of the blood vessels at the back of your eye. This procedure requires you to sit down with your head on a comfortable support. You will be required to look straight ahead and you will see a flickering light for short periods of time. This will not harm your eyes but may leave you with an afterimage for a few seconds.

Due to the flickering light it is important that you tell us if you have a known history of epilepsy. Although the camera poses very little harm, there is a small risk of triggering an episode of epilepsy in those not diagnosed.

**At the end of session 3:**

• You will be advised not to drive a motor vehicle, ride a bicycle or operate moving machinery for at least 4 hours after the appointment due to the insertion of the eye drop.

5. **Are any risks associated with this project?**

All the devices used in this study are commercially available and CE marked to ensure they comply with European Health and Safety requirements. We have worked hard to limit the risks associated with this study and all participants will be selected carefully. Despite this it is still important that you are aware of the possibility of adverse effects.

Leanne Smewing is a qualified optometrist and is fully trained in the techniques being performed. In addition first aiders will be present on both sites.

**Use of Proxymetacaine 0.5% and fluorescein**

This is a drop that is used when measuring eye pressure. It is a drop routinely used by Optometrists and Eye Hospitals so it possible you may have had it before. You may experience mild stinging when the drop is first put into your eyes, but this will subside very quickly. The drops are a type of local anaesthetic and take about 60 seconds to work and the effect may last for up to 20 minutes. You must not rub your eyes and try to avoid getting dust or grit in your eye as the anaesthetic would prevent you feeling any effect of this until after the drops wear off.

In the unlikely event that you experience any unusual symptoms such as pain and soreness in or around your eyes or your vision seems blurred after the appointment,
contact a member of the research team, your optometrist or seek medical advice as you
might be experiencing an adverse reaction to the drops.

**Use of Tropicamide 0.5%**

This is the drop that is used with the retinal camera to make your pupils larger. It takes
approximately 15 to 30 minutes to work and up to 6 hours to wear off. Again, it is a drop
routinely used by Optometrists and Eye Hospitals so it possible you may have had it
before.

As mentioned previously, the enlargement of the pupil may cause a reduced quality of
vision temporarily and you may experience some glare in bright sunlight or artificial light
for a few hours afterwards. You are therefore advised not to drive a motor vehicle, ride a
bicycle or operate moving machinery for at least 6 hours after the appointment. You may
also wish to use sunglasses during this time to alleviate any increased light sensitivity.
You may experience mild stinging when the drop is first put into your eyes, but this will
subside very quickly.

There is a very small chance that using this drop can trigger unusual symptoms such as
pain and redness around the eyes or ‘misty’ vision, usually immediately after drop
insertion. This is very unlikely but if any of these symptoms do occur you are advised to
speak to the research team and seek medical advice immediately as you may be
experiencing an adverse reaction to the drops.

**Use of the retinal camera**

The retinal camera is a non-invasive device that poses very little harm. As it uses a
flickering light it is important to be aware that there is a small risk of it triggering an
episode of epilepsy in those not diagnosed.

**Taking a blood sample**

There can be a mild short irritation when taking a blood sample. Only a small amount of
blood is taken during this study so you shouldn’t feel any significant after-effects.
However some people may feel dizzy and faint during and after the test. If this has
happened to you in the past, or happens during the test please tell the person carrying
out the test so they’re aware and can help you feel more comfortable. After the test, you
may have a small bruise where the needle went in. Bruises can be painful, but are
usually harmless and fade over the next few days.

A small blood sample of 9mL will be taken. This blood sample will provide us with
information about oxidative stress. The sample we store will not contain any of your DNA
and will be stored and destroyed following the human tissue act procedures. The sample
will be kept in the -80 degree C freezer in the locked laboratory in the Human
Metabolism Laboratory at the Food and Nutrition Unit, Link Building, Plymouth
University. They will be stored until all analysis is completed, stored for maximum of 2
years. Only the research team and technical staff will have access to the samples.

Any abnormal findings found from this study would be sent in a letter to your GP and a
copy would also be sent to you.
6. **What are the benefits of participation?**
This study is not likely to benefit you directly, but it will help researchers at Plymouth University evaluate whether AGE levels influence the function of the blood vessels in your eye or contribute to the development of the eye disease glaucoma. In the future it may open up the possibility of new diagnostic and treatment options in the form of dietary, lifestyle or targeted therapeutic interventions.

7. **Who has reviewed the study?**
All research is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and approved by the Health Research Authority and Plymouth University’s Faculty Research Ethics Committee.

8. **Do I have to take part?**
No. You are free to decide whether or not to participate in this study. You should take time to consider your participation in this research and ask questions if there are aspects which you do not understand or if you need further information. If you decide to take part you will be asked to sign three copies of a consent form in the presence of an appropriate member of the research team. You will be given a signed copy of the consent form and an information sheet for your own records. You may decide not to participate or withdraw at any time without giving a reason or without it affecting your relationship with Plymouth University, or your current or future treatment in any way. If you decide to withdraw from the study all identifiable data and samples collected from you would be withdrawn. The samples would be destroyed as per human tissue authority guidelines. The lead investigator may also remove you from the study if they believe it to be in your best interest.

9. **Is there remuneration for participation?**
Unfortunately, you will not get paid to participate in this project.

10. **Is taking part confidential?**
If you decide to participate in the study we will inform your GP you are doing so.

All of the information you provide as a participant and any data collected as a result of your participation in this study will remain confidential. Your records may be looked at by the research team involved in this study and the monitoring or audit team approved by the university. All information will be stored electronically on a computer which is password protected, in a document file that is also password protected. All information will be handled in compliance with the Data Protection Act (1998).

Your name and address (which we need in order to contact you) will be stored separately from the other information you supply during the study so that you cannot be identified from your study records. After 10 years, all of your records will confidentially be disposed of in accordance with the guidelines laid out by Plymouth University.

Any data collected in this investigation may be submitted for publication or used in presentations. Neither your name nor information disclosing your identity will be released or published without your explicit consent to the disclosure.
11. What will happen to the results of the research study?
The results will be available through University of Plymouth, if you require a copy of the final report please write to the address given below.

12. What if I have any further questions or require further information?
If you have any questions about our project, either now or in the future, please feel free to contact:
Leanne Smewing;
Email: leanne.smewing@plymouth.ac.uk
Phone: (0)1752 587541

13. What if I have a complaint?
Should you have reason to complain about the way you have been treated at any stage during the study you can contact;

Dr Stephanie Mroczkowska;
Email: stephanie.mroczkowska@plymouth.ac.uk
Telephone: 01752 587549
Alternatively, you can make your complaint directly to Leanne Smewing, (contact details as above).

In the unlikely situation that you experience any unusual side effects to any of the procedures carried out you are advised to seek immediate medical advice. You will be issued with a list of any drugs administered to you and this should be shown to any medic that you consult.

If you have any concerns about the way in which the study has been conducted, please contact the Secretary of the Health & Human Sciences University Research Ethics Committee Sarah C Jones by email, or telephone (details below) as well as contacting the Patient Advice and Liaison Service (PALS) on 0800 328 3205.

Sarah C Jones;
Email: hhsethics@plymouth.ac.uk
Telephone: 01752 585339

If there is negligent harm during the clinical trial, Plymouth University owes a duty of care to the person harmed.

If you decide to participate in this study you will be asked to sign a consent form. A copy of this consent form can be found at the end of this information pack.

Thank you for taking the time to read this information sheet
Appendix 2.6 – OHT consent form

Declaration of informed consent

Title of Project: Advanced glycation end products as a biomarker for accelerated ageing in glaucomatous optic neuropathy

Name of researcher: Leanne Smewing

(Please initial the boxes, if you agree)

I confirm that I have read and understood the patient information sheet for the above study and have had an opportunity to ask questions and have received acceptable answers.

I understand that the information collected during this study will remain strictly confidential and accessible only to appropriate members of the research team.

I understand that appropriate members of the research team will review my NHS records as part of a 5 year longitudinal aspect of this study.

I understand that my participation in this study does not replace or constitute a complete eye examination in any way. During the study and after completion of the scheduled study visit I agree to continue eye care at my regular eye care practitioner.

I am aware that this study has been reviewed and approved by the Health Research Authority and Plymouth University’s Faculty Research Ethics Committee, and that if I have any concerns or questions about my participation in this study I may contact Leanne Smewing, leanne.smewing@plymouth.ac.uk or at (0)175257541.

I agree that the research team, auditors, monitors, regulatory authorities and ethics committees may have restricted access to my medical records.

I am aware that I may withdraw from the study at any time without giving any reason and without affecting my medical or care or legal rights. I understand that the investigator reserves the right to discontinue my participation from the study at any time, with regards to the research or the health of my eyes.

I am aware that my participation in this study is voluntary. I agree of my own free will, to participate in this study. I also consent to the release of information from the study to my eye care practitioner, where relevant.

Signature of participant: .......................................................... Date: ..........................

Printed name of participant: ........................................................................................................

As the chief investigator responsible for this research or a designated deputy, I confirm that the nature and purpose of this research have been explained to the participant named above.

Signature of person explaining consent: .......................................................... Date: ..........................

Printed name of person explaining consent: ...................................................................................

(Three copies to be signed, one copy is to be kept by the participant and one copy is kept by the research team)
Appendix 3 – Demographic questionnaire

PERSONAL INFORMATION QUESTIONNAIRE

All information contained within this questionnaire is strictly confidential. Please complete all of the questions on both sides of this questionnaire as accurately as possible. Please tick (✓) all of the boxes that apply to you.

Date: [ ]
Participant number: [ ]

Gender: [ ] Male  [ ] Female

DOB: [ ]

SECTION 1: ETHNICITY AND COUNTRIES LIVED IN

1A. HOW WOULD YOU DESCRIBE YOUR ETHNICITY?

- [ ] White British
- [ ] White Irish
- [ ] White Other (Please specify)
- [ ] Black British
- [ ] Black African
- [ ] Black Caribbean
- [ ] Mixed: White and Black African
- [ ] Mixed: White and Black Caribbean
- [ ] Mixed: White and Asian
- [ ] Mixed Other (Please specify)
- [ ] Pakistani
- [ ] Bangladeshi
- [ ] Chinese
- [ ] Other Asian (Please specify)
- [ ] Other ethnic group (Please specify)
- [ ] Do not wish to specify

1B. HAVE YOU LIVED IN THE UK ALL OF YOUR LIFE? Yes [ ] No [ ] (If no please give details of the other countries you have lived in with relevant dates).

SECTION 2: EDUCATION AND INCOME

2A. WHAT IS THE HIGHEST LEVEL OF EDUCATION WHICH YOU HAVE CURRENTLY ACHIEVED?

- [ ] PhD
- [ ] Masters/other post-graduate
- [ ] Degree
- [ ] Other (Please specify)
- [ ] Other higher education (eg diploma)
- [ ] A levels/Higher
- [ ] ONC/National BTEC
- [ ] GCSE (A-C)
- [ ] GCSE (D-G)
- [ ] None

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### SECTION 3: SMOKING AND ALCOHOL INTAKE

<table>
<thead>
<tr>
<th>Q.</th>
<th>A.</th>
<th>B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A. Do you currently smoke?</td>
<td>☐ Yes (Go to 3D)</td>
<td>☐ No</td>
</tr>
<tr>
<td>3B. Have you smoked in the past?</td>
<td>☐ Yes</td>
<td>☐ No (Go to 3F)</td>
</tr>
<tr>
<td>3C. How many years has it been since you last smoked?</td>
<td>☐ Yes</td>
<td>☐ No (Go to 3F)</td>
</tr>
<tr>
<td>3D. For how many years have you/did you smoke?</td>
<td>☐ Yes</td>
<td>☐ No (Go to 3F)</td>
</tr>
</tbody>
</table>

#### 3E. What do you/did you smoke?
- ☐ Cigarettes
  - Number per day
- ☐ Cigars
  - Number per day
- ☐ Pipe
  - Number per day

#### 3F. Do you drink alcohol?
- ☐ Yes (Continue to 3G)
- ☐ No (Go to 4A)

#### 3G. How many units do you drink on average in one week?
- Pint of low-strength beer/lager/cider = 2 units
- 125ml glass wine = 1.5 units
- 250ml glass of wine = 3 units
- Pint of high-strength beer/lager/cider = 3 units
- 175ml glass wine = 2.1 units
- Single shot of spirits = 1 unit

### SECTION 4: MEDICAL AND OCULAR HEALTH

#### 4A: Have you ever been diagnosed or had treatment for any of the following?
- Stroke
- Arrhythmia
- Diabetes
- Overactive thyroid
- Angina
- Transient ischemic attacks
- Epilepsy
- Underactive thyroid
- Coronary artery disease
- Peripheral vascular disease
- Asthma
- COPD
- Heart failure
- Severe dyslipidaemia
- Other (Give Details)

#### 4B: Have you ever been diagnosed or had treatment for high blood pressure?
- Yes
- No (If No continue to 4D)
- Not sure
- Other (Give Details)

#### 4C: Is your blood pressure now under control?
- Yes
- No

#### 4D: Have you ever been tested for diabetes?
- Yes
- No (If No continue to 4F)

#### 4E: Did this result in a diagnosis?
- Yes
- No

#### 4F: When was your last sight test?

#### 4G: Have you ever been diagnosed or had treatment for any of the following eye conditions?
- Macular Degeneration (AMD)
- Cataract
- Retinal detachment
- Glaucoma
- Macular hole
- Uveitis/Iritis/keratitis
- Corneal Ulcer
- Ocular hypertension
- Macular edema
- Retinal haemorrhaging
- Amblyopia (lazy eye)
- Eye Injury
- Epiretinal membrane
- Diabetic retinopathy
- Other (Give Details)

#### 4H: Are you aware of any direct family members who have been investigated/diagnosed with Glaucoma?
- Yes (Please detail which family member)
- No

#### 4I: Have you ever had a blood sample taken before?
- Yes
- No (If No continue 4H)

#### 4J: Did you have any after-effects? E.g. some people feel dizzy and faint during and after the test
- Yes
- No

#### 4K: Have you had a recent cholesterol test? (within the last 3 months)
- Yes (Please give details of reading if known)
- No

#### 4L: Have you ever had an adverse reaction to drops administered by an Optometrist?
- Yes (Please give details)
- No
<table>
<thead>
<tr>
<th>SECTION 5: MEDICATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A: Please list all of your medications (if you have a prescription copy with you the investigator will take a copy):</td>
</tr>
</tbody>
</table>

END OF QUESTIONNAIRE
Appendix 4.1 – Specifically designed FFQ

Dietary questionnaire

Questions about what you eat, how you cook and what you drink

Throughout this questionnaire the cooking method will also be described such as; frying, grilling, boiling and roasting. If you are unsure of the exact meaning of these terms please let the person going through this questionnaire with you aware and they can explain further.

When frying/roasting foods what type of oil/cooking fat would you use?

………………………………………………………………………………………………………………………………

For the first section you will be shown images of different portion sizes of food. Please put a (X) in the box which best describes (on average) how much you would usually have eaten at a main meal in the last 12 months.

The images will be of a food pictured on its own, although this may not be the way you usually eat this food please try to answer as best you can.

For example:

Breaded fish

Which option best describes what you ate?

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than A</td>
<td>A</td>
<td>Between A &amp; B</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>60g</td>
<td>110g</td>
<td>170g</td>
</tr>
</tbody>
</table>
If you have eaten any of these foods which option best describes what you ate?

1. **Breaded fish**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than A</td>
<td>60g</td>
<td>110g</td>
<td>170g</td>
</tr>
<tr>
<td>Between A &amp; B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between B &amp; C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. **Thin crust pizza**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than A</td>
<td>50g</td>
<td>200g</td>
<td>400g</td>
</tr>
<tr>
<td>Between A &amp; B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between B &amp; C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. **Sausages**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than A</td>
<td>45g</td>
<td>90g</td>
<td>135g</td>
</tr>
<tr>
<td>Between A &amp; B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between B &amp; C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Continued, which option best describes what you ate?

4. Fried skinless chicken

<table>
<thead>
<tr>
<th>Less than A</th>
<th>A</th>
<th>Between A &amp; B</th>
<th>B</th>
<th>Between B &amp; C</th>
<th>C</th>
<th>More than C</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>

4. Fried skinless chicken

5. Breaded oven chicken

<table>
<thead>
<tr>
<th>Less than A</th>
<th>A</th>
<th>Between A &amp; B</th>
<th>B</th>
<th>Between B &amp; C</th>
<th>C</th>
<th>More than C</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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</tbody>
</table>

6. Roast beef

<table>
<thead>
<tr>
<th>Less than A</th>
<th>A</th>
<th>Between A &amp; B</th>
<th>B</th>
<th>Between B &amp; C</th>
<th>C</th>
<th>More than C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>
This questionnaire will ask you about your usual eating habits over the last 12 months. Examples of food portion sizes can be found on the final page.

Please put a number in the box for the number of portions which best describes how much you eat/drink of the items listed below.

Please put an (X) in the box which best describes (on average) how often you eat/drink the items listed below.

For example:

<table>
<thead>
<tr>
<th>Frequency you have eaten</th>
<th>Portion</th>
<th>Never</th>
<th>Less than once</th>
<th>1 to 3 time</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feta cheese</td>
<td>1</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Over the last 12 months on average how often did you drink the following?

All drinks 250ml

<table>
<thead>
<tr>
<th>Frequency you drank</th>
<th>Portion</th>
<th>Never</th>
<th>Less than once</th>
<th>1 to 3 time</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
</tr>
</thead>
<tbody>
<tr>
<td>White wine</td>
<td></td>
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<td></td>
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<tr>
<td>Red wine</td>
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<td></td>
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<tr>
<td>Spirits</td>
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<tr>
<td>Beer</td>
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<tr>
<td>Apple juice</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Cranberry juice</td>
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<tr>
<td>Orange juice from concentrate</td>
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<tr>
<td>Fresh squeezed orange juice</td>
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<tr>
<td>Cola</td>
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<tr>
<td>Sugar free cola</td>
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<tr>
<td>Lemonade</td>
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<td></td>
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<tr>
<td>Sugar free lemonade</td>
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</tr>
<tr>
<td>Instant coffee</td>
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<td></td>
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<tr>
<td>Instant decaf coffee</td>
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<tr>
<td>Filter coffee</td>
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</tr>
<tr>
<td>Tea</td>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Decaf tea</td>
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<tr>
<td>Fruit flavoured tea</td>
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<td></td>
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</tbody>
</table>

How do you drink your tea/coffee?

E.g. Tea with milk and 1 sugar
Over the last 12 months on average how often did you eat/drink the following?

### Milk/milk products - All drinks 250ml

<table>
<thead>
<tr>
<th>Frequency you drank/ate:</th>
<th>Portion</th>
<th>Never</th>
<th>Less than once</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot chocolate (packet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot chocolate sugar free (packet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ice cream, vanilla</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Whole milk</td>
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<tr>
<td>Semi-skimmed milk</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skimmed milk</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Warmed milk</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Custard (127g - 1average pat)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Plain low fat yoghurt</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Flavoured low fat yoghurt (127g - 1 average pat)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Other milk/milk product</td>
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</tr>
</tbody>
</table>

Over the last 12 months on average how often did you eat the following?

### Dairy/eggs/meat substitutes - All products 30g unless otherwise stated

<table>
<thead>
<tr>
<th>Frequency you ate:</th>
<th>Portion</th>
<th>Never</th>
<th>Less than once</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheddar (have 30g on bread)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced fat cheddar (have 30g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brie</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cottage cheese</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>(125g - 1average serving)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Feta</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>(130g - 1average serving)</td>
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</tr>
<tr>
<td>Mozzarella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(125g - 1average serving)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Parmesan</td>
<td></td>
<td></td>
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<tr>
<td>(135g - 1average serving)</td>
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</tr>
<tr>
<td>Swiss processed</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(130g - 2average serving)</td>
<td></td>
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<td>Cream cheese</td>
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Dairy/eggs/meat substitutes continued - All products 30g unless otherwise stated

Advanced glycation end products as a biomarker for accelerated ageing version 1.0, 24/06/16
### Frequency you ate:

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<tr>
<th>Food Item</th>
<th>Never</th>
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<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
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<td>Boiled egg (1 medium, 1 egg)</td>
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<td>Boiled egg (12 small, 1 egg)</td>
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<td>Tofu (10g)</td>
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### Meat & fish - All products 90g unless otherwise stated

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<th>1 to 3 times</th>
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<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
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<th>3 or more times</th>
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<td>Stir fried steak strips</td>
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<td>Meatballs boiled in sauce (10g)</td>
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<td><strong>Meat &amp; fish continued</strong></td>
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<td>Bacon fried (15 min.)</td>
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<td>Bacon grilled (15 min.)</td>
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<td>(150g = 2 slices)</td>
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<td>Smoked deli ham (150g = 2 slices)</td>
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<td>(7 slices = 120g)</td>
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<td>Pork sausage fried (15 slices / 115g</td>
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<td>Pork sausage grilled (15 slices / 115g)</td>
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<tr>
<td>Chicken breast cubed pan fried (15 min)</td>
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<td>(46g = 1 slice)</td>
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<tr>
<td>Chicken breast cubed, pan fried (15 min) and boiled</td>
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<td>(115g = 1 small breast)</td>
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<tr>
<td>Boiled chicken breast (120g = 1 small breast)</td>
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<tr>
<td>Grilled chicken breast (15 min = 1 small breast)</td>
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<tr>
<td>Pan fried chicken breast (3 slices)</td>
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<tr>
<td>Roasted chicken breast in barbecue sauce (1 slice)</td>
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<tr>
<td>Roasted breaded chicken breast (15 min = 1 breast, one picket)</td>
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<tr>
<td>Roasted chicken breast with the skin (1 small breast)</td>
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<tr>
<td>Breaded chicken breast with the skin oven fried (15 min = 1 breast, one picket)</td>
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Advanced glycation end products as a biomarker for accelerated ageing version 1.0, 24/06/16
<table>
<thead>
<tr>
<th>Meat &amp; fish continued</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frequency you ate:</strong></td>
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<tr>
<td><strong>Chicken curry</strong></td>
</tr>
<tr>
<td><strong>Chicken casserole</strong> (1 kg)</td>
</tr>
<tr>
<td><strong>Chicken nuggets</strong> (100g)</td>
</tr>
<tr>
<td><strong>Other chicken</strong></td>
</tr>
<tr>
<td><strong>Turkey breast steak, skinless</strong> (150g - 1 small breast)</td>
</tr>
<tr>
<td><strong>Roasted turkey breast</strong> (75g - medium portion shown)</td>
</tr>
<tr>
<td><strong>Turkey mince</strong></td>
</tr>
<tr>
<td><strong>Other Turkey</strong></td>
</tr>
<tr>
<td><strong>Poached salmon fillet</strong> (125g)</td>
</tr>
<tr>
<td><strong>Smoked salmon</strong> (50g - 3 slices)</td>
</tr>
<tr>
<td><strong>Roasted trout</strong> (55 slices)</td>
</tr>
<tr>
<td><strong>Roasted tuna</strong> (55 slices)</td>
</tr>
<tr>
<td><strong>Canned tuna</strong> (80g - 1 can)</td>
</tr>
<tr>
<td><strong>Oven cooked breaded whiting</strong> (55 slices) (150g - medium portion)</td>
</tr>
<tr>
<td><strong>Oven cooked breaded cod</strong> (150g - medium portion)</td>
</tr>
<tr>
<td><strong>Battered cod</strong> (100g - piece)</td>
</tr>
<tr>
<td><strong>Other Fish</strong></td>
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</table>

Any meat/fish dishes you eat often not yet mentioned?

...
### Carbohydrates - All products 30g unless otherwise stated

<table>
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<tr>
<th>Frequency you ate:</th>
<th>Portion</th>
<th>Never</th>
<th>Less than once</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 or more times</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Per month</td>
<td>Per week</td>
<td>Per day</td>
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<tr>
<td>Sliced white bread</td>
<td>1/2 slice</td>
<td>Never</td>
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<tr>
<td>Toasted white bread</td>
<td>1 slice</td>
<td>Never</td>
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<tr>
<td>Sliced wholemeal bread</td>
<td>1 slice</td>
<td>Never</td>
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<tr>
<td>Toasted wholemeal bread</td>
<td>1 slice</td>
<td>Never</td>
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<tr>
<td>White bread, baguette</td>
<td>1 slice (approx.)</td>
<td>Never</td>
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<tr>
<td>Pitta bread</td>
<td>1/2 pitta</td>
<td>Never</td>
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<tr>
<td>Bagel (1 slice)</td>
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<tr>
<td>Toasted bagel</td>
<td>Never</td>
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<tr>
<td>Other bread</td>
<td>Never</td>
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</tbody>
</table>

### Bran flakes (30g – see pic)

### Corn flakes

### Frosted flakes

### Fruit and fibre

### Shreddies

### Shredded wheat

### Porridge (1/2 cup – medium bowl)

### Porridge with honey (1/2 cup – medium bowl)

### Rice crispies

### Weetabix

### Muesli

### Other cereal | Never |       |                |             |         |         |              |                |

### Canned red kidney beans (1/2 cup – 1 heaped tbsp)

### Cooked (1/2 cup – 1 heaped tbsp)

### Spiral pasta (125g) | 150g – see pic

### Boiled rice (125g) | 150g – 1 medium portion

### Other grains | Never |       |                |             |         |         |              |                |

### Roasted white potato (120g) | 150g

### Boiled white potato (120g) | 150g

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Advanced glycation end products as a biomarker for accelerated ageing version 1.0, 24/05/16
<table>
<thead>
<tr>
<th>Frequency you eat:</th>
<th>Portion size</th>
<th>Less than once</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
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<tbody>
<tr>
<td>Roasted sweet potato</td>
<td>1/2</td>
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<tr>
<td>Homemade chips</td>
<td>1/4 portion</td>
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<tr>
<td>Fast food chips</td>
<td>1/4 portion</td>
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<td>Jacket potato</td>
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<td>Granola bar</td>
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### Carbohydrates continued - All products 30g unless otherwise stated

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<th>1 to 3 times</th>
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<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
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<th>2 times</th>
<th>3 or more times</th>
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<td>Per week</td>
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<td>Doritos (corn crisps) (1 packet)</td>
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<td>Mini cheddars (15g – 1 packet)</td>
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<td>Crackers (8 crackers)</td>
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<td>Melba toast (3 pieces)</td>
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<td>Rice cake (4 rice cakes)</td>
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<td>Hummus (1.25g – 1 heaped tbsp)</td>
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<td>Other savoury snacks</td>
<td>Per month</td>
<td>Per week</td>
<td>Per day</td>
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### Fruits, vegetables and miscellaneous - All products 100g unless otherwise stated

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<th>Portion</th>
<th>Less than once</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
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<tbody>
<tr>
<td>Apple (1 small apple)</td>
<td>Per month</td>
<td>Per week</td>
<td>Per day</td>
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<td>Baked apple (1 small apple)</td>
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<tr>
<td>Banana (1 medium banana)</td>
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<td>Cantaloupe melon (117g) (1 small melon)</td>
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<td>Raisin (29g – 1 tbsp)</td>
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<tr>
<td>Other fruits</td>
<td>Per month</td>
<td>Per week</td>
<td>Per day</td>
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<td>Raw Onion (70g – 1 medium onion)</td>
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<td>Raw Tomato (108g – 1 medium tomato) (100g of tomatoes)</td>
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<td>Avocado (65g – 1 large avocado)</td>
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<td>Other raw vegetables</td>
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<tr>
<td>Other grilled vegetables</td>
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<td>Cooked Green beans (159g – 1 medium portion)</td>
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<td>Cooked carrots (165g – about 3 heaped tbsp)</td>
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*Advanced glycation end products as a biomarker for accelerated ageing version 1.0, 24/06/16*
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<th>Frequency you eat:</th>
<th>Portion Size</th>
<th>Less than once</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
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<th>3 or more times</th>
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<td></td>
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<td>Per month</td>
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<td>Fruit ice lolly</td>
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<td>Roasted almonds</td>
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<tr>
<td>Roasted walnuts</td>
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<td>Salted peanuts</td>
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<td>Butter</td>
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<td>Mayonnaise</td>
<td>(5g – 10g)</td>
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<td>Mayonnaise low fat</td>
<td>(5g – 10g)</td>
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Advanced glycation end products as a biomarker for accelerated ageing version 1.0, 24/08/16
<table>
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<th>Ingredient</th>
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<th>1 to 3 times</th>
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<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salad dressing (1 tsp = 2.5 g)</td>
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<tr>
<td>Ketchup (2 tbsp = 3.5 g)</td>
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<tr>
<td>Mustard (1 tsp = 3 g)</td>
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<td>Soy sauce (1 tsp = 5 g)</td>
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<tr>
<td>Balsamic vinegar (1 tbsp = 2.5 g)</td>
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<tr>
<td>White vinegar (1 tbsp = 2.5 g)</td>
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<tr>
<td>Pickle (1 tsp)</td>
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<tr>
<td>Other condiments...</td>
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Questionnaire portion guidance

Below are some examples of portions to help when knowing our own portion size:

- 250 ml of water
- 250 ml of milk
- 35g bagel – ½ a plain bagel
- 35g bread – 1 slice
- 30g cheese
- 30g cornflakes
- 30g walnuts
- 100g sweet potato
- 90g chicken breast – 1 small breast

100g boiled pasta
200g boiled pasta

Advanced glycation end products as a biomarker for accelerated ageing version 1.0, 24/06/16
Appendix 4.2 – FFQ prompt flow chart

**FFQ prompt flow chart for clinician**

Explain the principle of the FFQ and remind the participant to be as honest as possible

Ask if there has been a significant change to their diet in the last 12 months

Ask the participant to read the instructions

Prompt the first question, ‘What sort of cooking fat would you use?’

Talk the participant through the worked example for the portion questions and show the plate used to take the photographs. If they would never eat a food photographed put a line through the foods name.

Show the FFQ itself and remind the participant that it covers foods eaten over the last 12 months. Explain that this is to encompass foods eaten in different seasons e.g. more salads in the summer.

Talk the participant though the worked example (feta cheese)

Explain that if a portion size isn’t written next to the food it should be at the top of its ‘group’ e.g. all cheese 30g unless otherwise stated, such as cream cheese 1tbsp.

There are rows within the questionnaire which say ‘other’, explain that this is intended to allow the recording of foods they eat often that haven’t been listed in the questionnaire

Note all drinks are 250ml and show the reference page at the end of the FFQ. Show the cups which amount to 250ml.
Begin the FFQ, allow the participant to fill in the questionnaire themselves and offer assistance if required.

When beginning cheese section show the 30g portion of cheddar on the reference page. If their portion is larger than this adjust the portion to account for this.

When reaching ‘other cheese’ provide prompt ‘e.g. stilton?’

Explain timing’s on soft/hard boiled eggs is from when they go into cold water (Americanism)

‘Other egg’ provide prompt ‘e.g. scrambled? Omelette?’

Bacon portion ‘one rasher or more?’

Sausages portion, 2 sausages = 1 portion, therefore if they eat 3 sausages record as 1.5

‘Other pork’ prompt ‘e.g. roast pork?’

‘Other lamb’ prompt ‘e.g. roast lamb?’

If they are uncertain between ‘chicken breast cubed and pan fried’ and ‘chicken breast cubed, pan fried and boiled’ explain that the second would be where the chicken once cooked is added to a sauce and boiled e.g. sweet & sour chicken.

If they put never to battered cod prompt ‘do you think you have had fish and chips at all in the last 12 months?’ it may be they have a different type of battered fish.

‘Other fish’ provide prompt ‘e.g. mackerel (tinned or fresh)’
When beginning the ‘Carb’s’ section remind the participant each slice of bread = 1 portion.

When beginning ‘cereals’ remind the participant to look at the reference page for what 30g ‘looks like’

When reaching fruits and vegetables address the fact that not many fruits and vegetables are listed in the FFQ. Reiterate that they should fill the ‘other’ sections with fruits/veg they eat often.

‘Raw veg’ prompt ‘for example this would include salad – what raw vegetables would go into your salad?’

‘Other condiments’ prompt ‘e.g. jam, marmalade?’

Finally, make sure to add the date the FFQ was completed and the participant’s code to the FFQ.
Appendix 5 – 4 day weighed food diary

4 - Day Weighed Food Diary

Use this diary to record **everything** you **eat and drink** for 4 days – 3 weekdays and 1 weekend day. The purpose of filling out these food records is to help better understand the foods you eat, when you eat and your portion size.

- Please record your food items and portions in a real time, do not wait until the end of the day, as you may forget some items and the analysis will be unreliable.
- Do not try to modify your dietary habits and the food choices just because you are recording, as it will not represent your usual diet. Similarly, please avoid recording during feasts such as Birthdays for the same reason.
- If you eating out, please try to estimate portion and best list the meals ingredients. Record the place of eating (name of the restaurant) and the foods eaten.

You will be loaned a set of digital kitchen scales to allow you to weigh your foods. Drinks can be measured either by pouring into a measuring jug, or by ‘zeroing’ your mug/cup on the digital scales and weighing the fluid, in international units (1 gram (g) = 1 millilitre (ml)).

**Example**

<table>
<thead>
<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>Weight (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Home</strong></td>
<td>2pm</td>
<td>Ham sandwich: 2 slices of hovis granary bread, 1 tsp of butter, 3 wafer thin slices of ham</td>
<td>Total weight: 110g Bread = 35g per slice Ham = 35g</td>
</tr>
<tr>
<td><strong>OR example if out</strong></td>
<td></td>
<td>OR example if out</td>
<td></td>
</tr>
<tr>
<td><strong>Out shopping</strong></td>
<td>2pm</td>
<td>Boots shapers ham sandwich</td>
<td>110g</td>
</tr>
</tbody>
</table>
Before beginning the food diary please can you fill out the table below

<table>
<thead>
<tr>
<th>What do you regularly eat for….</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
</tr>
<tr>
<td>Lunch</td>
</tr>
<tr>
<td>Evening meal</td>
</tr>
<tr>
<td>Snacks</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kitchenware sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinner plate across (cm)</td>
</tr>
<tr>
<td>Side plate across (cm)</td>
</tr>
<tr>
<td>Bowl across (cm)</td>
</tr>
<tr>
<td>Bowl down (cm)</td>
</tr>
<tr>
<td>Bowl (ml) full capacity</td>
</tr>
<tr>
<td>Mug/cup (ml) full capacity</td>
</tr>
<tr>
<td>Glass (ml) full capacity</td>
</tr>
</tbody>
</table>
# 4 - Day Weighed Food Diary

**Day 1**

<table>
<thead>
<tr>
<th>Date:</th>
<th>Day of the week:</th>
<th>Activity:</th>
</tr>
</thead>
</table>

## Before Breakfast

<table>
<thead>
<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>Weight (g/ml)</th>
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<tbody>
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</table>

## Breakfast

<table>
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<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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</table>

## Between breakfast and lunch

<table>
<thead>
<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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</table>
### Lunch

<table>
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<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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### Between lunch and evening meal

<table>
<thead>
<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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</table>
### Evening meal

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<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
</tr>
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</table>

### Between evening meal and breakfast

<table>
<thead>
<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
</tr>
</thead>
</table>
### Day 2

<table>
<thead>
<tr>
<th>Date:</th>
<th>Day of the week:</th>
<th>Activity:</th>
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</table>

#### Before Breakfast

<table>
<thead>
<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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#### Breakfast

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<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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#### Between breakfast and lunch

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<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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<tr>
<td>Lunch</td>
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<td>Item, portion, description</td>
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<tr>
<td>Place eaten</td>
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<td>Time</td>
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<td>g/ml</td>
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<th>Between lunch and evening meal</th>
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<th>Item, portion, description</th>
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<td>Place eaten</td>
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</table>
### Evening meal

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<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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### Between evening meal and breakfast

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<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
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Day 3

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<tr>
<th>Date:</th>
<th>Day of the week:</th>
<th>Activity:</th>
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</table>

**Before Breakfast**

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<tr>
<th>Place eaten</th>
<th>Time</th>
<th>Item, portion, description</th>
<th>g/ml</th>
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**Breakfast**

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<th>Place eaten</th>
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**Between breakfast and lunch**

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<th>Place eaten</th>
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### Between lunch and evening meal

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Appendix 6 – Lab analysis methods

6.1 ASCORBATE MEASUREMENT

HPLC electrochemical detection of ascorbate

SOLUTIONS

Standard Ascorbate – make fresh on the day

Ascorbate: 0.018 g ascorbic acid/100 ml [0.009 g/50 ml] in H₂O gives a 1.0 mM stock solution.
Dilute in PBS to standards 10μM, 30μM and 50μM.

10% Metaphosphoric acid [MPA] containing 2mM EDTA

Make up a solution of 2 mM EDTA in H₂O. e.g 0.186 g EDTA disodium salt made up in 250 ml H₂O. Use this to make up the MPA solution.
16 g of MPA (33.5 – 36.5%) diluted with 50 ml of HPLC H₂O containing 2 mM EDTA.

5% MPA containing 1 mM EDTA

A 1:2 dilution of the above in H₂O

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

0.100 g made up in 1.0 ml of 5% MPA in 1 mM EDTA. Make up e.g. 0.025g plus 250μl 5% MPA in 1mM EDTA.
* Make this first as needs to dissolve *

MOBILE PHASE

50 mM Phosphate buffer containing 540 μM EDTA and 2% Methanol.

Weigh out 3.9 g of sodium dihydrogen orthophosphate HPLC grade [MW 156.01] and place in a 500 ml flask.

Weigh out 0.1 g EDTA disodium salt [MW 372.2] and add to the flask.

Make up to about 300 ml with HPLC grade H₂O.

Add 10 ml of methanol.

Make up to 500 ml with HPLC grade H₂O.

Take to pH 2.8 with 6.0 M HCl.

Filter and degas (Millipore glassware)
ASCORBATE MEASUREMENT PROCEDURE

1. Take 200 µl of plasma and add to 200 µl of 10% MPA containing 2.0 mM EDTA. Mix well and centrifuge at 20,000 x g at 4°C for 10 minutes.

2. Take 2 x 90 µl aliquots of supernatant.

3. To one add 10 µl of 5% MPA containing 1 mM EDTA. Add a further 200 µl of 5% MPA containing 1 mM EDTA. Mix well. This is for measurement of reduced ascorbate. From this sample remove 50 µl and add to a tube containing 450 µl 5% MPA containing 1 mM EDTA. Mix well. This is for measurement of urate.

4. To the other sample add 10 µl 350 mM TCEP in 5% MPA containing 1 mM EDTA. Incubate for 20 minutes at room temperature. This is to reduce the oxidised dehydroascorbate back to ascorbate. Add a further 200 µl of 5% MPA containing 1 mM EDTA. Mix well. This sample is for measurement of total ascorbate

Suggested procedure: Inject the reduced sample, and the sample for urate while the total ascorbate sample is incubating. Then inject the total sample.

Standards: Prepare standards as follows:
- Take 200 µl of standard and add to 200 µl of 10% MPA containing 2 mM EDTA & ‘Buzz’
- Take a 90 µl aliquot and add 10 µl of 350 mM TCEP in 5% MPA containing 1 mM EDTA.
- Incubate for 20 minutes at room temperature.
- Add a further 200 µl of 5% MPA. Mix well.
- Inject into HPLC system
- Use HPLC H₂O to wash between each standard and samples
6.2 MEASUREMENT OF MALONDIALDEHYDE

Remember! – Don’t put the weighed out crystals back in the tub!!

SOLUTIONS

1) Butylated hydroxytoluene [BHT] (found in top cupboard) 0.05% in 95% ethanol (kept in yellow cupboard). To be made up on the day.
   Weigh out 0.05 g in 100 ml HPLC water, for this study used 0.0025 g (with small amounts measure with test tube on the scales) in 5 ml of 95% ethanol. i.e 4.750ml in 250 µl water.

2) Phosphoric acid (0.44 M)
   Take 1.0 ml of concentrated orthophosphoric acid and make up to 100 ml with distilled water

3) TBA (42 mM)
   Weigh out 0.303 g of TBA and make up to 50 ml with water. Heat at 50-55 C to ensure that it dissolves.

4) Stock solution of MDA (1mM)
   Weigh out 0.00313 g of MDA and make up to 10 ml with distilled water store at (-80c). Dilute down in pbs to a range of standards from 500 nM to 10 µM

5) Phosphate buffered saline [PBS] PH 7.4
   [137 mM NaCl; 2.7 mM KCl; 8 mM Na₂HPO₄; 1.46 mM KH₂PO₄]
   Using a 250 ml volumetric flask weight out the following:
   2.0 g of NaCl (MW 58.44), 0.05 g of KCl (74.56),0.716 g of Na₂HPO₄ (MW 358.14),
   0.05 g of KH₂PO₄ (MW 136.09) dissolved in about 200 ml distilled water, adjust PH to 7.4 make up to 250 ml storage in Keep this solution at 4°C.

6) Mobile phase – the solution that carried the sample through the machine
   Potassium dihydrogen orthophosphate [KH₂PO₄] (50 mM)/MeOH 80/20 v/v
   Weigh out 3.403 g KH₂PO₄ and dissolve in 500 ml HPLC grade water. Add 125 ml MeOH. Adjust to pH 6.8 using 5 M sodium hydroxide. Filter and degass.
   HPLC wavelengths: Excitation 515 nm, Emission 553 nm
MEASUREMENT OF MALONDIALDEHYDE PROCEDURE

In 2.0 ml centrifuge tube with screw caps, put 50 µl of Plasma or standard or blank add 50µl of BHT solution then add 400 µl of phosphoric acid and 100 µl of TBA solution.

1) Cap the tubes, mix well on a vortex mixer. Heat for 1 hour at 100° C in a dry block.
2) Remove tubes and place on ice to cool.
3) Add 300 µl of n-1- butanol. Mix well using the vortex mixer.
4) Centrifuge the mixture for 5 minute at 13,000 × g. to separate the aqueous and butanol phases.
5) Carefully remove 200 µl of the butanol extract and place in tubes inject the butanol extract direct into HPLC system.
6.3 MEASUREMENT OF ALPHA-TOCOPHEROL (VITAMIN E) IN PLASMA

SOLUTIONS

Mobile phase: 6% tetrahydrofuran (THF) in methanol

15 ml THF made up to 250 ml with MeOH. Don’t pH or filter.

Vit E standards, make up in methanol: 5, 10, 20, 50 ug/ml, in MeOH.

Weigh out a tiny blob and add MeOH to mix, e.g:

0.0215 g vitamin E + 215 ul MeOH = 100 mg/ml

Sample preparation

To 100 ul plasma, standards and blank (use MeOH for blank) add 200 ul acetronitrile-THF [3 parts acetronitrile : 2 parts THF].

Vortex each sample for 2 minutes to get the full extraction.

Centrifuge 12,800 g for 20 mins, 4 degrees C using refrigerated centrifuge.

Remove supernatant for injection.

HPLC set up

THF destroys some plastics (though seems OK with our Eppendorf tubes), and so the rheodyne and tubing need to be changed prior to measuring vitamin E. The column and guard column are also changed.

Absorbance is read at 292 nm

1 ml/min pump setting. Comes off at ~ 5 min, do a 7 min run.

Use MeOH for between injection washes.

Use a programmed wash after standards and after each batch of samples.
## Appendix 7 – Full list of parameters measured by the Corvis ST

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOP (mmHg)</td>
<td>Intraocular pressure (based on A1)</td>
</tr>
<tr>
<td>CCT (µM)</td>
<td>Central corneal thickness by optical pachymetry</td>
</tr>
<tr>
<td>Time A1 (ms)</td>
<td>Measures the time from the initiation of the air puff to the first inward corneal movement</td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>Measures the time to second applanation / outward corneal movement</td>
</tr>
<tr>
<td>Length A1 (mm)</td>
<td>Length of the applanated flat cornea surface at first applanation</td>
</tr>
<tr>
<td>Length A2 (mm)</td>
<td>Length of the applanated flat cornea surface at second applanation</td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>Velocity of corneal surface movement at the first applanation</td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>Velocity of corneal surface movement at the second applanation</td>
</tr>
<tr>
<td>Deformation amplitude A1 (mm)</td>
<td>The magnitude of the movement of the corneal surface at the first applanation</td>
</tr>
<tr>
<td>Deformation amplitude A2 (mm)</td>
<td>The magnitude of the movement of the corneal apex at the second applanation</td>
</tr>
<tr>
<td>Highest deformation amplitude (mm)</td>
<td>The magnitude of the movement of the corneal surface at the highest concavity</td>
</tr>
<tr>
<td>Highest concavity time (ms)</td>
<td>Time from the initiation of the air puff to the highest concavity of the deformation of cornea</td>
</tr>
<tr>
<td>HC radius (mm)</td>
<td>The radius of curvature at highest concavity of the deformation of the cornea.</td>
</tr>
</tbody>
</table>
## Summary of AGE and Ocular disease studies

### Diabetic retinopathy (DR)

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Findings</th>
</tr>
</thead>
</table>
| Ghanem et al. (2011)                 | 30 HC  
Type 2 DM: 35 mild NPDR  
30 severe NPDR  
25 PDR | Serum levels of pentosidine and CML are related to severity of DR. In addition, aqueous humor level of CML increased with progression of DR. |
| Choudhuri et al. (2013)              | 95 HC  
Type 2 DM: 70 NPDR  
105 PDR  
102 DNR | Serum and vitreous CML levels were significantly elevated in PDR and NPDR subjects compared to DNR. ROS production was higher in NPDR and PDR compared to the DNR group. |
| Al-Mesallamy et al. (2011)           | 20 HC  
Type 2 DM: 14 DNR  
14 NPDR  
9 PDR | Soluble RAGE levels were significantly lower in patients NPDR and PDR compared to HC and DNR patients.                                    |
| Anitha et al. (2008)                 | 188 HC  
Type 2 DM: 188 DNR  
153 NPDR  
41 PDR | Advanced glycation index, an assay to detect serum AGEs was significantly associated with severity of DR.                                |
| Boehm et al. (2004)                  | HC 792  
Type 2 DM: 81 DNR  
56 DR | Higher serum CML levels were associated with advanced stages of DR.                                                                      |
| Beisswenger et al. (1995)            | 29 HC  
48 type 1 DM | Levels of collagen-linked AGEs, measured by ELISA, were correlated with preclinical stages of DR (not indicated by other methods).        |
| Miura et al. (2003)                  | Type 1 DM: 35 DNR  
22 NPDR  
28 PDR | Elevation of serum CML and non-specified AGE were associated with the severity of diabetic retinopathy in patients with Type 1 diabetes. |
| Murata et al. (1997)                 | (Immunohistochemistry)  
9 HC  
27 DR | Increased CML was associated with increased vascular endothelial growth factor. CML also found to be located in the thickened vascular wall. |
<p>| Yao et al. (2009)                    | Cultured human aortic endothelial cells | Evaluated RAGE and its ligands in human aortic endothelial cells. Proposed that ROS as well as being formed at the end of RAGE activation, ROS also create a positive feedback of RAGE facilitated pro-inflammatory signalling. |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zong et al. (2011)</td>
<td>Animal study: rats (n = 10)</td>
<td>Hyperglycaemia in vivo and in vitro induces upregulation of RAGE and its ligands, leading to RAGE signalling. This links to pro-inflammatory responses by retinal Müller glia.</td>
</tr>
<tr>
<td>Hirano et al. (2014)</td>
<td>111 HC Type 2 DM: 36 DNR 71 NPDR 31 PDR</td>
<td>SAF was increased in patients with DM to HC. SAF correlated with severity of DR, whereas single measured HbA1c did not.</td>
</tr>
</tbody>
</table>

**Age-related macular degeneration (AMD)**

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni et al. (2009)</td>
<td>32 HC 58 AMD</td>
<td>Higher levels of CML and pentosidine in AMD plasma protein.</td>
</tr>
<tr>
<td>Ishibashi et al. (1998)</td>
<td>Human donor eyes 8 AMD 12 aged HC</td>
<td>Soft, macular drusen and/or basal laminar and basal linear deposits showed CML accumulation. In choroidal neovascular membranes RPE cells showed CML accumulation in their cytoplasm.</td>
</tr>
<tr>
<td>Schutt et al. (2003)</td>
<td>Human donor eyes 10 pairs of donor eyes</td>
<td>Lipofuscin associated proteins were damaged by covalent modifications of MDA and AGEs in human RPE.</td>
</tr>
<tr>
<td>Glenn et al. (2011)</td>
<td>RPE recovered from 7 aged donor eyes</td>
<td>AGE-induced modifications to the basement membrane alters the RPE proteome.</td>
</tr>
<tr>
<td>Hollyfield et al. (2003)</td>
<td>Human donor eyes 18 HC 5 AMD</td>
<td>Protein cross-links and AGEs were observed in drusen and Bruch's membrane.</td>
</tr>
<tr>
<td>Mulder et al. (2009)</td>
<td>31 HC 73 neovascular AMD</td>
<td>SAF was increased in patients with neovascular AMD</td>
</tr>
</tbody>
</table>

**AGEs and diabetic keratopathy**

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sato et al. (2001)</td>
<td>13 HC Type 2 DM: 8 DNR 6 NPDR 12 PDR</td>
<td>The corneal AGE fluorescence values were significantly higher in the PDR group compared with HC.</td>
</tr>
</tbody>
</table>
Mori et al. (2009) | 8 HC 17 PDR | Corneal and lens autofluorescence significantly increased in PDR, compared with HC. Hyperglycemia associated with increasing corneal autofluorescence in PDR.

Sady et al. (1995) | Human donor eyes 11 HC 8 DM | Fluorescence and pentosidine were present at higher levels in DM corneas compared to age-matched HC corneas.

Kaji et al. (2000) | Human donor eyes 8 HC 8 DM | CML present in epithelial basement membrane of all DM corneas, only in one of 8 HC corneas.

Kim et al. (2010) | Animal study: rats 8 control rats 8 DM rats | Corneal thickness was increased in DM rats compared to controls. Increased AGE in corneal tissues of DM rats

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tezel at al. (2007)</td>
<td>Human donor eyes 30 HC 38 Glaucoma</td>
<td>Higher levels of AGEs and RAGE were detectable in the glaucomatous retina and ONH. In glaucomatous eyes AGE was predominantly extracellular in laminar cribriform plates in the ONH, RAGE was was predominant on glial cells.</td>
</tr>
<tr>
<td>Amano et al. (2001)</td>
<td>Human donor eyes (all elderly 6 HC 7 DM)</td>
<td>Pyrraline detected in sclera, pia mater, cribriform plates, connective tissues in the optic nerve. Also found around vessels in the optic nerve and pia mater.</td>
</tr>
<tr>
<td>Albon et al. (1995)</td>
<td>Human donor eyes Age range 3 to 92</td>
<td>Age-related changes included an increase in total collagen and a decrease in the proportion of type III collagen in the lamina cribrosa. Age-related increase of pentosidine.</td>
</tr>
<tr>
<td>Spoerl et al. (2005)</td>
<td>80 porcine eyes 24 pairs of human donor eyes</td>
<td>Glyceraldehyde and methylglyoxal increase the stiffness of the lamina cribrosa and the peripapillary sclera in human and in porcine eyes.</td>
</tr>
<tr>
<td>Park et al. (2012)</td>
<td>Cultured human trabecular meshwork cells</td>
<td>AGE enhances cellular deterioration with age in human trabecular meshwork cells, accompanied with increased oxidative stress.</td>
</tr>
<tr>
<td>Tezel et al. (1997)</td>
<td>HC 24 POAG 31</td>
<td>Small increase in aqueous humor endothelin levels in patients with POAG versus controls</td>
</tr>
<tr>
<td>Doganay et al. (2002)</td>
<td>14 HC 16 POAG</td>
<td>Decreased aqueous humor NO levels in patients with POAG compared to HC</td>
</tr>
<tr>
<td>Galassi et al. (2004)</td>
<td>46 HC 38 POAG</td>
<td>NO plasma levels and aqueous humor levels were decreased in POAG compared to HC</td>
</tr>
<tr>
<td>Himori et al. (2016)</td>
<td>36 HC 71 POAG 176 NTG</td>
<td>SAF negatively correlated with MD in the youngest subjects, not in the older subjects.</td>
</tr>
<tr>
<td>Study</td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Himori et al. (2015)</td>
<td>20 HC</td>
<td>73 NTG</td>
</tr>
<tr>
<td>Schweitzer et al. (2018)</td>
<td>424 HC</td>
<td>31 POAG</td>
</tr>
</tbody>
</table>
### Appendix 9 – Table summary of dietary AGE literature

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hofmann et al. (2002)</td>
<td>16 HC mice 20 DM mice</td>
<td>Mice fed a low dAGE diet had lower CML and MG levels compared with high dAGE fed mice.</td>
</tr>
<tr>
<td>Vlassara et al. (2002)</td>
<td>24 type 2 DM: 11 in 2wk 13 in 6wk</td>
<td>Participants on reduced dAGE diet had a reduction in circulating AGE level and reduction in both TNF-α and VCAM1.</td>
</tr>
<tr>
<td>Vlassara et al. (2002)</td>
<td>24 type 2 DM: 11 in 2wk 13 in 6wk</td>
<td>Participants on reduced dAGE diet had a reduction in circulating AGE level and reduction in both TNF-α and VCAM1.</td>
</tr>
<tr>
<td>Vlassara et al. (2009)</td>
<td>325 HC 66 chronic kidney disease patients</td>
<td>Reduction of dAGE lowers oxidative stress and inflammation in both healthy and chronic kidney disease patients.</td>
</tr>
<tr>
<td>Uribarri et al. (2011)</td>
<td>18 HC 18 type 2 DM</td>
<td>Restriction of dAGE may preserve natural defences and insulin sensitivity by maintaining lower basal oxidative stress.</td>
</tr>
<tr>
<td>Piroddi et al. (2011)</td>
<td>10 HC 10 chronic kidney disease patients 10 hemodialysis patients</td>
<td>Dietary intake of CML did not affect circulating levels in these groups of patients.</td>
</tr>
<tr>
<td>Luevano-Contreras et al. (2013)</td>
<td>34 type 2 DM</td>
<td>Restriction of dAGEs significantly decreased TNFα and malondialdehyde levels.</td>
</tr>
<tr>
<td>Semba et al. (2014)</td>
<td>24 healthy volunteers 12 high dAGE 12 low dAGE</td>
<td>No significant changes in serum and urinary CML concentrations from baseline to follow-up in the high-AGE diet group. A high- or low-diabetic AGE had no significant impact on inflammatory mediators or peripheral arterial tonometry after 6wk intervention.</td>
</tr>
<tr>
<td>Mark et al. (2014)</td>
<td>74 overweight women 37 high dAGE 37 low dAGE</td>
<td>The low-AGE diet decreased urinary AGEs, fasting insulin concentrations, and Homeostasis model assessment of insulin resistance, compared with the high-AGE diet.</td>
</tr>
<tr>
<td>Study</td>
<td>Participants</td>
<td>Outcome Description</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-----------------------------------</td>
<td>-------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Macias-Cervantes et al. (2015)</td>
<td>43 overweight 15 low dAGE and exercise 14 low dAGE 14 exercise (with habitual food intake)</td>
<td>AGE-restricted diet reduces serum. The addition of exercise to the restricted diet has the same effects but also improves lipid profile.</td>
</tr>
<tr>
<td>Barbora de Courten et al. (2016)</td>
<td>20 healthy volunteers</td>
<td>A diet that is low in AGEs may reduce the risk of type 2 diabetes by increasing insulin sensitivity.</td>
</tr>
<tr>
<td>Scheijen et al. (2018)</td>
<td>450 volunteers</td>
<td>Higher intake of dAGEs is associated with higher levels of AGEs in plasma and urine.</td>
</tr>
</tbody>
</table>
Appendix 10 - Statistical tests

Appendix 10.1 – Median and IQR for non-parametric data in Chapter 4

<table>
<thead>
<tr>
<th>Median</th>
<th>IQR</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urribari (average CML kU/day)</td>
</tr>
<tr>
<td>Plasma</td>
<td>91.40</td>
<td>77.42</td>
</tr>
<tr>
<td>CML</td>
<td>124.05</td>
<td>p = 0.038*</td>
</tr>
</tbody>
</table>

Table 10.4.2: Median, IQR and Spearman’s correlations between plasma CML and dAGEs calculated with each database. Interquartile range: IQR. *p < 0.05

<table>
<thead>
<tr>
<th>Spearman’s correlation coefficients (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>1. Urribari (average CML kU/day)</td>
</tr>
<tr>
<td>2. Hull CML (average mg CML/day)</td>
</tr>
<tr>
<td>3. Scheijen-CML (average mg CML/day)</td>
</tr>
<tr>
<td>4. Scheijen-CEL (average mg CEL/day)</td>
</tr>
<tr>
<td>5. Scheijen-MGH1 (average mg MG-H1/day)</td>
</tr>
</tbody>
</table>

Table 10.4.4: Medians, IQR and Spearman’s correlations between dAGEs for each database measured with FFQ1 (n = 70). Interquartile range: IQR. *p < 0.05. **p < 0.01.
### Appendix 10.2 – Median and IQR for non-parametric data in Chapter 5

<table>
<thead>
<tr>
<th>Median</th>
<th>IQR</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uribari (average CML kU/day)</td>
</tr>
<tr>
<td>SAF</td>
<td>2.10</td>
<td>2.00, 2.47</td>
</tr>
<tr>
<td></td>
<td>p = 0.545</td>
<td>p = 0.085</td>
</tr>
</tbody>
</table>

Table 10.5.1: Median, IQR and Spearman’s correlations between SAF (AU) and dAGEs calculated with each database. *p<0.05

<table>
<thead>
<tr>
<th>Median</th>
<th>IQR</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SAF (AU)</td>
</tr>
<tr>
<td>Ascorbate (µM)</td>
<td>44.89</td>
<td>36.83, 62.65</td>
</tr>
<tr>
<td>MDA (µM)</td>
<td>0.59</td>
<td>0.48, 0.72</td>
</tr>
<tr>
<td>Alpha tocopherol (µM)</td>
<td>40.41</td>
<td>31.81, 50.37</td>
</tr>
</tbody>
</table>

Table 10.5.2: Median, IQR and Spearman’s correlations between SAF (AU) and each oxidative stress biomarker: ascorbate (vitamin C), malondialdehyde (MDA) and alpha tocopherol (form of vitamin E). *p<0.05
### Table 10.5.3: Median, IQR and Spearman’s correlations between all parameters which correlated with SAF.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median</th>
<th>IQR</th>
<th>Spearman’s correlation coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R values</td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.00</td>
<td>53.00, 67.50</td>
<td>0.435</td>
</tr>
<tr>
<td>Dietary AGE MG-H1 (average mg MG-H1/day)</td>
<td>29.43</td>
<td>22.28, 36.55</td>
<td>0.333</td>
</tr>
<tr>
<td>Alpha tocopherol (µM)</td>
<td>40.41</td>
<td>31.81, 50.37</td>
<td>0.252</td>
</tr>
</tbody>
</table>

Table 10.5.3: Median, IQR and Spearman’s correlations between all parameters which correlated with SAF.

### Appendix 10.3 – Median and IQR for non-parametric data in Chapter 7

<table>
<thead>
<tr>
<th>Artery</th>
<th>Median</th>
<th>IQR</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDF</td>
<td>2.11</td>
<td>1.49, 3.02</td>
<td>0.007, p = 0.956</td>
</tr>
<tr>
<td>DA</td>
<td>3.51</td>
<td>2.21, 5.71</td>
<td>0.144, p = 0.272</td>
</tr>
<tr>
<td>BFR</td>
<td>1.30</td>
<td>0.49, 3.20</td>
<td>0.165, p = 0.207</td>
</tr>
<tr>
<td>MD %</td>
<td>2.23</td>
<td>1.26, 3.76</td>
<td>0.131, p = 0.319</td>
</tr>
<tr>
<td>RT (secs)</td>
<td>15.00</td>
<td>12.42, 19.00</td>
<td>-0.033, p = 0.803</td>
</tr>
<tr>
<td>MC %</td>
<td>1.31</td>
<td>0.63, 2.01</td>
<td>0.123, p = 0.349</td>
</tr>
<tr>
<td>CT (secs)</td>
<td>22.00</td>
<td>17.25, 28.50</td>
<td>0.215, p = 0.098</td>
</tr>
</tbody>
</table>

Table 10.7.1: Artery dynamic parameters, median, IQR and spearman’s correlation with SAF. AU: arbitrary units, BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.
<table>
<thead>
<tr>
<th>Vein</th>
<th>Median</th>
<th>IQR</th>
<th>Spearman's correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SAF (AU)</td>
<td></td>
</tr>
<tr>
<td>BDF</td>
<td>1.82</td>
<td>1.51, 3.04</td>
<td>-0.140, ( p = 0.287 )</td>
</tr>
<tr>
<td>DA</td>
<td>4.82</td>
<td>3.44, 7.36</td>
<td>-0.075, ( p = 0.570 )</td>
</tr>
<tr>
<td>BFR</td>
<td>2.82</td>
<td>1.94, 4.17</td>
<td>0.008, ( p = 0.950 )</td>
</tr>
<tr>
<td>MD %</td>
<td>3.98</td>
<td>2.95, 6.16</td>
<td>-0.098, ( p = 0.458 )</td>
</tr>
<tr>
<td>RT (secs)</td>
<td>18.00</td>
<td>16.75, 25.83</td>
<td>-0.004, ( p = 0.978 )</td>
</tr>
<tr>
<td>MC %</td>
<td>0.71</td>
<td>0.31, 1.45</td>
<td>0.015, ( p = 0.912 )</td>
</tr>
<tr>
<td>CT (secs)</td>
<td>33.25</td>
<td>25.83, 37.25</td>
<td>-0.147, ( p = 0.262 )</td>
</tr>
</tbody>
</table>

Table 10.7.2: Vein dynamic parameters, median, IQR and spearman’s correlation with SAF. AU: arbitrary units, BDF: baseline diameter fluctuation, DA: dilation amplitude, BFR: baseline corrected flicker response, MD%: maximum percentage dilation, RT: reaction time, MC%: minimum percentage constriction, CT: constriction time.
<table>
<thead>
<tr>
<th>Corneal parameters</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Spearman’s correlation coefficients (R values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAF (AU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time A1 (ms)</td>
<td>7.27</td>
<td>7.09, 7.59</td>
<td>0.140, p = 0.394</td>
</tr>
<tr>
<td>Time A2 (ms)</td>
<td>21.99</td>
<td>21.66, 22.26</td>
<td>-0.105, p = 0.526</td>
</tr>
<tr>
<td>Velocity A1 (m/s)</td>
<td>0.15</td>
<td>0.14, 0.17</td>
<td>-0.227, p = 0.165</td>
</tr>
<tr>
<td>Velocity A2 (m/s)</td>
<td>-0.27</td>
<td>-0.29, -0.24</td>
<td>0.290, p = 0.074</td>
</tr>
<tr>
<td>Deformation amp. A1 (mm)</td>
<td>0.13</td>
<td>0.13, 0.14</td>
<td>0.033, p = 0.840</td>
</tr>
<tr>
<td>Deformation amp. A2 (mm)</td>
<td>0.44</td>
<td>0.41, 0.49</td>
<td>0.113, p = 0.492</td>
</tr>
<tr>
<td>Highest deformation amp. (mm)</td>
<td>1.09</td>
<td>0.99, 1.15</td>
<td>-0.265, p = 0.103</td>
</tr>
<tr>
<td>Highest concavity time (ms)</td>
<td>16.40</td>
<td>16.17, 16.86</td>
<td>0.012, p = 0.942</td>
</tr>
</tbody>
</table>

### Appendix 11 – Gantt chart

<table>
<thead>
<tr>
<th>Activity</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
</tr>
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<td>Introduction to study and training (DVA, SAF, Bloods)</td>
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<td>Literature Review</td>
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<td>Thesis writing</td>
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### Appendix 12 – Research training activities

- Introduction to end note seminar
- Introduction to ethical approval seminar
- Library services seminar
- ITL – Introduction to learning
- Good clinical practice (GCP) training
- Research writing session
- Human tissue training session
- Phlebotomy training (Derriford hospital)
- Basic laboratory training
- Pipette training
- Preparing for the transfer seminar
- Lab analysis training
- Postgraduate statistics course – multiple seminar sessions
- Post-graduate writing workshop
- Preparing for the viva seminar
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