# A Prospective study of type 1 and type 2 Diabetics examining the correlation between antioxidant levels and neuronal layer loss. (DECAN)

## An exploratory study

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A Prospective study of type 1 and type 2 Diabetics examining the correlation between antioxidant levels and neuronal layer loss. (DECAN study)- An exploratory Study

Diabetic retinopathy has long been considered as a microvascular disease of the retina with complex pathways that contribute to the pathogenesis. There is significant evidence for the role of oxidative stress development of diabetic retinopathy and there is increasing interest in the role of antioxidant micronutrients in the prevention of diabetic complications. There is also growing evidence from clinical and laboratory studies to support neuronal damage in the retina of diabetic patients even before there are any fundal changes clinically. Studies have also examined the associations between macular pigment optical density and glycated haemoglobin (HbA1C) in type 2 diabetics with and without retinopathy. Although various theories have been proposed about neurodegeneration in diabetic retinopathy it remains unclear to what role it plays in the development and progression of diabetic retinopathy and if there is an early detectable relationship with antioxidant intake.

The aim of this study was to examine this further by examining the relationship between retinal neuronal layer changes assessed using spectral domain optical coherence tomography (SD-OCT) with dietary intake of foods. The development of diabetic retinopathy was then further explored to determine the relationship between severity of diabetic retinopathy with neuronal layer loss in individuals with type 1 and type 2 diabetes. The study also explored the relationship of HbA1C and serum lipid levels with macular pigment optical density measurements. Although a significant number of potential susceptible genes have previously been identified, the results of many studies have been limited by a number of negative findings so the exact roles in this area is still uncertain. Some potential single nucleotide polymorphisms (SNPs) were selected based on previous studies for analysis as part of this an exploratory study.

204 participants were recruited to the DECAN study. Outcome measures were dietary intake assessed using a food frequency questionnaire (FFQ), optical coherence tomography (OCT), macular pigment optical density (MOPD), glycated haemoglobin (HbA1c), serum lipids and deoxyribonucleic acid (DNA).

The results of the study showed that both outer nuclear layer (ONL) and the inner nuclear layer (INL) showed statistically significant associations with Vitamin B12, Pantothenic acid, copper and selenium. Selenium was seen to be associated with a thinner INL. The ONL showed a statistically significant association with vitamin B12, indicating that an increase in Vitamin B12 results in a thicker ONL whereas an increase in pantothenic acid is also associated with a thinner ONL.

At 12 months no correlations were evident between the FFQ data and severity of retinopathy or maculopathy. The study found that higher levels of MPOD values were positively associated with lower levels of triglycerides. There was no association was seen between MOPD scores, FFQ and OCT. Statistical analysis of the genotyping of 3 selected single nucleotide polymorphisms showed no statistically significant association between any of the groups. This study has added to the growing evidence that oxidative stress, antioxidants and neurodegeneration are critical factors in the pathogenies of diabetic retinopathy early on.

The study showed promising results for further research that could lead to an interventional trial using a supplementation of selenium which has not been previously reported.

Keywords: diet, diabetes, oxidative stress, optical coherence tomography, neurodegeneration.

To my children, my world

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List of abbreviations

| | |
|---|---|
| ACCORD | Action to Control Cardiovascular Risk in Type 2 Diabetes |
| ADVANCE | Action in Diabetes and Vascular Disease |
| AGES | advanced glycation end products |
| ALR2 | Aldose reductase gene |
| AMD | age related macular degeneration |
| AREDS | Age-Related Eye Disease Study |
| ATP | adenosine triphosphate |
| BDNF | brain-derived neurotrophic factor |
| BDR | Background diabetic retinopathy |
| BM | bruch's membrane |
| BMI | Body Mass Index |
| BP | Blood pressure |
| CC | choriocapillaris |
| CS | choroidal stroma |
| CSMO | Clinically significant macula oedema |
| CTU | Clinical Trials Unit |
| CWS | Cotton wool spots |
| DAG | diacylglycerol |
| DCCT | Diabetes Control and Complications Trial |
| DCRnet | The Diabetic Retinopathy Clinical Research Network |
| DES | Diabetic Eye screening |
| DM | Diabetes mellitus |
| DMO/DME | Diabetic macula oedema/edema |
| DNA | Deoxyribonucleic acid |
| DR | Diabetic retinopathy |
| DVLA | Driving and Vehicle Licensing Agency |
| EDIC | Epidemiology of Diabetes Interventions and Complications |
| ELM | external limiting membrane |
| ETDRS | Early treatment diabetic retinopathy study |

| | |
|---|---|
| FAME | Fluocinolone acetonide intravitreal implant for diabetic macular |
| FFA | Fundus Flourescein Angiography |
| FFQ | Food Frequency Questionnaire |
| FIELD | Fenofibrate Intervention and Event Lowering in Diabetes |
| GCL | ganglion cell layer |
| GSH | glutathione |
| HbA1c | Glycated haemoglobin |
| HDL | High density lipoprotein |
| HFP | heterochromatic flicker photometry |
| IDDM | Insulin Dependent Diabetes Mellitus |
| IFG | Impaired fasting glucose |
| IGT | Impaired glucose tolerance |
| ILAM-1 | intercellular cell adhesion molecule-1 |
| ILM | inner limiting membrane |
| INL | inner nuclear layer |
| IPL | inner plexiform layer |
| IRMA | Intraretinal micovascular abnormalities |
| IVTA | Intravitreal injection |
| LADA | Latent Autoimmune Diabetes of Adulthood |
| LDL | Low density lipoprotein |
| M | Maculopathy |
| MnSOD | Manganese superoxide dismutase |
| MOPD | Macular Pigment Optical Density |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NHS | National Health Service |
| NPDR | Non proliferative diabetic retinopathy |
| NSC | National screening committee |
| NVD | new vessels at the disc |
| NVE | new vessels elsewhere |
| NVI | new vessels at the iris |
| NVU | neurovascular unit |

| | |
|---|---|
| OCT | Optical Coherence Tomography |
| ONL | outer nuclear layer |
| OPL | outer plexiform layer |
| P | Photocoagulation |
| PARP | poly(ADP-ribose) polymerase |
| PDR | Proliferative diabetic retinopathy |
| PKC | protein kinase C |
| PR | photoreceptors |
| PRP | Laser pan retinal photocoagulation |
| PUFA | polyunsaturated fatty acid |
| R | Retinopathy |
| RAS | Renin-angiotensin-aldosterone system |
| RCO | Royal College of Ophthalmology |
| RGC | retinal ganglion cells |
| RNFL | retinal nerve fibre layer |
| RNFLT | retinal nerve fibre layer thickness |
| ROS | reactive oxygen species |
| RPE | retinal pigment epithelium |
| SCG | Scottish Collaborative Group |
| SD-OCT | Spectral Domain- Optical Coherence Tomography |
| SNP | Single nucleotide polymorphism |
| SOD | superoxide dismutase |
| TC | Total cholesterol |
| TD-OCT | time-domain optical coherence tomography |
| U | Ungradeable |
| UKPDS | United Kingdom Prospective Diabetes Study |
| VA | visual acuity |
| VEGF | Vascular endothelial growth factor |
| WESDR | Wisconsin Epidemiologic Study of Diabetic Retinopathy |

Food Frequency Questionnaire nutrient abbreviations

| | |
|---|---|
| Alcoh_g | Alcohol (g) |
| Biotn_ug | Biotin (ug) |
| CHO_G | Carbohydrate (g) |
| Ca_mg | Calcium (mg) |
| Caffeine | Caffeine (g) |
| Carot_ug | Carotene (ug) |
| Chol_mg | Cholesterol (mg) |
| Cl_mg | Chlorine (mg) |
| Cu_mg | Copper (mg) |
| Energ_kJ | Energy (kJ) |
| Fat_g | Fat (g) |
| Fe_mg | Iron (mg) |
| Fibre_g | Fibre (NSP) g |
| FolAC_ug | Folic acid (ug) |
| I_ug | Iodine (ug) |
| Kcal | Energy (kcal) |
| K_mg | Potassium (mg) |
| MUFA_g | Monounsatured fat (g) |
| Mg_mg | Magnesium (mg) |
| Mn_mg | Manganese (mg) |
| Na_mg | Sodium (mg) |
| Niacin_mg | Niacin (mg) |
| PUFA_g | Polyunsaturated fats (g) |
| P_mg | Phosphorous (mg) |
| PanAc_mg | Pantethenoic acid |
| PotNia_mg | Potential niacin |
| Protn_g | Protein (g) |
| Retin_ug | Retinol (ug) |
| Ribof_mg | Riboflavin (mcg) |
| SFA_g | Saturated fat (g) |

| | |
|---|---|
| Se_ug | Selenium (ug) |
| Starch_g | Starch (g) |
| Sugars_g | Total sugars (g) |
| Thiam_mg | Thiamin (mg) |
| Vt_B12_ug | Vit B12 (ug) |
| Vt_B6_mg | Vit B6 (mcg) |
| Vt_C_mg | Vit C (mg) |
| Vt_E_mg | Vit E (mg |
| Vt_K_mg | Vit K (mg) |
| Vt_d_ug | Vit D (ug) |
| Zn_mg | Zinc (g) |

# Chapter 1: Background

The following review was written based on Medline and Embase searches in 2015 and 2018. The initial search the following subheadings were applied 'Diabetic Retinopathy' in Medline – diagnosis, genetics, diet therapy, blood, complications and classification – and then limited the search to English Language articles and those discussing human patients. In Embase, 'Diabetic Retinopathy' and 'Antioxidants' were the focus of the articles, and also limited those to English language articles included animal and human patients.

Further searches were carried out on antioxidants and diabetic retinopathy focussing on dietary supplements, type 2, type 2, OCT and neuronal layer loss, genetics and diabetic retinopathy.

Subheading used were (DIABETIC RETINOPATHY and ANTIOXIDANTS) and (DIETARY SUPPLEMENTS or DIABETES MELLITUS, GENETICS AND DIABETIC RETINOPATHY, TYPE 1 or DIABETES MELLITUS, TYPE 2 or TOMOGRAPHY, OPTICAL COHERENCE or "neuronal layer loss").

Searches for Macular pigment optical density and diabetic retinopathy (focussing on serum lipids, diet, OCT) Used subheadings DIABETIC RETINOPATHY and ("macular pigment optical density" or (MACULAR PIGMENT and "optical density").

Searches for Serum lipids and diabetic retinopathy successful searches used subheadings ("serum lipids" or LIPIDS-blood) and (DIABETIC RETINOPATHY).

Limits applied: English Language to present and using full articles cited in the references of journal articles.

## 1.1 Definition of Diabetes

Diabetes mellitus is a group of conditions which causes either decreased production of insulin or systemic resistance to insulin effects which occurs when there is elevation and dysregulation of blood glucose levels. [1] When the body cannot produce enough insulin or use it effectively it can result in the damage of several other tissues that are sensitive to insulin such as the kidneys, nerves and the retina which can lead to heart attack, stroke, kidney failure, lower limb amputation and blindness. [2] It is a chronic condition and managing the disease can be a substantial burden for patients. The World Health organisation aims is to try and prevent type 2 diabetes and reduce the rates of complications and therefore improve the quality of life of individuals living with diabetes. [3]

## 1.2 Type 1 and Type 2 Diabetes

Type 1 diabetes – Insulin Dependent Diabetes Mellitus (IDDM)/ NO insulin

Type 1 diabetes is an autoimmune disease that whereby the body is not able to produce enough insulin to regulate the blood glucose levels. It is associated with a classic trio of symptoms at disease onset are polydipsia (excessive thirst), polyphagia (excessive appetite) and polyuria (excessive passage of urine) which have been described as the diagnostic hallmarks. [1, 2, 3].

It has historically been considered a disorder in children and adolescents, but this opinion has now changed as overt hyperglycaemia is also been identified in adults although to a greater extent in children and adolescents. [1] When it develops in adults over 35 years old it is sometimes referred to as Latent Autoimmune Diabetes of Adulthood (LADA). [2, 4]

However, the incidence of type 1 diabetes in young children (age <6 years) is rising. Type 1 diabetes (T1D) between 40-50% of children and young adults with type 1 diabetes will go on to develop microvascular complications. [5]

Additionally, there are still many gaps in knowledge regarding associations among and implications of nutrition, physical activity, glycaemic control, and hypoglycaemia in young children with type 1 diabetes. [6]

The deficit of insulin production requires daily administration of insulin. Symptoms of weight loss, vision changes and fatigue can also occur and can present suddenly. Individuals with type 1 diabetes need exogenous insulin replacements as without these they would not survive. Studies have shown that it is the most common childhood disease, is more common on boys and men. [6,7]

The cause of type 1 diabetes is still relatively unknown, it has been suggested that the autoimmune destruction of insulin- secreting pancreatic B cells of the pancreas causes type 1 diabetes. It is suggested that a series of functional effects in the bone marrow and thymus, immune system and β cells all contribute to the pathophysiology although some studies have shown that both type 1 and type 2 are both caused by a combination of genetic and environmental risk factors. [1, 5, 6, 8] and has been linked to seasonal changes and birth months. [8]

Patterson et al. (2015) examined the patterns of seasonal variations in the 23 EURODIAB registers of childhood diabetes in 19 European countries during the 20-yr. period 1989 to 2008. They also assessed seasonal variances and sunshine hours.

Unlike other studies they did not detect any impact of sunshine hours or in average temperature from the monthly norm in explaining the variances in the numbers of cases diagnosed each month. [9]

Type 2 Diabetes – Insufficient insulin

Type 2 diabetes is said to be caused by the ineffective use of insulin and studies have shown that it is usually as a result of excess body weight and physical inactivity. The symptoms are similar to type 1 but often the diagnosis is made much later than the actual onset by which time complications have occurred. Although usually only found in adults it is also now seen in children the contributing factor

is thought to be mainly due to being overweight and obese. The numbers are said to be on the rise and children as young as 4 yrs. old have been diagnosed as Type 2. The Diabetes UK estimates that there are 31,500 children and young people with diabetes, under the age of 19, in the UK.

Type 2 diabetes is increasingly diagnosed in obese children and adolescents. Evidence suggests that this disease commonly progresses more rapidly in youth compared to adults, and is associated with high rates of early microalbuminuria, hypertension and dyslipidaemia. [(10)]

Both forms of diabetes can cause long term complications such as cataract, neuropathy (peripheral nerve damage), nephropathy (progressive loss of renal function) and retinopathy.

## 1.3 Prevalence of Diabetes

In the European region the number of people with diabetes is 60 million [(303)] with some member states reaching a prevalence of 10-12% of the population. Diabetes UK reports that in the UK there are around 3.8 million people with Diabetes. Worldwide numbers are increasing; 422 Million adults for people are being affected by diabetes with 1.6 million deaths yearly being attributed to it.

This prevalence is thought to be associated with the increasing trends of unhealthy diets, physical inactivity and socioeconomic disadvantage leading to people being overweight and obese.

High blood glucose kills about 3.4 million people worldwide each year. Almost 80% of these deaths occur in low- and middle-income countries, and almost half are people aged less than 70 years. It is projected that the number of deaths due to diabetes will double between 2005 and 2030. It is also expected that the number of type 2 diabetics will double by 2030. [(11)]

The likelihood of developing diabetes is also affected by whether there is a family history. Genetics plays a strong role along with lifestyle and environment. [(12)]

## 1.4 Risk Factors

Diabetes is a disease that is strongly associated with both microvascular and macrovascular complications, including retinopathy, nephropathy, and neuropathy (microvascular) and ischemic heart disease, peripheral vascular disease, and cerebrovascular disease (macrovascular), and has been found to result in organ and tissue damage in approximately one third to one half of people with diabetes. [13]

All forms of diabetes that results in disease-specific microvascular changes are characterised by chronic hyperglycaemia. [1] When there is an excessive amount of glucose circulating the blood plasma this is referred to as Hyperglycaemia. Glucose levels will vary before and after meals, and continue to vary throughout the day. However, the normal blood glucose level in fasting adults is 4 – 7 mmol/L. If someone consistently measures above 7 mmol/L they will be considered hyperglycaemic, and one who consistently measures below 4 mmol/L is considered hypoglycaemic. [2]

The microvascular changes from hyperglycaemia can result in nephropathy, a variety of debilitating neuropathies and retinopathy. Accelerated macrovascular atherosclerotic change is also associated with diabetes. Studies have shown that people with diabetes are at higher risk of myocardial infarction, stroke, and limb amputation. [13]

Furthermore, those with diabetes are at increased risk of diabetic complications this is particularly in those with one or more components of the metabolic syndrome including dyslipidaemia, abdominal obesity, hypertension, prothrombotic state (Abnormality of blood coagulation that increases the risk of blood clots in blood vessels) and a proinflammatory state(increased cellular inflammation).

[11, 12, 13] Studies have shown that having a high body mass index (BMI) increases the risk of developing diabetes. [11, 15]. Whereas other studies found no association with BMI and complications of diabetes but concluded that the association was between BMI and blood pressure. [16]

Atchison and Barkmeier (2016) described the effects of blood glucose control and its effect on the clinical course of diabetes. Studies cited by Atchison and Barkmeier have shown that those with well controlled blood sugars are also at significantly lower risk for microvascular complications. [1] People with diabetes are also susceptible to developing cardiovascular disease which has also been associated with risks of hypertension (raised blood pressure). Different theories have been proposed as to why this happens which is described. It has been suggested that it is possibly due to hormonal control of blood sugar levels interacting with the renin-angiotensin-aldosterone system (RAS) at multiple levels and in both directions. Those with hypertension have increased rates of developing diabetes and those with diabetes have elevated RAS leading to hypertension. The risk of this has been shown to be reduced by using a pharmacologic blockade of the RAS. [1]

Mortality rate increases have been seen if there is a combination of hypertension and diabetes due to cardiovascular disease. Those with poorly controlled hypertension are twice as likely to die due to associated cardiovascular disease compared to those with diabetes with good pressure control. [1, 16]

Studies have also shown that there is a difference in the pattern of the disease in different ethnic groups. However, the findings amongst studies have been inconsistent and often multifactorial. The large-scale UK Prospective Diabetes Study (UKPDS) included White Caucasian, Asian Indian and Afro-Caribbean participants who were followed for a median of 18 years from entry to the end of post-study monitoring. They all had with newly-diagnosed type 2 diabetes.

Their findings showed that White Caucasian group, Asian Indian participants had reduced rates of all-cause death, and especially peripheral vascular disease. However, there was no between-group difference in fatal or non-fatal myocardial infarction. They found that the Afro-Caribbean ethnicity was associated with a substantially reduced risk of diabetes-related death, all-cause mortality, fatal

or non-fatal myocardial infarction and peripheral vascular disease compared with White Caucasian participants. They also found no differences in the rates of stroke or microvascular disease between the three ethnic groups. From these findings the authors suggested that UK-dwelling Asian Indian participants with Type 2 diabetes carry the greatest disease burden, but that White Caucasian participants have the worst prognosis. Whereas Afro-Caribbean participants enjoy relative protection from macrovascular disease. [17]

Several prospective studies have shown an increased risk for diabetes in men and women that smoke, which is also, associated with an increased risk in people with type 2 diabetes than type 1. [18] Smoking is also well-known risk factor for conditions such as atherosclerotic cerebrovascular and cardiovascular diseases which has been shown to be a leading cause of death in people with diabetes. [20] Smoking has also been shown to therefore have a harmful effect on people with diabetes by altering the lipid profile which in turn has an effect on cardiovascular health. [19]

Also, patients with diabetes have been shown to have higher triglyceride levels with lower high-density lipoprotein (HDL). [21] Triglycerides are a form of dietary fat found in dairy produce, meats and cooking oils. Lipoproteins are the form in which lipids are transported in the blood. Low density lipoprotein transports cholesterol from the liver to the tissues of the body. LDL is often referred to as the bad cholesterol" because too much is unhealthy. Whereas HDL is considered the "good cholesterol" as it is protective. Further explanation of cholesterol results are explained in table 1.1.

Table 1.1. Adapted from https://heartuk.org.uk/health-and-high-cholesterol/cholesterol-tests---know-your-number.

| Name | Description | Measure in mmol/L (millimoles per litre) |
|---|---|---|
| Total cholesterol (TC) | total amount of cholesterol in your blood. | Ideally it should be 5 or less |
| Non-HDL | total cholesterol minus your HDL-cholesterol (good cholesterol) and is the sum of all the “bad" cholesterols added together (including LDL cholesterol) | ideally it should be 4 or less |
| LDL | amount of LDL-cholesterol | ideally it should be 3 or less |
| HDL | the amount of good cholesterol | ideally it should be over 1 (men)<br>Over 1.2 (women). |
| Triglyceride | represent your body's ability to clear fat from the blood after a meal. | Ideally it should be less than 1.7 on a fasting sample or less than on a non-fasting sample) |

Legend:

TC= Total Cholesterol; Non-HDL=Non High-density lipoprotein; LDL = Low-density lipoprotein; HDL = high-density lipoprotein

NICE recommends all of the following once per year as part of the care process:

Blood Pressure

Body Mass Index calculated from weight and height to classify under, normal and overweight.

Serum creatinine – this blood test is used as measure kidney function.

Urinary albumin – this urine test which will detect earliest stages of kidney disease.

Cholesterol – to measure the fat in blood that can damage blood vessels.

Foot check – this checks the blood supply and sensation (feeling) in the feet.

Smoking Status

Digital Retinal Screening

HbA1c – this is a blood test for average blood glucose levels during the previous two to three

months. [22]

## 1.5 Diabetic Control

Type 2 diabetes accounts for 90% of people with diabetes worldwide and unlike Type 1 is largely preventable. To help prevent type 2 diabetes and its complications, people of all ages should achieve and maintain healthy body weight, be physically active, eat a healthy diet and avoid tobacco use (smoking increases the risk of cardiovascular diseases). It has been shown that obese men in comparison to women are at greater risk of developing complications such as cardiovascular, renal and ocular. [1] Many of the health risks associated with increasing body weight may first appear in children and young people and therefore advice on a healthy diet and exercise is crucial.

Individuals with impaired glucose tolerance (IGT) or impaired fasting glycaemia (IFG) are in the intermediate stage between normality and diabetes and are at high risk of developing type 2 diabetes. This risk can also be drastically reduced through intensive lifestyle modification and pharmacological intervention. Drug treatments may be required such as tablets or injectable medication. [23]

For people with type 1 diabetes the usual treatment is insulin delivered by either a pen or a pump but staying healthy and active is also important to minimise the risk of long-term complications.[23] However new treatment options are now available as described by Diabetes UK and include islet transplantation. Type 1 diabetes results from the destruction of insulin-producing cells in the islets of the pancreas. Islet cell transplantation involves extracting islet cells from the pancreas of a deceased donor and implanting them in the liver of someone with type 1 diabetes.

Diabetes UK provides information on the operation which is minor procedure performed under local anaesthetic that is usually done twice for each transplant patient. The first government-funded islet transplant programme in the world was performed in the UK and now 2008 Islet cell transplants have been available in the UK through the NHS. Since the launch hundreds of islet transplants have

been performed in the UK. Islet transplants have been shown to reduce the risk of severe hypos. (Hypoglycaemia). Hypoglycaemia can be triggered when a person with diabetes has too little food or too much insulin and that causes the blood sugar levels to fall dangerously low. Common symptoms associated with hypoglycaemia are feeling hungry, dizzy, sweaty, and trembling, a change in mood or finding it hard to concentrate. Results from UK islet transplant patients showed that the frequency of hypos was reduced from 23 per person per year before transplantation to less than one hypo per person per year afterwards as reported by Diabetes UK, there was also improved awareness of hypoglycaemia, less variability in blood glucose levels, improved average blood glucose and improved quality of life.

The Long-term results have proven to be good as the majority of transplant patients have been reported to now expect to have a functioning transplant after six years and some people have had more than 10 years success but many still need to take a low dose of insulin.

There are some risks however associated with the transplant such as of certain cancers, severe infections and other side effects related to the medication needed to prevent the islets from being rejected by the body have been associated with Islet transplants.

Other research is also being carried out looking at encapsulated islet cells as a treatment for type 1 diabetes which has been described as an advanced form of transplantation. Other treatment options in the research stages include the use of artificial pancreas systems and diabetes vaccines.

Treating diabetes is expensive. It costs the NHS £10 billion each year. However this is mainly because of the increased number of complications, things like amputation, blindness, kidney failure and stroke. It is anticipated that these cost pressure from diabetes for the NHS will get worse.

Preventions are needed to stop these complications developing and by treating them as early as possible both limits their impact on the person's life and saves the NHS money.

## 1.6 Diabetic complications

Diabetes remains an increasing health problem worldwide and is associated with premature coronary artery disease, renal failure and amputations but also blindness. There are several associated complications associated with diabetes which include cataracts, nerve palsies and glaucoma. The World Health Organisation have reported that the most common eye disorder associated with blindness if left untreated is diabetic retinopathy.

The management of systemic disorders is vital to preventing diabetic eye complications as well as other serious complications. Control of hypertension and hyperlipidaemia (abnormally elevated levels of any or all lipids or lipoproteins) has both been associated with delayed or reduced onset of diabetic retinopathy. [(1)]

Hypertension is said to coexist with diabetes with an incidence of 3 times greater chance of having hypertension in type 2 diabetics compared to those without diabetes. [(16)]

Studies have also shown that 50% of people with diabetes die of cardiovascular disease (primarily heart disease and stroke), and 10-20% of people with diabetes die of kidney failure. The overall risk of dying among people with diabetes is at least double the risk of their peers without diabetes. [(20)]

Long-term accumulated damage to the small blood vessels in the eye leads to diabetic retinopathy, an important cause of blindness. The World Health Organisation suggested that after 15 years of diabetes, approximately 2% of people become blind, and about 10% develop severe visual impairment.

## 1.7 Definition of Diabetic Retinopathy (DR)

Diabetic retinopathy is a disease of the retina, and is the leading cause of acquired blindness. Diabetic retinopathy has been classically considered a microvascular complication of diabetes that

threatens all individuals with diabetes. However other mechanisms are thought to also play a part. The microvasculature of the retina becomes damaged, the blood vessels swell and leak fluid, and if not prevented, new vessels start to grow (neovascularisation), and ultimately lead to the detachment of the retina It is a sight threatening disease that has been described to be characterised by both neuronal and vascular dysfunction in the early stages followed by neovascularisation in the later stages that further damages the vision. [24]

If left untreated it can lead to vision loss or blindness. This is then a leading cause of visual impairment in the working age population. [25]

## 1.8 Definition of Diabetic Maculopathy (DMO)

Diabetic maculopathy or diabetic macular oedema (DMO or DME) results from diabetic retinopathy and is where the macula area is affected mainly by disruption to the blood-retinal barrier. [26]

Diabetic macular oedema (DMO) is described as a thickening of the central retina, or the macula, and is associated with long-term visual loss in people with diabetic retinopathy. Clinically significant macular oedema (CSMO) is the most severe form of DMO. [27]

This could be in different forms but most commonly due to leakage of fluid or protein onto the macula causing the diabetic macula oedema.

The 3 basic patterns of oedema which can occur are:

- diffuse,
- cystoid
- serous detachment.

The macula is responsible for our central vision and if there is build-up of fluid or exudates (deposits of fat from blood) then symptoms of blurred vision are noticed. The decreased vision is caused by the fluid distorting the retinal architecture. It builds up due to the breakdown in the barriers within

the retinal blood vessels. This results in blurred vision which can occur at any stage in diabetic retinopathy. The diabetic macula oedema is detected by retinal thickening which can occur with or without the accumulation of the lipids (seen clinically as hard exudates) and proteins. [24] Therefore, the developments of non-invasive imaging of the retina and macular have contributed significantly to the diagnosis and monitoring of this condition.

## 1.9 Classification of Diabetic Retinopathy and maculopathy

Diabetic retinopathy progresses through different levels and the features at each stage have been described. Historically the classification and grading of diabetic retinopathy and maculopathy has been based on clinicians viewing the retina ophthalmoscopically and describing the signs based on increasing severity. Diabetic retinopathy can be categorised as either non-proliferative (NPDR) or proliferative (PDR) based on the absence or presence of neovascularisation. NPDR can then be subdivided into levels of severity mild, moderate or severe. [28]

The features of non-proliferative diabetic retinopathy (NPDR).

**Microaneurysm**s which are said to be the early signs of diabetic retinopathy are small circular red lesions (dots) in the retina. They are usually localised capillary dilatations and are saccular (round). They occur in isolation or in clusters and are associated with mild signs of diabetic retinopathy.

**Intraretinal haemorrhages** are larger more irregular red lesions that are generally 'dot' or 'blot' and are referred to as dot/blot haemorrhages but they may also be flame shaped depending on where they are within the retina. If within the nerve fibre layer, they are usually flame shaped they are superficial whereas in the deeper inner retinal layer they are the dot/blot ones. They are not thought to be vision threatening as they usually resolve in 3-4 months.

**Hard exudates** are yellow- white irregular shaped lesions that are intraretinal deposits of lipids and protein. They can occur as a single hard exudate or larger patches. They may also become ring shaped known as circinates or form into much larger confluent plaques. They are largely made up of

extracellular lipid which has leaked from abnormal retinal capillaries which can cause retinal oedema that is not visible using direct ophthalmoscopy. However, the suspicion of the underlying problem is often apparent as the exudates will form a ring or 'circinate' pattern around a cluster of micro aneurysms which are the leaking vessels, which can then be confirmed now using the advanced techniques of optical coherence tomography. When exudates form in the macula area it is termed macula oedema and the vision can be severely affected. [28, 29]

**Cotton wool spots** are distinct fluffy patches that have been said to appear as retinal nerve fibre layer due to ischemia, however this has been debated. The initial theory was that the pathophysiology of the development of CWS is an occlusion of precapillary arterioles, which causes a focal area of ischemia. However experimental models have clearly established this disruption of axoplasmic flow. [30]

They are described by many to be cystoid bodies because they look like cells. The disruption to the axoplasmic flow results in accumulation of organelles, mitochondria, secretory and enzymatic vesicles, and other axoplasmic constituents. [31]

The Early Treatment of Diabetic Retinopathy Study Report described them as "soft exudates" as "localized superficial swellings in the nerve fibre layer. They are round or oval in shape, white, pale yellow-white, or greyish-white in colour, and have ill-defined (feathery) edges, frequently with striations parallel to the nerve fibres." [29]. They are also associated with a number of other conditions such as arterial hypertension, central and branch retinal vein and artery occlusions, ischaemic optic neuropathies, carotid artery occlusion plus many infections such as toxoplasmosis, HIV, AIDS, and immune and collagen vascular diseases such as systemic lupus erythematous, dermatomyositis, rheumatoid arthritis, giant cell arteritis Waldenstroms macroglobulinemia ,Embolic phenomena. Many other conditions include IV drug abuse, Purtscher and Purtscher-like retinopathy, Hypercoagulability or hyperviscosity syndromes, Malignancy, Papillitis, Papilledema,

Transient hypoperfusion, Anaemia (severe), High altitude retinopathy, Radiation retinopathy and Epiretinal membrane. [31]

Venous beading, dilation, loops and duplication occurs adjacent to areas of no perfusion and reflects retinal ischemia. They have been described as predictors for advanced stages of diabetic retinopathy. [28]

Retinal pallor has been described as a non-specific feature that is only seen on red-free photos and fluorescein angiography. [28]

Intraretinal microvascular abnormalities (IRMA), areas of capillary dilatation. They are described as shunt vessels that appear as abnormal branching or dilation of existing blood vessels that act to supply areas of non-perfusion within the retina in diabetic retinopathy. They are described as vessels that "represent either new vessel growth within the retina or remodelling of pre-existing vessels through endothelial cell proliferation stimulated by hypoxia bordering areas of capillary nonperfusion". They may also be a variant of collateral formation and maybe seen in association with cotton wool spots or localised arterial occlusion. They may also be confused with dilated telangiectasia which can be seen in young patients with hyperaemia. [28]

Features of proliferative diabetic retinopathy (PDR).

Proliferative diabetic retinopathy results due to extensive capillary closure as the angiogenic response of the retina. [28] Neovascularisation, new vessels develop that originate from large veins grow at the interface of non-perfused retina and perfused retina, these can be seen either as new vessels at the disc (NVD) or described as new vessels elsewhere (NVE) due to the retina becoming more ischaemic. They are usually seen as fine tufts of fragile new vessels that are on the surface of the discs or coming from vessels of the major arcades on the surface of the retina. As they are very fragile, they can easily bleed. [28] Vascular endothelial growth factor (VEGF) which is a growth factor has been described as a contributing factor due to its response to multiple stimuli causing excessive

production of VEGF that induces the formation of new blood vessels and the permeability of existing vessels increases. [32]

They may also occur on the iris (NVI- proliferative). It is fairly uncommon but if seen can indicate more advanced ischemic changes. However they can also occur in association with other conditions such as central retinal artery/vein occlusion. New vessel formation can also occur on the anterior hyaloid when insufficient laser has been applied to the peripheral retina post vitrectomy. [28]

Preretinal or sub hyaloid haemorrhages can be the result if the new vessels bleed into the space between the retina and vitreous gel. They can appear as pockets of blood between the posterior hyaloid face and the retina. [28, 33]

Vitreous Haemorrhages that develop into the vitreous as a result of the new vessels bleeding can cause clumps of blood to be seen that causes a diffuse haze or floaters. The new vessels can form abnormal adhesions and when there is localised detachment blood can form which is said to have a boat shaped appearance, sub hyaloid haemorrhage. [28, 33]

The vitreous haemorrhage will often clear the visual axis as the blood collects inferiorly and forms a posterior vitreous detachment. If the blood doesn't do this then surgical intervention is required to have the blood removed (vitrectomy). [28]

Retinal fibrosis happens as the new vessels that grow on a platform of glial cells mature or regress. When there is repeated vitreous haemorrhage, the associated glial cells are at risk of scarring. Gliosis (fibrosis) may occur which results in fibrous tissue spreading across the inner surface of the retina, or optic disc or into the vitreous cavity. Studies have described serious complications due to the formation of the fibrotic membrane including retinal traction or detachment resulting in vision loss.

However just describing the signs have been shown to be unreliable as severe visual loss due to maculopathy may occur with only moderate signs of retinopathy.

The risk to sight in patients with diabetic retinopathy is known to come from 2 routes:

- growth of new vessels leading to intraocular haemorrhage and possible retinal detachment with profound global sight loss.
- and localised damage to the macula / fovea of the eye with loss of central visual acuity. [28]

The classification of the full disease has been developed from the original Airlie House Classification [33-35] .This was later modified by the Diabetic Retinopathy Study. It consisted of seven field stereo photo's that were standard images and compared them with the patient's findings in the corresponding fields. [34] The same classification was modified and used in the Early Treatment Diabetic Retinopathy Study (ETDRS) and remained the gold standard for many years. [35] In research however the complexity of it was seen to be too time consuming for everyday clinical practice. [36] The modified Airlie House Classification of diabetic retinopathy classifies diabetic retinopathy into 13 complex levels ranging from level 10 (absence of retinopathy) to level 85 (severe vitreous haemorrhage or retinal detachment involving the macula) by the grading of stereo photographs of 7 fields. [35]

Various versions have since been used in clinical research, clinical practice or reduced versions of the ETDRS have been adapted for countries without diabetic retinopathy screening programmes.

As diabetic retinopathy is vision threatening there was a need to develop a classification that could be used by clinicians and graders that would reflect the risk. The classifications all recognise the retinopathy with risk of new vessels and maculopathy as risk of damage to the foveal area.

The classification system that was adopted by the National Screening Committee (NSC) for use in England and Wales is aimed at detection and onward referral. The classification flags the level of the disease that is sufficiently severe to merit referral of the patient for expert ophthalmological opinion and possible treatment. The classification is simple and is based on identifiable features that an

appropriately trained photographer/grader can follow. It identifies four types of presentation assigns a letter which indicates the disease, treatment or not able to grade and a score that indicates the severity. R (retinopathy), M (maculopathy), P (photocoagulation) and U (unclassifiable). A similar classification was introduced by the Scottish Diabetic Retinopathy Grading Scheme. [37]

The different classifications described can be approximately mapped to each other as shown in Table 1.2 as cited in RCO guidelines. [28]

Table 1.2 Summary of different classifications adapted from Royal College of Ophthalmology diabetic retinopathy guidelines

| NSC | ETDRS | SDRGS | AAO international |
|---|---|---|---|
| R0 None | 10 None | R0 None | No apparent retinopathy |
| R1 background | 20 microaneurysms only | R1 Mild background | Mild NPDR |
| | 35 Mild NPDR | | Mod NPDR |
| R2 preproliferative | 43 Moderate NPDR | R2 Moderate BDR | |
| | 47 Moderate severe NPDR | | |
| | 53 A-D severe NPDR | R3 severe BDR | Severe NPDR |
| | 53 E very severe NPDR | | |
| R3 proliferative | 61 Mild PDR | R4 PDR | PDR |
| | 65 Moderate PDR | | |
| | 71, 75 High risk PDR | | |
| | 81,85 Advanced PDR | | |

Legend:

ETDRS = Early Treatment Diabetic Retinopathy Study; AAO = American Academy of Ophthalmology; NSC = National Screening Committee; SDRGS = Scottish Diabetic Retinopathy Grading Scheme; NPDR = non-proliferative diabetic retinopathy; BDR = background diabetic retinopathy; PDR = proliferative diabetic retinopathy.

## 1.10 Prevalence of Diabetic Retinopathy

Diabetic retinopathy is the leading cause of blindness of patients of working age in the UK. At least 2% of the UK population are known to have diabetes. Currently the diagnosed population with diabetes is 2.9 million, with 13% of those having sight threatening diabetic retinopathy. By 2025 it is estimated that five million people will have diabetes. Zhang et al. (2010) looked at the number of cases in the United States between 2005-2008 and found diabetic retinopathy to be the leading cause of new cases of legal blindness amongst adults aged between 40 years and older. They also found the prevalence to be much higher in non-Hispanic black individuals. However, they did not distinguish between type 1 and type 2 of diabetes and specific risk of diabetic retinopathy complications and the small sample sizes could have been a limiting factor in their analysis. [38]

There are major negative public health and economic consequences due to the epidemic rise of diabetes particularly in low and middle-income countries. In the sub-Saharan Africa (SSA) region there is the highest predicted percentage growth in diabetes. In a recent study by Bastawrous et al. (2017) they found that in a population of 1.6 Million in Nakuru County, Kenya: out of 150,000 they estimated that 1650 people over the age of 50 will develop diabetes mellitus per year, and 450 will develop diabetic retinopathy. [39]

Although some studies have described the association of possible risk factors such as hypertension and hyperlipidaemia for retinopathy in people without diabetes it still remains inconsistent across different populations as cited by Swetha et al. (2010). [40] Differences in the prevalence between men and women without diabetes have also been seen. Swetha et al. (2010) in their study found in all age groups that more men than women were found to have retinopathy. However, for both men and women aged between 50 and 69 years there was a peak which then declined. [40]

Some estimates predict that the worldwide prevalence of diabetes by 2030 will exceed 366 million. As diabetic retinopathy is a common complication for individuals with diabetes there is a huge risk of blindness amongst this population.[41, 42,43,44] The vision loss occurs due to a variety of mechanisms

including retinal detachment, pre retinal or vitreous haemorrhage, macular oedema, capillary non perfusion and is also associated with neovascular glaucoma. [38]

The World Health Organization estimates that diabetic retinopathy accounts for approximately 5% of the global prevalence of blindness, with estimates of 15% to 17% in developed countries. It has been suggested that despite all the advances in diabetic care, 90 % of people with diabetes for over 15 years will have some form of diabetic retinopathy. [45, 46]

It has also been shown that signs of diabetic retinopathy may precede the actual clinical diagnosis of diabetes in high risk populations. Studies have found that as high as 5-10 % of people without diabetes are found to have signs of diabetic retinopathy but it is argued they are mild. [47]

Swetha et al. (2010) carried out a population based cross sectional study of an Asian population aged between 40-80yrs old in Singapore. They found that 2500 of the 3280 patients that they studied did not have diabetes but 6% of those did have signs of diabetic retinopathy. [40] Other studies such as the Hoorn study, Blue Mountains study and Beaver Dam study have shown similar results as cited by Olafsdottir et al (2014). [46, 48]

## 1.11 Risk Factors for Diabetic Retinopathy

Studies have shown a lower incidence and slower progression of diabetic retinopathy with tight blood glucose. The risk of vision loss due to diabetic retinopathy has been said to be reduced by its early detection and treatment but also by the effective control of serum, glucose and blood pressure. [49]

Hypertension is also a well-known risk factor for many chronic conditions and by lowering the blood pressure there have been beneficial effects. [50] However, in regards to the development or progression of diabetic retinopathy the findings have been variable.

Liu et al. (2013) investigated the progression and regression of diabetic retinopathy and the effects of population risk factors on the rates of transition across retinopathy stages They found that the

risk of progression from no diabetic retinopathy to mild, mild to observable, and from observable background diabetic retinopathy to severe non proliferative/proliferative diabetic retinopathy was strongly positively associated with glycaemic exposure. They also found a significant risk effect with blood pressure with a slight difference between diastolic and systolic point on the state of diabetic retinopathy transition. [51]

There was no evidence in this study that other risk factors such as sex, smoking status, BMI, serum creatine or HDL influenced the diabetic retinopathy after adjustment for glycaemic control and HbA1C. Saif et al. (2014) in their study concluded that both systolic and diastolic blood pressure are important risk factors for the progression of retinopathy in normotensive patients with type 2 diabetes. However, they also considered the risk of atherosclerosis and found a strong correlation between carotid intima-media thickness with both blood pressure and diabetic retinopathy I the same patients and which they concluded could be a valuable marker for carotid atherosclerotic lesions. [52]. Patients with proliferative diabetic retinopathy showed both a higher diastolic and systolic blood pressure along with a higher carotid intima-media thickness in comparison to patients with non-proliferative diabetic retinopathy.[52]

Studies have demonstrated the importance of blood glucose control to delay or slow down the progression of ocular complications due to diabetes but findings that have advocated blood pressure control for the same reason have supported varying conclusions. [53]

A Cochrane review by Do et al. (2015) examined 15 RCTs that were conducted in North America and Europe examining the effects of blood pressure control in type 1 and type 2 diabetics looking at evidence that could further support the theory that reducing blood pressure had a beneficial effect in preventing or slowing the rate of progression of diabetic retinopathy. The review found that although there was considerable variability amongst the trials that they identified with regards sample size, eligibility, outcomes reported etc., lowering blood pressure in several chronic conditions does show beneficial effects. They found that with regards diabetic retinopathy the available

evidence supports the effects of preventing diabetic retinopathy for up to 4-5 years with the intervention of lowering the blood pressure but lacked support to conclude that lowering blood pressure would solely prevent diabetic retinopathy however a close relationship with the severity of diabetic retinopathy in a large number of type 1 and type 2 diabetics has been seen so advocating good control could prevent damage to vision. [54]

One study did find that for every 10mmHg increase in systolic blood pressure the risk of diabetic retinopathy was increased by 1.23 times and a 1.19 increased risk of vision-threatening retinopathy. Whereas with increasing diastolic blood pressure the risk reduced. [1]

In the UKPDS study a 34% reduction in progression of retinopathy was seen with tight blood pressure control using angiotensin- converting enzyme inhibitor or a β-blocker. However, the ACCORD study showed no significant difference between intensive blood pressure control vs. standard blood pressure control. [55, 56]

Atchison and Barkmeier (2016) examined the existing literature to review the links between glycaemic control, high cholesterol and hyperlipidaemia, arterial hypertension, obesity, inflammatory markers, sleep disordered breathing and exercise and the risk of developing diabetic retinopathy but they also looked at prevention of diabetic retinopathy. Whilst lots of literature discuss the benefits of good glycaemic control and some argue also blood pressure control, the effects of cholesterol and lipid control, inflammatory markers, sleep disordered breathing, obesity and exercise are less well established. [1]. Whilst the above have all been studied intensively as major risk factors there are still variations in consistency and pattern of these factors. Some suggest that it is actually the duration of diabetes that is an important predictor for diabetic retinopathy but not necessarily type as cited in Lima et al. (2016).[57] Whereas Forga et al. (2016) in their study found that rate of diabetic retinopathy was increased with age in type 1 diabetics. They also confirmed the influence of HDL-cholesterol, glycaemic control and diastolic blood pressure on the occurrence of retinopathy. [58]

Body mass Index (BMI) in diabetics is a well-known risk factor. In the Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetics Study (SN-DREAMS-I study) they investigated the association of obesity and diabetic retinopathy. They carried out a cross sectional study between 2003-2006 of an urban Indian population. They found the prevalence of generalised obesity was more in women than men. Obesity was defined as generalised obesity which was by the BMI and waist circumference and abdominal obesity which was the waist to hip ratio. Whereas the abdominal obesity was more in men than women, a high waist to hip ratio was found to be associated with diabetic retinopathy but not with severity. When considering diabetic retinopathy, they found that the prevalence of diabetic retinopathy and sight threatening diabetic retinopathy was more in the isolated abdominal group. [59]

Other studies have found that BMI either had no relationship or showed an inverse relationship with diabetic retinopathy when BMI was considered as a continuous variable. [59-61]

A Cochrane review by Zhou et al. (2017) identified 27 articles that examined the risk of diabetic retinopathy and BMI as shown below. Although there were limitations to the analysis, they found no association between being overweight and the risk of diabetic retinopathy. [63]

In support of this Chan et al. (2018) found in a population-based cohort of Asian adults of Malay and Indian ethnicities, they found that a higher BMI was associated with an increased incidence of diabetes mellitus, but lower incidence of diabetic retinopathy. This was independent of potential cofounders such as age, gender, ethnicity, duration of diabetes and HbA1c. When BMI was analysed either as a categorical or continuous variable the associations were consistent. In addition, when stratified by gender and ethnicity, the analysis showed that the associations were consistent. [61]

In other studies ethnicity has been shown to be a significant risk factor associated with the development of diabetic retinopathy. In America African Americans were found to have a high prevalence of type 2 diabetes and seemed to have an associated high risk for microvascular

complications including diabetic retinopathy. Some studies have found that this risk is higher than in Caucasians after adjusting for other clinical risk factors.

Penman et al. (2016) [66] however looked specifically at proliferative diabetic retinopathy and found an increased risk of association in a case-controlled study of African Americans with type 2 diabetes along with duration of diabetes, systolic hypertension and insulin use but not glycaemic control. In their study the participants that were using insulin had much higher odds of having proliferative diabetic retinopathy than those not on insulin. This has been seen in other ethnic groups. [66]

Glycated haemoglobin (HbA1c) reflects the blood glucose concentration and is used to monitor the glycaemic control in patients. Some studies have found HbA1c to be the most important factor associated with prevalence of diabetic retinopathy. This was shown in a study of Type 2 diabetic patients in Taiwan but only in those who had been diabetic for a fixed duration. Stewart et al. (1993) found that duration of diabetes influenced the prevalence of diabetic retinopathy. [45]

In the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study (DCCT/EDIC) studies showed that intensive treatment reduced the risk of onset and progression of diabetic retinopathy in patients with type 1 diabetes compared with conventional therapy. Furthermore, in the EDIC study they concluded that the early effects of metabolic control continue to accrue over many years affecting the future development of diabetic retinopathy which they described as metabolic memory. [67]

In the DCCT and EDIC the concept of the “metabolic memory” phenomenon is described where there has been hyperglycaemia in the months and years following the onset of diabetes and it is “remembered” despite improvement of subsequent glycaemic control. [69]

In the DCCT, patients with type I diabetes were randomized to receive either conventional insulin therapy intensive insulin therapy. Those receiving intensive insulin therapy showed significantly fewer diabetic complications such as nephropathy, neuropathy, or retinopathy after a mean of 6.5

years. [68]. The conventional insulin therapy group was then also offered intensive insulin therapy during the follow-up in the EDIC study. It was demonstrated in this study that patients previously allocated to the intensive insulin therapy group continued to have a lower incidence of diabetic complications compared to the conventional group. This was also despite there being no subsequent difference in treatment or HbA1c levels between the groups. [68]

In a similar study of patients with type II diabetes, the UK Prospective Diabetes Study (UKPDS) demonstrated similar long-term benefit of intensive glycaemic control on diabetic complications which they termed the 'legacy effect'. [70, 71]

Both these studies emphasize that in patients with diabetes it is important to optimize glycaemic control as early as possible. [67] However also conversely highlighting the damaging effects of long-term hyperglycaemia which can persist even after it has been controlled. [72]

Wang et al. (2017) and other cited authors have also suggested that further analysis of data from the entire DCCT cohort suggested that HbA1c levels and duration of diabetes only account for approximately 11% of the variation in risk of diabetic retinopathy, suggesting that other components play a role. [72]

Studies have examined the optimal levels for HbA1c and the association with the development of diabetic retinopathy. Thresholds of 48mmol/mol (6.5%) have been suggested but this has been the topic of debate for many years. [73] The importance of good control is well documented. [73] A more recent study by Nakagami et al. (2017) support this as their study recommended that values of 6.5% HbA1c (48mmol/mol and fasting plasma of 7.0mmol/L could be used as diagnostic thresholds for diabetes. [74]

In 2015, the American Diabetes Association reported that according to the Clinical Practice, glycosylated haemoglobin (HbA1c) was recommended as an excellent predictive marker for the diagnosis of diabetes. They advised that each 1% reduction in HbA1c lessens the risk of developing

systemic and ocular complications by 40 %.3 .The measurement of HbA1c is considered as important as blood glucose measurement. The level of HbA1c is a reflection of average plasma glucose over the past 2–3 months. It can be carried out without patients fasting and can be measured at any time. Recently, there has been an increased interest to use HbA1c as a marker for screening of those at high risk of developing diabetes as it shows high sensitivity and specificity.[10] The use of HbA1c is considered as one of the most discriminative and effective tools for the diagnosis of diabetic patients who are vulnerable to develop complications including retinopathy. [(75)]

The World Health Organization defines diabetes mellitus as a fasting venous plasma glucose level that is equal to or higher than 7.0 mmol/L or venous plasma level is equal to or greater than 11.1 mmol/L, measured 2 hours after oral intake of 75 g glucose.

However, many studies have shown that other risk factors such as glycaemic control, hypertension, and hyperlipidaemia all may contribute but genetic factors are also said to play an important part and several papers have looked at the roles that they may play in the development of diabetic retinopathy. In a study by Roy and Klein (2001) they reported that sex, blood pressure, HbA1c, proteinuria and peripheral vascular disease were all associated with the presence of hard exudates on African Americans with type 1 diabetes. Whilst other studies have found relationships with blood pressure, renal function and the presence of hard exudates not all authors were able to replicate the same associations. Serum lipid levels such as total cholesterol levels and LDL cholesterol have been seen to consistently be associated with hard exudates. [(76)]

However, the reported association between hard exudates and triglycerides has been quite inconsistent and somewhat controversial. Chew et al. (1996) reported that the presence of hard exudates was not associated with serum triglycerides but was associated with the rapid onset of hard exudates. [(77)]

The Early Treatment of Diabetic Retinopathy Study found that by lowering the lipids it may also reduce the risk of hard exudates and therefore prevent vision loss in patients with diabetic retinopathy. [78]

Most studies usually assess the presence of hard exudates qualitatively whereas in a study by Sasaki et al. (2013) they found that this could be done quantitively using a semi-automated method. They investigated the association between hard exudates and diabetic risk factors using multiple linear regression models and found that those with high lipid levels and higher triglyceride levels demonstrated a higher risk of central macular involvement. [79] The exact role of serum lipids in the pathogenesis of diabetic retinopathy remains controversial, although they have been reported as a risk factor for both diabetic retinopathy and diabetic macular oedema. [80]

Agroiya et al. (2013) found a strong correlation between serum lipids levels and the severity of diabetic retinopathy. [81] This suggested that a much simpler and also precise method of analysing hard exudates in the eye could be used but further research was required in this area to confirm the central location and overall total hard exudate area and the association with triglycerides in a quantitative way. [79] In a study of a Chinese patients with type 2 diabetes they found a similar association of higher triglyceride levels being independently associated with an increased risk of diabetic retinopathy which could suggest that by controlling serum lipid levels it could reduce the risk of diabetic retinopathy. [82] In the FIELD study (Fenofibrate Intervention and Event Lowering in Diabetes Study of type 2 diabetics, those treated with fenofibrate compared to controls were less likely to need laser treatment. [78]

Cigarette smoking has also been suggested as a risk factor for the incidence and progression of diabetic retinopathy but the results have been inconsistent with both positive and negative associations described. [83-88] However smoking in diabetics is associated with other complications such as cardiovascular disease which is a leading cause of death. [20]

It has been proposed that if the modifiable risks such as blood glucose. Systolic blood pressure, weight and tobacco use are controlled in patients with long term diabetes, the risk for developing diabetic retinopathy could decrease. [89]

Overall, as proposed by Stitt et al. (2016) there is clear clinical evidence that demonstrates that good blood glucose control if implemented early in the course of Type 1 and Type 2 diabetes it could possibly reduce the development and progression of retinopathy and other vascular complications of diabetes. However as the duration of diabetes increases, the longer-term exposure to "'environmental stresses (as experienced by the macro and micro-vasculature) associated with the presence of diabetes, continued injury mediated by 'glycaemic memory', and the establishment of cumulative structural vascular damage, may mean that the benefits of achieving better glycaemia decline as patients age." [90]

Table 1.3 Summary of Modifiable risks and Non modifiable risks adapted from Jenkins et al. (2015) [91]

| Non -Modifiable | Modifiable |
|---|---|
| Genetics | Hypertension |
| Family History | Dyslipidaemia |
| Ethnicity | Other diabetic complications |
| Age | Obesity |
| Duration of diabetes | Smoking |
| Type of diabetes | Anaemia |
| | Pregnancy |
| | Social factors |

In the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) Study the results showed that HbA1c, blood pressure, and total cholesterol only accounted for about 10% of the variability in retinopathy risk and that other factors contribute. [36]

However, it has been suggested that biomarkers related to these major retinopathy risk factors can be used to guide retinopathy screening intervals so as to increase the cost-efficiency of this

resource. Recent reviews support the extension of retinal screening intervals from the generally recommended annual review to every two years for people with well-controlled type 2 diabetes and no diabetic retinopathy. There are many suggestions of introducing personalized screening intervals. This would be bases on a mathematical calculation to consider gender, type and duration of diabetes, glycaemic control, blood pressure, and current retinopathy grade. [36]

Whilst major risk factors for the development and progression of diabetic retinopathy are known it has been proposed that biomarkers could be used as already in other conditions.

A biomarker can be defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention ". For diabetic retinopathy the biomarkers in could include HbA1c levels, carotid intima media thickness, serum creatinine levels.

The terms biomarker, risk factor, and determinant are often used interchangeably. The World Health Organization defines a risk factor "as any attribute, characteristic, or exposure of an individual that increases the likelihood of developing a disease or injury" so include for smoking, hypertension, and hyperglycaemia.

HbA1c is a measure of glycaemic control and is strongly associated with diabetic retinopathy therefore is a risk factor. Other aspects of glucose control include glycaemic variability and alternate assays. These aspects may also be regarded as biomarkers however they are not well studied in relation to diabetic retinopathy. Other biomarkers with a similar process can be observed for lipid levels as high total and LDL-cholesterol (LDL-C), triglycerides, and low HDL-C levels are traditional risk factors for cardiovascular disease, and some have suggested also for diabetic retinopathy. (91)

It has been suggested that biomarkers could also guide treatment choice. This could include the identification of subgroups of patients with diabetes and retinopathy according to their different

responses to treatment such as intraocular anti-VEGF treatment and examine those that respond or not at all. [91]

## 1.12 Screening for Diabetic Retinopathy

The aim of screening is to reduce risk from a particular condition in an identified population. [28]

As defined by https://www.gov.uk/guidance/diabetic-eye-screening-programme-overview

"screening is the process of identifying people who appear healthy but may be at increased risk of a disease or condition).

Even with good or adequate control individuals with diabetes are still at risk of developing diabetic retinopathy. Early detection and treatment of visual complications is vital and therefore screening for diabetic eye disease is essential. As about 5% of the UK population are diagnosed with diabetes are the numbers are rising. [92]

Screening is a way of detecting diabetic retinopathy before the individual notices any changes. It is offered to everyone over the age of 12 years. Over 12 years of age has been suggested as it is extremely unusual to see any sign of diabetic retinopathy in a child under this age and of those recorded most have been mainly Type 1 with very little reported on Type 2. [28]

The diabetic screening programme is a system of well-defined parameters that follow rigorous quality assurance processes. The four nations offer national screening programmes.

Due to the changing epidemiology of the condition, the UK National Screening Committee (UK NSC) constantly review the population eligible for screening. This is to ensure that the screening model and key policies such as the screening intervals are also regularly reviewed to ensure improvement in care for people with diabetes but that it also remains a cost effective program to reduce the risk of sight loss. [28]

The UK NSC recommendation sets out clear guidelines to follow for diabetic eye screening programmes for standardisation across the UK. Initially screening is performed every year, however following a review in 2016 the evidence from a large observational study led to a change of the strict criteria to allow those at low risk to be screened every two years rather than annually. However, those at high risk should remain. The large observational study carried out by Leese at al. (2015) observed 354,549 patients over 4 years. They were able to identify patients within diabetes retinal screening programs who were at low, medium (further stratified into subgroups) or high risk of progressing towards referable diabetic eye disease and those that needed referral to an ophthalmologist. [92]

The UK NSC Programme is overseen by a Programme Advisory who gave guidance on the recommended software to be used and methods of imaging the eyes. The method of two field digital photography with the minimum grading classification was implemented. The screening test uses technician screeners or photographers at fixed locations or mobile locations using a mobile van at GP surgeries or other locations. The programme board oversee the screening in any area is and has representation from Ophthalmology, Public Health, Commissioners and the local Screening Team. There are slight differences between the other 3 nations but the monitoring and quality assurance is key to all screening programmes.

The National pathway standards for the NHS diabetic eye screening (DES) programme are clear guidelines for the programme which details the standards and performance objectives, the monitoring pathways, quality assurance and failsafe documents all available at https://www.gov.uk/government/publications/diabetic-eye-screening-standards-and-performance-objectives.

Other methods of screening have been suggested that include computer aided system

Sandhu et al. (2018) assessed the feasibility and accuracy of a computer-assisted diagnostic (CAD) system to diagnose and grade nonproliferative diabetic retinopathy (NPDR) from optical coherence

tomography (OCT) images the study detailed the use of a robust approach for segmentation of 12 distinct retinal layers. They also used a two-stage, deep fusion classification network to classify patients as normal, subclinical stage diabetic retinopathy, or mild/moderate diabetic retinopathy based on three discriminant features, namely curvature, reflectivity, and thickness, across all the segmented retinal layers. The results showed that the system achieved an average of 94% total diagnostic accuracy. They suggested that further studies could be undertaken using OCT angiographic data which could improve accuracy. [93]

Early studies using this technology have already been used in other conditions such as AMD and are showing promising results but still with some simplicity. [93]

## 1.13 Prevention of Diabetic Retinopathy

Modifiable risk factors for diabetic retinopathy include glycaemia, blood pressure and lipid levels. Therefore, modifying lifestyles could have a significant impact on the associated risk factors.

Strict glycaemic control and regular diabetic screening for diabetic retinopathy among diabetics is typically the standard of care for prevention. Strict blood pressure has been recommended for diabetics by many because of its known beneficial effect on the prevention of cardiovascular events, stroke, and nephropathy but its effect on diabetic retinopathy remains controversial. It has been previously reported that keeping HbA1c below 7.6% (60 mmol/mol) as a treatment target seems to prevent PDR for up to 20 years in type 1 diabetic patients. [94]

Both the United Kingdom Prospective Diabetes Study (UKPDS) and the Diabetes Control and Complications Trial (DCCT) showed a lower incidence and slower progression of diabetic retinopathy following tight blood glucose control. [95, 96]

Although studies have shown that tight control of blood pressure is essential for preventing or arresting diabetic retinopathy, other studies have shown ambiguous results because no benefits for

retinopathy were found with intensified management of blood pressure. However, it should be noted that patients in the UKPDS were required to be hypertensive at baseline to enter the blood pressure control arm of the study, unlike ADVANCE or ACCORD. Therefore, the effect of lowering blood pressure was therefore greater in UKDPS than in either ADVANCE or ACCORD studies. Also, the duration of follow-up in the UKPDS was much longer than in either the ADVANCE or ACCORD. [99-101]

Other researchers that are involved in clinical trials as part of the Diabetic Retinopathy Clinical Research group also have reported that treatment used either alone or combined with laser treatment is required to reduce loss of vision, [102] but those risk factors that are modifiable should be addressed to reduce the prevalence of diabetic retinopathy. However other epidemiological studies carried out following these trials showed the prevalence still to be high.

It has also been suggested that insulin therapy is beneficial in delaying onset and progression of diabetic retinopathy as it improves retinal blood flow and vascular tone of retinal vasculature and therefore helps achieve good glycaemic control. [103] Good glycaemic control has been seen to reverse early retinopathy signs if initiated early in the course of diabetes but equally intensive glycaemic control has been reported to have adverse effects including worsening the retinopathy. This has been thought to be due to a raid reduction of plasma glucose levels and the up regulation of the insulin growth factor could be attributed to early worsening of the retinopathy. [104]

There is little evidence that any particular class of anti-diabetic drug independent of glycaemic control is either detrimental or beneficial in relation to diabetic retinopathy. In the Casteldaccia Eye Study (2004) they reported an association with duration of diabetes, duration and type of antidiabetic treatment and duration of alcohol intake. However, their results showed that only duration of antidiabetic treatment was the variable that was independently associated with diabetic retinopathy. They concluded that this was therefore the most important risk factor more so than duration of diabetes. [105]

Ramachandran et al. (2016) showed the overall inference from various trials with regards the effects of systemic control in diabetic retinopathy. [107]

An experimental study by Avogaro and Fabini (2014) showed that some of the newer anti-diabetic drugs like sitagliptin (dipeptidyl peptidase-4 inhibitor) may possibly show a protective effect on diabetic retinal cells by decreasing the retinal inflammatory state and neuronal apoptosis. [107] Others have also explored the effects of antidiabetic drugs. [108,109] However there have been no clinical studies that have evaluated the retinopathy endpoints whilst taking gliptins.

Clinicians are however advised to use caution with glitazones if a patient has diabetic macular oedema. This is a class an oral hypoglycaemic agents which are associated with fluid retention in 5-15% of patients using them. They result in the activation of peroxisome proliferator-activated receptor –γ, which is a transcription factor located in the adipose tissue and retina. Stopping the drug seemed to resolve the diabetic oedema. [110]

However, the potential ocular effects of thiazolidinedione's which are used to control blood sugar has been somewhat controversial. [111] Some studies have suggested that they may contribute towards macular oedema however the ACCORD trial saw no effect in those with and without exposure to the drug in patients with macular oedema. [112]

Many studies have reported Calcium dobesilate monohydrate (CaD) is indicated for treating diabetic retinopathy and is approved for use in several countries. [113, 114] Reviews of the evidence suggest that CaD has proven beneficial in the very early stages of diabetic retinopathy but its effectiveness in more advanced stages is unclear. [115] However, further research is still needed to further elucidate its various possible effects in the diabetic retina. [101]

A relatively new therapy for diabetes is pancreas transplantation. It has been said to have dramatic effects on blood sugars resulting in many patients being left in a euglycemic (normal level of sugar in

the blood) state. Case reports have demonstrated stabilisation and even regression of diabetic retinopathy after a pancreas transplant. [1]

Serum lipids have been reported by some to have less influence in the development of PDR or DME. [116,117] However, Kawasaki et al. (2018) felt that lipid lowering medication and statin use may also have a potential protective association as seen in type 2 diabetics with non-proliferative diabetic retinopathy. [118]

Randomised controlled trials have already demonstrated the effectiveness of supplementation for age-related macular degeneration in the AREDS study [119] and therefore there is a growing need to evaluate the impact of antioxidants whether by diet or supplementation on the development of diabetic retinopathy.[120] Therapies with vitamins and supplements have already been used in diabetic animal studies using a diet supplemented with antioxidant vitamins C and E, however clinical trials on human diabetics in this area are few and those that have demonstrated beneficial effects against oxidative stress have focused more on outcome measures relating to complications rather than the development of retinopathy. [121,122]

A systematic review by Bartlett and Eperjesi (2008) [123] identified 50 randomized trials investigating nutritional supplementation in type 2 diabetes. A variety of nutritional supplements were identified such as alpha-lipoic acid, chromium, folic acid, isoflavones, magnesium, selenium, vitamin C, vitamin E and Zinc. Oxidative stress was shown to reduce when 200mg of Vitamin E was taken due to its antioxidant capacity.

Various trials have also identified positive effects of some nutrients on outcome measures relating to insulin resistance and cardiovascular factors in diabetics but the evidence on the beneficial effects on the development of retinopathy is limited.

## 1.14 Management and treatment of diabetic retinopathy and maculopathy.

### Diabetic Retinopathy

Laser therapy has been used for many years to delay the progression of diabetic retinopathy and the prevent loss of vision in both proliferative diabetic retinopathy and for diabetic macula oedema.

Laser pan retinal photocoagulation(PRP) uses laser (light energy) delivered to the peripheral retina to usually seal off blood vessels. The energy is absorbed by the retinal pigments which heat up and cause thermal damage to the retinal tissues. The aim of the photocoagulation is to slow down the growth of new blood vessels in the retina and prevent progressive visual loss. [27]

A significant number of randomised control trials have examined the use of lasers in the treatment of PDR and NPDR. [124-135]

However, it was the two landmark randomised clinical trials the Diabetic Retinopathy Study (DRS) and the Early Treatment for Diabetic Retinopathy Study (ETDRS) that paved the way for the initial treatment protocols comparing treatment of PDR against no treatment. And only those with high risk PDR were treated due to the adverse events. [35] Since these pivotal trials numerous studies have examined different laser strategies to reduce the impact on vision form the laser burns and laser is sometimes done before the development of PDR as a result of modern lasers. [127, 132]

The current recommendations by the Royal College of Ophthalmology is no treatment for mild non proliferative diabetic retinopathy (R1) or moderate non proliferative diabetic retinopathy(R2) but when moderate becomes severe and it starts to approach the proliferative stage (R3) the laser should be considered. The decision to treat or not can be aided by the use of fundus fluorescein angiography (FFA) which is a technique used to examine the bloody vessels and circulation in the eye using a dye called fluorescein injected into a vein in the hand or arm. [28]

Full PRP is indicated for NVD or NVE, but in advanced cases with vitreous haemorrhage a vitrectomy may be indicated. [28]

As studies have shown using such laser can cause side effects. [133, 134, 141, 142] Field defects and impaired night blindness can have an impact on other aspects of life such as driving. [138,144] In the UK individuals who have had laser to both eyes or to one eye if they only have sight in one are, they are required by law to advise the DVLA. The Royal College of Ophthalmology guidelines state that after full PRP about 40-50% of individuals have some reduction in visual fields, which may have an impact on their driving, although the newer lasers do give smaller lighter burns. [28]

A Cochrane review by Evans (2014) provided evidence that lasers still play a huge part in the treatment of PDR but there is considerable interest in intravitreal therapy as an adjunct therapy to it. [27]

Maculopathy

Laser treatment for maculopathy or DMO like for retinopathy has been used for a significant number of years following the DRS and ETDRS trials and defined treatment protocols emerged using focal and grid patterns of delivery of laser which were in place for quite some time. The introduction of optical coherence tomography (discussed in detail in chapter 2) however resulted in some changes of practice following further studies and modified treatment protocols and patterns of application emerged as seen following the study carried out by The Diabetic Retinopathy Clinical Research Network (DCRNet) [102,148]. Results from studies on the use of micropulse laser which is considered even less aggressive than standard macular laser have been variable. [145, 146]

Numerous studies have examined the use of corticosteroids (triamcinolone) delivered by intravitreal injection ( IVTA) into the eye following the implication of some of the complex pathways involved on the pathogenesis of macular oedema but the results were inconsistent. [ 151,152]

Other studies examined the combined use of IVTA with focal laser with no additional benefit being seen. [150] Another treatment option is the use of intravitreal slow release biodegradable drug delivery systems have been explored such as Dexamethasone (available as Ozurdex ® Allergan)

although they allow longer periods between treatments. As they are delivered by a sustained release implant, they are reported to improve the vision by interfering with the ischaemic and inflammation mechanisms that damage the retinal blood barrier. They may also inhibit neovascularisation. [153-156]

The use of Illuvian ® in the Fluocinolone acetonide intravitreal implant for diabetic macular oedema (FAME) study showed benefits in patients that had DMO for greater than 3 years and this is now licenced for use in the UK for the treatment of DMO [28] and several studies have shown that some patients do respond favourably to corticosteroids when they have not responded to anti-VEGF corticosteroids. [158].

Over the last few years intravitreal injections of anti-VEGF agents have gradually replaced many requirements for laser treatment. VEGF is a major driver of the vascular hyperpermeability that leads to DMO, and anti-VEGF therapies have transformed the treatment of this condition.

The three key anti-VEGF therapies used are ranibizumab (Lucentis®, Genentech USA, Inc., CA, US), aflibercept (Eylea®, Regeneron Pharmaceuticals, Inc., NY, US) and bevacizumab (Avastin®, Genentech USA, Inc., CA, US). Currently only ranibizumab and aflibercept are licenced for use in the UK, however bevacizumab is currently used off licence. [28]

Aflibercept is a soluble VEGF receptor fusion protein that binds to all isoforms of the VEGF-A family unlike ranibizumab and bevacizumab. It has also been shown to be longer acting due to its higher binding affinity. There have been many trials investigating the safety and efficacy of these anti-VEGF therapies either used alone, in comparison to another or with laser as an adjunct. Massin et al. (2010) in the RESOLVE study showed that ranibizumab was effective in improving visual acuity and was well tolerated in patients with DME. [158]

Mitchell et al. (2011) in the RESTORE study of Ranibizumab used alone and combined with laser found a bigger visual acuity gain over standard laser in patients with visual impairment due to DME. However, at 1 year, no differences were detected between the groups. [159]

Nguyen et al. (2012) study in the RISE and RIDE study showed improved vision and reduced risk of further vision loss with improved macular oedema with a good safety profile. They also found the visual acuity gains to be maintained for longer with a decreased frequency of treatment. [160]

However there have already been much debate around those that insufficiently respond to all of these therapies as it has been suggested that there are a proportion of patients with diabetic retinopathy that also respond poorly to anti-VEGF therapies due to the multifactorial nature of the disease. [161, 162] In general the use of intravitreous aflibercept, bevacizumab, and ranibizumab have been shown to be effective in a large number of cases and relatively safe treatments for diabetic macular oedema but they are not with very few complications.

Table 1.4 Summary of current management options for diabetic retinopathy (DR) and diabetic macula oedema (DMO) adapted from Wilkinson (2003) and Wang et al (2017). [27, 163]

| Diabetic retinopathy (DR) | Presence or absence of diabetic macula oedema (DMO) | Management Options |
|---|---|---|
| Mild NPDR<br><br>Moderate NPDR<br><br>Severe NPDR | | None<br><br><br><br>Consider photocoagulation |
| Any of above with | Mild DMO Some retinal thickening or hard exudates in posterior pole but distant from centre of macula<br><br>Moderate DMO Retinal thickening or hard exudates approaching but not involving the centre of the macula<br><br>Severe DMO Retinal thickening or hard exudates involving the centre of the macula | Focal or grid laser<br><br><br><br>Anti-VEGF drugs, intraocular corticosteroids<br><br><br>As above |
| PDR | Severe DMO Retinal thickening or hard exudates involving the centre of the macula | Pan retinal laser photocoagulation, anti-VEGF drugs |

Legend:

NPDR = non-proliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy; DMO = diabetic macula oedema; VEGF = vascular endothelial growth factor.

Other studies have investigated the effects of Treatment with light in the far-red to near-infrared region of the spectrum (photobiomodulation) ameliorated lesions of diabetic retinopathy in streptozotocin-induced diabetic rats and reduced oxidative stress and cell death in vitro. [163] Tang et al. (2014) also showed that in 4 diabetic patients during their pilot study, that photobiomodulation caused a significant reduction in focal retinal thickening with non-central diabetic macula oedema. [164] The Cleopatra study investigated an alternative therapy for diabetic macula oedema, It was a phase III randomized controlled single-masked multicentre clinical trial to test the clinical efficacy of light-masks in preventing dark-adaptation in the treatment of non-central diabetic macula oedema

[166] however the light mask did not show any long term therapeutic benefits and its indication for use was not indicated for treatment for diabetic macula oedema.

Studies are on-going for anti-VEGF therapy including treatment strategies and investigation into other novel treatments continue to evolve.

Summary

As described, there are generally three options of treatment available for diabetic retinopathy, laser, intravitreal injections or surgery treatment options which mainly delay the progression of diabetic retinopathy and maculopathy. However often the nature of the disease has caused devastating loss of unrecoverable vision loss in some cases so there are probably other aspects of the pathogenesis of diabetic retinopathy in the complex pathways that warrant further attention and could be possible targets for therapy much earlier on in the disease process.

In the next chapter the literature will be reviewed and the research rationale will be proposed.

# Chapter 2 Literature review

## 2.1 Aetiology Diabetic Retinopathy

The retina is described as a transparent layer of neural tissue between the retinal pigmented epithelium (RPE) and the vitreous.[167] It conforms to the nearly spherical shape of the eye and has an inner transverse diameter of approximately 22mm.It is described as consisting of 10 layers which include 9 layers within the sensory retina and pigment epithelium. This pattern of layers is present throughout the retina except in the fovea. The fundamental functions of the retina are to capture light, convert the photochemical energy into electrical energy potentials, and transmit them to the brain, and turn into recognizable images. [167] The retina at the posterior pole is termed the macula which is responsible for detailed central vision and colour and operates in moderate to bright light conditions, whereas the peripheral retina which operates in dim-light conditions, detects motion, and peripheral vision.

The retina therefore operates in bright light to near darkness, and photoreceptor sensitivity has been shown to vary over nine orders of magnitude. [168] The macula and peripheral retina operate together, therefore the macula is more sensitive than the periphery in bright light, and the reverse occurs in dim light.

Normal vision is therefore dependent on intact cell to cell communication among the neuronal, glial, microglial, vascular, and pigmented epithelial cells of the retina in these areas. [167]

The macula, which is approximately 6 mm in diameter in humans, is located between the vascular arcades temporal to the optic disc. The macula has the thickest ganglion cell layer and a high number of horizontal neuronal connections resulting in the ability to resolve fine detail and discriminate contrast between objects. The centre of the macular can be identified by locating the foveal reflex. In this area the photoreceptor layer is composed mostly of cones especially in the very centre at the fovea. The inner segments are separated by Muller cells. [167.168]

Müller cells and astrocytes are two types of glial cells which provide nutritional and regulatory support for the neurons. The müller cells span the retina from the retina pigment epithelium (RPE) to the internal limiting membrane. This is a basement membrane formed by Müller cell end-feet that interfaces with the vitreous gel. Müller cells contact neurons and blood vessels in the plexiform and nerve fiber layers, and astrocytes envelop blood vessels in the nerve fiber and ganglion cell layers and contact ganglion cells and amacrine cells. Müller cells and astrocytes convey substrates from the circulation to neurons and regulate blood-retinal barrier properties and synaptic function. Müller cells also store the glycogen for conversion to lactate, which synthesize retinoic acid from retinol, and regulate extracellular ion concentrations to modulate plasma membrane polarization/depolarization. This supplies the neurons in the glutamate/glutamine cycle to control neurotransmission, and protect neurons from glutamate excitotoxicity. [(169).] Studies by Barber and Rungger-Brandle as cited in Gardner (2002) have shown that astrocytes undergo prominent changes in diabetes and also muller cells have been long known to proliferate in the formation of epiretinal membranes in proliferative retinopathy. [(5,167,170)]

The Glial cells are the interface between the neurons and the vasculature and are thus key regulators of neuronal nutrition and metabolism. The lamellar cellular architecture of the retina has been described as having alternating layers of neurons (outer and inner nuclear layers and ganglion cell layer) interposed with two plexiform layers, where neurons communicate at synapses between dendrites and between axons and dendrites. The retina includes five major cell types that perform sensory, regulatory, nutritional, and immunomodulatory functions. The neurons perform sensory functions and define color perception, spatial resolution, and contrast discrimination. [(167, 168)] Beyond the macula, the retina has mostly rod photoreceptors and associated inner neurons, with fewer horizontal interconnections. [(171)]

The RPE cell layer of the retina serves as a selective conduit of substrates. In particular the outer blood-retinal barrier and allows oxygen diffusion from the choroidal circulation to the outer retina. It

partitions the neural elements of the retina from the circulation to protect it from circulating inflammatory cells and their cytotoxic products to allow the retina to regulate its extracellular chemical composition, particularly that of ions important for neuronal activity as described by Gardner et al.(2002). [167] Thus, this epithelial cell layer plays a crucial role in vision, although its role in diabetic retinopathy is not clear.

Microglial cells are the fourth class of cells and are described as cells related to tissue microphages. [167] They are said to monitor the local environment by interacting with neurons, glia, and endothelium and that react to stresses, including infection and trauma, by release of pro-inflammatory cytokines and clearance of necrotic or apoptotic cells via phagocytosis. Microglial cells become activated and help to resolve local injury, but unrelenting stresses cause persistent inflammatory responses. [172]

The fifth class of cells includes vascular endothelial cells and pericytes that line them. They provide nutritional support and waste product removal for the inner retina and have been the focus of much research in diabetic retinopathy as they have been found to be lost in the early stages of diabetic retinopathy. [173,174]

The pericytes are smooth muscle cells of capillaries that regulate retinal vasculature and control the blood flow of the retinal microcirculation. [57]

Pericytes loss has been described as one of the earliest histological signs in diabetic retinopathy. It is documented that it is likely that their function depends on as-yet-undefined signals from the neural retina. Blood vessels are the only structures that are visible by clinical examination because they convey erythrocytes containing the visible pigment hemoglobin. However, it is reported that each of the retinal cells described is affected by diabetes. [167] It has been suggested that all the retinal cellular elements are involved in diabetic retinopathy which includes the glial cells, neurons, bipolar cells, amacrine cells and ganglion cells. [175]

The scale of retinal vascular changes that occur in diabetic retinopathy has already been described in detail in chapter 1 and includes microaneurysms, intraretinal hemorrhages, intraretinal accumulation of lipids and proteins (hard exudates), focal venous dilatation (venous beading), intraretinal microvascular abnormalities and neovascularization. [24]

Despite their prominent appearance by clinical examination, the vasculature constitutes less than 5% of the retinal mass, so the retina is a vascularized neural tissue however it has been suggested that the increase in vascular permeability in diabetic retinopathy may be due to effects of diabetes that alter the neural components of the retina giving rise to a breakdown in the interactions between neurons, glia and endothelial cells. [170] Most studies of diabetic retinopathy have focused on the pathogenesis resulting from the impact of the microvascular injury, breakdown of the inner blood-retinal barrier, microvascular occlusion, retinal ischemia and ischemia-induced neovascularization. [90]

The inner blood-retinal barrier is thought to be compromised in diabetic retinopathy, largely attributable to an increase in the permeability of retinal endothelial cells. Many have described this change is due to elevated levels of hypoxia-related growth factors and inflammatory cytokines. Also, the accumulation of advanced glycation end products (AGEs), hyperglycemia, and the loss of pericytes. Numerous mechanisms then play a part in the transcellular and paracellular transport across the retinal capillary walls resulting in changes in endothelial cells and the opening of intercellular junctions. [176]

As a consequence of these changes there is leakage of macromolecules from blood vessels into the interstitial spaces of the retina and structural changes in the capillary basement membrane as well as changes in pericytes and glial cells. This then accelerates leakage from the inner blood-retinal barrier, all contributing to fluid accumulation in the retina. Clinically, this then presents as diabetic macular oedema. [176]

The unique retinal structure imparts special physiologic constraints compared with other nervous system tissues because of the requirement for transparency, and these features may also contribute to its susceptibility to diabetes. For example, retinal axons are not ensheathed by myelin, since myelin is opaque and blocks light transmission. Unmyelinated nerves require more energy to maintain membrane potentials than myelinated axons. [177]

Secondly Antonetti et al. (2004) also described previous reports that the density of blood vessels that would absorb light is relatively low, so the oxygen tension of the inner retina is relatively hypoxic with a $pO_2$ of only approximately 25 mm. The $pO_2$ gradient of the retina declines from the outer retina to the inner retina. Thirdly, the inner retina possesses relatively fewer mitochondria that contain light-absorbing cytochrome proteins of the electron transport chain. Müller cells are relatively enriched in mitochondria and are found in regions of higher $pO_2$ in plexiform layers and ganglion cells but not as prominently in the nuclear layers. [178]

Thus, previous studies as cited in Antonetti et al. (2006) have shown that the inner retina relies heavily on glycolysis, a less efficient means of generating adenosine triphosphate (ATP) than oxidative phosphorylation, which predominates in the outer retina, where the $pO_2$ is approximately 80 mmHg. They outlined that in spite of this sparse vascularity and low $pO_2$, the retina has one of the highest metabolic demands of any tissue. Phototransduction requires ATP to maintain ion gradients across cell membranes, for neurotransmission at synapses, to replenish photoreceptor outer segment membranes, and to transport proteins and neurotransmitters anterograde and retrograde via axons to the optic nerve and lateral geniculate body of the thalamus. Is has been stated that combination of high metabolic demand and minimal vascular supply may limit the inner retina's ability to adapt to the metabolic stress of diabetes. Whereas the outer retina receives its oxygen and nutrients by diffusion from the choroid through the pigmented epithelium and is relatively spared from the early insults of diabetes. [174]

Many studies have demonstrated that diabetic retinopathy is strongly associated the duration of diabetes and hyperglycemia. [17, 55, 71, 96, 99,179,180] The pathogenesis of diabetic retinopathy remains very complex and somewhat unclear. While it is known that hyperglycemia is a major factor, diabetes is associated with changes not only in insulin and OGF-1 but also in many hormones and metabolites including free fatty acid, amino acids, advanced glycation end products, and components of the oxidative stress pathway. [181]

The development of diabetic complications from Hyperglycemia is thought to involve various pathways. As cited in Wang et al. (2018) they described the pathways that are a result of vivo and in vitro studies. The five biochemical pathways include accumulation of advanced glycation end products (AGEs), activation of the polyol pathway, the hexosamine pathway, the protein kinase C pathway, as well as poly (ADP-ribose) polymerase activation. [24, 182]See Fig 2.1 as produced by Wang et al. (2018) demonstrates the complexity of the pathways.

Polyol pathway

The polyol pathway (also known as the sorbitol-aldose reductase pathway) is a glucose- metabolic pathway that has been reported to be linked to the progression of diabetic retinopathy. The first enzyme in the polyol pathway is aldose reductase and the second is dehydrogenase which is activated as the blood glucose level rises. [183] The high blood glucose results in excessive production of sorbitol, this is due to the unused glucose entering the pathway and causing aldose reductase to reduce it so sorbitol. The aldose reductase uses a cofactor, nicotinamide adenine dinucleotide phosphate (NADPH). [183] The reduction of glucose to sorbitol results in the consumption of NADPH, this is required for regenerating reduced glutathione and nitric oxide. Nitric oxide is also known as ‘endothelium-derived relaxing factor’, and signals dilation of blood vessels. Glutathione acts as an antioxidant. [123]

Glutathione is an antioxidant that is found in the body which plays a vital role in removing the free radicals that are formed during different processes. [183]

It is therefore as a result of hyperglycemia that the polyol pathway is activated which leads to the increased accumulation of sorbitol along with increased production of ROS with accompanying reduced levels of glutathione and nitric acid. All of which are thought to contribute to the development of diabetic retinopathy. [123]

Accumulation of advanced glycation end products (AGEs)

During the polyol pathway fructose is produced which acts as one of the prime precursors of advanced glycation end products (AGES) formation. These are end products of glycation reaction of various sugar molecules. They accumulate much slower in individuals without diabetes but in individuals with diabetes the accumulation is much faster due to the varied availability of glucose. The glycation rate of glucose is slow, fructose however is high and is therefore one of the glycation agents responsible for AGEs which has been shown in animal studies. It has been suggested that it is critically involved in the pathogenesis of diabetic retinopathy as the AGEs bind to their receptors causing damaging effects. The receptors are known as receptors for advanced glycation end products (RAGEs). A combination of oxidative stress and hyperglycemia increases formation of AGEs whish augments the expression of RAGE resulting in damage to the retinal cells and hence progression of diabetic retinopathy. [183]

The hexosamine pathway

The hexosamine pathway is also involved in the metabolism of glucose, by excess amounts of glucose it has been suggested that this may also contribute to the complications of diabetes. [184] Also, the hyperglycemic activation of this pathway may contribute to the pathogenesis of diabetic complications as a result in changes to gene expression and protein function. [123, 185]

### The activation of protein kinase C

The protein kinase C (PKC) family is made up of 11 isoforms, nine of which are activated by a lipid second messenger called diacylglycerol (DAG). Hyperglycemia may indirectly increase the activation of PKC by joining of the AGE receptors and increased activity of the polyol pathway. [186]

Craven et al. (1994) as cited in Bartlett et al. (2008) suggested that abnormal activation of PKC is involved in the decreased production of nitric oxide in experimental diabetes. Bartlett et al also reported evidence of decreased production of nitric oxide in smooth muscle cells that is induced by hyperglycemia and that inhibition of nitric oxide production may also contribute to increased microvascular matrix protein accumulation. [123,187]

### Poly (ADP-ribose) polymerase activation

Zheng et al. (2004) investigated the role of poly (ADP-ribose) polymerase (PARP) in the development of diabetic retinopathy in animal studies. Activity of PARP was increased in whole retina and in endothelial cells and pericytes of diabetic rats. It has previously reported that Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme which is involved in the cellular response to DNA injury. When the DNA the damage is mild, poly (ADP-ribose) facilitates cell survival. However, when the DNA damage is severe, there is cell dysfunction or death as PARP activation can induce cellular energetic disturbances. In the study by Zheng et al. (2004) they demonstrated that PARP activity in retina as seen to be increased in diabetes and that inhibition of PARP slows the development of early lesions of diabetic retinopathy. [188]

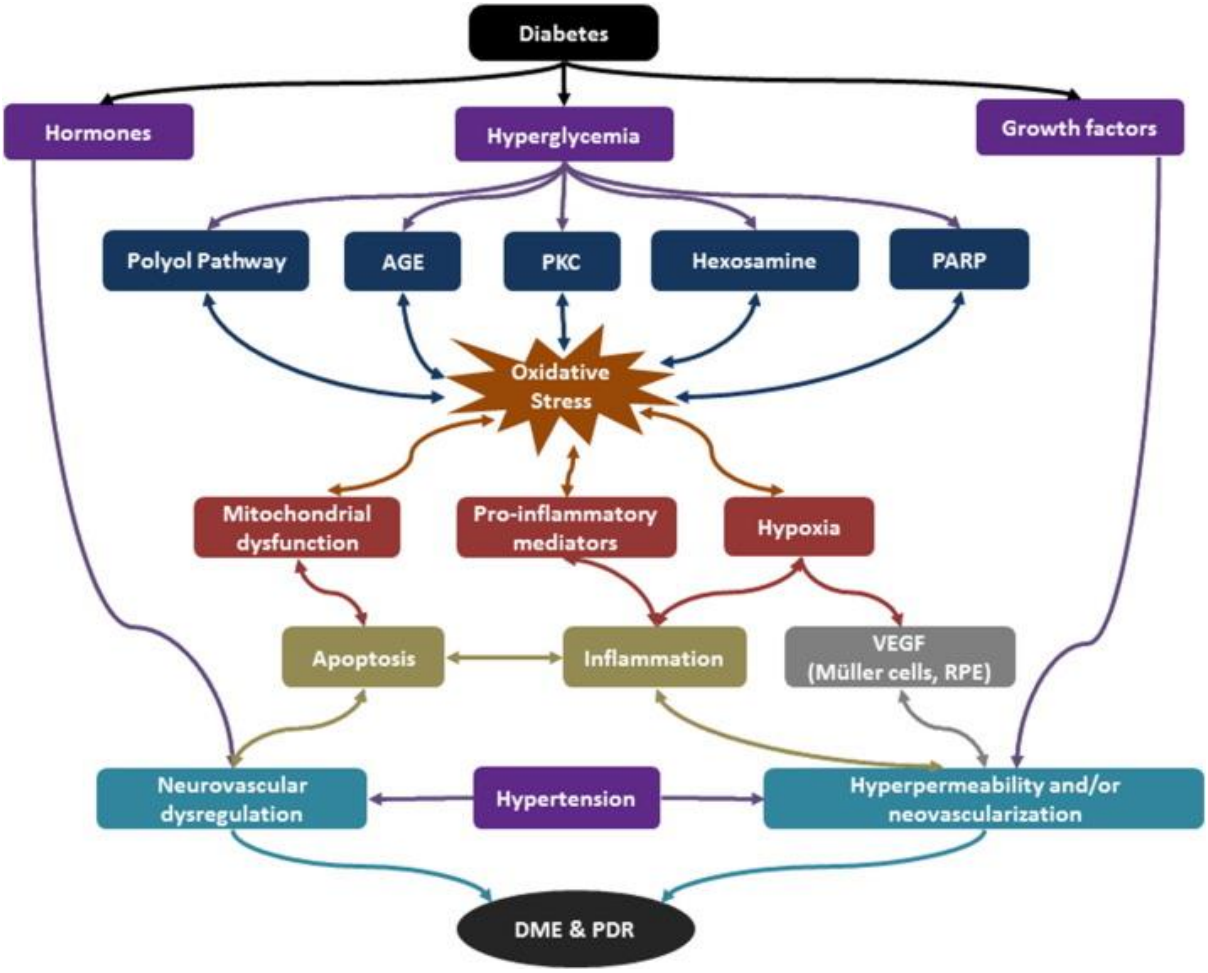



Fig 2.1 Reproduced from Wang et al. (2018) described the hyperglycemia – induced pathways that contribute towards the pathogenesis of diabetic retinopathy. [24]

Together these complex pathways have been said to contribute to the production of oxidative stress, inflammation, microvascular dysfunction and mitochondrial damage. This in turn up-regulates pro-inflammatory mediators, transcription factors, chemokine's, and adhesion molecules. [189]

In addition to this causing widespread microvascular injury, there is now accumulating evidence that hyperglycemia may result in preceding neuronal dysfunction, which may contribute to visual loss. [190]

Taking all these findings together there is a strong suggestion that there is the unique anatomic and physiologic specialization required for vision demands intact cell-cell communication. This specialization it is felt may, in turn, predispose the retina to diabetes-induced damage if the

metabolic derangements typical of diabetes interfere with the generation of neurotransmitters, macromolecule synthesis, or induce proapoptotic or proinflammatory responses. [174]

Although microvascular changes are undeniably integral to retinopathy, the retina is a vascularized neural tissue, not a network of blood vessels. Histopathologic studies emphasized the loss of neurons in human diabetic retinopathy over 40 years ago. [191]

Since such studies, numerous reports using electroretinography, dark adaptation, contrast sensitivity, and color vision tests have conclusively demonstrated that neuroretinal function is compromised before the onset of vascular lesions in humans. [192,193]

Also, it is shown that loss of oscillatory potentials on electroretinograms can predict the onset of proliferative retinopathy better than vascular lesions seen on fundus photographs or capillary non-perfusion visualized by fluorescein angiograms as described by Antonetti et al. (2006). [174]

Furthermore, it has been shown that electroretinograms and psychophysical tests, including, short-wave automated perimetry and frequency doubling perimetry, reveal field defects in patients with little or no vascular retinopathy, and visual fields predict the severity of retinopathy better than visual acuity. [194]

As discussed at the cellular level, diabetes alters the function and structure of all retinal cell types. Studies have shown that postmortem human diabetic retinas exhibit increased markers of apoptosis in ganglion cells. Animal studies show accelerated apoptosis of retinal neurons, glial activation, impaired glial cell metabolism, and microglial cell activation. [195,196]

Together, it is felt that these studies leave little doubt that neural retinal defects are among the earliest detectable changes in diabetes. Regardless of whether the initial events begin in blood vessels or neural cells, the clinical stages of diabetic retinopathy manifest cellular, histological, and functional features of a retinal neuropathy .[167] It is documented that there is no clear evidence that

a primary, selective defect in vascular cells is sufficient to cause diabetic retinopathy. Clearly, then it is essential to treat both the vascular and neural elements of the retina to preserve vision. It is felt that this concept permits a new paradigm for understanding the mechanism of vision impairment in diabetes and provides therapeutic targets that are directly linked to vision. [197]

Studies have already shown that that diabetes impairs insulin receptor signaling in retina, brain [198] and peripheral nerve [199] as well as in classic insulin-sensitive tissues. There is also the suggestion that the metabolic impact of diabetes on "complications" prone tissues and peripheral tissues forms a continuum. Whereas muscle and adipose respond acutely to fluctuating insulin levels and change rapidly after diabetes onset, retina and brain insulin action has a higher set point and responds less rapidly after diabetes onset. Plasma insulin penetrates the eye and brain more slowly than peripheral tissues. Thus, it is also important to consider the role of impaired insulin action in the development of complications, even in tissues where insulin does not regulate glucose uptake. "Complications" then, are the manifestations of altered diabetes metabolism in organs that are associated with clinical impairment. [174]

Research has suggested that micronutrients may potentially alter the risk of diabetic retinopathy by interfering with these pathologic mechanisms. Richter et al. (2018) have also studied the effects of inflammatory reactions and diabetic retinopathy. [200] They cited several studies that have already described well-established correlations between elevated inflammatory parameters and the development of diabetic retinopathy. [200]

Inflammation has been described as central to the pathogenesis of diabetic retinopathy. Although very complex the event has been reported to involve inflammatory cytokines, chemokine's, adhesion molecules, prostaglandins, as well as inflammatory cells including neutrophils and macrophages participate. [201] VEGF is broadly considered as a pro-angiogenic factor, but has also been identified as a critical contributor to the progression of inflammation in diabetic retinopathy. As described in chapter 1 VEGF promotes the expression of intercellular cell adhesion molecule-1

(ICAM-1) and initiates leukostasis in the diabetic retina, this process has been reported to then lead to retinal ischemia in diabetic retinopathy. [202]

Furthermore, studies have shown that there may be the ability of an antioxidant combination to arrest retinal abnormalities and lipid per oxidation even in the presence of poor glycaemic control.

The Hoorn study [203] found that diabetic retinopathy was positively associated with cholesterol and triglyceride levels, and that retinal hard exudates were associated with LDL cholesterol. In the DCCT/EDIC cohort, retinopathy was positively associated with serum triglycerides and negatively with HDL. Whereas other studies have not found the same association. [203]

Lipoproteins however may play an important role in the complex process of retinopathy, but it is still under-recognised because of relatively weak associations of plasma levels with retinopathy severity. As described by Stitt et al. (2016) by understanding the role of lipoproteins, and their capacity once developed to impact on the cycles of retinal injury holds promise for the development of new and effective treatments to stop the progression of diabetic retinopathy. [90]

Some literature has indicated that there is an association between oxidative stress and the development of complications from diabetes including retinopathy. [204]

Studies have also shown that when oxidative stress is increased in the retina in diabetes; the levels of oxidatively modified DNA and nitrosylated proteins are elevated, and antioxidant defence enzymes are impaired. Therefore, by gaining an understanding of the role of mitochondria in the development of retinopathy in diabetes this should help identify therapies that can neutralize super oxides and inhibit their dysfunction and ultimately, the development of retinopathy. [205-207] Further studies have shown that strategies that aim to decrease the oxidative stress may decrease or even prevent the retinal abnormalities and hence control the development of diabetic retinopathy. This therefore leads us to the assumption that there is a key role of direct oxidative damage and the protective action of antioxidants in retinal alterations associated with diabetic retinopathy. [208]

These studies strongly suggest that alterations associated with oxidative stress therefore offer many potential therapeutic targets making this an area of great interest to the development of safe and effective treatments for diabetic retinopathy.

## 2.2 Oxidative stress and diabetic retinopathy

Over the years the mechanistic contribution of oxidative stress and the role in the development of complications in diabetes has been of great interest. The complications are equally associated with both type of diabetes. [209] Poor glycaemic control, hypertension, lipoprotein abnormalities, inflammation advanced glycation end products (AGES) and oxidative stress are all reported to be mediators of the vascular damage in diabetes. [209]

Oxidation is needed to maintain cell viability and survival and is important as part of the normal cellular metabolism. During the process of oxidation some types of free radicals otherwise known as reactive oxygen species (ROS) are produced which can be by products of normal metabolism or exogenous exposure. ROS includes hydrogen peroxide, superoxide anion and single oxygen and are scavenged by endogenous antioxidant systems. [120] Damage to membrane lipids, proteins, nucleic acids and carbohydrates can occur from oxidation. [183,210]

Oxidative stress occurs when there is an imbalance between the formation of free radicals and then the destruction of them. Free radicals are the chemical species that contain an unpaired single electron in their outermost atom shell. They have the capability to exist independently. [210] Oxidative stress can therefore arise when there is large excess of the free radicals or the number is not controlled by antioxidants such as vitamins A, C and E, carotenoids, glutathione or antioxidant minerals such as zinc, copper, manganese and selenium. [210]

Authors have described the many effects of hyperglycaemia and the complex pathways which are indirect, e.g. through the formation of advanced glycation/lipoxidation products but also with increasing oxidative stress. However, authors have suggested that other factors related to these

processes may also play critical roles such as variations in the antioxidant defence status, or the nature of substrates exposed to oxidative stress. [211-213]

Antioxidants that are exogenous and endogenous substances can provide protection from ROS damage. Singlet oxygen is reported to be scavenged by vitamin A and other retinoid, as well as beta-carotene and other carotenoids. The role of the enzyme superoxide dismutase (SOD) is to remove superoxide anion radical which contains manganese in the cell mitochondria, and copper and zinc is in the cytosolic compartment. Hydrogen peroxide is metabolised by the selenium-containing enzyme, glutathione peroxidase. [122]

The enzymatic antioxidant systems such as copper, zinc, manganese, superoxide dismutase (MnSOD) may remove the ROS sequentially or directly that will then prevent the excessive accumulation and as a consequence the adverse effects. [183]. When the amounts of ROS being produced and the amount of exogenous and endogenous antioxidants available are not balanced, a state of oxidative stress exists. [123]

Therefore, an increased production of reactive oxygen species (ROS) and a reduction in antioxidant defences then leads to the diabetic complications due to oxidative stress. [214] Another source of oxidative stress is nicotinamide adenine dinucleotide phosphate oxidase (NOx) enzymes. The primary function of NOx is to produce ROS. [215] The AGE protein induces intracellular generation of ROS by NOx which is associated with VEGF expression. ROS which is produced by the isoforms Nox2 and Nox4 has been shown in an experimental study using mice to facilitate retinal neovascularization by producing a model of hypoxia-induced angiogenesis (oxygen-induced retinopathy). [216]The elevation of ROS which occurs as consequence of mitochondrial dysfunction in diabetes has been shown to cause nitrative stress, and have also been linked to neurodegeneration.

Other studies as cited by Wang et al. (2017) have found evidence that suggests that neurotrophic factors are essential for the survival, growth and regulation of retinal neurons and glial cells. [24] They described the key retinal neurotrophic factors that are produced by neurons and glial cells in

the retina. These then help to protect and maintain neurons including brain-derived neurotrophic factor (BDNF), nerve growth factor, neurotrophin-3 and neurotrophin-4. It has also been reported by Dai, Xia, & Xiong (2012) as cited in Wang et al. (2017) that BDNF plays a particularly important role in guarding against neurodegeneration in diabetic retinopathy. [24] It has been described that it does this by promoting an increase of glutamate uptake which causes up-regulation of the glutamate transporter and glutamine synthetize in Müller cells under hypoxic conditions. [24]

A few other studies as cited by Wang et al. (2017) have suggested that the reduced level of BNDF correlates with reduced glucose, lipid metabolism and insulin resistance, as well as increased food consumption which further promotes diabetes. [24]

Experimental studies in animals and humans have reported decreased levels of antioxidant enzymes and antioxidant vitamins which may then be implicated in diabetic retinopathy due to the relationship between oxidative stress and dysmetabolism. When oxidative stress is increased the levels of oxidatively modified DNA and nitrosylated proteins rise which impairs the antioxidant defence enzymes. [209]

It has been suggested that some antioxidants could also inhibit the formation of reactive oxygen species (ROS) or act at other levels such as scavenging free radicals or increasing enzyme capabilities to give better antioxidant defence. [209, 211]

The retina is particularly susceptible to damage caused by oxidative stress because of intense exposure to light and oxygen and its abundance of polyunsaturated fatty acid (PUFA) which is prone to lipid peroxidation. [217]

Research has also shown that administration of long term of lipoic acid prevents the development of diabetic retinopathy in rats. The apoptotic capillary cells and acellular capillaries were seen to decrease. [206]

The pivotal role of oxidative stress in the pathogenesis of microvascular dysfunction aspect in diabetic retinopathy has been described by many, however it has also been proposed that it contributes to retinal neuronal degeneration. [24]

## 2.3 Neuronal loss and diabetic retinopathy

Diabetic retinopathy has long been considered as a microvascular disease of the retina as suggested in many studies as the clinically visible signs of microaneurysms, capillary non perfusion, haemorrhages and lipoexudates are seen ophthalmoscopy. [219] However, some of the early signs of diabetic retinopathy, neural apoptosis, loss of ganglion cell bodies, glial reactivity and reduction in thickness of the inner retinal layers have been seen in many studies as cited by Van Dijk et al. (2010) [218]

There continues to be much debate about whether vascular diabetic retinopathy causes diabetic retinal neuropathy or if hyperglycaemia causes neurological damage directly. However, there is growing evidence suggesting that retinal neurodegeneration contributes to the development of the microvascular abnormalities as an early event in the pathogenesis of diabetic retinopathy. [101,174, 220]

It has been reported that early signs of neuroretinal damage may precede vascular signs of diabetic retinopathy. [57] Experimental studies have shown that retinal ganglion cells (RGCs) are extensively damaged in diabetes. This suggests that the pathogenesis of diabetic retinopathy has an underlying neuronal component although not yet fully understood. [221]

It has been suggested that there are different mechanisms that underlie the development of the neuronal damage. They involve the release of local factors from the glial cells to modulate the retinal blood flow which is essential for the integration of vascular and neuronal activity in the retina. Mechanisms of retinal neurodegeneration have already been identified in diabetes which

includes extracellular glutamate excitotoxicity, neuro-inflammation oxidative stress, and the loss of neurotrophic factors. [101,170,197]

There is also clinical and laboratory evidence to support neuronal damage in the retina of diabetic patients prior to any fundal changes clinically.

The overall importance of neuronal and glial dysfunction has been discussed and led to a proposed ‘‘feed-forward” hypothesis in which dysfunction in neural cells are involved in the breakdown of the blood retinal barrier which then promotes an inflammatory and oxidative environment that results in vascular dysfunction. [174]

Further experimental evidence has reinforced this finding by demonstrating that reduction of some neuronal cells occurred in diabetic rodent models. This was possibly even prior to appearance of obvious microvascular lesions. Histologically is has been suggested that apoptosis of the retinal ganglion cells (RGCs) may be the initial losses observed in the diabetic retina. This may account for the reduced thickness of the retinal nerve fibre layer that has been detected in diabetic patients without retinopathy or with only background disease. [219, 223]

Despite their prominent appearance by clinical examination, the vasculature constitutes less than 5% of the retinal mass. It has been suggested that the increase in vascular permeability in diabetic retinopathy may be due to effects of diabetes that alter the neural components of the retina, leading to a breakdown in the interactions between neurons, glia and endothelial cells. [13] It has also been shown that shortly after the onset of diabetes, the ability of muller cells in their ability to convert glutamate to glutamine was reduced causing the accumulation of glutamate. This then leads to neurotoxicity due to the uncontrolled influx of intracellular calcium ions as described by Ng et al. (2016).[221]

There has also been shown to be an increased expression of the vascular endothelial growth factor (vegf) in animal studies of diabetic retinas by the glial cells and retinal ganglion cells. This results in

the blood-retinal barrier being broken down causing damage to the neuronal retina by harmful circulatory agents. [221] The functional coupling and interdependency of neurons, glia and highly specialised vasculature has been described by others as the neurovascular unit (NVU) in the central nervous system. The impairment of the NVU in the context of the retina has been described as a contributing event in the pathogenesis of diabetic retina. [24,101, 174] It is now recognised that retinal glia cells, neural and microvascular dysfunction is interdependent and essential in diabetes for the development of diabetic retinopathy. [101] Therefore the retinal circulation may be normalised by maintaining retinal glia and neuronal functions to prevent or delay diabetic progression. [224]

The effects of hyperglycaemia as a result of the production of ROS cause oxidative damage of the neurons. All of these finding therefore suggest that the integrity of the vascular and neuronal elements are affected. [221]

It is reported that each of the retinal cells described is affected by diabetes and studies have shown that these neural retinal defects are among the earliest detectable changes in diabetes. [225] Regardless of whether the initial events begin in blood vessels or neural cells, the clinical stages of diabetic retinopathy manifest cellular, histological, and functional features of a retinal neuropathy. [167] However there appears to be no clear evidence that a primary, selective defect in vascular cells is sufficient to cause diabetic retinopathy.

Clearly, then it is essential to treat both the vascular and neural elements of the retina to preserve vision. It is felt that this concept permits a new pattern for understanding the mechanism of vision impairment in diabetes and provides therapeutic targets that are directly linked to vision as is already know in glaucoma, that visual field and ganglion cell loss are the hallmark of the disease. Retinal nerve fibre (RNFL) thickness maps using Spectral domain optical coherent topography (SD-OCT) has been shown to be reliable and reproducible and has been used clinically to evaluate the loss. [227] So, it is reasonable to assume that there is an associated OCT thickness reduction in diabetic associated neuronal loss.

The introduction of new methods of assessment has meant that the evaluation of neurodegeneration has gained clinical significance. [223] Clinical studies have already shown that the measurement of retinal nerve fibre layer (RNFL) and ganglion cell layer thickness in patients with diabetes assessed by OCT could be a simple way of detecting the early signs of neurodegeneration in diabetic retinopathy. Advances in OCT with new algorithms have also enabled the structural changes in the retina to be objectively quantified. This is in comparison to routine fundus examination techniques using a slit lamp. [228]

OCT is a non- invasive imaging test based on low coherence tomography. It enables images of the retina to be taken using light waves to take cross- section pictures. The use of long wavelength of light allow for it to penetrate into the scattering medium. Optical coherence topography was initially time domain (TD) which collected 400 axial measurements per second with an axial resolution of approximately 10µm, it is now spectral domain(SD) using a higher scan resolution and reduced motion artefacts. The scan rate of SD-OCT is now at least 18,000 axial measurements per second with an axial resolution of 5µm and now giving three dimensional ( 3D ) images that are close to the isotropic volumetric scans that make fewer assumptions about the tissues in between, Therefore the high resolution of the SD-OCT gives an enhanced definition of the neuronal layers. The use of SD-OCT allows for identification of the retinal layers which could be used to explain those that are affected by diabetes and help identify mechanisms that cause changes early on in the disease. Using automated segmentation software individual layers can be quantitively assessed.

Heidelberg Engineering have described at least 13 retinal layers using SD OCT. These are as follows:

ILM (Internal limiting membrane) is transparent and very thin acellular membrane on the surface of the retina. It is formed by astrocytes and the end feet of muller cells.

RNFL (Retinal Nerve fibre layer) is formed by the expansion of fibres of the optic nerve and is thickest near the optic disc. Studies have already shown correlations of thinning of the RNFL in retinitis pigmentosa and glaucoma. [220] Shi et al. (2018) indicated in their study that patients with type 2 diabetes with diabetic retinopathy have shown that RNFL loss which has been suggested could be one of the earliest structural changes in the retina in these patients. [230, 233]

GCL (Ganglion Cell Layer) is a layer of the retina that consists of ganglion cells and displaced amacrine cells. Studies have shown loss of ganglion cell layer in the macula of patients with diabetes before vascular changes occur. [231]

IPL(Inner Plexiform Layer) is layer of synapses between the bipolar, amacrine and ganglion cells. Some associations have been reported correlating changes in IPL thickness and severity of diabetic retinopathy. [233]

INL (Inner Nuclear Layer) is made up of closely packed bipolar, horizontal cells, muller cells and amacrine cells and thinning of the INL has been seen in patients with type 1 diabetes and minimal diabetic retinopathy. [231]

OPL (Outer Plexiform Layer) is also known as the synaptic layer as it consists of a network of synapses between dendrites of horizontal cells and photoreceptor cell inner segments. It has been suggested that changes occur in the OPL in patients with Type 2 diabetes. [234]

ONL (Outer Nuclear Layer) contains the cell bodies of photoreceptors, both rods and cones.

ELM (External limiting Membrane) is the outer membrane with a network like structure and is situated at the base of the rods and cones.

PR (Photoreceptor Layers) rods and cones.

RPE (Retinal Pigment Epithelium) is the pigmented cell layer and is key to nourishing the retinal visual cells, has been suggested that it plays a causal role in diabetic retinopathy and hyperglycaemia can have detrimental effects on the RPE. [235]

BM (Bruch's Membrane) is a unique pentalaminar structure which acts an elastin and collagen rick extracellular matrix.

CC (Choriocapillaris) layer of capillaries.

CS (Choroidal Stroma) is a thin pigmented vascular layer of connective tissue.

The IRL (inner retinal layer) is described as consisting of the ILM through to the ELM. The ORL (outer retinal layer) consists of the photoreceptor layers through to the choroid. [307]

Although some studies had previously shown changes in retinal thickness in patients with type 1 diabetes, they used TD-OCT and therefore would have been unable to differentiate between the individual layers. Ng et al. (2016) investigated the association of diabetes and diabetic retinopathy with retinal ganglion cells (RGC) loss in participants with type 2 diabetes compared to age and gender matched controls without diabetes. They found that using OCT RGC loss was present in patients with diabetes with no diabetic retinopathy and was progressive in those with moderate or severe diabetic retinopathy. [221] However other studies have found thicker inner layers of the retina in patients with diabetes. [223] Demir et al. ( 2014) and Park et al. (2011) as cited by Ng et al. (2016) also found no association with RGC loss and increased severity of diabetic retinopathy. [221]

Van Dijk et al. (2012) found that in both Type 1 and Type 2 diabetes patients there was thinning of both the GCL and the RNFL in those that had existing signs of diabetic retinopathy. This was also correlated with duration of diabetes. In their study patients with minimum diabetic retinopathy had duration of 8 years longer than those without diabetic retinopathy. [218, 219 ,231] This indicates that both diabetic retinopathy and neurodegeneration could be closely linked as the processes develop

slowly over time as late complications of diabetes. Vujosevic et al. (2013) also found no RGC loss in patients with no apparent diabetic retinopathy. [236,237]

Fahmy et al. (2018) carried a study to evaluate the effect of diabetes, diabetic retinopathy, and HbA1c on peripapillary retinal nerve fibre layer thickness (RNFLT) using optical coherence tomography.

In their study they found that there was a correlation between the level of HbA1c and RNFL. The RNFL was affected in the superior quadrant of diabetic patients in relation to glycaemic control as compared to healthy patients. This is consistent with the outcome of previous studies cited by Fahmy et al. (2018) which demonstrated that RNFLT was decreased in patients with preclinical DR in all 4 quadrants, but the difference was significant only at the superior quadrant.

However other studies have reported significant changes in other quadrants as well. [75]

Chen at al. (2015) in their study showed that the average peripapillary RNFL thickness in diabetic patients without clinical diabetic retinopathy was significantly decreased compared to age-matched healthy controls. They found that the peripapillary RNFL thickness was significantly reduced in diabetic patients without detectable diabetic retinopathy. They suggested that measurement of peripapillary RNFL thickness may become a novel way to evaluate and monitor early retinal changes in diabetic patients. [238]

Simo (2018) hypothesised that as studies have shown in both experimental and human studies that as neurodegeneration is an early event in the retina in diabetes, glial activation and some degree of neural apoptosis exists in the retina of most individuals with long term diabetes. This microvascular disease may only develop in a subset when the apparent changes are seen that are initially triggered by glial activation and neurodegeneration. However, there may be two pathophysiological events in the later stages that evolve independently. [101]

However, many of the findings from the studies cited in Ng et al. (2016) showed that there have been inconsistent results and data from animal and post mortem human studies could not agree with the evidence of RGC damage preceding the onset of the apparent microvascular diabetic retinopathy lesions. [221]

Although the neurodegenerative process is found at very early stages in both experimental and clinical settings, its relevance in the pathogenesis of retinal microvascular abnormalities remains to be elucidated. Vujiosevic at al. (2013) described how hyper reflective intraretinal spots (HRS) seen using SD-OCT have been identified in diabetic eyes even without diabetic retinopathy. They proposed that the HRS are mainly located in the inner retina, where the resident microglia is present and that these may represent microglial activation in the early stages of diabetic retinopathy. [236]

In another in vivo study, SD-OCT was used to assess the changes in thickness of selected retinal layers both in the macula and the peripapillary area in diabetic patients without diabetic retinopathy or with early stages of DR versus normal patients. They reported a decrease in RNFL thickness in the macula of diabetic eyes even without any clinical sign of retinopathy. The author suggested that the "reduced RNFL thickness may be due to progressive ganglion cells and astrocytes loss induced by diabetes but that it may depend on direct toxicity of hyperglycaemia or on Müller cells dysfunction, which are unable to maintain an adequate osmotic equilibrium between the intra- and the extracellular matrices with consequent apoptosis of neuronal cells and progressive axonal degeneration". [237]

The results supported the theory that neural changes may occur before clinical signs as they found thinning of the inner neural retina in diabetic patients without clinically detectable retinopathy and with mild and moderate nonproliferative retinopathy without macular oedema. They found that retinal thinning is mainly due to the selective thinning of inner retinal layers in the central retina which they suggested strongly indicates an early neuronal loss in DR. They also reported that

automatic intraretinal layering by SD-OCT could be a useful tool to diagnose and monitor early intraretinal changes in diabetic retinopathy. [237]

Different authors however have reported the thinning of RNFL and, in some cases, of the GCL + IPL complex. [239]

DeBuc et al. (2010) in their study found that the RNFL and GCL+IPL complex was thinner in diabetes mellitus and moderate diabetic retinopathy eyes than in normal healthy eyes. However, they used a different method of assessment by comparing thickness measurements of diabetic eyes without the presence of retinopathy to eyes with minimal diabetic retinopathy. The suggested that the results of the study may reflect neurodegenerative changes in the diabetic retina. The authors proposed that the findings also have possible implications for early detection of macular damage in diabetes as the macular region is rich in retinal ganglion cells, and so they suggested that diabetic damage of this central region might occur early in the disease process. They also supported the indication of using intraretinal layer quantification on OCT images to give a better understanding of macular pathophysiology in health and disease. [239]

The authors proposed that the results of the study revealed the preceding step of manifest diabetic retinopathy may be a neurodegeneration of the retina which seems to be detectable in vivo by OCT mapping of the local retinal abnormalities and corresponds to previous experimental results. [239]

Changes in OCT seen in the inner neuronal layers could provide parameters that appear before the clinically apparent signs of diabetic retinopathy. These could provide biomarkers that potentially clinicians could use to help detect the very early stages of diabetic retinopathy and may offer a way of monitoring response to treatment interventions. [221]

Current treatments target later stages of diabetic retinopathy when vision usually has already been affected. [101] By having a better understanding of the pathogenesis of diabetic retinopathy more novel and more efficient preventions and interventions could be explored.

The FIELD and ACCORD studies did not use OCT to assess DME and neither had diabetic retinopathy as a primary endpoint. Therefore, it would be of great interest to analyse the effect of such proposed interventions in controlling changes related to diabetic retinopathy and maculopathy using OCT. [80] OCT is now widely used for monitoring many conditions as it is easy and quicker to use and provides anatomical and structural information.

At present, the presence of neurodegeneration can be measured by SD-OCT which permits the examination of the individual layers. However, there are other means of doing this that have been reported and could also provide additional information by means of functional methods such as multifocal electroretinography (mfERG), standard automated perimeter (SAP) frequency doubling perimeter (FDP), or microperimetry. [101]

As already mentioned, neurodegeneration is an early event in the pathogenesis of diabetic retinopathy which could participate in microvascular impairment. Therefore, the identification of patients in whom retinal neurodegeneration could play a key role for the development of diabetic retinopathy is a challenge but with studies investigating treatments with interventions such as neuroprotective agents this could undoubtedly give additional crucial information to monitor early changes. The recent Euro condor study which was a trial to assess the efficacy of neuroprotective drugs administered topically to prevent or arrest diabetic retinopathy failed to show any effect of two neuroprotective drugs that were eye drops ( brimonidine and somatostatin) in preventing or arresting the microvascular changes associated with diabetic retinopathy. Their results did nonetheless find thinning of the GCL-IPL and RNFL layers which increased with the presence of diabetic retinopathy. Despite this they also found that 32% of the patients with visible diabetic retinopathy on fundus photos did not show any functional or structural abnormalities related to neurodegeneration. However, there were limitations to this study in that the follow up was short and they included lots of patients with no or mild disease with good control. [241] The complex pathology observed in the diabetic retina reflects the inter-connected nature of all the component

cells in this neural tissue. Most current therapies however remain focused on late-stage PDR and DME, but it is suggested that widening the appreciation of the diabetes-associated pathophysiology in the neurovascular unit could provide opportunities to prevent disease in the earlier stage stages of the disease. The evidence suggests that in the coming years new treatments based on an understanding of the causative mechanisms of diabetic retinopathy will be developed and address the need for both vascular and neuroprotection. [241]

Despite significant advances towards the understanding of retinal neurodegeneration in diabetic retinopathy, it remains to be clarified whether retinal neuronal abnormalities are indicators for developing microvascular dysfunction, or whether microvascular and neuronal dysfunction develop at the same time in the early stages of diabetic retinopathy. [24]

Table 2.1 A summary of the studies investigating the changes in the retinal layers of patients with diabetes.

| | EVIDENCE | |
|---|---|---|
| Retinal Layer | Positive association | No association |
| Ganglion cell layer | [218,219,223,231,236,237,239,241] | [221,233,294] |
| Inner plexiform layer | [218,233,239,241] | |
| Inner nuclear layer | [218,231] | |
| Outer plexiform layer | [234] | |
| Retinal pigment epithelium | [235] | |
| Retinal nerve fibre layer | [75,218,219,227,228,230,231,237,238] | |

## 2.4 Antioxidants, supplements and diabetic retinopathy

The role of minerals and vitamins which are antioxidant micronutrients have been investigated as it is thought that they may help reduce the oxidative stress. [214] As animal models of diabetic retinopathy have shown beneficial effects of antioxidants on the development of retinopathy, it is clear that the limited clinical trials that have been carried out have provided some ambiguous results. However, as antioxidants are being used for other chronic diseases it certainly warrants further investigation to investigate potential beneficial effects of antioxidants in the development of retinopathy in diabetic patients. [214,215,245]

Antioxidants that are derived from the diet that have been studied extensively include vitamins E, C, A and carotenoids and studies have shown that they may act at different levels by increasing the antioxidant defence enzyme capabilities or decreasing the formation of ROS or scavenge free radicals. Da Silva et al summarised the findings in a table which is reproduced below:

Table 2.2 Summary of antioxidants previously produced by Da Silva et al. (2010) that have been reported to have high therapy potential in diabetic retinopathy. [209]

| ANTIOXIDANT | MECHANISM OF ACTION |
|---|---|
| Amino guanidine | Inhibits the accelerated death of retinal capillary cells and development of retinopathy, inhibits lipid peroxidation and AGEs formation |
| Ascorbic acid, acetate, α- tocopherol, trolox cysteine, NAC, β-carotene and selenium | Reduce PKC in the retina, and lipid peroxides, prevents the decrease of SOD, GR and catalase |
| Benfotiamine | Inhibits MnSOD, increases the inhibition of acellular capillaries in the retina blocking the major pathways involved in hyperglycaemia induced retinal dysmetabolism; |
| Caffeic acid | Anti-angiogenic activity in retinal endothelial cells and retinal neovascularization, suppression of the ROS induced and VEGF expression |
| Calcium dobsilate | Decreases retinal permeability, stabilized BRB and reduced overexpression of VEGF |
| Curcumin | Ocular anti-inflammatory multipotent activities |
| Lipoic acid | Attenuates the apoptosis of retinal capillary cells and acellular capillaries, decreases the levels of nitrotyrosine, VEGF and oxidatively modified proteins, activation of NF-kB |
| Nicanartine | Inhibits pericyte loss |
| Pycnogenol | Free radical scavenger, anti-inflammatory properties |
| Rosmarinc acid | Anti-angiogenic activity to retinal neovascularization, inhibits the proliferation of retinal endothelial cells, and the angiogenesis of tube formation |
| Selenium | Down-regulate VEGF production in the retina of diabetics |
| Trolox | Partially prevents the loss of pericytes via reducing membrane lipid peroxidation |
| Vitamin C and E | Reduce neovascularization, prevent the inhibition of retinal GR, GPx and SOD activities |
| Vitamin E | Restores retinal blood flow, free radical scavenger, normalize diabetic retinal hemodynamic |
| Zinc | Prevents diabetes-induced GSH loss in the retina |

Studies have already investigated oxidative stress in other conditions such as stroke as it has been suggested that the pathogenesis of stroke is partly due to oxidative stress which is reduced by some of the compounds in several vegetables and fruits. [214] A study by Hariri et al. (2013) investigated the role of B vitamins and antioxidants with the risk of stroke. They examined 69 stroke patients matched to a control group of 60 unaffected patients. They assessed the dietary intake using a validated self-administered food frequency questionnaire(FFQ). Their findings supported previous studies that had shown that higher consumptions of certain fruits and vegetables are associated with a lower risk of stroke. In particular deficiencies in B vitamins and antioxidant vitamins E and C have suggested a protective mechanism from stroke. [247]

Studies have discussed many antioxidants such as vitamins C, E A and carotenoids which are also an important class of antioxidants. [209] These non-enzymatic antioxidants systems consist of scavenging molecules that are endogenously produced and also include lipoic acid and selenium. It has also been suggested that enzymatic antioxidant systems such as copper (Cu), Zinc (Zn), manganese superoxide dismutase (MnSOD), GPx, GR and catalase may remove the ROS sequentially or directly which would then prevent excessive accumulation and resulting adverse effects. [209]

Antioxidants such as N-acetylecysteine (NAC), Vitamin C and α -lipoic acid have also been shown to be effective in reducing diabetic complications indicating that it may be beneficial either by ingestion of natural antioxidants or through dietary supplementation. [209] Antioxidants such as lipoic acid is capable of thiol-disulphide exchange and is able to scavenge ROS. By doing this it reduces the metabolites such as glutathione which will then help maintain a healthy cellular state. [215].

Zinc has antioxidant properties as it contains the antioxidant enzyme superoxide dismutase and has been shown to reduce glutathione loss in the retina. [215]

Vitamin E exists is 4 common forms, alpha-tocopherol, beta-tocopherol, gamma-tocopherol, and delta-tocopherol. Alpha – tocopherol is the most effective antioxidant of the vitamin E group and is reported to protect against lipid peroxidation. Experimental studies have suggested that vitamin E is

effective in both the prevention and development of complications in diabetes. Its actions consist of lowering the lipid peroxidation and lowering ROS concentration with less effect in protein and DNA oxidation. It is normally found in high concentrations in the retina but is reported to be below standard limits in individuals with type 2 diabetes. [250]

Supplementation with vitamin E has been found to reduce glycated haemoglobin in both type 1 and type 2 diabetes. This is seen even more so if the individuals have poor glycaemic control and already low level of vitamin E. [251] In patients with diabetic retinopathy the role of vitamin E has been related to increased levels of oxidative stress in the vascular endothelium. [250]

Taurine and vitamin E supplementation along with selenium has also been reported and showed a reduced biochemical retinal alteration in diabetic rats. [209]

Pycnogenol is a natural pine bark extract with strong antioxidant and hyperglycaemic properties. [123] It has been shown to be a compound with both free radical scavenging and anti-inflammatory properties and has been reported in earlier studies to have beneficial effects on the progression of retinopathy in diabetic patients as described by Kowrulu (2007). [207]

Domanico et al. (2015) also described the influence of an antioxidant supplementation of pycnogenol 50mg, Vit E 30mg and co enzyme Q10 20mg on the circulating levels of reactive species (ROS) in patients with non-proliferative diabetic retinopathy over a period of 6 months. [252]

Vitamin C (ascorbic acid) prevents the propagation of chain reactions by scavenging ROS directly and therefore preventing a reduction in protein glycation. It also prevents the breakdown of nitric oxides, and decreases the oxidation of low-density lipoproteins (LDL). It is therefore described as chain breaking antioxidant. [123]

Therapies with different vitamins and supplement combinations have been used to treat diseases associated with mitochondrial dysfunction. In experimental studies the administration of a diet complemented with antioxidant vitamins C and E was shown to inhibit the impairment of

antioxidant defence system. Better results were however seen using vitamins C+E mixture complemented with Trolox, N-acetyl cysteine, beta-carotene and selenium, or with beta-carotene, zinc and copper. Combinations of micronutrients are being used for other ocular disease such age-related macular degeneration. [122]

A number of animal studies have shown the effects of antioxidant supplementation in diabetic rats. Benfotianine which is a lipid soluble thiamine (Vit B1) inhibits MnSOD and has been shown to inhibit the increase in acellular capillaries in the retina of diabetic rats. It is shown that it does by blocking the major pathways involved in hyperglycaemia induced retinal dysmetabolism, including AGEs, PKC and hexosamine pathways. [123, 253,255]

Green tea is rich in polyphenols which is described as a potent antioxidant is said to inhibit peroxidation and scavenges hydroxyl and superoxide radicals. Other studies as cited by Kowrulu reported that green tea supplementation in diabetic rats improved the levels of superoxide dismutase (SOD) and glutathione(GSH) and reduced the glucose levels. It was also shown to improve retinopathy which was evident by reductions in acellular pericyte ghosts and capillaries.

Several other animal studies have also used the systemic administration of antioxidants such as green tea, cocoa, vitamin E, SOD mimetics, or nutritional supplements containing zeaxanthin, lutein, lipoic acid, or omega-3 fatty acids have been effective in preventing retinal neurodegeneration and early microvascular impairment. [205, 256]

Richter et al. (2018) evaluated the effects of beta-glucan and vitamin D supplementation on inflammatory markers in patients with diabetic retinopathy and found Vitamin D with beta-glucan significantly lowered inflammatory markers in patients with diabetic retinopathy over 3 months but further work is needed in this area. [200]

Lutein and zeaxanthin are carotenoid pigments found in the retina but more so in the macula in high concentrations. They are antioxidants and reduce oxidative stress by scavenging free radicals. They

have been studied extensively in many eye diseases especially in age related macular degeneration (AMD). Macular pigment is the term used to describe the three isomeric carotenoids meso-zeaxanthin (MZ ) a synthesis product of lutein), lutein (L) and zeaxanthin (Z) which all accumulate in the macula. The high levels of macular pigment protect against photo oxidative damage by filtering blue light and provide antioxidant protection the retina by inhibiting the peroxidation of long chain polyunsaturated fatty acids. [257]

Macular carotenoids have been reported as very effective antioxidants that are capable of quenching singlet oxygen and triplet state photosensitizers this then inhibits the peroxidation of membrane phospholipids, scavenging ROS, and results in reduction of lipofuscin formation. These essential functions of macular pigment decrease oxidative stress in the retina and enhance vision in both normal and abnormal retinas. [258]

Several experimental studies also demonstrated a reduction in retinal oxidative damage after carotenoid supplementation in diabetic rats. However, just a few studies evaluated the association between diabetic retinopathy and macular pigment optical density (MPOD), and the results were not consistent. Results from studies have been inconsistent with some indicating that diabetic patients with retinopathy had lower levels of macular pigment whilst others showed the reverse. [257]

Since lutein and zeaxanthin are entirely of dietary origin and cannot be synthesized by the human body, foods rich in those elements, such as green leafy vegetables, corn, squash, Chinese wolfberry, and egg yolks have been reported to increase levels of macular pigment. It is also reported that macular pigment levels are also affected by multiple other factors, including genetics, age, gender, smoking status, and body mass index. [257]

Several objective techniques have been used to measure macular pigment optical density (MPOD) indirectly and noninvasively. They are divided into either psychophysical heterochromatic flicker photometry (HFP), HFP and motion photometry or optical methods (auto fluorescence spectrometry reflectometry, imaging reflectometry, and Raman spectrometry. They have all been previously well

described in the literature and each one has certain merits and limitations. In conjunction with many of the MPOD techniques, HFP is based on the spectral absorption properties and retinal location of macular pigment, it determines the MPOD by presenting a light stimulus of two alternating wavelengths at the fovea and at a parafoveal area, one that is short and maximally absorbed by macular pigment, and another longer which is not absorbed by macular pigment. [258]

The relationship between macular pigment and macular disease such as AMD has been extensively studied. Equally studies have shown that lower levels of macular pigment have been associated with known risk factors such as smoking and elevated lipid profiles for developing age-related macular degeneration. There has been growing evidence that higher levels of carotenoids in the diet and in the blood, plasma are associated with a lower risk for age related macular degeneration. Other studies have shown that by increasing carotenoids in the diet or by providing lutein and/or zeaxanthin the macular pigment levels can be manipulated.

However, there have been limited studies with conflicting results evaluating the association between macular pigment levels and diabetic retinopathy. [258]

One study found that MPOD level is not statistically significantly influenced by the onset of diabetes or early stage of DR in the studied population. However, the MPOD level has been shown to be positively associated with thicker central foveal thickness and higher intake of foods containing carotenoids. [258]

Humans cannot synthesize macular pigment, but must absorb lutein and zeaxanthin from the diet. Many studies have demonstrated that diet, especially carotenoid intake, influences macular pigment levels. Ford et al. (1999) as cited by Lima et al. (2010) found that serum levels of macular carotenoids in diabetic patients were significantly lower than normal patients, implying a deficiency of lutein and zeaxanthin in diabetic diet or poor absorption from the gut in diabetic patients. They also suggested that it is possible that the dietary habit may interact with other factors such as

hyperglycaemia, could influence the MPOD levels, which could partially explain the results of no association between diabetic retinopathy and MPOD. [258]

Equally a study by Bilen et al. (2016) compared macular pigment optical density (MPOD) in diabetic and non-diabetic patients by using heterochromatic flicker photometry and to investigate the correlation of MPOD with glycosylated haemoglobin (HbA1C), serum lipid levels and vitamin D level.Their results found no correlation was found between MPOD, HbA1C, serum lipid levels and vitamin D levels. [259]

There have been many studies investigating the role of antioxidants, some of the same nutritional antioxidants that have been demonstrated to slow the progression of AMD, have shown exciting results in preventing the pathogenesis of retinopathy in diabetic rodents. It has previously been postulated that this would merit the further testing of antioxidants in a clinical trial to prevent the development and/or progression of diabetic retinopathy.

However, although some studies using dietary antioxidants have provided some positive results, clinical studies have been limited, and there have been many variable results. Further studies are required.

Table 2.3 Summary of the studies investigating the relationship of single or combinations of antioxidants and other nutrients with the development of diabetic retinopathy.

| Antioxidant/other nutrient | Evidence |
|---|---|
| Vitamin E | [122,205,209,250,251,252,256] |
| Vitamin C | [122,123,205,206,209] |
| Vitamin A | [209] |
| Carotenoids | [205,206,209,256,257,258] |
| Zinc | [122,209] |
| Vitamin B | [123,205,206] |
| Copper | [122,209] |
| Manganese superoxide dismutase (MnSOD) | [205,209,256] |
| N-acetyl cysteine (NaC) | [209] |
| α -lipoic acid | [205,209,256] |
| Taurine | [209] |
| Pycnogenol | [123,207,252] |
| Selenium | [122] |
| Vitamin D | [200,259] |
| Beta-Glucan | [200] |

## 2.5 Genetics

Many studies have investigated the environment wide associations and genetic variants with the development of diabetes with some evidence in relation to the development of diabetic and limited in relation to diabetic macular oedema. Despite this a significant number of potential susceptible genes have been identified. [260,261]

In contrast to genome-wide association approach, the candidate gene approach focuses on associations between genetic variation within pre-specified genes of interest and phenotypes of disease states. Polymorphisms of many genes have also been studied, and some have been associated with DR to date. [261] Single nucleotide polymorphisms (SNPs) in an array of genes involved in the complex pathways for diabetic retinopathy have been reported to have an effect on the development and progression of diabetic retinopathy in different populations. Those that have been studied are groups of genes with known roles in the metabolism or function of diabetes. [262]

Therefore, as suggested by Broadgate et al. (2017) some of the genes studied may be linked to more than one of the processes in the complex pathways of diabetic retinopathy. [262]

Several large-scale candidate gene and genome wide association studies have been performed. Some of the associations are summarised in Table 2.4. The full function of many of the genes is still not fully understood.

SLCA08

SLC30A8 a zinc transporter gene is expressed in pancreatic islets and localized in insulin secretory granules of islet b cells. Studies have described its function appears to be to modulate insulin secretion and storage. [260] Other studies have found diet-dependent glucose intolerance and insulin secretion abnormalities in SLC30A8 knockout mice. [221] Thus, this SNP may be important in the pathogenesis of diabetic retinopathy.

ALR2

Also, the Aldose reductase gene (ALR2) which has been shown to have a possible association with diabetic retinopathy was also identified in both patients. Alr2 has been reported to be the first and rate-limiting enzyme of the polyol pathway and is involved in the pathogenesis of diabetic retinopathy. ALR2 is known to convert glucose to sorbitol. It has been shown that in the presence of hyperglycaemia, sorbitol accumulates intracellularly leading to osmotic stress. (260) This has been shown to result in the formation of microaneurysms, basement membrane thickening and pericyte loss in animal models. [(260)]

Recent studies have implicated the aldose reductase gene as being important in initiating diabetic retinopathy. Studies in differing ethnic populations have associated single nucleotide polymorphisms (SNP's) within the gene promoter region with the susceptibility to diabetic retinopathy. However, the risk allele could be different amongst different ethnic groups and type of diabetes. [(263)]

Therefore, the results of some studies have been limited by a number of negative findings and therefore many failed to find efficacy of ALR2 in preventing incidence or progression of diabetic retinopathy so its role in this area is still uncertain.

MnSOD

Other studies aimed at reducing oxidative stress have also reported some success. Kowrulu et al showed in their studies that retinal mitochondrial dysfunction has a crucial role in the apoptosis of capillary cells. This occurrence is thought to precede the development of retinal histopathology features of diabetic retinopathy and equally the proposed metabolic phenomenon. The enzyme MnSOD which is responsible for scavenging mitochondrial superoxide, was inhibited. [(206)]

Others have found that histone methylation of Sod2 which is the gene that encodes MnSOD showed a potentially important role in the development and progression of diabetic retinopathy. In a study using streptozotocin treated rats, the transgene expression of AAV-delivered MnSOD, the key

antioxidant enzyme in mitochondria, elevated the level and activities of retinal MnSOD and was shown to reduce basement membrane thickening and retinal capillary apoptosis by catalase This was then associated with restoration of retinal MnSOD and catalase activity, which succeeded in counteracting hyperglycaemia-induced oxidative stress. [212].

The challenges in designing a genetic association study in relation to diabetic retinopathy are multiple. The genetic contribution to diabetic retinopathy appears to be relatively modest, requiring larger sample sizes to achieve sufficient statistical power. Determining the number of patients requires complex statistical planning that is required to achieve sufficient statistical power. [261]

The use of candidate gene studies to examine diabetic retinopathy therefore has revealed several limitations. All genetic studies require replication in at least one other cohort, but few replicative studies have been performed. Reported associations have been weak, and conflicting results have been published. Meta-analyses are hampered by lack of standardization for categorizing diabetic retinopathy and DME, analysis across different ethnicities, and small sample sizes. Additionally, incorrect assumptions regarding gene importance or involvement in pathophysiology may lead to inconsistent, or negative results. The results that have been reported in the vast literature have been conflicting. This has been due to many factors using small sample sizes, different ethnicity, different type of diabetes, variations in study design, differences in clinical end points, and underlying genetic differences between study groups. [261]

Many individual studies have reported statistically significant associations between various polymorphisms and features of diabetic retinopathy. However, it is difficult to draw conclusions as many of the results are conflicting in the available literature. [261]. Gene therapy for diabetic retinopathy although slowly emerging as a possible therapeutic approach it is still in its infancy. There are considerable numbers of studies that have and are still being carried out but they are still in vitro and in vivo studies. [24]. In animal studies rats and mice tend to be used more than other

species however they do not possess a macula so are not able to replicate all the features of diabetic retinopathy such as DMO. [101]

Table 2.4 Summary of Genes reported to have a positive association with diabetic retinopathy adapted from Broadgate et al. (2017). [262]

| GENE | GENE |
|---|---|
| ACE | L1PG |
| AD1POQ | LOC101928923 |
| AGER | LOC105369178 |
| AKR1B1 | miR-126(EGFL7) |
| ARHGAP22 | MiRNA-146a |
| CLL2 | MRPL14 |
| CDKAL1 | MTHFR |
| CFB | NFE2L2 |
| CFH | NOS3 |
| CHN2 | LOC105369178 |
| CNR1 | PON1 |
| CRP | $PPAR_{\gamma}$ |
| CXCL8 | PPARGC1A |
| CXCL10 | ROMO1 |
| EDN1 | $RXR_{\gamma}$ |
| EPO | SELP |
| FGF2 | SERPINE1 |
| FNDC5 | SERPINF1 |
| GLO1 | SLC30A8 |
| GR1K2 | SLMAP |
| GSTM1 | SOD2 |
| GSTT1 | SORD |
| HFE | TCF7L2 |
| H1F1A | TGFβ |
| HMGA1 | TLR4 |
| ICAM1 | TMEM217 |
| IDUA | TNFα |
| IGSF21- KLHDC7A | TNFRSF11B |
| IL6 | TXNRD2 |
| IL10 | UCP1 |
| ITGA2 | UCP2 |
| ITGB3 | UTS2 |
| KCNJ11 | VDR |
| KDR | VEGFA |
| LEKR1-CCNL1 | PON1 |

## 2.6 Research Rationale

The World Health Organization estimates that diabetic retinopathy accounts for approximately 5% of the global prevalence of blindness, with estimates of 15% to 17% in developed countries. Long term glycemic control and optimal blood pressure levels may possibly delay or prevent retinopathy, and treating retinal vascular changes in patients with diabetes prevents visual loss. However, all require significant clinical input or specialist ophthalmologic care.

Review of the literature provides evidence for the role of oxidative stress in the pathogenesis of diabetic retinopathy and there is increasing interest in the role of antioxidant micronutrients in the prevention of diabetic complications. [214, 209]

Micronutrients may potentially alter the risk of diabetic retinopathy. In particular, the role of nutritional supplementation is of increasing interest with regard to ocular disease. [123] Randomised controlled trials have demonstrated the effectiveness of supplementation for age related macular degeneration however conclusive clinical trials on diabetes in this area are lacking. Various trials have identified positive effects of some nutrients on outcome measures relating to insulin resistance and cardiovascular factors in diabetics but the evidence on the beneficial effects on the development of retinopathy is limited. [123]

Furthermore, as described there are many clinical and laboratory evidences to support neuronal damage in the retina of diabetic patients even before there are any fundal changes clinically. Studies have also shown a correlation between macular pigment optical density and HbA1C in type 2 diabetics with and without retinopathy. [258] However although various theories have been proposed about neurodegeneration in diabetic retinopathy it remains unclear however to what role it plays in the development and progression of diabetic retinopathy and if there is an early detectable relationship with antioxidant intake. More importantly, how it would affect vision long term. Further research in the area is necessary.

This study aimed to explore this further by examining the relationship between retinal neuronal layer loss assessed using SD-OCT with dietary intake of foods using a food frequency questionnaire. The development of diabetic retinopathy will be further explored to determine if there is a relationship between the severity of diabetic retinopathy with neuronal layer loss in individuals with type 1 and type 2 diabetes. No previous studies appear to have explored such relationships between dietary intake, neuronal layer loss and diabetic retinopathy. The study also explored the relationship of HbA1C and serum lipid levels with macular pigment optical density measurements.

Diabetic Retinopathy is a leading cause of visual loss therefore, it is important to be able to reduce the number of patients who may be developing this disorder as early as possible so any potential treatment can be offered. This research will aimed to deepen our understanding of why diabetic retinopathy occurs and may offer possibilities of a new therapeutic approach to prevention that can be explored by way of an interventional clinical trial. The outcomes of this research could increase our understanding of the beneficial effects of antioxidants in the prevention of the development of retinopathy in patients with diabetes.

Also, whilst many studies have investigated the environment wide associations and genetic variants in diabetic retinopathy but there still remains little evidence in relation to the early development of diabetic retinopathy and diabetic macular oedema. A significant number of potential susceptible genes have been identified. [262] However, the results of some studies have also been limited by a number of negative findings and therefore many failed to find efficacy of preventing incidence or progression of diabetic retinopathy so the exact roles in this area is still uncertain. Studies have suggested that genetic analysis may reveal a gene mutation which may predispose to diabetic retinopathy even if the patient is asymptomatic. Some potential SNP's that have previously been identified were selected for analysis as part of this pilot study. [262]

The future benefits from an observational study could be generalised to patients with diabetic retinopathy. If we find that levels of antioxidants or other components of food intake play a key part

in diabetic retinopathy then there will be scope to carry out a large multi-centre clinical trial looking at antioxidant or dietary supplements, which may help to prevent progression of diabetic retinopathy and prevent visual impairment.

## 2.7 Summary

As antioxidants are being used for other chronic diseases it certainly warrants further investigation by way of a clinical trial to investigate potential beneficial effects of antioxidants in the development of retinopathy in diabetic patients. If successful this project could result in radically altered management and visual outcomes in patients with diabetic retinopathy as early intervention could prevent the devastating disease much sooner.

The aim of this thesis is to explore the relationship of neuronal layer loss with dietary intake of antioxidants in patients with Type 1 and Type 2 diabetes, including the possible association with severity of diabetic retinopathy. Literature has been reviewed and a research rationale put forward. In the next chapter the protocol for the DECAN study will be described.

# Chapter 3 Trial design

The aim of this thesis was to investigate the effect dietary antioxidant intake using a food frequency questionnaire and explore the possible correlation with neuronal layer loss in patient with type 1 and type 2 diabetes with and without diabetic retinopathy. In the previous chapter's reviews of the literature provides evidence for the role of oxidative stress in the pathogenesis of diabetic retinopathy and there is increasing interest in the role of antioxidant micronutrients in the prevention of diabetic complications. [214,209] As also described evidence was reviewed that suggested that neuronal damage in the retina of diabetic patients occurs even before any fundal changes are seen clinically. [218,221]Although various theories have been proposed about neurodegeneration in diabetic retinopathy the evidence remains unclear to what role it plays in the development and progression of diabetic retinopathy and if there is an early detectable relationship with antioxidant intake and changes in the neuronal layers.

The research rationale was put forward and the protocol for the DECAN study was developed as described below.

## 3.1 Research objectives

A Prospective study of type 1 and type 2 Diabetics examining the correlation between antioxidant levels and neuronal layer loss as an exploratory study (DECAN study).

To assess dietary antioxidant intake level using the self-administered Semi quantitative food frequency questionnaire (FFQ) [308] developed and validated by the Scottish Collaborative Group (SCG).

Perform genetic analysis to identify if certain DNA sequence variants predisposes patients to oxidative damage using the restriction fragment length polymorphism (RFLP) approach, so that

several potentially important single nucleotide polymorphisms (SNPs) within each pathway would be targeted. A cross sectional study with a longitudinal element was undertaken.

## 3.2 Recruitment

Patients with type 1 and type 2 diabetes patients were recruited from the diabetic eye clinics at Frimley Park Hospital. They were either sent a patient information leaflet (see appendix 6) and an introduction letter to complete if they wished to attend. Alternatively, during their routine outpatient appointment, the patient was asked by the clinical team if they wished to take part.

## 3.3 Inclusion/exclusion criteria

For inclusion all patients were aged over 18 years and able to give written consent, male or female with no prior treatment of diabetic retinopathy. Patients with Type 1 or at least 5 years duration of Type 2 Diabetes were included in the study. They also had no other retinal disease. Those with other systemic conditions and on medication were included; this was captured using a personal data questionnaire.

Exclusion criteria were patients with active intraocular or periocular inflammation, history of any intraocular surgery within the last 2 months or current treatment with any other investigational products were excluded.

## 3.4 Sample size calculation

In order to meet the study primary objective of showing 2-sided statistical significance at 5% significance level, with 90% power, of a 0.1 correlation between neuronal layer thickness and antioxidant intake at 6 months a total of 1028 patients were to be included in the study.

The change in thickness of the retinal neuronal layer as measured by OCT were to be analysed in a general linear mixed model using SPSS neuronal layer thicknesses as dependent variables, with

independent variables the antioxidant intake questionnaire score, including as necessary covariates any risk factors of diabetic retinopathy.

Additionally, the just mentioned analysis for the primary objective will be carried out with the addition of data for the baseline visit and 12 months post baseline with a categorical variable for visit and a visit by antioxidant questionnaire score interaction. The antioxidant FFQ score analysis will be carried out by the Scottish Collaborative Group (SCG FFQ). [308] To examine the correlation between the retinal neuronal layer loss and development of diabetic retinopathy an exactly analogous approach will be used to that just described for analysing the correlation between antioxidant intake and neuronal layer loss.

The sample size was recalculated due to recruitment problems as follows:

In order to detect a correlation coefficient r=0.25, with Type I error 0.05 and Type II error 0.05, a sample size of 202 was required.

## 3.5 Baseline data

**Personal Data questionnaire (PDQ)**

A personal data questionnaire (PDQ) was used to record demographic data, lifestyle data and general health status. The PDQ included duration of diabetes, type of treatment with oral antidiabetics or insulin, age, weight, smoking status and level of physical activity at the first visit as well as recorded. See appendix 5

**The Scottish Collaborative Food Frequency Questionnaire (SCG FFQ)**

A food frequency questionnaire (SCG FFQ) was given to each participant on enrolment. See appendix 1d. [308]

The SCG FFQ is a research tool that has been validated and used in a number of large-scale epidemiological studies to assess the dietary intake of a wide range of nutrients. It contains 19

sections that detail 175 commonly eaten foods or drink that estimate habitual diet over the previous 2-3 months therefor it is semi-quantitative as the participants estimate the amounts of each food and frequency of eating it. A standard operating procedure (SOP) was issued with the questionnaires and was followed for the duration of the study (see appendix 1e). [308]

Patients completed the FFQ at their first visit for the preceding 2 months and then for each follow up visit. Participants were not given any advice on current dietary intake or asked to make any changes to their diet.

**Visual acuity**

This included a visual acuity check using Logmar. Distance VA only was measured using EDTRS log MAR charts with glasses if worn. Log MAR charts have 5 letters and 0.1 log MAR progression per line. The advantage of using these charts is that they provide an equal-interval scale, and there are five letters per line. The visual acuity was then recorded in total number of letters identified.

Standard Snellen charts do not provide a linear scale and have a decreasing number of letters per line as the letter size increases.

**Macular pigment optical density (MOPD)**

Macular pigment optical density measurements were taken using the Maculux for both eyes.

Method described later in the chapter.

**Slit lamp examination**

A detailed eye examination was carried out at every visit. Slit lamp examination of the front of the eye, and then a dilated examination of the back of the eye using a 78D lens. The presence of any diabetic retinopathy was then determined and any other pathology noted.

**Fundus Photography**

The retina was then photographed using the Optos wide field camera.

Grading was carried out using the fundus examination and optos photos. Each participant was graded using the Early Treatment Diabetic Retinopathy Study (ETDRS) severity scale.

**Optical Coherence Tomography (OCT)**

The following parameters were set and repeated at each visit following a devised standardised imaging protocol. The Heidelberg spectralis SD-OCT was used for both scans; all patients were dilated for the scans and appropriately focused for refractive error. Refractive error was not recorded. The transverse software was enabled with automated segmentation.

Macular F2 High Density Volume Scan: TRANSVERSE SCAN

Resolution mode: High speed

ART: 16 Fames

Pattern: 97 scans, 15°x10°, 30µ separation

Centred: Anatomical fovea

Macular F2 Dense Volume Scan:

Resolution mode: High speed

ART: 24 Fames

Pattern: 49 scans, 20°x20°, 120µ separation

Centred: Anatomical fovea

3.6 Outcome measures

**Bloods**

Blood samples for DNA, HbA1c and serum lipids were taken at baseline.

**SCG FFQ**

The SCG food frequency questionnaire (FFQ) has been designed as a research tool for the assessment of habitual diet and was used to obtain quantitative estimates of nutrient intake to determine antioxidant levels in these patients at baseline, month 6 and month 12. [308]

**SD OCT**

A central macular thickness scan and transverse section scan was taken at baseline and then follow up scans at 6 and 12 months.

Fundus photography (Optos widefield imaging)

Fundus photographs of the eye were taken using the Optos camera. The DR screening classification system was used to grade all images. Grading was performed by either L North or another clinician.

Images were graded for diabetic retinopathy using the NSC screening classification.

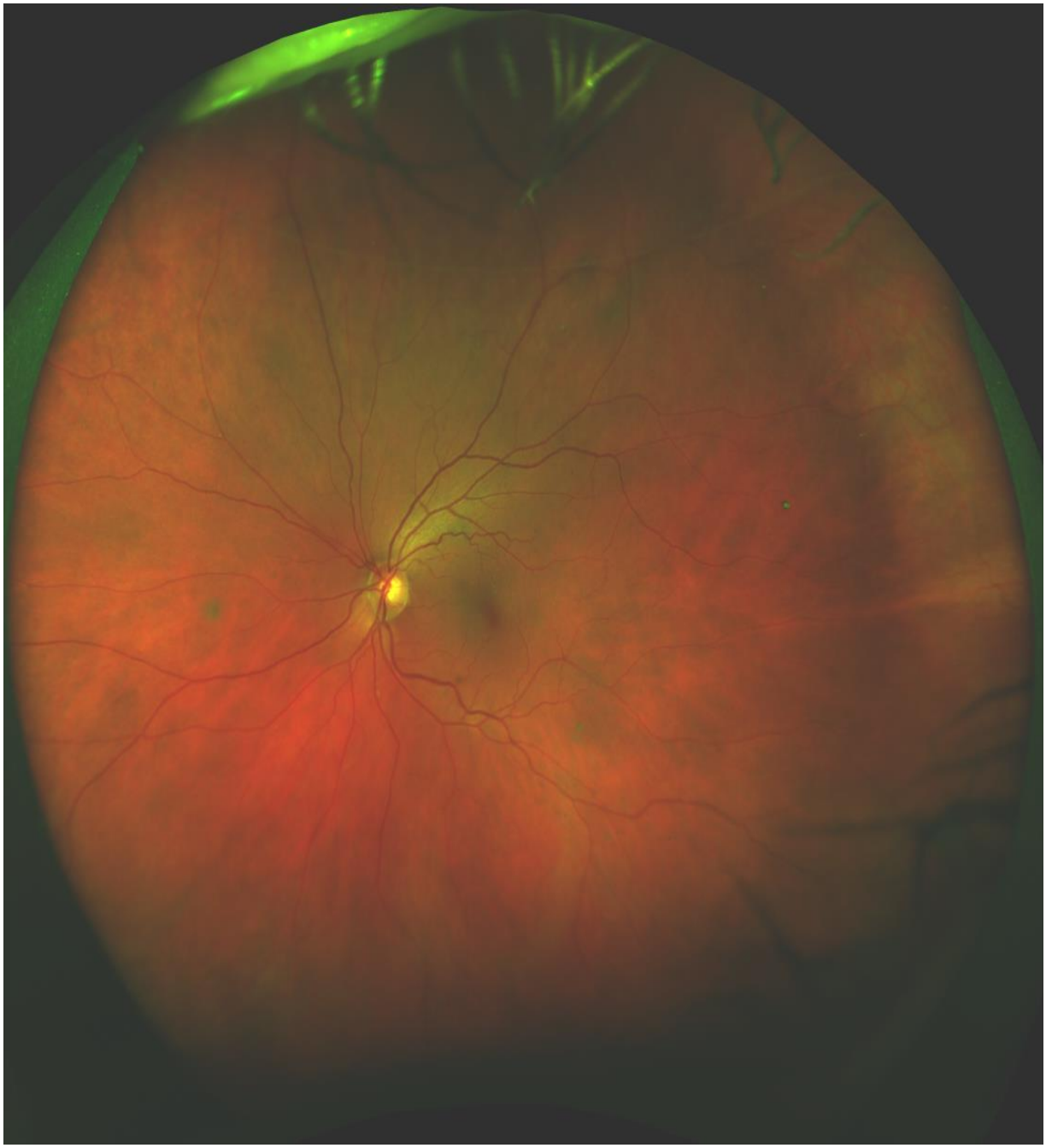


Fig 3.1. Widefield image of subject 1 demonstrating images used for grading

**Macula ®Praxis (MOPD)**

The MacuLux® was used to assess macular pigment optical density in each participant. It is based on the principles of heterochromatic flicker photometry (HFP).

MacuLux®Profiler is used to analyse the most centrally located area of the macula, i.e. at eccentricity of 0.5° and 0. 25°.The optical density of this foveal zone is determined in relation to the base line on an eccentric location (7°). (Egiba vision)


Fig 3.2 Screen shot image of the MacuLux from ebiga vision.

The MacuLux®PROFILER is a densitometer for the assessment of the macular density of the eye. Densitometers by the American manufacturer MACULARMETRICSIIMethod reproduced from https://www.ebiga-vision.com/en/maculux/

Method

1. Subject looks through the ocular.

2. he/she will always see a non-flickering test field.

3. When the patient perceives a flicker, he acknowledges this by pressing the test button.

Result

This flickering sensation is generated by alternating flashes of blue and green light, specifically between 460 nm (blue) of the wave length with maximum absorption by the macular pigment and 550 nm (green) of the reference wave length, which is not being absorbed by the macular pigment. Each participant underwent a test series for the fovea and for the parafovea, (see Fig 3.4).

The increase in the amount of blue light necessary for absorption by the pigment is the measure of the optical density. After the determination and the calculation of the reference in the pigmented area of the Parafovea at 7°, the device indicates the optical density of the macular pigment (MPOD) (Egiba- vision).

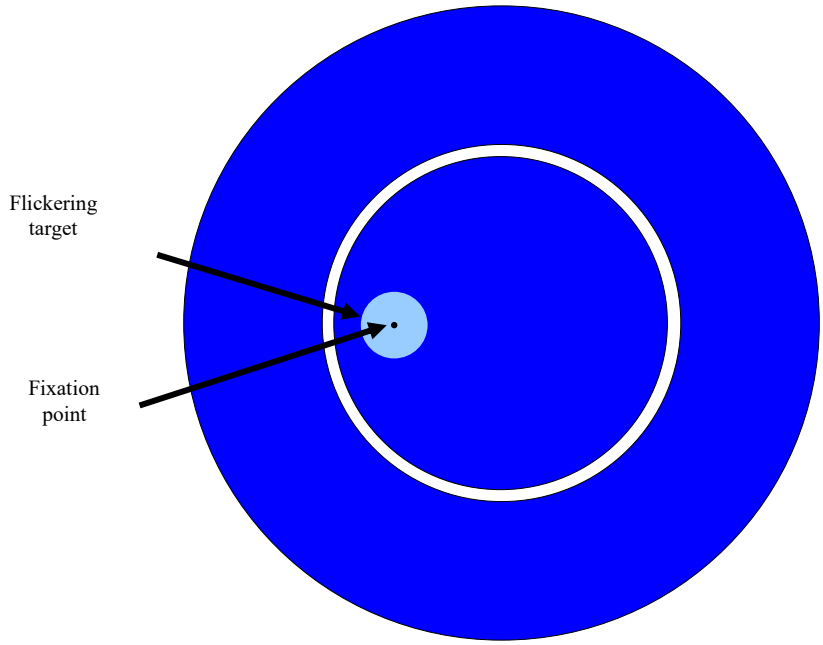
Each participant in the study performed the test undilated for either eye.

The MOPD values were considered of good quality based on the standard deviation as shown in table 3.1.

Table 3.1 showing acceptable values for MOPD

| Standard deviation | Evaluation |
|---|---|
| 0,03 -0,06 | Very good |
| 0,06-0,09 | Good and repeatable |
| 0,1 | Repeat the test |

FOVEA (0, 5°) – TEST




PARAFOVEA (7°) – TEST

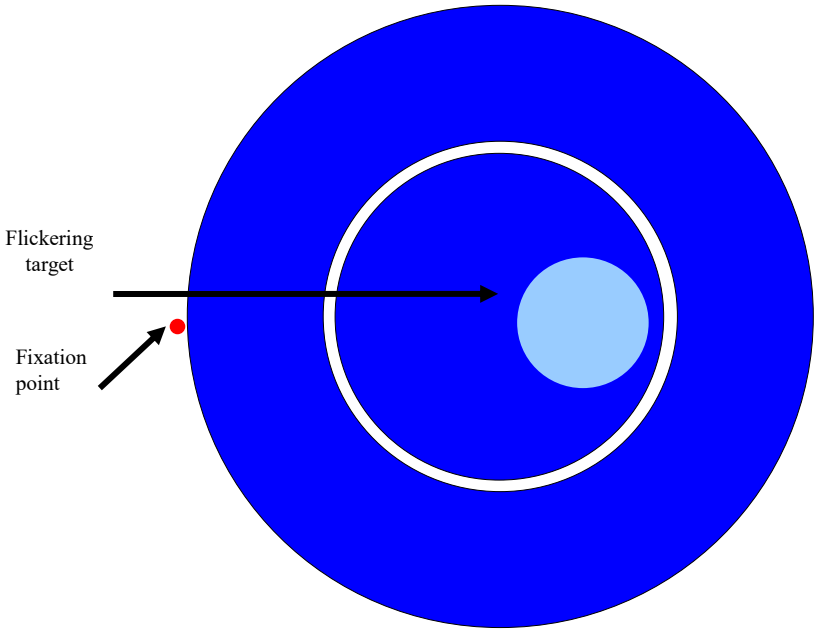



Fig 3.3 Image of MOPD test instructions

Blood samples

Methods for HbA1C and serum lipids

2 sets of blood were obtained from each subject at baseline and set 1 was repeated at month 12.

Set 1 The HbA1c (small purple top) and serum (Gold top).

Samples were labelled as per protocol and pathology request printed form and attached as standard care.

Set 2: DNA Baseline only

The DNA samples were collected and the immediately placed in a -20 Freezer, they were then transported to Oxford labs on dry ice for analysis.

Methods for DNA testing

Genomic DNA preparation from whole blood.

Genomic DNA was extracted from whole blood samples using QIAamp Blood kit (Qiagen) as per the manufacturer's instructions but briefly, 2mls of whole blood and 200μlof Qiagen protease solution were added to a 15ml centrifuge tube and mixed before adding 2.4ml buffer AL. The samples were inverted 15 times and shaken vigorously for 1 minute. The samples were incubated for 10 minutes at 700C before 2mls of 100% ethanol was added and the samples vigorously shaken. Half the sample was applied to a QIAamp midi column placed in a 15 ml centrifuge tube. The sample was centrifuged at 1850g for 3 minutes, the column removed, the filtrate discarded and the column replaced in the tube. The remainder of the sample was applied and spun as before. The filtrate was discarded and 2mls of buffer AW1 applied to the column, then centrifuged at 4500g for 1 minute. Then 2mls of buffer AW2 applied and centrifuged for 15 minutes. The filtrate was discarded and the column

placed in a clean collection tube before 300µl of distilled water was applied and the column incubated at room temperature for 5 minutes. The column was centrifuged at 4500g for 2 minutes to elute the sample. To gain maximum concentration the eluted sample was reapplied to the column and spun again as before.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) allows the amplification of specific DNA sequences, using two oligonucleotide primers which hybridise to opposite strands and flank the DNA target region. PCR reactions were performed in a final volume of 25µl, using 2x MyTaq PCR mix (Bioline, UK), 10pmol of each oligonucleotide (Sigma, UK) and 20-40ng of genomic DNA. A thermocycler (BioRad) was used to amplify the products as follows; an initial denaturation at 950C for 3 minutes followed by 40 cycles of denaturation at 950C for 30 seconds, annealing at 570C (SLC30A8 and AKR1B1) or 64.50C (SOD2) for 15 seconds and an extension at 720C for 30 seconds. After completion of the cycles, a final extension at 720C for 5 minutes was performed. The products were visualised by running 10µl of each product on a 1% agarose gel.

PCR purification

If required the PCR products were purified using a QIAquick PCR purification kit (Qiagen, Germany) as per the manufacturer's instruction but briefly, five volumes of buffer PB was added to 1 volume of PCR product. This was applied to a QIAquick spin column and centrifuged for 1 minute at 17,000g. The flow through was discarded. Then 750µl of buffer PE was applied to the column and centrifuged as before. The flow through was discarded and the column spun again as before to remove residual buffer. The column was transferred to a clean eppendorf tube and 30µl of double distilled water was applied. After incubating at room temperature for 1 minute the column was centrifuged at 17,000g for 1 minute to elute the purified sample.

Restriction Endonuclease Digestion

Restriction endonuclease digests were carried out using the reaction buffer supplied by the enzyme manufacturer. Table 3.2 below lists the conditions required for each reaction and table 3.3 show the primer sequences.

Table 3.2 reactions with RFLP digestion conditions, P- purified product, UP- unpurified product.

| Gene | Enzyme | Buffer | Total Volume | PCR Volume | Enzyme Volume | Buffer Volume | Water Volume | Incubation conditions |
|---|---|---|---|---|---|---|---|---|
| ALR2 | *BseYI* (NEB,UK) | 3.1 | 20µl | 15µl P | 0.5µl | 2µl | 2.5µl | $37^0$C for 1 hour |
| MnSOD | *BsaWI* (NEB,UK) | cutsmart | 50µl | 15µl UP | 0.4µl | 5µl | 29.6µl | $60^0$C for 1 hour |
| SLC30A8 | *PstI* (Invitrogen) | Anza Red Buffer | 20µl | 4µl UP | 1µl | 2µl | 13µl | $37^0$C for 1 hour |

Table 3.3 showing primer sequences.

| Primer Name | Primer Sequence (5'->3') |
|---|---|
| ALR2_rs759853_F | TTGTTGAGCAGGAGACGGCT |
| ALR2_rs759853_R | GCTGAACCACACCTAGTATG |
| SCL30A8_rs11558471F | ACGCCACATTAGAACTTGGTTC |
| SCL30A8_rs11558471R | CTGTGGTTGACAATCTTCTAGA |
| SOD2rs4880F | CAGCACTAGCAGCATGTTGA |
| SOD2rs4880F | ACAACAGTAAGGCAAGCTCC |

Agarose Gels and Visualisation

Agarose gel electrophoresis was used to separate DNA fragments. In each case 1% gels were used. The appropriate amount of agarose (Sigma, UK) (w/v) was dissolved in 1X TAE buffer by heating in a microwave oven. When cooled to below 600C ethidium bromide (Sigma, UK) was added to a final concentration of 0.5μg/ml. The solution was poured into a gel former with an appropriate comb. The gel was allowed to set completely before being placed in a horizontal gel tank and completely covered with 1X TAE buffer. The comb was removed. The samples to be run had 1/10 volume of loading dye added and were loaded in to the wells. A commercial marker (1Kbplus, Invitrogen, UK) was also loaded for size determination. The DNA was electrophoresed horizontally along a voltage gradient of 1-5V/cm until the required separation was achieved. During electrophoresis the ethidium bromide in the gel intercalates with the DNA, this enables visualisation on a UV transilluminator (320nm). The gels were imaged using a UGENIUS (Syngene) gel imager.

Sanger DNA sequencing

Any genotypes needing to be confirmed were Sanger sequenced. 5μl of purified PCR product diluted to a concentration of 10ng/μl and 5μl of the primer used for amplification (3.2pmol/μl) were commercially sequenced by Source Biosciences (UK).

3.7 Follow up

Data collection took place at baseline, 6 and 12 months and started in 2014.

Follow-up food frequency questionnaires were provided for completion prior to attending for their next visit. Participants were encouraged not to change their diet for the duration of the trial.

All other data was collected at the study visit. Recruitment was planned to be complete by 2016 to allow all participants to complete follow-up, finishing in 2017. However, the low number of participants prompted the decision to continue recruitment for a further 6 months and the sample size was recalculated.

Full details of the amendments are provided in the next chapter.

## 3.8 Data collection procedure

Table 3.4 Summary of data collection

| Assessment | Baseline | 6 months | 12 months |
|---|---|---|---|
| Informed consent | X | | |
| PDQ | X | | |
| Visual Acuity | X | X | X |
| SCG-FFQ | | | |
| Macular pigment optical density measurement both eyes | X | X | X |
| Dilation | X | X | X |
| Optos Fundus photos | X | X | X |
| SD -Optical coherence tomography | X | X | X |
| Slit lamp examination (including grading) | X | X | X |
| Serum HbA1C | X | | X |
| Blood sample for DNA | X | | |

## 3.9 Analyses

For each outcome measure the change between baseline, and then baseline, 6 months, and 12-month values were calculated. Descriptive statistical analysis of quantitative data was made by the determination of mean and standard deviation. For qualitative data the analysis of frequency and percentage in each category was used. The distribution of each continuous variable was inspected via the histogram. No extreme deviations from normality were seen. Normality tests were not run.

Differences were examined using either the chi-squared or Kruskal-Wallis test or one-way ANOVA test. To explore the associations between the variables over the three study visits a non-parametric spearman's rank correlation coefficient was used.

The study eye was selected based on best visual acuity. Where both eyes were the same the eye was randomly selected using SPSS software.

## 3.10 Ethical approval

The study was approved by the Aston Ethical Committee and Research and Development Committee at Frimley Park Hospital NHS Foundation Trust. Ethical approval was given by the NHS Research Ethics Committee (REC). The tenets of the Declaration of Helsinki were followed.

## 3.11 Summary

This chapter described the protocol for the DECAN study. A Prospective study of type 1 and type 2 Diabetics examining the correlation between antioxidant levels and neuronal layer loss as an exploratory study (short name -DECAN study).

The outcome measures used were dietary intake assessed using a validate semi- quantitative food frequency questionnaire (SCG –FFQ), OCT layer thickness, grading of diabetic retinopathy, macular pigment optical density scores (MOPD), serum lipid and HbA1C levels.

The next chapter will describe the results of the study.

# Chapter 4 Results

## 4.1 Baseline characteristics

Out of the 210 people that were recruited into the study 4 decided to discontinue immediately, 13 withdrew immediately after baseline. Further withdrawals occurred immediately after subsequent visits. The remaining participants completed the study; a breakdown is shown in table 4.1.

Table 4.1: Summary of enrolment and follow-up figures.

| | Type 1 | | Type 2 | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| Enrolled but failed to elicit sufficient* Baseline data [n=4] | 0 | 0 | 4 | |
| Baseline data only (Discontinued before first follow-up at 6 months) [n=13] | 3 | 0 | 6 | 4 |
| Baseline + Attended first follow-up (6 months) [n=13] | 2 | 4 | 6 | 1 |
| Baseline + Attended second follow-up (12 months) [n=4] | 0 | 0 | 4 | 0 |
| Baseline + Attended both follow-ups (6 and 12 months) [n=176] | 20 | 21 | 99 | 36 |

* = Age, gender, diabetes type, blood pressure, macular pigment and OCT layer data

Of the 210 recruited, data were only available for 206 for baseline statistical analysis. The follow up statistical analysis, utilising data across all 3 visits, was performed only on 176 participants and does not include those participants that discontinued or missed visits.

## 4.2 Baseline characteristics

The participants were split into 4 groups, Type 1 males, type 1 females, Type 2 males and type 2 females. The baseline characteristics of the 204 at baseline showed that they were predominantly Caucasian (89.4%) across the groups, however there were 15 type 2 males that were Asian (13%)

There was a statistically significant difference in gender between the diabetes groups (chi-squared test: p=0.002).

Table 4.2 Summary of gender of participants for Type 1 and Type 2

| | | GENDER | | TOTAL |
|---|---|---|---|---|
| TYPE OF DIABETES | | MALE | FEMALE | |
| TYPE 1 | n = | 25 | 25 | 50 |
| | % within TYPE | 50.0% | 50.0% | 100.0% |
| TYPE 2 | N = | 41 | 115 | 156 |
| | % within TYPE | 26.3% | 73.7% | 100.0% |
| TOTAL | | 66 | 140 | 206 |
| % within TYPE | | 32.0% | 68.0% | 100.0% |

Table 4.3 Summary of ethnicity between type 1 and type 2 males and females

| | Type 1 | | Type 2 | | % |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | |
| Caucasian | 24 | 23 | 98 | 39 | 89.3 |
| Asian | 0 | 0 | 15 | 1 | 7.8 |
| Black | 1 | 1 | 1 | 0 | 1.5 |
| Mixed | 0 | 1 | 0 | 0 | 0.5 |
| Nepalese | 0 | 0 | 0 | 1 | 0.5 |
| Other | 0 | 0 | 0 | 1 | 0.5 |

The numbers were too small in the subgroups for statistical analysis for ethnicity.

Table 4.4 Summary of ages between the type 1 and type 2

| | Diabetes Type | N | Mean | Std Deviation |
|---|---|---|---|---|
| AGE ( years) | Type 1 | 50 | 52 | 15 |
| | Type 2 | 156 | 65.5 | 11.3 |

Statistically significant using unpaired t-test: $P < 0.0005$

Table 4.5 Summary of age for Type 2 only

| | Gender | N | Mean | Std Deviation |
|---|---|---|---|---|
| AGE ( years) | Female | 41 | 65.3 | 12.2 |
| | Male | 115 | 65.5 | 11.0 |

Not statistically significant using unpaired t-test: $P = 0.921$

Table 4.6 Summary of age for Type 1 only

| | Gender | N | Mean | Std Deviation |
|---|---|---|---|---|
| AGE ( years) | Female | 25 | 48.4 | 15 |
| | Male | 25 | 55 | 15 |

Not statistically significant using unpaired t-test: $P = 0.12$

Table 4.7 Summary of the differences in baseline characteristics between the 4 groups using one way ANOVA

| | TYPE 1 MALE N =25 | | | TYPE 1 FEMALE N =25 | | | TYPE 2 MALE N =113 | | | TYPE 2 FEMALE N =41 | | | Stat Test ANOVA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Demographic features | N | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD | P value |
| Age (years) | 25 | 54.8 | 14.8 | 25 | 48.4 | 14.6 | 115 | 65.5 | 10.9 | 41 | 65.3 | 12.2 | <0.0005 * |
| HbA1c (mmol/mol) | 25 | 73.3 | 16.7 | 23 | 64.8 | 11.7 | 109 | 65.4 | 17.8 | 39 | 66.6 | 21.9 | 0.247 |
| BP systolic (mmHg) | 23 | 141.3 | 17.1 | 23 | 134.1 | 18.5 | 109 | 142.7 | 20.6 | 38 | 144.3 | 22.2 | 0.256 |
| BP Dias (mmHg) | 23 | 76.3 | 7.3 | 23 | 71.1 | 8.5 | 109 | 75.2 | 10.5 | 38 | 72.3 | 7.2 | 0.090 |
| Duration of diabetes (years) | 22 | 26.5 | 11.3 | 24 | 27.7 | 11.4 | 103 | 16.3 | 6.5 | 33 | 16.3 | 9.1 | <0.0005 * |

The distribution of each continuous variable was inspected via the histogram. Normality tests were not run. No extreme deviations from normality were seen. If p value is > 0.05 then there is no statistical difference in mean between the 4 groups. Where p is < 0.05 a higher mean was identified in the type 1 groups as shown in table 4.7

In table 4.8 below the statistically p values identified a significantly higher mean in the type 1 females compared to the other 3 groups.

Table: 4.8 Summary of the differences in baseline characteristics (LIPIDS) between the 4 groups using one way ANOVA

| | TYPE 1 MALE N =25 | | | TYPE 1 FEMALE N =25 | | | TYPE 2 MALE N =113 | | | TYPE 2 FEMALE N =41 | | | Statistical Test ANOVA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Demographic features | N | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD | P value |
| Cholesterol | 23 | 4.3 | 0.6 | 23 | 5.0 | 1.0 | 106 | 4.0 | 1.0 | 38 | 4.4 | 1.0 | 0.002* |
| HDL | 23 | 1.5 | 0.5 | 23 | 1.8 | 0.6 | 105 | 1.2 | 0.3 | 37 | 1.4 | 0.4 | <0.0005* |
| Non-HDL | 15 | 3.0 | 0.8 | 17 | 3.0 | 0.8 | 79 | 3.0 | 1.0 | 26 | 3.1 | 0.1 | 0.733 |
| Triglyceride | 13 | 1.6 | 1.0 | 10 | 1.3 | 0.7 | 49 | 2.0 | 1.2 | 20 | 2.0 | 1.0 | 0.184 |

Table 4.9 Summary of those on medication for both blood pressure and hypertension at baseline (categorical data)

| | TYPE 1 MALE N =25 | | | TYPE 1 FEMALE N =25 | | | TYPE 2 MALE N =113 | | | TYPE 2 FEMALE N =41 | | | Statistical Test $X^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Demographic features | N | n | % | N | n | % | N | n | % | N | n | % | P value |
| Hypertension | 24 | 14 | 58.3 | 24 | 8 | 33.3* | 112 | 69 | 61.6 | 40 | 27 | 67.5 | 0.045* |
| Statins | 24 | 11 | 45.8 | 24 | 11 | 45.8 | 113 | 74 | 65.5 | 40 | 28 | 70 | 0.073* |

When exploring those on medications, type 1 females were seen to have a statistically lower prevalence of being on medication as seen in table 4.9

Baseline characteristics were analysed using Kruskal- Wallis test to compare the 4 subgroups for the ordinal variables smoking, BMI, Exercise and type of diabetic medication/control

Table 4.10 Summary of smoking status at baseline- ordinal outcomes

| Demographic features | TYPE 1 MALE | TYPE 1 FEMALE | TYPE 2 MALE | TYPE 2 FEMALE | Total |
|---|---|---|---|---|---|
| Never smoked | 12 (48%) | 17 (68%) | 68 (60.2) | 23 (57.5) | 120 (59.1%) |
| Ex-smoker | 8 (32%) | 4 (16%) | 35 (31%) | 14 (35%) | 61 (30) |
| Current smoker | 5 (20%) | 4 (16%) | 10 (8.8%) | 3 (7.5%) | 22 (10.8 %) |
| Total | 25 (100%) | 25 (100%) | 113 (100%) | 40 (100% | 203 (100%) |

Not statistically significant p value= 0.515 using Kruskal-Wallis test.

Table 4.11 Summary of baseline characteristics for BMI- ordinal outcomes

| Demo features | TYPE 1 MALE N =25 | | | | TYPE 1 FEMALE N =25 | | | | TYPE 2 MALE N =113 | | | | TYPE 2 FEMALE N =41 | | | | Kruskal - Wallis test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BMI | N | O | OB | MO | N | O | OB | MB | N | O | OB | MB | N | O | OB | MB | P value |
| N = | 7 | 5 | 8 | 0 | 3 | 10 | 8 | 0 | 12 | 43 | 29 | 3 | 6 | 12 | 9 | 4 | 0.475 |
| % = | 35 | 25 | 40 | 0 | 14.3 | 47.6 | 38.1 | 0 | 13.8 | 49.4 | 33.3 | 3.4 | 19.4 | 38.7 | 29 | 12.9 | |

BMI was categorised as: U =Underweight <18.5 N =Normal 18.5 – 25, 0 =Over weight 25 -30, OB =Obese 30-40, MO =morbidly obese 40+

No participant was recorded as underweight so not included above.

Table 4.12 Summary of baseline characteristics for exercise

| Demo feature | TYPE 1 MALE N =25 | | | | TYPE 1 FEMALE N =25 | | | | TYPE 2 MALE N =113 | | | | TYPE 2 FEMALE N =41 | | | | Kruskal - Wallis test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exercise | NA | FA | MA | VA | NA | FA | MA | VA | NA | FA | MA | VA | NA | FA | MA | VA | P value |
| N= | 1 | 11 | 3 | 6 | 0 | 12 | 9 | 3 | 14 | 39 | 38 | 21 | 2 | 21 | 12 | 5 | 0.854 |
| % | 4.2 | 45.8 | 25 | 25 | 0 | 50 | 37.5 | 12.5 | 5 | 52.5 | 30 | 12.5 | 8.5 | 41.5 | 32.5 | 17.5 | |

Exercise was categorised as: NA =Not active FA = fairly active MA =moderately active VA =Very Active

Table 4.13 Summary of baseline characteristics for diabetic control-ordinal outcomes

| Demo feature | TYPE 1 MALE N =25 | | | | TYPE 1 FEMALE N =25 | | | | TYPE 2 MALE N =113 | | | | TYPE 2 FEMALE N =41 | | | | Kruskal -Wallis test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Diabetic medication | Ins | IT | T | D | Ins | IT | T | D | Ins | IT | T | D | Ins | IT | T | D | P value |
| N = | 19 | 5 | 0 | 0 | 23 | 1 | 0 | 0 | 4 | 40 | 65 | 3 | 3 | 13 | 21 | 3 | <0.0005 |
| % = | 79.2 | 20.8 | 0 | 0 | 95.8 | 4.2 | 0 | 0 | 3.6 | 35.7 | 58 | 2.7 | 7.5 | 32.5 | 52.5 | 7.5 | |

Diabetic medication/control was recorded as: INS = Insulin, IT =Insulin and tablets, T = tablets, D = Diet

There was no significant difference between groups for any of the baseline characteristics except for age and duration of diabetes which were both statistically significant p value <0.0005 as shown in table 4.7.This is expected as the range 24 -88 years across the groups. The type 1 group ranged from age 24 – 82 and the Type 2 group age ranged from 41 – 88.

Both cholesterol and HDL were also statistically significant, HDL p value <0.0005 and cholesterol p value 0.002. The type 2 males had statistically significant lower levels of cholesterol and HDL compared to the other groups, see table 4.8.

4.3 Results

All participants were asked to fill out a food frequency questionnaire in order to assess their dietary habits over the study period. The forms were completed at the baseline visit to reflect their diet intake for the preceding 2 months. The data from the questionnaire was then inputted manually into a web-based data entry system and the outputs from the data were computer generated.

The data was analysed using the Mc cance and Widdowson's Composition of Foods (5th Edition plus related supplements) found at http://foodfrequency.org/services by the University of Aberdeen.

The output provides the nutrient intake from each food and drink for each patient was produced. This is calculated as:

- Total nutrient intake per day
- Nutrient intake per food per day

The questionnaire also collects data about supplements taken and the analysis of this was carried out separately.

In table 4.14a below the baseline characteristics of total intakes across the 4 groups was compared. Note that no p value was < 0.05 therefore means and standard deviations for the individual groups are not listed, seen table 4.14b There was no statistically significant differences in the baseline food intakes between the groups with the exception of the Type 2 females for alcohol which had a p value = 0.027. As demonstrated in Fig 4.1.

Table 4.14a Summary of the baseline characteristics of total intakes with test result ANOVA to compare the 4 groups

| Dietary n=176 | Mean | SD | P VALUE | Dietary | Mean | SD | P VALUE |
|---|---|---|---|---|---|---|---|
| Water | 2008.609 | 833.1158 | 0.692 | Mg | 316.45 | 127.101 | 0.542 |
| Prot | 83.194 | 34.9689 | 0.372 | P | 1515.64 | 607.659 | 0.424 |
| Fat | 80.683 | 39.4816 | 0.107 | Fe | 12.0901 | 5.11359 | 0.364 |
| CHO | 219.550 | 97.6008 | 0.687 | Cu | 1.3241 | .61167 | 0.472 |
| kCAL | 1932.58 | 827.234 | 0.318 | Zn | 10.291 | 4.2624 | 0.212 |
| Energkj | 8122.54 | 3469.636 | 0.328 | Cl | 4211.91 | 1855.268 | 0.222 |
| SFA | 31.511 | 15.8903 | 0.090 | Mn | 3.267 | 1.7831 | 0.386 |
| MUFA | 28.224 | 14.4418 | 0.097 | Se | 54.77 | 27.595 | 0.273 |
| PUFA | 13.674 | 7.1776 | 0.197 | I | 217.64 | 108.031 | 0.873 |
| TRANS | 2.043 | 1.1699 | 0.090 | Retin | 534.22 | 523.987 | 0.457 |
| Sugars | 95.468 | 53.1694 | 0.943 | Carot | 4280.55 | 3729.281 | 0.331 |

Table 4.14b Summary of the differences in baseline characteristics of total intakes with test result ANOVA to compare the 4 groups.

| Dietary n = 179 | Mean | SD | P VALUE | Dietary | Mean | SD | P VALUE |
|---|---|---|---|---|---|---|---|
| Vit D | 4.1398 | 3.33533 | 0.979 | Vit B12 | 6.889 | 4.2427 | 0.769 |
| Vit E | 9.6255 | 4.88188 | 0.715 | FoIAC | 293.36 | 145.182 | 0.748 |
| Thiam | 1.6832 | .72494 | 0.626 | PanAC | 5.8863 | 2.37019 | 0.799 |
| Ribof | 2.0057 | .87464 | 0.969 | Biot | 45.308 | 19.4177 | 0.442 |
| Niacin | 20.550 | 8.7754 | 0.407 | Vit C | 111.70 | 81.923 | 0.299 |
| PotNia | 17.910 | 7.3948 | 0.459 | Vit K | 79.207 | 126.1051 | 0.364 |
| Vit B6 | 2.1856 | .94889 | 0.707 | Alcohol | 7.179 | 10.4929 | 0.027* |

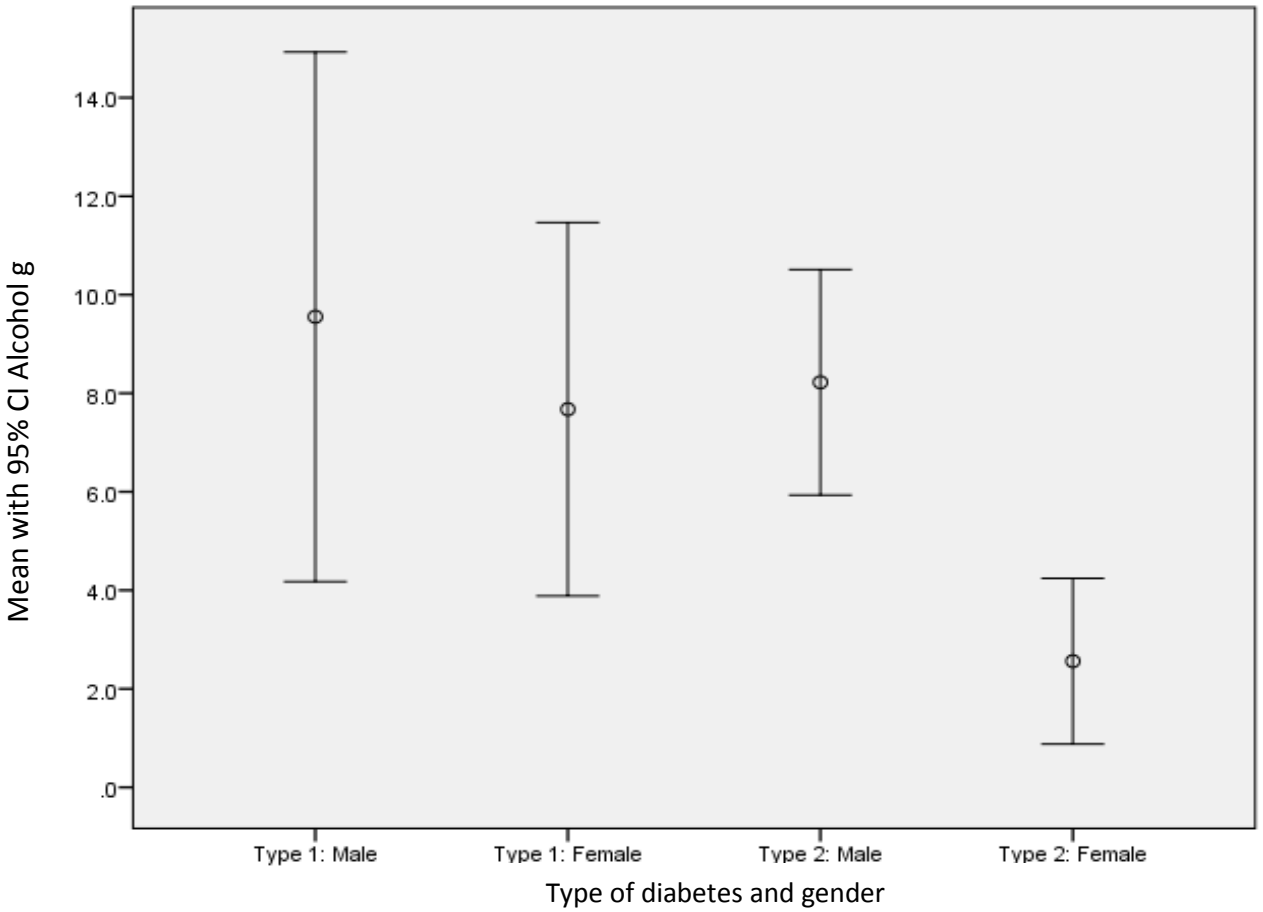



Fig 4.1. Graph of Alcohol (the one FFQ variable not equal across subgroups): means with 95% confidence intervals for each subgroup

Table 4.15 Summary of participants grading at baseline for retinopathy of the study eye using

| Retinopathy n = 202 | Type 1 | | Type 2 | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| RO (NO DIABETIC RETINOPATHY) | 3 | 0 | 9 | 7 |
| R1 | 17 | 20 | 82 | 28 |
| R2 | 5 | 5 | 19 | 4 |
| R3 | 0 | 0 | 2 | 1 |

Not statistically significant p value= 0.287 using Kruskal-Wallis test

Table 4.16 Summary of participants grading at baseline for maculopathy of the study eye

| Maculopathy | Type 1 | | Type 2 | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| MO | 20 | 21 | 87 | 34 |
| M1 | 5 | 4 | 25 | 6 |

Not statistically significant p value= 0.739 using test $\chi^2$

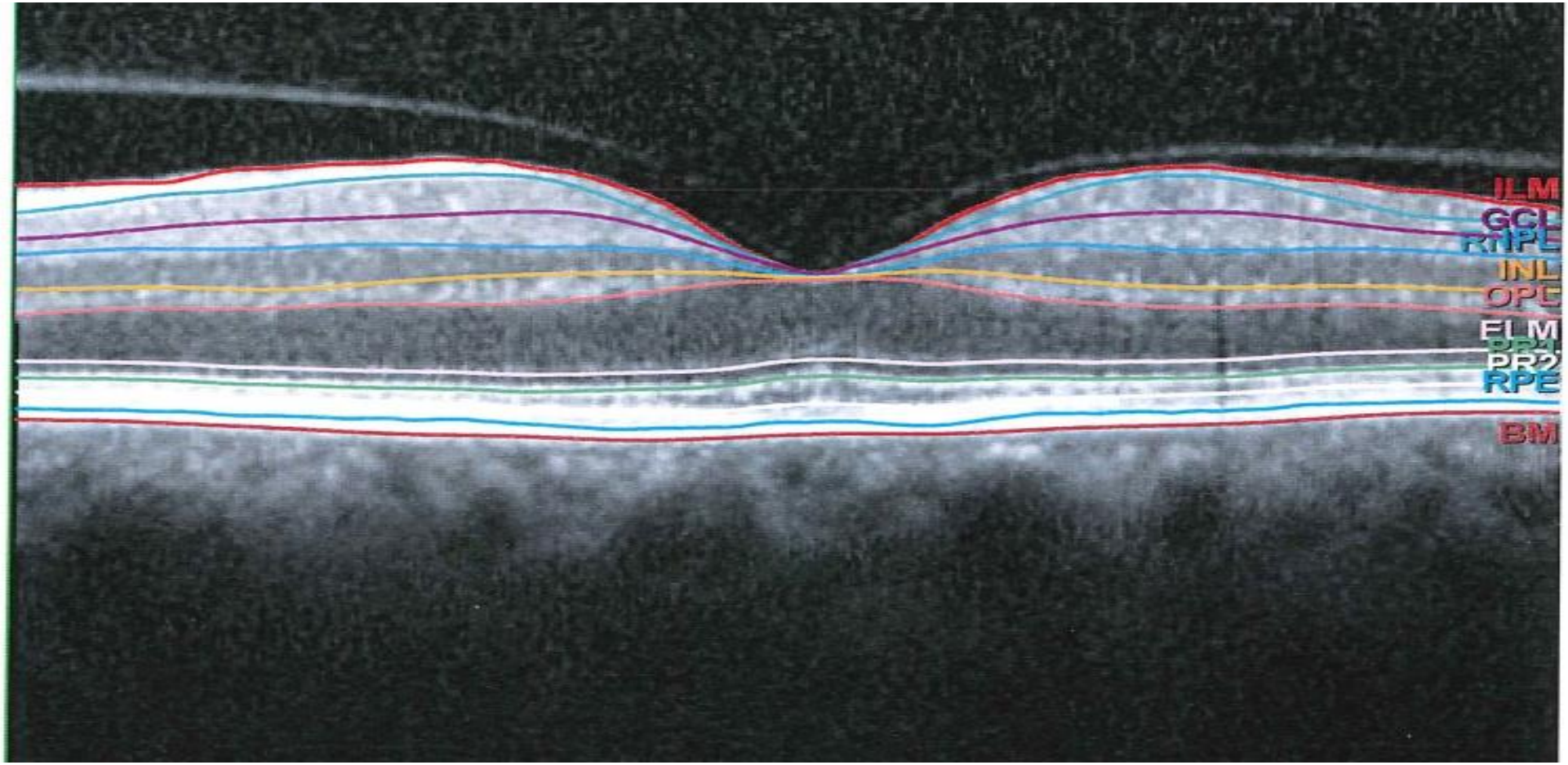



Fig 4.2 showing automated segmentation.

Table 4.17 Summary of n = 202 total macular pigment scores (MOPD) and total OCT layers scores using ANOVA test to compare the 4 groups at baseline.

| MOPD AND OCT LAYERS | Mean | SD | P value |
|---|---|---|---|
| MOPD | .3031 | .21357 | 0.776 |
| CMT | 285.8350 | 30.50889 | 0.018* |
| RETINA | 286.0976 | 30.19009 | 0.038* |
| RNFL | 13.6780 | 3.50962 | 0.115 |
| GCL | 17.5268 | 6.50682 | 0.374 |
| IPL | 22.7220 | 4.58533 | 0.042* |
| INL | 24.4341 | 11.13032 | 0.021* |
| OPL | 27.8585 | 5.76879 | 0.239 |
| ONL | 92.7512 | 12.68091 | 0.243 |
| RPE | 17.5366 | 1.85897 | 0.156 |
| IRL | 198.2195 | 30.37278 | 0.015* |
| ORL | 88.0637 | 4.59609 | 0.011* |

There was no statistically significant difference between MOPD score between the 4 groups. However, as shown in table 4.17, when examining the OCT layers , CMT Scan p value =0.018 showed that the Type 1 females had thinner central macular thickness in comparison to the other groups. When looking at the transverse scan the Retina thickness scan p value = 0.038, Inner plexiform layer thickness (IPL) p value = 0.042, and Inner nuclear layer (INL) p value 0.021 all showed statistical significance for the Type 2 males having lower readings indicating thinning. Similarly, when examining the outer retinal layer thickness (ORL) scores there appeared to be thinning of the ORL in the type 2 males compared to the type 1 females, p value =0.011 which is statistically significant. All of the comparisons (one way ANOVAs) deliver a statistically significant result and looking at the means with 95% confidence intervals plots underneath see Figures 4.3 -4.7 , it's always the Type II Males being higher scores than the Type I Females. Except for the last one (ORL) where the Type I Females are higher than the Type II Males.

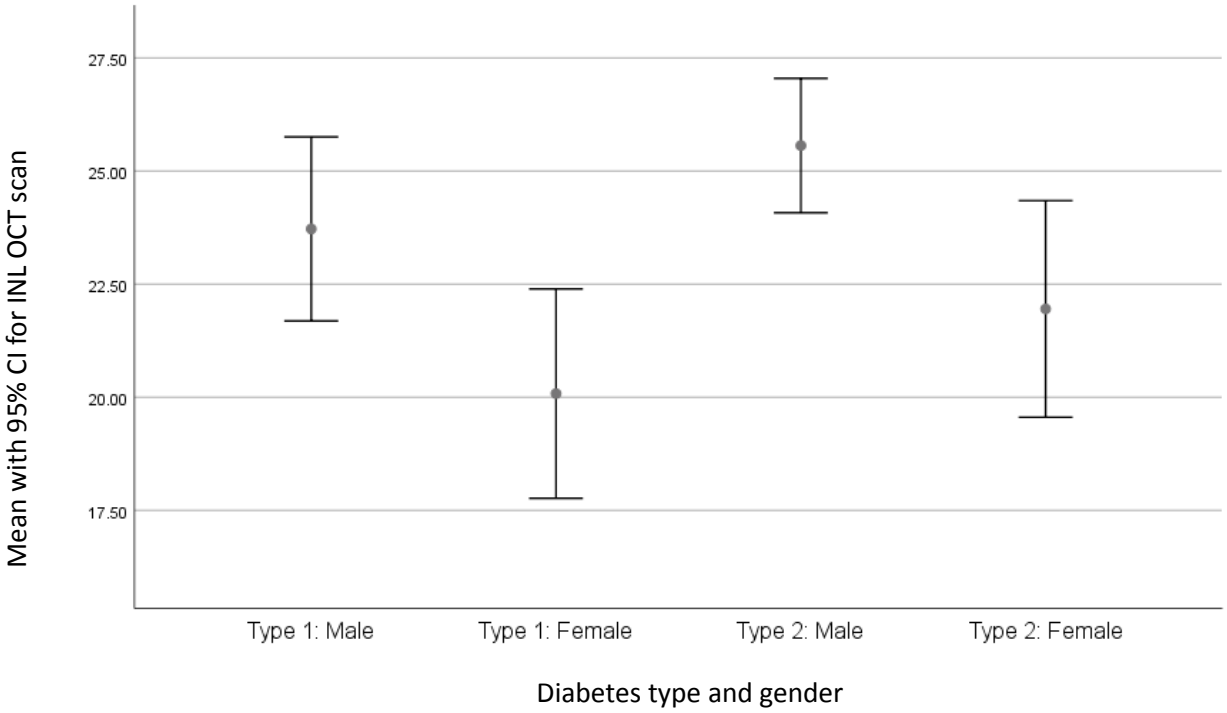



Fig 4.3 Graph of INL SD-OCT scan means with 95% confidence intervals for each subgroup

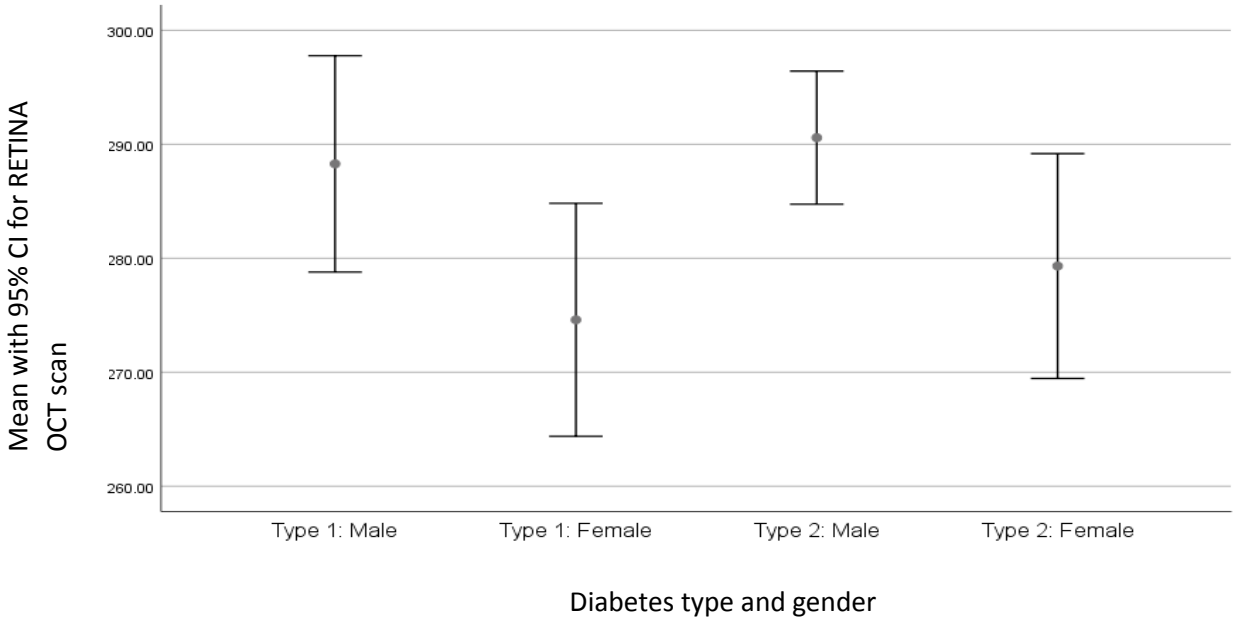



Fig 4.4 Graph of RETINA SD-OCT scan means with 95% confidence intervals for each subgroup

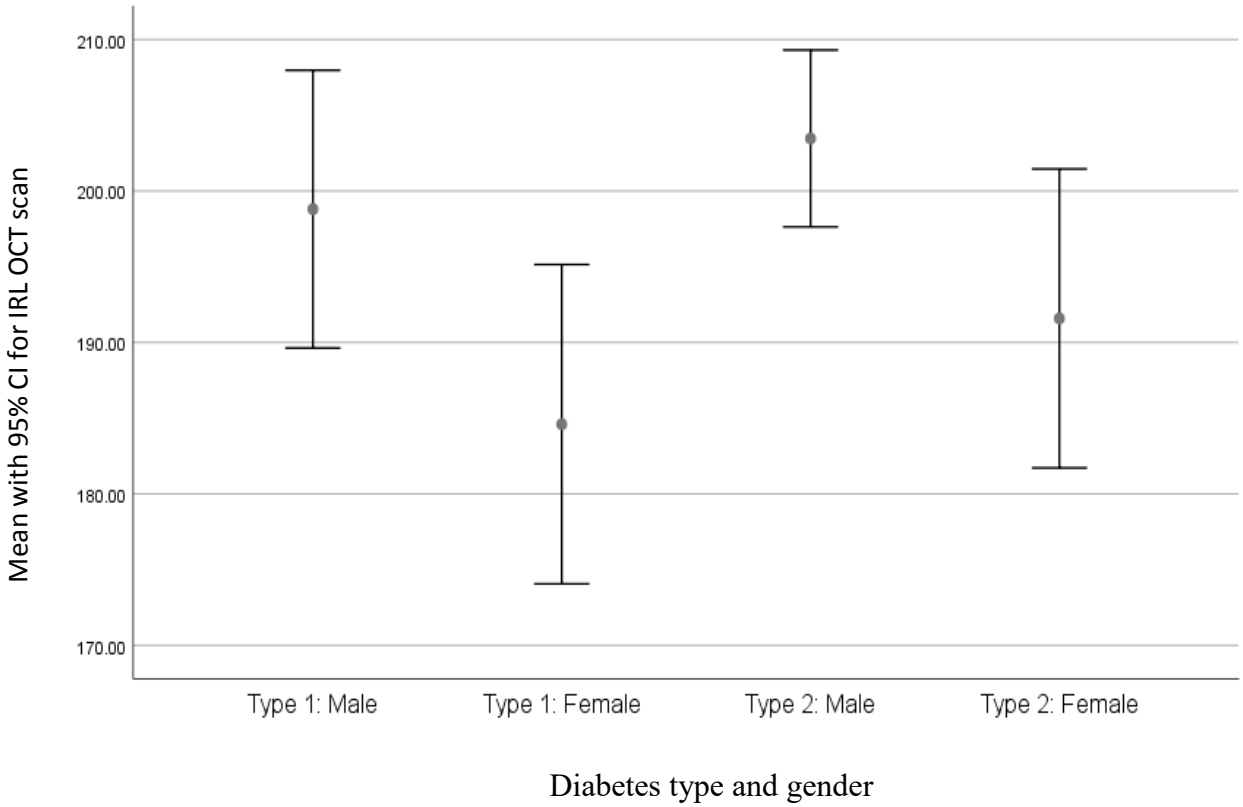



Fig 4.5 Graph of IRL SD-OCT scan means with 95% confidence intervals for each subgroup

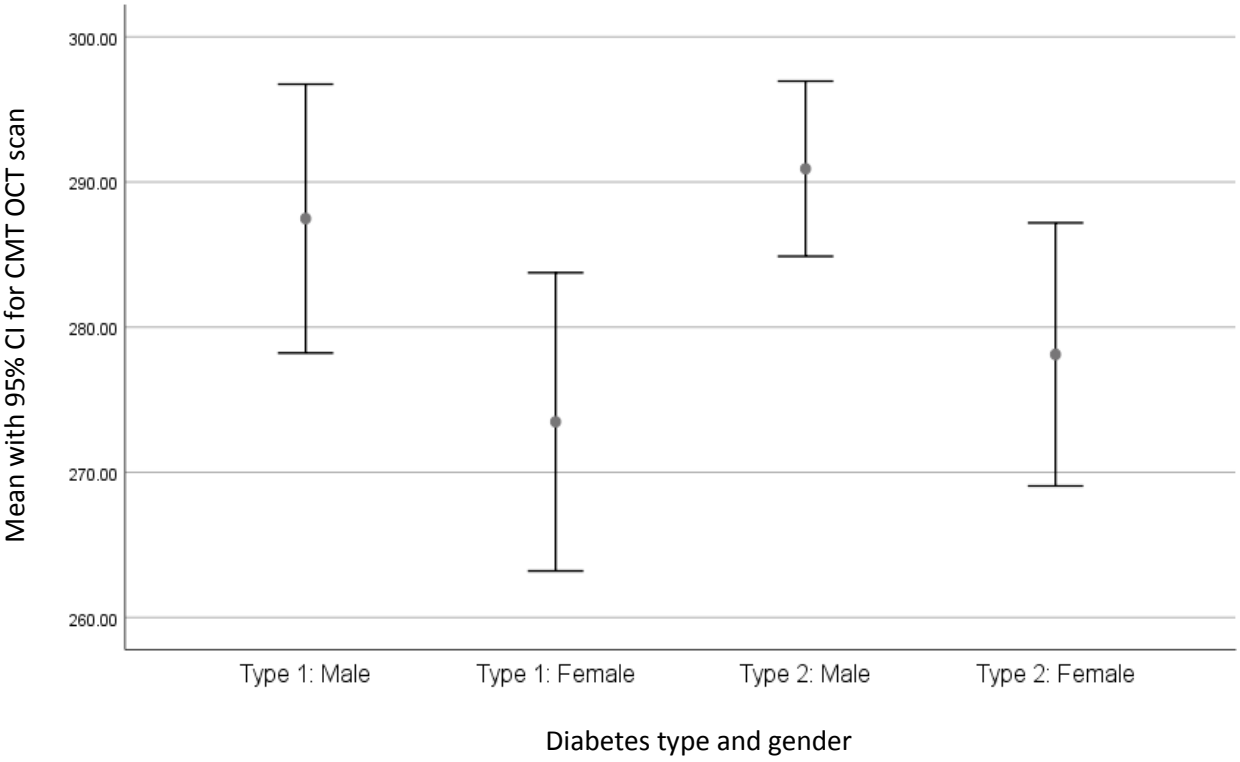



Fig 4.6 Graph of CMT SD-OCT scan means with 95% confidence intervals for each subgroup

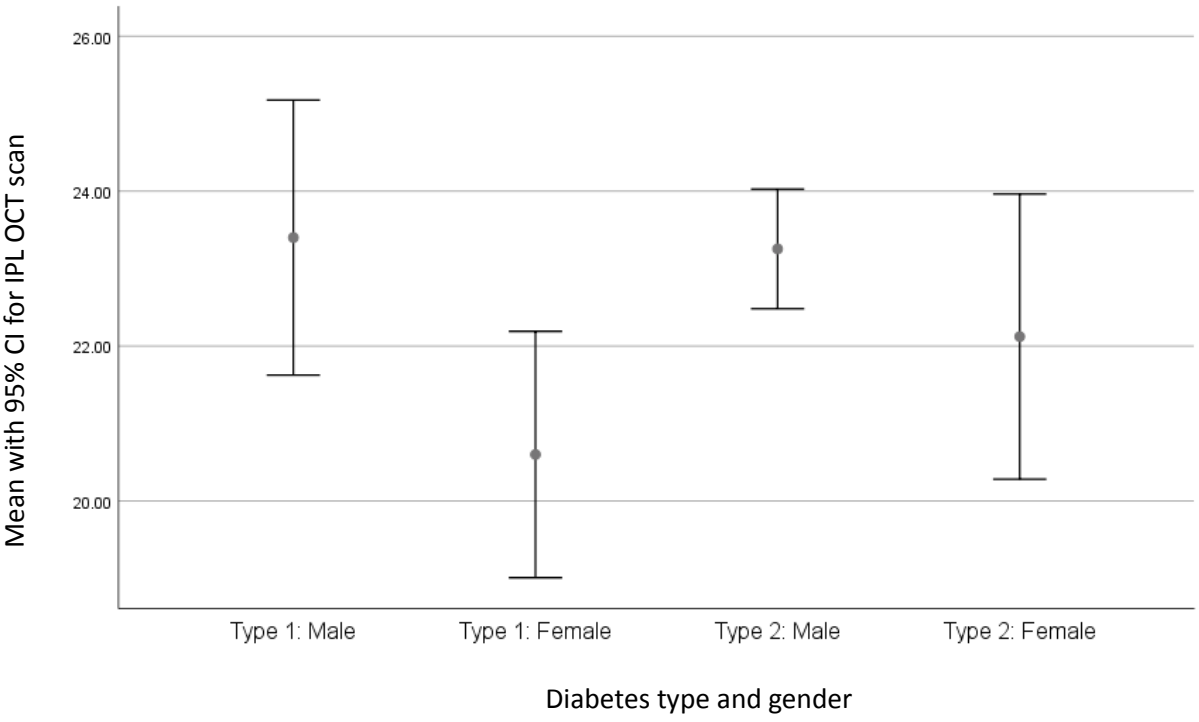



Fig 4.7 Graph of IPL SD-OCT scan means with 95% confidence intervals for each subgroup

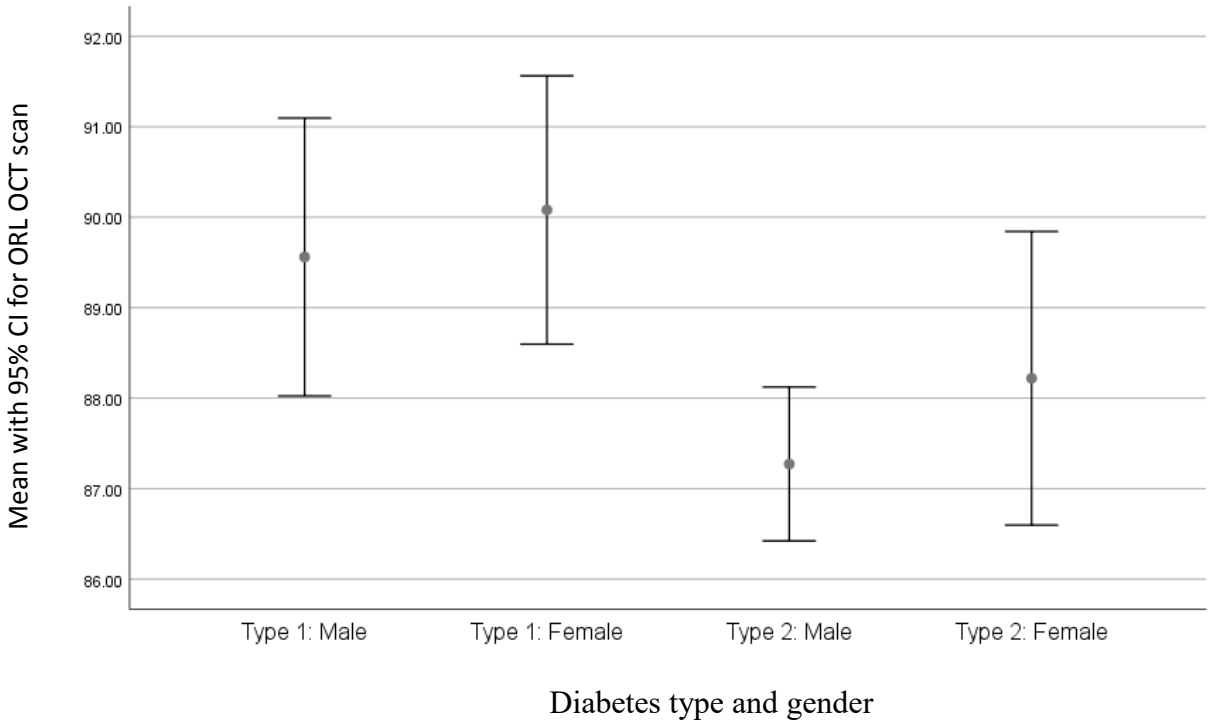



Fig 4.8 Graph of ORL SD-OCT scan means with 95% confidence intervals for each subgroup

Baseline outcomes

For each outcome measure the association between each proposed relationship was assessed at baseline and then the difference between baselines and follow up at 6 months and 12 months were explored.

The primary outcome was to assess antioxidants and OCT to determine if there was a relationship between dietary intake assessed using a food frequency questionnaire at baseline and over a period of 12 months with neuronal layer loss assessed using SD – OCT using automated segmentation software.

Participants were also asked to provide details on the FFQ of any additional nutritional supplementation at baseline and throughout the study duration on subsequent questionnaires. Not all participants were able to specify the amounts of their supplements and some quantities appeared excessive.

The relationship between FFQ and OCT was explored using the Pearson test at baseline as two continuous variables which were both normally distributed, The results showed statistically significant correlations between Protein, Fat, saturated fat, monosaturated fat, magnesium, phosphorous, Iron, Zinc, Iodine, Vitamin D, Thiamine, Riboflavin, Potential niacin, Vitamin B12, Folic acid, pantothenic acid and biotin food and OCT layer thickness mainly seen in the IPL and the RNFL at baseline across the 4 groups. As some of the variables are correlated by definition applying any correction would be inappropriate. In table 4.18 the rows of correlations enable the reader to select a variable of particular interest e.g. fat, monosaturated etc. and examine its correlation with other variables. (See appendix 1 a, 1b, 1c).

However, when the supplement intake was explored using the same analysis it appeared that pantothenate and biotin were associated with an increased CMT thickness p value =0.001 and p value = 0.020 respectively. Vitamin E showed a statistically significant association with thinning of the ONL on the transverse scan p value =0.001

Table 4.18 Summary of supplements at baseline vs. OCT layers p values. (Full details in appendix 2a, 2b)

| P values | MOPD | CMT | RETINA | RNFL | GCL | IPL | INL | OPL | ONL | RPE | IRL | ORL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VIT A | 0.248 | 0.745 | 0.581 | 0.112 | 0.685 | 0.320 | 0.600 | 0.990 | 0.615 | 0.400 | 0.505 | 0.457 |
| VIT D | 0.849 | 0.582 | 0.233 | 0.084 | 0.713 | 0.243 | 0.543 | 0.889 | 0.089 | 0.940 | 0.266 | 0.485 |
| VIT E | 0.803 | 0.201 | 0.135 | 0.621 | 0.323 | 0.289 | 0.550 | 0.991 | **0.011*** | 0.597 | 0.163 | 0.378 |
| VIT C | 0.520 | 0.925 | 0.948 | 0.820 | 0.970 | 0.956 | 0.814 | 0.564 | 0.480 | 0.406 | 0.972 | 0.565 |
| VIT B1 | 0.235 | 0.643 | 0.473 | 0.300 | 0.578 | 0.368 | 0.408 | 0.902 | 0.540 | 0.704 | 0.450 | 0.826 |
| VIT B2 | 0.244 | 0.735 | 0.479 | 0.317 | 0.586 | 0.377 | 0.425 | 0.868 | 0.513 | 0.716 | 0.456 | 0.843 |
| VIT B3 | 0.246 | 0.749 | 0.480 | 0.320 | 0.587 | 0.379 | 0.428 | 0.863 | 0.509 | 0.717 | 0.457 | 0.845 |
| VIT B6 | 0.229 | 0.578 | 0.468 | 0.287 | 0.569 | 0.359 | 0.393 | 0.928 | 0.562 | 0.686 | 0.443 | 0.807 |
| FOLIC ACID | 0.077 | 0.752 | 0.222 | 0.309 | 0.531 | 0.315 | 0.253 | 0.844 | 0.260 | 1.000 | 0.279 | 0.283 |
| VIT B12 | 0.324 | 0.665 | 0.533 | 0.455 | 0.644 | 0.458 | 0.565 | 0.667 | 0.371 | 0.811 | 0.516 | 0.960 |
| VIT K | | | | | | | | | | | | |
| PantAC | 0.303 | **0.001*** | 0.670 | 0.286 | 0.747 | 0.575 | 0.371 | 0.323 | 0.472 | 0.885 | 0.660 | 0.766 |
| BIO | 0.336 | **0.020*** | 0.746 | 0.458 | 0.917 | 0.760 | 0.556 | 0.471 | 0.676 | 0.745 | 0.770 | 00.910 |
| Cu | 0.136 | 0.726 | 0.692 | 0.342 | 0.772 | 0.251 | 0.367 | 0.262 | 0.603 | 0.169 | 0.542 | 0.140 |
| Ca | 0.799 | 0.553 | 0.409 | 0.365 | 0.654 | 0.537 | 0.407 | 0.467 | 0.513 | 0.524 | 0.385 | 0.626 |

As can be seen in table 4.18 no statistically significant relationships were found except for PantAC with CMT (r = + 0.511 p = 0.001), BIO with CMT (r = + 0.372 p= 0.020) and Vit E with ONL (r = - 0.406 p=0.011) which were all statistically significant

Table 4.19 Group statistics- M0 vs. M1 and OCT outcomes at baseline

| | Maculopathy | Number | mean | Std. Deviation | Unpaired t-test: p-value |
|---|---|---|---|---|---|
| CMTscan | M0 | 162 | 283.0741 | 26.83549 | 0.053 |
| | M1 | 40 | 295.0500 | 35.89725 | |
| RETINAscan | M0 | 162 | 282.7901 | 25.79841 | 0.022* |
| | M1 | 40 | 297.2250 | 36.51939 | |
| RNFLscan | M0 | 162 | 13.4136 | 2.68801 | 0.016* |
| | M1 | 40 | 14.9000 | 5.58753 | |
| GCLscan | M0 | 162 | 17.0000 | 6.01241 | 0.057 |
| | M1 | 40 | 19.0500 | 6.21805 | |
| IPLscan | M0 | 162 | 22.3025 | 4.29660 | 0.015* |
| | M1 | 40 | 24.2250 | 4.95357 | |
| INLscan | M0 | 162 | 23.2284 | 6.59406 | 0.017* |
| | M1 | 40 | 26.1250 | 7.68010 | |
| OPLscan | M0 | 162 | 27.6173 | 5.81937 | 0.204 |
| | M1 | 40 | 28.9000 | 5.14806 | |
| ONLscan | M0 | 162 | 91.5556 | 11.27246 | 0.068 |
| | M1 | 40 | 96.5750 | 16.03504 | |
| RPEscan | M0 | 162 | 17.6790 | 0.93578 | 0.019* |
| | M1 | 40 | 17.0250 | 1.44093 | |
| IRLscan | M0 | 162 | 194.1235 | 27.23210 | 0.004* |
| | M1 | 40 | 209.5000 | 36.51203 | |
| ORLscan | M0 | 162 | 88.2716 | 4.64521 | 0.328 |
| | M1 | 40 | 87.4750 | 4.40854 | |

The relationship between the severity of diabetic retinopathy and maculopathy and OCT thicknesses were explored at baseline and whilst no association was seen with retinopathy there was a statistically significant association with the presence of maculopathy see table 4.19.

The association between dietary intake using the food frequency questionnaire and the severity of diabetic retinopathy was also explored using a spearman's rank non-parametric correlation test. As the severity of diabetic retinopathy was an ordinal variable (none/mild/moderate/severe) rather than a continuous one, and the association of this with the food frequency questionnaire, the non-parametric Spearman's Rank correlation test was used.

A positive association was seen with riboflavin that was statistically significant at $\rho = -0.163$ p value = 0.031 indicating the higher the riboflavin the more chance of no diabetic retinopathy. See appendix 3a, 3b, and 3c.

The association between HbA1c, serum lipids and MOPD were then explored at baseline, see table 4.20. A positive association was seen between higher HBa1c levels with lower MOPD scores $\rho = -0.146$, P = 0.044* and triglycerides with MOPD $\rho = -0.226$, p = 0.035* which was also statistically significant.

However, when a repeated measures ANOVA was run using data across all 3 visits, only triglyceride was statistically significant (p=0.012), with a negative coefficient indicating that lower levels of triglyceride were associated with higher levels of macular pigment. The predicted model was: -

MOPD = 0.373 - 0.045 x Triglyceride.

Table.4.20 Correlations between HBA1c, lipids and macular pigment (MOPD)

| | MOPD | Pearson correlation P value |
|---|---|---|
| HBA1C | N=190 | r=-0.146, p=0.044* |
| TRIGLYCERIDE | N=87 | r=-0.226, p=0.035* |
| HDL | N=182 | r=-0.014, p=0.856 |
| NONHDL | N=132 | r=0.012, p=0.887 |
| cholesterol | N=184 | r=0.005, p=0.943 |

**Follow up – baseline, 6 months and 12 months**

In order to investigate the effect of time on dietary intake measures, OCT neuronal layers and severity of diabetic retinopathy and maculopathy as determined by grading, a repeated measures ANOVA was used and multiple linear regression analysis.

FFQ was explored with the individual neuronal layers and the results showed that the following OCT layers INL and ONL showed an association producing the following models of probability:

**Inner Nuclear layer**

INL = 23.768 + 2.275 (if Diabetic Type 2) [ p =0.112]

- -4.112 (if Female) [ p =0.002 ]
- -1.061 * Cu mg [p = 0.038 ]
- +0.024 * Se ug [p =0.034 ]

For INL a significantly significant association was found for copper and selenium. The model gives an assumption that a low intake of selenium was associated with a thinner layer in the INL whereas a high intake of Cu was associated with a thinner layer in the INL. All other FFQ constituents were not statistically significant.

INL has been seen to be positively correlated with the presence of M1 (see earlier unpaired t-test: p=0.017).

Note that as Diabetes Type and gender were also statistically significant, identifying the need to adjust for these 2 demographics/clinical features when assessing the impact of nutrients.

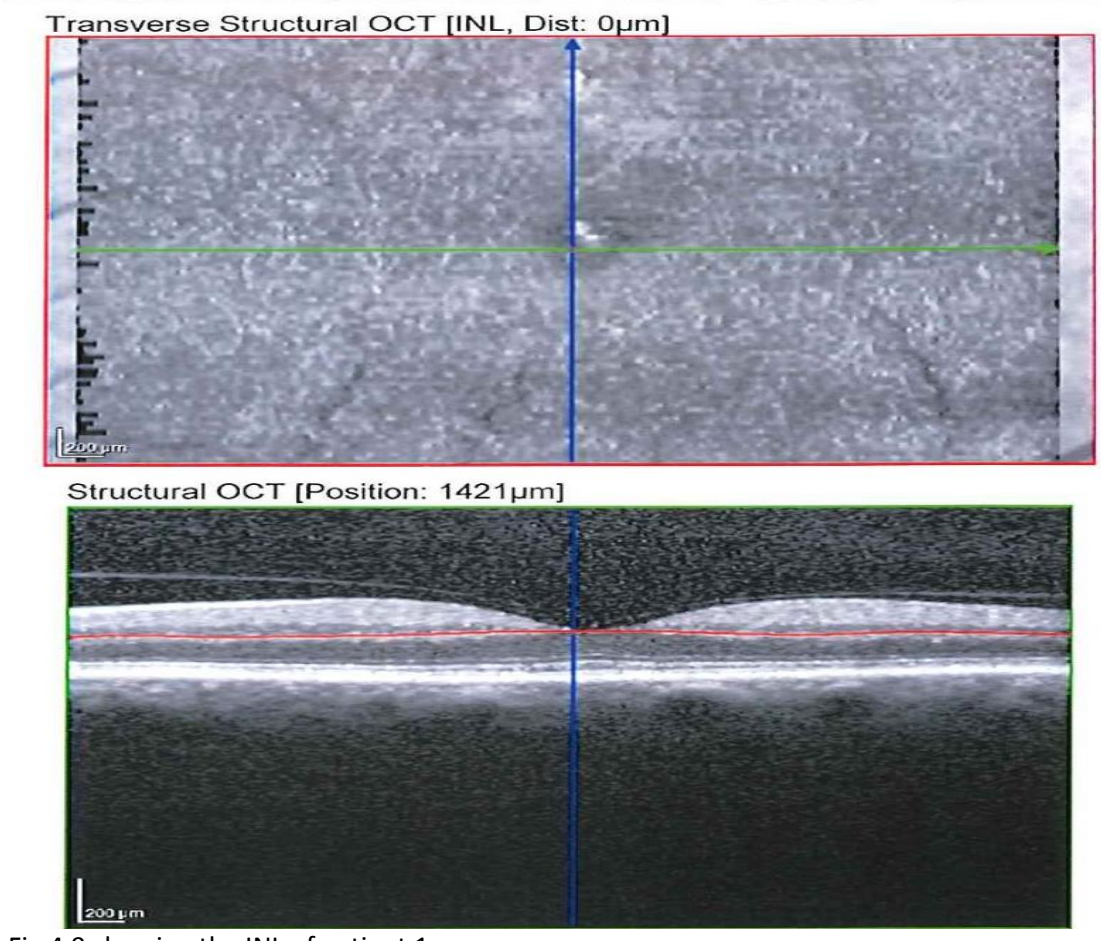


Fig 4.9 showing the INL of patient 1

**Outer nuclear layer**

ONL = 93.500 + 1.981 (if Diabetic Type 2) [p = 0.358 ]

- -2.921 (if Female) [p = 0.137 ]
- +0.197 * Vit B12 [p =0.048]
- -0.418 * PanAc [p = 0.044 ]

For ONL there was a statistically significant association with the model showing an increase in thickness of ONL with Vitamin B12, whereas an increase in PanAc resulted in a lower ONL score. (Pantothenate (or pantothenic acid) is Vitamin B5.

**Outer retinal layer**

Showed no association with the FFQ however a statistically significant association was seen with type of diabetes and gender.

ORL = 87.019 – 1.912 (if Diabetic type 2) [p = 0.006]

- +1.010 (if Female) [p = 0.112]

No correlations were evident between the intake of food nutrients on the food frequency diet questionnaire data and the severity of diabetic retinopathy or maculopathy when scoring the grading on a linear scale as follows: R0 (No diabetic retinopathy) = 0, R1 = 1, R2 = 2, R3 – 3, M0 = 0, M1 = 1

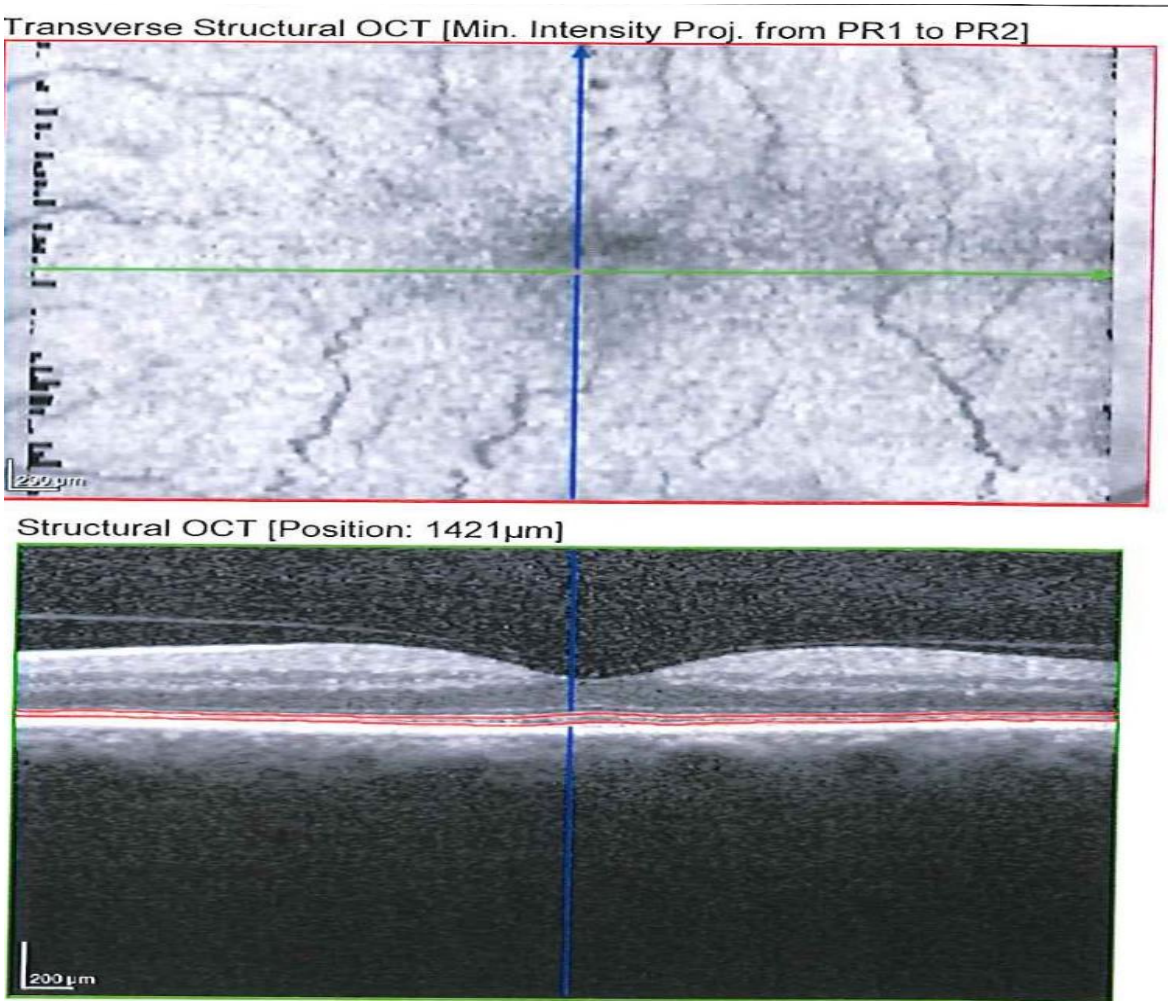
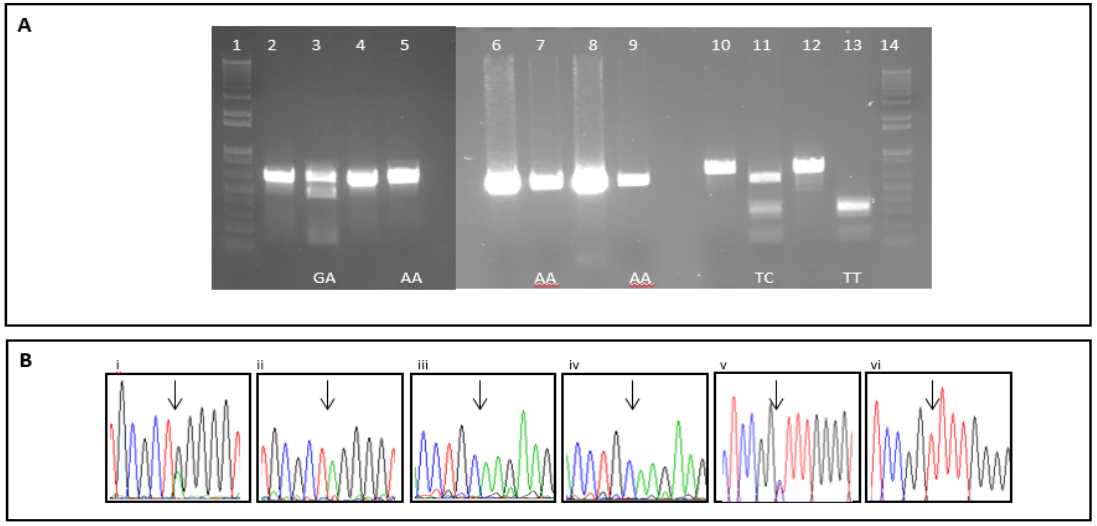



Fig 4.10 showing the ORL of patient 1

Genetics

Based on previous possible associations with diabetic retinopathy in the literature SNPs in 3 candidate genes were chosen to be evaluated to explore whether the genotypes were associated with diabetic retinopathy in our subject groups.




**Restriction Fragment Length Polymorphism (RFLP) genotyping of two patients.**

A. Agarose gel electrophoresis of undigested and digested PCR products with the patient's genotype noted in the digested lanes. **1**. 1Kbplus marker, **2**. undigested *ALR2* product patient 1, **3**. digested *ALR2* product patient 1, **4**. undigested *ALR2* product patient 2, **5**. digested *ALR2* product patient 2, **6**. undigested *SLC30A8* PCR product patient 1, **7**. digested *SLC30A8* product patient 1, **8**. undigested *SLC30A8* PCR product patient 2, **9**. digested *SLC30A8* product patient 2, **10**. undigested *SOD2* PCR product patient 1, **11**. digested *SOD2* product patient 1, **12**. undigested *SOD2* PCR product patient 2, **13**. digested *SOD2* product patient 2, **14**. 1Kbplus marker.

B. The undigested, purified PCR products were sequenced to verify the genotype of each patient. ***i***. *ALR2* patient 1 (GA), ***ii***. *ALR2* patient 2 (AA), ***iii***. *SLC30A8* patient 1 (AA), ***iv***. *SLC30A8* patient 2 (AA), ***v***. *SOD2* patient 1 (TC), ***vi***. *SOD2* patient 2 (TT).

Fig 4.11 Showing the RFLP genotyping of 2 patients in the study

Statistical analysis of the genotyping of all 3 SNP's showed no statistically significant association between any of the groups.

ALR 2

Table 4.21 showing the genotyping for ALR2 SNP

| | Genotype | | NO DR | R1 | R2 | R3 | Total | Mean ranks | P value |
|---|---|---|---|---|---|---|---|---|---|
| ALR2 | AA | n = | 2 | 20 | 3 | 0 | 25 | 90.58 | |
| | | % | 8 | 80 | 12 | 0 | 100 | | |
| | GA | n = | 6 | 76 | 8 | 3 | 93 | 92.19 | |
| | | % | 6.5 | 81.7 | 8.6 | 3.2 | 100 | | |
| | GG | n = | 5 | 50 | 17 | 2 | 74 | 103.91 | |
| | | % | 6.8 | 67.6 | 23 | 2.7 | 100 | | |
| Total | | | 13 | 146 | 28 | 5 | 192 | | 0.144 |
| % | | | 6.8 | 76 | 14.6 | 2.6 | 100 | | |

SLC3AO8

Table 4.22 showing the genotyping for SLC3AO8

| | Genotype | | NO DR | R1 | R2 | R3 | Total | Mean ranks | P value |
|---|---|---|---|---|---|---|---|---|---|
| SLC | AA | n = | 8 | 72 | 12 | 2 | 94 | 90.58 | |
| | | % | 8.5% | 76.6% | 12.8% | 2.1% | 100.0% | | |
| | GA | n = | 3 | 63 | 14 | 2 | 82 | 92.19 | |
| | | % | 3.7% | 76.8% | 17.1% | 2.4% | 100.0% | | |
| | GG | n = | 2 | 11 | 2 | 1 | 16 | 103.91 | |
| | | % | 12.5% | 68.8% | 12.5% | 6.3% | 100.0% | | |
| Total | | | 13 | 146 | 28 | 5 | 192 | | 0.434 |
| % | | | 6.8% | 76.0% | 14.6% | 2.6% | 100.0% | | |

MnSOD

Table 4.23 showing the genotyping for MnSOD

| | Genotype | | NO DR | R1 | R2 | R3 | Total | Mean ranks | P value |
|---|---|---|---|---|---|---|---|---|---|
| MnSod | CC | n = | 1 | 41 | 7 | 1 | 50 | 99.16 | |
| | | % | 2.0 | 82.0 | 14.0 | 14.0 | 100 | | |
| | TC | n = | 9 | 68 | 17 | 2 | 93 | 94.13 | |
| | | % | 9.7 | 73.1 | 15.1 | 2.2 | 100 | | |
| | TT | n = | 3 | 37 | 7 | 2 | 49 | 98.29 | |
| | | % | 6.1 | 75.5 | 2814.3 | 4.1 | 100 | | |
| Total | | | 13 | 146 | 28 | 5 | 192 | | 0.741 |
| % | | | 6.8 | 76 | 14.6 | 2.6 | 100 | | |

## 4.4 Adverse events and serious adverse events

***Adverse event*** means any untoward medical occurrence including occurrences which are not necessarily caused by or related to the procedure.

***Adverse reaction*** means any untoward and unintended response in a subject to an investigational procedure which is related to that procedure.

***Serious adverse event***, ***serious adverse reaction*** or ***unexpected serious adverse reaction*** means any adverse event, adverse reaction or unexpected adverse reaction, respectively, that:

- results in death,
- is life-threatening,
- requires hospitalisation or prolongation of existing hospitalisation,
- results in persistent or significant disability or incapacity.
- Is otherwise considered medically significant by the investigator

Important medical events that may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the outcomes listed in the definition of serious will also be considered serious.

***Unexpected adverse reaction*** means an adverse reaction the nature and severity of which is not consistent with what is known about the procedure.

**Serious adverse event (SAE):**

1. any systemic event which requires hospitalisation

2. death

3. severe visual loss

**Adverse event (AE):**

1. any systemic event that does not meet the criteria for an SAE

2. ocular adverse events:

- For DECAN other ophthalmic conditions that could affect the outcome such as but not limited to:

intraocular pressure over 40mm Hg requiring treatment/surgery, sight-threatening adverse event e.g. retinal vascular occlusion, retinal detachment.

As this was an observational study there were no associated SAEs or AEs with the study. All other non-study related SAEs were reported in the correct timely manner to the sponsor and R & D department.

## 4.5 Summary

Statistically significant associations have been demonstrated with dietary intake using a food frequency questionnaire and neuronal layer loss. Further discussion of the results plus the secondary objective findings will be discussed in the next chapter.

# Chapter 5 Discussion

## 5.1 Main outcomes

The primary objective of this study was to determine if there was a relationship between food intakes in particular antioxidants assessed using a food frequency questionnaire and neuronal layer loss assessed using SD-OCT. Although many food components showed statistically significant association at baseline including magnesium, phosphorous, iron, zinc, iodine, Vit D, thiamine, Riboflavin, potential niacin, Vit B12, Folic acid, pantothenic acid and biotin all mainly in the IPL, however these were not significant over the 3 visits. When a repeated measure ANOVA test was run the IPL did not appear to be affected by diet.

The ONL and INL however did show statistically significant associations with Vit B12, Pantothenic acid, copper and selenium. All the other FFQ variables were not statistically significant.

Selenium was seen to be associated with a thinner INL; therefore, it is suggested that a low intake of selenium may be associated with a thinner INL. Whereas a high intake of copper was associated with a thinner layer in the INL.

It has previously been reported that selenium is essential for regulating the activity of the enzymes involved in protection against oxidative stress, which suggests that selenium could be useful for the treatment in ocular pathologies such as diabetic retinopathy. [265,266].

Selenium is currently reported to be used for diseases such as heart disease, including stroke and atherosclerosis. It has also been suggested that selenium is used by individuals for other conditions such as under-active thyroid, osteoarthritis, rheumatoid arthritis , hay fever, infertility, cataracts, grey hair, abnormal pap smears, chronic fatigue syndrome (CFS), mood disorders, arsenic poisoning, and preventing miscarriage, plus many more have been described.[299]

Previous studies of diabetes have found that the level of selenium intake varies among different populations but there have been conflicting reports with regard its impact with diabetes with some suggesting that that a high level of selenium could reduce the prevalence of diabetes whereas others suggested an increased prevalence of diabetes was related to that a high level of serum selenium. The results of the Selenium and Vitamin E Cancer Trial (SELECT) found no significant relationship between supplementary selenium and the risk of type 2 diabetes. [265]

Marty (2005) describes the anecdotal report many years ago from an ophthalmologist who used a mixture of Vitamin E and C along with a high dose of selenium and found by chance that the antioxidants appeared to slow down vision loss due to diabetic retinopathy. [266]

The results of the DECAN study certainly warrant further investigation into the use of selenium as a supplement in patients with diabetic retinopathy.

Other findings showed that the ONL showed a statistically significant association with vitamin B12, indicating that an increase in Vitamin B12 results in a thicker ONL whereas an increase in pantothenic acid is associated with a thinner ONL.

The INL was also seen to be positively correlated with the presence of M1 (unpaired t-test: $p=0.017$). So, this would indicate a possible association with severity of diabetic but with maculopathy. A larger sample and further analysis would be required to confirm this.

Automated segmentation on SD OCT offers an objective and efficient method to quantify thickness of the individual retinal layers. As this study revealed, identification of retina layers and measurement of their thickness demonstrates that individual layers of the retina may be affected differently in diabetic retinopathy.

Equally this study shows that the dietary intake from individuals such as antioxidants appear to contribute to the changes in the retinal layers and therefore supports the concept that early diabetic retinopathy includes a neurodegenerative component. This has not previously been examined.

The secondary objectives were to examine the relationship between dietary intake and development of diabetic retinopathy. The results of the present study show that only riboflavin was associated with diabetic retinopathy at baseline explored using a spearman's rank correlation non parametric test $\rho$= -0.163 p value – 0.031. This would indicate that the more riboflavin the more chance there is being no diabetic retinopathy. However, when a repeated measure ANOVA test was run over the 3 visits no correlations were evident between the FFQ data and severity of retinopathy or maculopathy when scoring the diabetic grading on a linear scale.

The association between HbA1c, serum lips and MOPD were also explored at baseline and over the 3 visits. At baseline a positive association was seen between higher HBa1c levels with lower MOPD scores $\rho$ = - 0.146, P = 0.044* and triglycerides with MOPD $\rho$ = -0.226, p = 0.035* which was also statistically significant. However, when a repeated measures ANOVA was run using data across all 3 visits, only triglyceride was statistically significant (p=0.012), with a negative coefficient indicating that lower levels of triglyceride were associated with higher levels of macular pigment.

The data in this study suggest that MPOD level is not statistically significantly influenced by the level of HbA1C but higher levels of MPOD values are positively associated with lower levels of triglycerides. No evident association was seen between MOPD scores, FFQ and OCT.

Chung et al. (2017) investigated the effect of statin administration and dyslipidaemia on the development of diabetic macular oedema in patients with type 2 diabetes. They found that lipid lowering therapy with statins protected against the development of diabetic macular oedema and progression of diabetic retinopathy in patients with type 2 diabetes. [(268)] In this study triglycerides showed a significant correlation with MOPD but further research is needed to investigate the subgroups with maculopathy and those on statins. Other studies found that statin use is associated with a small but significant increased risk for development of diabetes. However, the effect of statin use on the development of diabetic macular oedema and progression of diabetic retinopathy in patients with pre-existing type 2 diabetes is unknown. [(268, 269)]

This study also does not support previous studies that have found MPOD levels to be positively associated with thicker central foveal thickness and higher intake of foods containing carotenoids. [257] Another study found that Type 2 diabetic patients, with or without retinopathy, had reduced MPOD when compared with that in nondiabetic patients. In addition, a significant inverse correlation between MPOD and HbA1C levels was observed however dual-wavelength auto fluorescence imaging was used investigate differences in MP optical density (MPOD) and a control group of nondiabetics .[258]

In this study there were statistically significant differences in gender (more men than women), age (older with type 2 diabetes), duration of diabetes and cholesterol and HDL. Although not statistically significant there was predominantly more Caucasians in the study (89.3%)

There have been many possible associations of SNPs with diabetic retinopathy reported to date, however due to the complexity of the disease it is difficult to draw conclusions due to the conflicting reports of significance in many of the studies. As seen in this study no association was seen with diabetic retinopathy and either SNP but numbers were significantly low, the disease states were altogether and there were no matched controls used.

Restriction fragment length polymorphism (RFLP) was used in this study as it is considered the simplest and earliest method to detect SNPs and then Tetra-primer amplification refractory mutation system PCR was carried out. Genotyping with Sequenom may be considered as an alternative. Sequenom's iPLEX SNP genotyping method, which uses a MassARRAY mass spectrometer, is designed in such a way that 40 different SNP assays can be amplified and analysed in a PCR together. [270]

As previously reported, there is also accumulating evidence that suggests that the metabolic memory phenomenon and vascular dysfunction may be related to epigenetic modification, despite achieving normal blood sugar levels. [24] It has been suggested by Wang et al. (2017) that the key

issue to be addressed would be gene-environment interaction with persistent epigenetic modification. [24]

## 5.2 Limitations

The main limitation of the study was the low number of participants that took part. The initial aim of the study was to recruit 2028 type 1 or type 2 participants with no or some level of diabetic retinopathy.

Over 3000 individuals with diabetes are screened every month in Surrey alone. Of these over 500 per month are referred to attend the eye unit at Frimley Park Hospital. The hospital eye service serves 3 screening programmes; Surrey, Hampshire and Berkshire. Therefore, the intension to recruit 1028 was considered achievable based on numbers attending the hospital eye clinic alone. It was soon apparent that a lot of participants did not want additional visits to their usual outpatient attendances so where possible the visits were matched to their usual appointment. Also, as there was a significant number that were working age so the DNA (did not attend) rate for clinics was particularly high also making recruitment difficult. To try and alleviate this, appointments were offered for the evening for those that did not want to take time off work to attend.

Amendments were made to both the inclusion/exclusion criteria and visit schedule. Despite this the recruitment was much more difficult than anticipated therefore the sample size was recalculated and reduced to 202 as shown in See table 5.1 below.

Table 5.1 Summary of amendments during recruitment

| | Summary of amendments |
|---|---|
| Amendment 1 | The first amendment was a change to the exclusion criteria to any glaucoma as this would affect the RNFL and to request consent on the same day. The OCT was added to the appendix. Other changes were for clarification. |
| Amendment 2 | This was in response to the above amendment with regards to consent and also to extend the duration of the study. We also removed bloods at 6 months so that it was in line with standard care for the patients. |
| Amendment 3 | This was the request to reduce the sample size from 1028 to 400 |
| Amendment 4 | This was in response to the above request as statistical justification was required and therefore this was provided but allowed the sample size to be reduced to 202. This was approved by ethics |
| Amendment 5 | Trial duration extended from 36 months to 42 months |

Whilst the aim of the genetic analysis was to explore the possible association of SNP's as a pilot study the resulting number recruited affected the results. It has been reported that a single SNP analysis the number of samples required would be 248 increasing to 1, 205 for 500 SNPs. If a sample size of 248 is used then a control group is required if a 1:1 ratio to achieve 80% statistical power under the assumption that the prevalence is 5%.

Therefore, to explore the association in greater detail for diabetic retinopathy a much larger study sample would be needed. The reasoning behind the selection of the SNPs was based on their previously reported function and how they may contribute to the disease state. There have been various associations reported by previous authors but there are still a lot of conflicting evidence between genome wide association studies and candidate genes studies that have not been as successful.

However as this was a pilot study it was felt that the association of the chosen genes could still be explored in this study as previous associations had been made in other candidate gene studies with diabetic retinopathy but further analysis would be carried at a later date with a larger samples obtained from a multi-centre study and participants were consented accordingly for this.

Another limitation is the fact that the baseline characteristics of the study population may not accurately reflect those of the general population. It could be that those people with diabetic retinopathy, who volunteer for research projects such as this, are generally better informed and have a greater interest in their diabetic control and general health and could be more interested in prevention strategies.

For example, the potential risk factors such as BMI, smoking, exercise could change significantly over the course of the study by stopping smoking, losing weight and becoming more active. Diabetic control and blood pressure control may have altered although HbA1c and BP were recorded at baseline and 6 months follow up.

Equally despite informing participants that dietary advice was not being given some participants did comment that they had made significant changes since starting and were following a much more health balanced diet and taking supplements on the other hand some participants failed to return questionnaires despite numerous reminders. Some data was incomplete either due to not being filled in correctly or participants just not wanting to fully complete it at that time. Any missing FFQ data would automatically in that situation mean a never score could have changed the dietary intake and confound the results. There can also be significant differences in diet between ethnic groups and this could have affected the results.

The use of food frequency questionnaires is commonly used in research on diet and disease to assess the usual food or nutrient intakes of individuals. The food frequency questionnaire used in this study was the SCG FFQ is based on commonly eaten UK foods and does not allow for a range of diversity between ethnic groups. There may also have been seasonal variations as the study was

conducted over 12 months with different start points for the patients. This has been investigated in other diseases as antioxidants from some food may be more readily available during different seasons and therefore the antioxidants that may prevent against disease by reducing the oxidative stress may be reduced at different times of the year. This would need to be investigated further. Also, the blood samples that were taken were not fasting bloods not all profiles were available for all patients. The grading that was performed was carried out by the same examiner although very experienced in grading of diabetic images there was no control for inter grader reliability.

## 5.3 Improvements

This needs to be further explored in a larger cohort of patients over a longer time frame with well-defined parameters to include ethnicity, gender and type 1 or type 2 and matched controls. Also, the severity of diabetic retinopathy including those with and without macular oedema should be explored in comparison to a control group. Future research would include microperimetry to detect early functional changes with a longer follow up period. As there are advances in imaging the use of OCTA would be valuable as it can be used to detect changes in the retinal vasculature.

Optical coherence tomography angiography (OCTA) is a new, non-invasive modality that visualizes retinal capillary blood flow without the need for intravenous dye. OCTA visualizes individual retinal capillary plexuses, including the superficial capillary plexus (SCP), deep capillary plexus (DCP), and more recently, the middle capillary plexus (MCP).

Early studies have shown promising results in its use in conditions such as diabetic retinopathy suggesting it could be used as a screening tool for diabetic retinopathy. [271, 272]

For future work examining the effects of antioxidants would require special monitoring through diet or through supplementation which can be assessed by a food frequency questionnaire however modifications are required to make them more user friendly as in this study many patients found the FFQ to be very cumbersome. A future study should will require careful analysis of data given, so that

all antioxidants that are possibly physiologically relevant as well as the concentrations required to alleviate diabetic complications, with minimal side effects can be established.

Furthermore, an oxidative stress test would be preferred in a future study as this would enable the researcher to evaluate the body's oxidative stress status and antioxidant reserve and would be valuable if considering an interventional study of supplementation of antioxidants. There are various sample methods that have been proposed for this. For a future study the test to be considered could require either a 5ml unpreserved urine sample or a blood sample, two serum samples of 4ml each and one whole blood sample of 7ml. The samples would be collected after fasting for 10 hours overnight. The urine sample measures the free radical damage, including oxidative damage to cell membranes i.e. Lipid peroxides and the oxidative damage to DNA (8-hydroxy-deoGuanosine). The blood sample measures the anti-oxidant reserve and enzyme function which includes total antioxidant capacity, whole blood glutathione, the enzymes glutathione and superoxide dismutase and also blood markers of damage measure lipid peroxides. There are other markers of oxidative stress that can be analysed such as in cells and tissues an example of these can be seen at https://www.cellbiolabs.com/selection-guide-oxidative-stress-assays-sample-type.

The genetic association with diabetic retinopathy and DMO equally needs to be further explored as separate entities with much larger well-defined cohorts addressing disease state and ethnic groups individually compared to matched controls. This will enable the investigators to determine if any true associations can be seen that will lead onto the possibility of identifying gene/genes that may predispose individuals to develop the complications of diabetic retinopathy or DMO. Genotyping with sequenom could be used as it is a powerful platform which provides a flexible method of assaying up to a few thousand markers and individual or alternatively the epigenetic processes as they may be more important in helping address this complex disorder. Future genetic analysis more appropriate to diseases has previously been described for diseases with such complex pathogenesis and multiple clinical manifestations and so could include a different approach when planning future

studies such as focusing on “the delivery of multiple transgenes, the modulation of transgene expression, and optomize patient selection”. [24] There are many on-going Phase III human clinical trials for hereditary retinal diseases other ocular diseases with complex pathophysiology, such as AMD. Gene therapy although still in its infancy may well prove a useful adjunct or alternative to conventional treatment for patients with diabetic retinopathy. [24]

## 5.4 Conclusions

Although further research is needed the results of the DECAN study certainly adds scope to the theory that early neurodegeneration changes may occur in diabetic retinopathy before the clinically detectable microvascular signs and that micronutrients may have some influence on this process and lead to the development of an interventional clinical trial. There was also no evidence shown in previous studies that examined the direct correlation of dietary intake with early changes in the neuronal layers using OCT in this cohort of patients.

Medical intervention and treatments for DR at present are focused on metabolic control of blood glucose and blood pressure and surgical procedures which includes laser photocoagulation, surgery and the commonly intravitreal injection (IVT) This study has added to the growing evidence that oxidative stress, antioxidants and neurodegeneration are critical factors in the pathogenesis of diabetic retinopathy early on. It suggests that intervention to protect the retinal neurons in diabetic retinopathy may protect against damage to the retinal vessels and delay or even inhibit the progression of diabetic retinopathy. The study shows promising results for further research that could lead to an interventional trial using supplementation.

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| | | MOPD | CMTscan | RETINAscan | RNFLscan | GCLscan | IPLscan | INLscan | OPLscan | ONLscan | RPEscan | IRLscan | ORLscan |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Water | Pearson Correlation | -.068 | .111 | .131 | .108 | .099 | .152 | .101 | .159 | .024 | .037 | .111 | .093 |
| | Sig. (2-tailed) | .377 | .140 | .082 | .152 | .189 | .043 | .181 | .034 | .754 | .619 | .139 | .216 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Protng | Pearson Correlation | -.094 | .066 | .100 | .152 | .117 | .191 | .037 | .117 | -.054 | .077 | .084 | .084 |
| | Sig. (2-tailed) | .217 | .377 | .186 | .043 | .118 | .010 | .625 | .118 | .476 | .306 | .265 | .263 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Fatg | Pearson Correlation | -.070 | .053 | .067 | .125 | .110 | .168 | .012 | .091 | -.079 | .048 | .055 | .091 |
| | Sig. (2-tailed) | .359 | .479 | .371 | .097 | .143 | .025 | .869 | .228 | .295 | .528 | .470 | .228 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| CHOg | Pearson Correlation | -.125 | .023 | .018 | .078 | .041 | .116 | .000 | .052 | -.112 | .044 | -.001 | .109 |
| | Sig. (2-tailed) | .102 | .765 | .811 | .300 | .589 | .124 | .995 | .487 | .136 | .559 | .993 | .146 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| KCal | Pearson Correlation | -.102 | .039 | .051 | .109 | .079 | .154 | .008 | .085 | -.094 | .056 | .035 | .102 |
| | Sig. (2-tailed) | .182 | .602 | .502 | .146 | .297 | .041 | .916 | .262 | .212 | .461 | .645 | .175 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| EnergkJ | Pearson Correlation | -.103 | .039 | .051 | .110 | .079 | .154 | .009 | .085 | -.094 | .055 | .035 | .101 |
| | Sig. (2-tailed) | .179 | .600 | .499 | .145 | .296 | .040 | .910 | .260 | .213 | .463 | .641 | .178 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| SFAg | Pearson Correlation | -.035 | .067 | .081 | .136 | .125 | .181 | .025 | .095 | -.063 | .029 | .073 | .072 |
| | Sig. (2-tailed) | .646 | .373 | .281 | .070 | .098 | .016 | .741 | .208 | .404 | .700 | .335 | .336 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| MUFAg | Pearson Correlation | -.081 | .044 | .061 | .118 | .105 | .162 | .012 | .089 | -.087 | .050 | .048 | .094 |
| | Sig. (2-tailed) | .288 | .557 | .415 | .116 | .161 | .030 | .879 | .237 | .249 | .511 | .524 | .210 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| PUFAg | Pearson Correlation | -.096 | .015 | .034 | .079 | .076 | .122 | -.018 | .072 | -.085 | .052 | .017 | .092 |
| | Sig. (2-tailed) | .209 | .839 | .656 | .297 | .315 | .106 | .808 | .340 | .257 | .494 | .826 | .222 |

Appendix 1a Showing correlations at baseline for FFQ vs OCT

aph Styles

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| TRANSg | Pearson Correlation | -.050 | .079 | .078 | .117 | .109 | .168 | .041 | .115 | -.086 | .020 | .064 | .120 |
| | Sig. (2-tailed) | .512 | .294 | .300 | .118 | .147 | .025 | .584 | .126 | .255 | .795 | .400 | .111 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Sugarsg | Pearson Correlation | -.102 | -.042 | -.031 | .015 | -.001 | .050 | -.031 | .034 | -.121 | .024 | -.044 | .074 |
| | Sig. (2-tailed) | .182 | .579 | .676 | .843 | .989 | .509 | .682 | .651 | .108 | .753 | .558 | .324 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Mgmg | Pearson Correlation | -.128 | .068 | .095 | .112 | .100 | .178 | .034 | .096 | -.031 | .014 | .075 | .096 |
| | Sig. (2-tailed) | .094 | .368 | .208 | .137 | .185 | .017 | .648 | .204 | .685 | .853 | .318 | .202 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Pmg | Pearson Correlation | -.075 | .100 | .126 | .149 | .129 | .203 | .044 | .115 | -.022 | .054 | .104 | .110 |
| | Sig. (2-tailed) | .327 | .182 | .093 | .048 | .086 | .007 | .555 | .128 | .773 | .470 | .168 | .143 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Femg | Pearson Correlation | -.112 | .063 | .061 | .113 | .081 | .159 | -.010 | .063 | -.060 | .054 | .042 | .102 |
| | Sig. (2-tailed) | .141 | .403 | .415 | .132 | .285 | .034 | .895 | .406 | .430 | .473 | .575 | .175 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Cumg | Pearson Correlation | -.136 | -.044 | -.030 | .047 | -.008 | .073 | -.065 | -.005 | -.109 | .014 | -.048 | .102 |
| | Sig. (2-tailed) | .075 | .555 | .688 | .535 | .914 | .332 | .392 | .946 | .147 | .858 | .523 | .176 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Znmg | Pearson Correlation | -.113 | .075 | .099 | .155 | .101 | .185 | .038 | .100 | -.038 | .056 | .081 | .097 |
| | Sig. (2-tailed) | .137 | .320 | .188 | .039 | .181 | .013 | .612 | .182 | .610 | .460 | .281 | .196 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Clmg | Pearson Correlation | -.127 | .067 | .071 | .117 | .078 | .146 | .027 | .100 | -.054 | .053 | .056 | .073 |
| | Sig. (2-tailed) | .095 | .373 | .347 | .121 | .300 | .051 | .719 | .185 | .477 | .482 | .455 | .332 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Mnmg | Pearson Correlation | -.188 | .036 | .027 | .094 | .056 | .118 | .004 | .103 | -.110 | .022 | .011 | .085 |
| | Sig. (2-tailed) | .014 | .633 | .721 | .211 | .462 | .118 | .954 | .172 | .143 | .771 | .883 | .258 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Seug | Pearson Correlation | -.075 | -.011 | .044 | .100 | .077 | .135 | .004 | .072 | -.085 | .088 | .027 | .089 |
| | Sig. (2-tailed) | .330 | .881 | .558 | .182 | .307 | .073 | .963 | .339 | .259 | .245 | .717 | .237 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Iug | Pearson Correlation | -.033 | .077 | .121 | .113 | .146 | .185 | .068 | .091 | -.004 | .044 | .115 | -.002 |

Appendix 1b Showing correlations at baseline for FFQ vs OCT

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sig. (2-tailed) | .665 | .304 | .109 | .132 | .052 | .014 | .370 | .228 | .959 | .560 | .127 | .982 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Retinug | Pearson Correlation | -.046 | -.016 | -.012 | .067 | .005 | .058 | -.039 | -.007 | -.054 | .040 | -.011 | -.012 |
| | Sig. (2-tailed) | .549 | .828 | .871 | .373 | .943 | .445 | .607 | .929 | .474 | .596 | .888 | .871 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Carotug | Pearson Correlation | -.068 | -.088 | -.067 | .000 | -.032 | -.029 | -.092 | .057 | -.128 | .153 | -.083 | .101 |
| | Sig. (2-tailed) | .376 | .243 | .375 | .997 | .672 | .696 | .220 | .447 | .089 | .042 | .268 | .178 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Vt_dug | Pearson Correlation | -.028 | .067 | .116 | .131 | .156 | .216 | .048 | .067 | .013 | .048 | .120 | -.051 |
| | Sig. (2-tailed) | .713 | .375 | .122 | .081 | .038 | .004 | .524 | .373 | .863 | .521 | .112 | .498 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Vt_Emg | Pearson Correlation | -.083 | .013 | .021 | .066 | .047 | .097 | -.042 | .101 | -.104 | .098 | -.005 | .151 |
| | Sig. (2-tailed) | .277 | .862 | .779 | .383 | .534 | .196 | .578 | .179 | .166 | .195 | .945 | .044 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Thiammg | Pearson Correlation | -.133 | .102 | .114 | .162 | .105 | .191 | .032 | .110 | -.002 | .032 | .098 | .071 |
| | Sig. (2-tailed) | .080 | .175 | .131 | .030 | .161 | .011 | .676 | .145 | .978 | .675 | .194 | .349 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Ribofmg | Pearson Correlation | -.065 | .162 | .181 | .190 | .168 | .248 | .074 | .151 | .029 | .024 | .165 | .039 |
| | Sig. (2-tailed) | .398 | .030 | .016 | .011 | .025 | .001 | .325 | .044 | .697 | .752 | .027 | .603 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Niacinmg | Pearson Correlation | -.048 | .111 | .140 | .177 | .139 | .217 | .045 | .149 | -.010 | .055 | .121 | .101 |
| | Sig. (2-tailed) | .533 | .140 | .063 | .018 | .063 | .004 | .555 | .047 | .895 | .463 | .108 | .181 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| PotNiamg | Pearson Correlation | -.087 | .069 | .107 | .146 | .120 | .196 | .053 | .101 | -.040 | .032 | .090 | .082 |
| | Sig. (2-tailed) | .257 | .359 | .156 | .051 | .111 | .009 | .481 | .180 | .595 | .675 | .230 | .276 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Vt_B6mg | Pearson Correlation | -.126 | .110 | .135 | .161 | .125 | .217 | .039 | .149 | .001 | .017 | .121 | .063 |
| | Sig. (2-tailed) | .099 | .143 | .073 | .031 | .096 | .004 | .602 | .047 | .988 | .818 | .107 | .404 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |
| Vt_B12ug | Pearson Correlation | -.064 | .061 | .104 | .174 | .131 | .213 | .048 | .062 | -.019 | .094 | .104 | -.034 |
| | Sig. (2-tailed) | .402 | .419 | .167 | .021 | .082 | .004 | .524 | .414 | .798 | .213 | .166 | .656 |
| | N | 173 | 179 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 | 178 |

Appendix 1c Showing correlations at baseline for FFQ vs OCT

| | | MOPD | CMTscan | RETINAscan | RNFLscan | GCLscan | IPLscan | INLscan | OPLscan | ONLscan | RPEscan | IRLscan | ORLscan |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VitAREug | Pearson Correlation | -.189 | .054 | -.092 | -.262 | -.068 | -.166 | -.088 | .002 | -.084 | .140 | -.112 | .124 |
| | Sig. (2-tailed) | .248 | .745 | .581 | .112 | .685 | .320 | .600 | .990 | .615 | .400 | .505 | .457 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitDug | Pearson Correlation | -.031 | .091 | .198 | .284 | .062 | .194 | .104 | -.024 | .280 | .013 | .185 | .117 |
| | Sig. (2-tailed) | .849 | .582 | .233 | .084 | .713 | .243 | .534 | .889 | .089 | .940 | .266 | .485 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitEmg | Pearson Correlation | -.041 | -.209 | -.247 | -.083 | -.165 | -.176 | -.100 | .002 | -.406 | .089 | -.231 | -.147 |
| | Sig. (2-tailed) | .803 | .201 | .135 | .621 | .323 | .289 | .550 | .991 | .011 | .597 | .163 | .378 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitCmg | Pearson Correlation | .106 | -.016 | -.011 | .038 | -.006 | .009 | -.039 | -.097 | .118 | .139 | .006 | -.096 |
| | Sig. (2-tailed) | .520 | .925 | .948 | .820 | .970 | .956 | .814 | .564 | .480 | .406 | .972 | .565 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitB1mg | Pearson Correlation | -.195 | .077 | -.120 | -.172 | -.093 | -.150 | -.138 | .021 | -.102 | .064 | -.126 | .037 |
| | Sig. (2-tailed) | .235 | .643 | .473 | .300 | .578 | .368 | .408 | .902 | .540 | .704 | .450 | .826 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitB2mg | Pearson Correlation | -.191 | .056 | -.118 | -.167 | -.091 | -.147 | -.133 | .028 | -.110 | .061 | -.125 | .033 |
| | Sig. (2-tailed) | .244 | .735 | .479 | .317 | .586 | .377 | .425 | .868 | .513 | .716 | .456 | .843 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitB3mg | Pearson Correlation | -.190 | .053 | -.118 | -.166 | -.091 | -.147 | -.132 | .029 | -.111 | .061 | -.124 | .033 |
| | Sig. (2-tailed) | .246 | .749 | .480 | .320 | .587 | .379 | .428 | .863 | .509 | .717 | .457 | .845 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitB6mg | Pearson Correlation | -.197 | .092 | -.121 | -.177 | -.095 | -.153 | -.143 | .015 | -.097 | .068 | -.128 | .041 |
| | Sig. (2-tailed) | .229 | .578 | .468 | .287 | .569 | .359 | .393 | .928 | .562 | .686 | .443 | .807 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| Folicacidug | Pearson Correlation | -.286 | -.052 | -.203 | -.170 | -.105 | -.168 | -.190 | -.033 | -.187 | .000 | -.180 | -.179 |
| | | | | | .309 | .531 | .315 | .253 | .844 | .260 | 1.000 | .279 | .283 |

Appendix 2a Supplements at baseline vs MOPD and OCT outcomes

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitB12ug | Pearson Correlation | -.162 | -.072 | -.104 | -.125 | -.075 | -.124 | -.096 | .072 | -.149 | .040 | -.109 | .008 |
| | Sig. (2-tailed) | .324 | .665 | .533 | .455 | .654 | .458 | .565 | .667 | .371 | .811 | .516 | .960 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| VitKug | Pearson Correlation | .[a] | .[a] | .[a] | .[a] | .[a] | .[a] | .[a] | .[a] | .[a] | .[a] | .[a] | .[a] |
| | Sig. (2-tailed) | . | . | . | . | . | . | . | . | . | . | . | . |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| Pantothenoicacidmg | Pearson Correlation | -.169 | .511 | -.071 | -.177 | -.054 | -.094 | -.149 | -.165 | .120 | .024 | -.074 | .050 |
| | Sig. (2-tailed) | .303 | .001 | .670 | .286 | .747 | .575 | .371 | .323 | .472 | .885 | .660 | .766 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| Biotinug | Pearson Correlation | -.158 | .372 | -.054 | -.124 | -.017 | -.051 | -.099 | -.121 | .070 | -.054 | -.049 | -.019 |
| | Sig. (2-tailed) | .336 | .020 | .746 | .458 | .917 | .760 | .556 | .471 | .676 | .745 | .770 | .910 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| Cuug | Pearson Correlation | -.243 | .058 | .066 | .159 | .049 | .191 | .151 | .186 | -.087 | -.228 | .102 | -.244 |
| | Sig. (2-tailed) | .136 | .726 | .692 | .342 | .772 | .251 | .367 | .262 | .603 | .169 | .542 | .140 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| Camg | Pearson Correlation | .042 | -.098 | -.138 | -.151 | -.075 | -.103 | -.139 | -.122 | -.110 | .107 | -.145 | .082 |
| | Sig. (2-tailed) | .799 | .553 | .409 | .365 | .654 | .537 | .407 | .467 | .513 | .524 | .385 | .626 |
| | N | 39 | 39 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |

Appendix 2b Supplements at baseline vs MOPD and OCT outcomes

Appendix 3a Nonparametric (Spearman's Rank) Correlations between FFQ and DR

**Correlations**

| | | | DRGrading |
|---|---|---|---|
| Spearman's rho | Water | Correlation Coefficient | .035 |
| | | Sig. (2-tailed) | .645 |
| | | N | 175 |
| | Protng | Correlation Coefficient | -.053 |
| | | Sig. (2-tailed) | .482 |
| | | N | 175 |
| | Fatg | Correlation Coefficient | -.048 |
| | | Sig. (2-tailed) | .531 |
| | | N | 175 |
| | CHOg | Correlation Coefficient | -.032 |
| | | Sig. (2-tailed) | .671 |
| | | N | 175 |
| | KCal | Correlation Coefficient | -.030 |
| | | Sig. (2-tailed) | .694 |
| | | N | 175 |
| | EnergkJ | Correlation Coefficient | -.032 |
| | | Sig. (2-tailed) | .676 |
| | | N | 175 |
| | SFAg | Correlation Coefficient | -.072 |
| | | Sig. (2-tailed) | .340 |
| | | N | 175 |
| | MUFAg | Correlation Coefficient | -.027 |
| | | Sig. (2-tailed) | .727 |
| | | N | 175 |
| | PUFAg | Correlation Coefficient | -.008 |
| | | Sig. (2-tailed) | .919 |
| | | N | 175 |
| | TRANSg | Correlation Coefficient | -.052 |
| | | Sig. (2-tailed) | .496 |
| | | N | 175 |
| | Sugarsg | Correlation Coefficient | -.118 |
| | | Sig. (2-tailed) | .120 |
| | | N | 175 |
| | Mgmg | Correlation Coefficient | -.040 |
| | | Sig. (2-tailed) | .595 |
| | | N | 175 |

Appendix 3b Nonparametric (Spearman's Rank) Correlations between FFQ and DR

| | | | |
|---|---|---|---|
| | Pmg | Correlation Coefficient | -.061 |
| | | Sig. (2-tailed) | .425 |
| | | N | 175 |
| | Femg | Correlation Coefficient | -.033 |
| | | Sig. (2-tailed) | .664 |
| | | N | 175 |
| | Cumg | Correlation Coefficient | .008 |
| | | Sig. (2-tailed) | .916 |
| | | N | 175 |
| | Znmg | Correlation Coefficient | -.018 |
| | | Sig. (2-tailed) | .811 |
| | | N | 175 |
| | Clmg | Correlation Coefficient | -.031 |
| | | Sig. (2-tailed) | .688 |
| | | N | 175 |
| | Mnmg | Correlation Coefficient | -.038 |
| | | Sig. (2-tailed) | .621 |
| | | N | 175 |
| | Seug | Correlation Coefficient | -.033 |
| | | Sig. (2-tailed) | .660 |
| | | N | 175 |
| | Iug | Correlation Coefficient | -.132 |
| | | Sig. (2-tailed) | .083 |
| | | N | 175 |
| | Retinug | Correlation Coefficient | .047 |
| | | Sig. (2-tailed) | .538 |
| | | N | 175 |
| | Carotug | Correlation Coefficient | -.069 |
| | | Sig. (2-tailed) | .364 |
| | | N | 175 |
| | Vt_dug | Correlation Coefficient | -.108 |
| | | Sig. (2-tailed) | .156 |
| | | N | 175 |
| | Vt_Emg | Correlation Coefficient | -.097 |
| | | Sig. (2-tailed) | .204 |
| | | N | 175 |
| | Thiammg | Correlation Coefficient | -.072 |
| | | Sig. (2-tailed) | .345 |
| | | N | 175 |
| | Ribofmg | Correlation Coefficient | -.163 |

Appendix 3c Nonparametric (Spearman's Rank) Correlations between FFQ

| | | | |
|---|---|---|---|
| | | Sig. (2-tailed) | .031 |
| | | N | 175 |
| | Niacinmg | Correlation Coefficient | -.044 |
| | | Sig. (2-tailed) | .562 |
| | | N | 175 |
| | PotNiamg | Correlation Coefficient | -.049 |
| | | Sig. (2-tailed) | .516 |
| | | N | 175 |
| | Vt_B6mg | Correlation Coefficient | -.091 |
| | | Sig. (2-tailed) | .233 |
| | | N | 175 |
| | Vt_B12ug | Correlation Coefficient | -.021 |
| | | Sig. (2-tailed) | .786 |
| | | N | 175 |
| | FolACug | Correlation Coefficient | -.117 |
| | | Sig. (2-tailed) | .123 |
| | | N | 175 |
| | PanAcmg | Correlation Coefficient | -.066 |
| | | Sig. (2-tailed) | .387 |
| | | N | 175 |
| | Biotnug | Correlation Coefficient | -.041 |
| | | Sig. (2-tailed) | .588 |
| | | N | 175 |
| | Vt_Cmg | Correlation Coefficient | -.070 |
| | | Sig. (2-tailed) | .355 |
| | | N | 175 |
| | VITKmg | Correlation Coefficient | -.028 |
| | | Sig. (2-tailed) | .718 |
| | | N | 175 |
| | Alcohg | Correlation Coefficient | .072 |
| | | Sig. (2-tailed) | .346 |
| | | N | 175 |

Scottish Collaborative Group
Food Frequency Questionnaire

**Version 6.5&6.6**
**University of Aberdeen**

# Standard Operating Procedures (SOP)

## SOP 1: Completing questionnaires

## SOP 2: Checking completed questionnaires

## SOP 3: Preparation of questionnaires for data entry

## SOP 4: Web based FFQ data entry

**Scottish Collaborative Group**
**Food Frequency Questionnaire**

**Version 6.5/6.6**
**University of Aberdeen**

# Standard Operating Procedure 1: Completing questionnaires

1. Before distributing the SCG FFQ to volunteers, it is recommended that the investigator fills in the questionnaire themselves to gain an understanding of how to complete it.

2. If face to face contact is possible, ask volunteers to carefully read the '*how to complete the questionnaire*' instructions on the first page of the FFQ.

It is important to highlight the need to respond to all questions and ensure they understand how to complete the questionnaire:

- For food eaten on average 1-7 days per week, '*measures per/day*' – how much of the food they ate in a day they ate the food - and *number of days/week*' – how many days a week they had the food.
- Foods eaten more than once per month but less than once per week, '*M*' should be circled and '*measures per/day*' should be reported.
- Foods either 'not eaten' or consumed less than once per month '*R*' should be circled and measures per day/month need not be reported.

This questionnaire is designed to be completed by the volunteers alone. Investigators are advised to give volunteers the opportunity to complete the questionnaire themselves, before checking its completeness

3. If the questionnaires are being mailed to volunteers, highlight the end to read these instructions on the covering letter. We also recommend asking for a contact telephone number in case questions arise.

**NB: Poorly completed questionnaires may yield inaccurate estimates of nutrient intake!**

**Scottish Collaborative Group**
**Food Frequency Questionnaire**

**Version 6.5/6.6**
**University of Aberdeen**

## Standard Operating Procedure 2: Checking completed questionnaires

1. Questionnaires should be checked as soon as possible after completion, ideally in the presence of the subject (e.g. at a clinic visit). For questionnaires returned by mail the subject should be contacted by telephone or mail if questions arise. **If the subject cannot be contacted, their answers should be left unchanged: intended responses should never be assumed.**

2. Check the 'subject code' is clearly marked on the front cover of the questionnaire and any loose pages.
3. Check there is an answer to every question (section) and sub-question (line) of the questionnaire.
    - For rarely eaten foods (i.e. the subject has recorded 'R' under 'number of days per week') no answer is required under 'measures per day'.
    - For all other foods, there should be 1 answer under 'measures per day' and 1 answer under 'number of days per week'. Measures for foods eaten less than once per week and more than once per month should also complete both sections.
        - Where there are no answers ask the subject to provide the missing answer.
        - Where there are 2 or more answers, ask the subject to select 1.

    **NB: PLEASE ENSURE <u>BOTH MEASURES AND FREQUENCY OF CONSUMPTION ARE COMPLETED</u> QUESTIONNAIRES WITH 10 OR MORE UNANSWERED QUESTIONS WILL NOT BE ANALYSED.**

4. Double check specific lines and sections for completeness.
    - 'Bread type' (line 1e)
    - 'Sugar consumption' (line 17d)
    - 'Fat spread and oil type' (lines 17e and g): full brand name and descriptions are provided.
    - 'Other foods' (section 20): full description and measures outlined
    - Supplements (section 21): full brand name(s); supplement strength and measures are specified.

Scottish Collaborative Group
Food Frequency Questionnaire

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# Standard Operating Procedure 3: Preparation of questionnaires for data entry

1. Selection and entry of 'official codes' for fats and spreads (line 17e) should be undertaken using the SCG fat coding sheet.

2. 'Other foods' reported in section 20 should be checked in collaboration with nutritionist or dietician. If you do not have easy access to a nutritionist or dietician, please contact us for further advice.

- Foods containing low energy or few nutrients of interest (e.g. sugar-free jelly, raw mushrooms, garlic, water chestnuts) can be ignored.
- Food nutritionally similar to foods already listed on the questionnaire can be added to the appropriate line. The 'measures per day' and 'number of days per week' should be edited to reflect reported total intake.

  e.g. A subject recorded 2 measures per day, 2 days per week, under 'other breads' (line 1d), then also reported - in the 'other foods' (section 20) - eating 1 measure per day, 2 days per week of ciabatta bread. In this case line 1d should be updated to the 3 measures per day, while days per weeks should remain unchanged.

  NB: A list of suggested lines on the questionnaire to add 'other foods' is available.

- Any changes to reflect addition of 'other foods' should be made using different coloured ink to that originally used by the subject.

- Foods which cannot be entered to existing lines should be given a food code and measure weight by a dietician / nutritionist. The measure weight per day should be calculated as:

$$\frac{\text{(measure* weight x measures per day x days per week)}}{7}$$

* measure provided by subject or estimated from typical food portion sizes

The nutrient content of the calculated daily consumption of the food should be derived, by entering the McCance & Widdowsons' food code and weight per day into a nutrient analysis package e.g. Windiets

**Please note the nutrient content calculated for 'other foods' not incorporated into appropriate lines of the questionnaire, should be added to the nutrient analysis output file, before undertaking analysis and interpretation of the complete FFQ nutrient data.**

# Scottish Collaborative Group Food Frequency Questionnaire

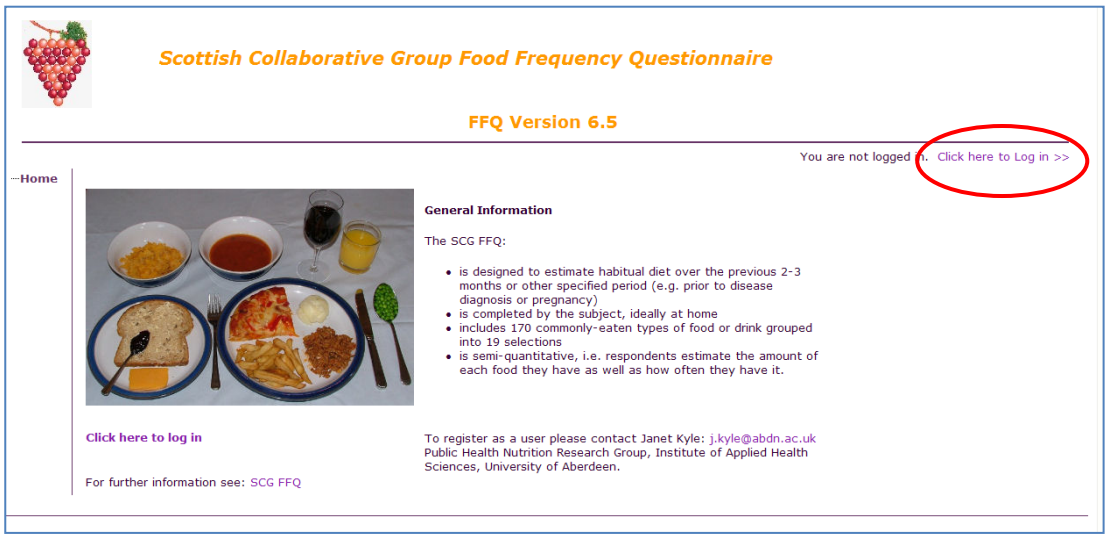
**Version 6.5/6.6**
**University of Aberdeen**

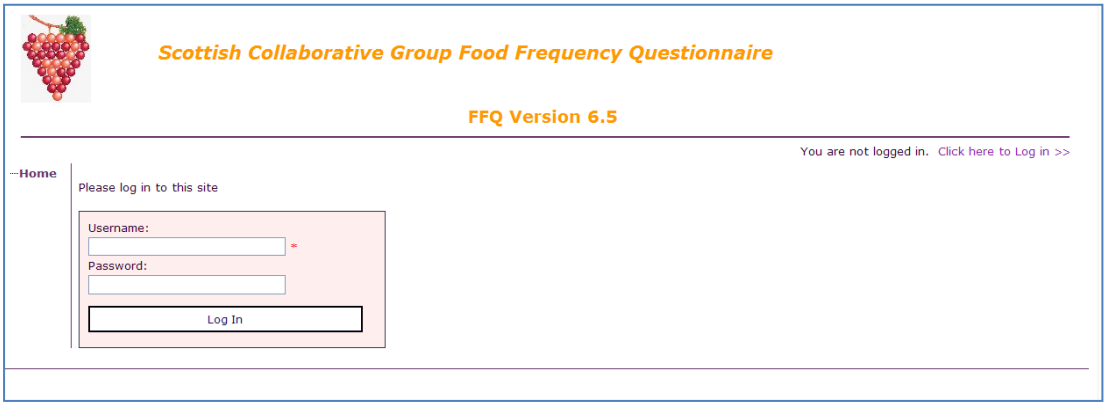
## Standard Operating Procedure 4: Web-based FFQ data entry

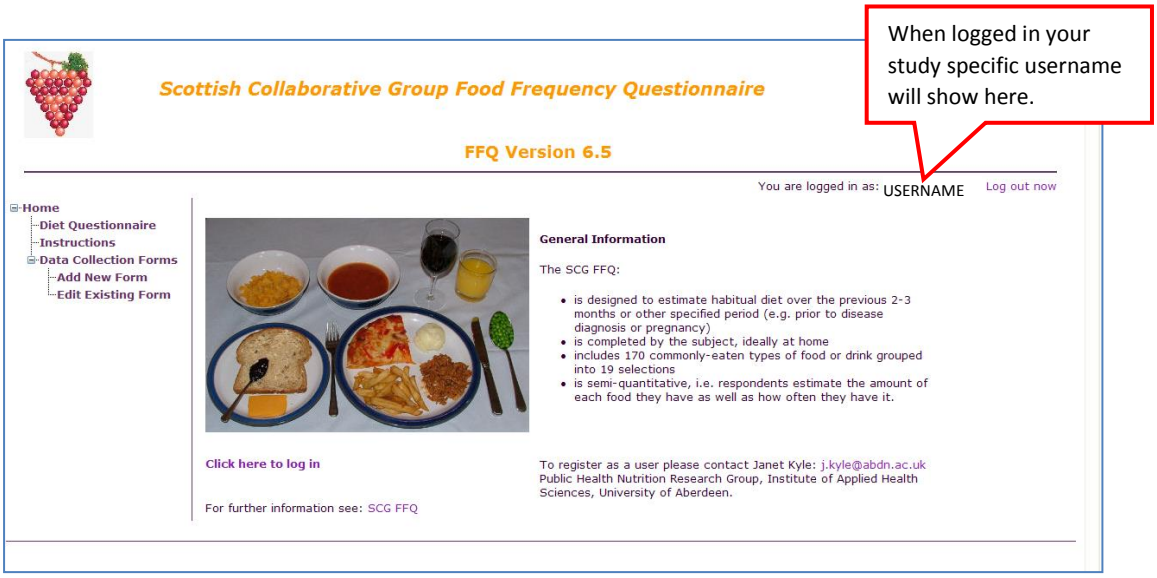
1. Before entering FFQ data ensure you have obtained the correct website address and login details from the SCG FFQ administrator.

2. You will receive an e-mail outlining the web address and link, study specific user name and password. Keep these details in a safe secure place.

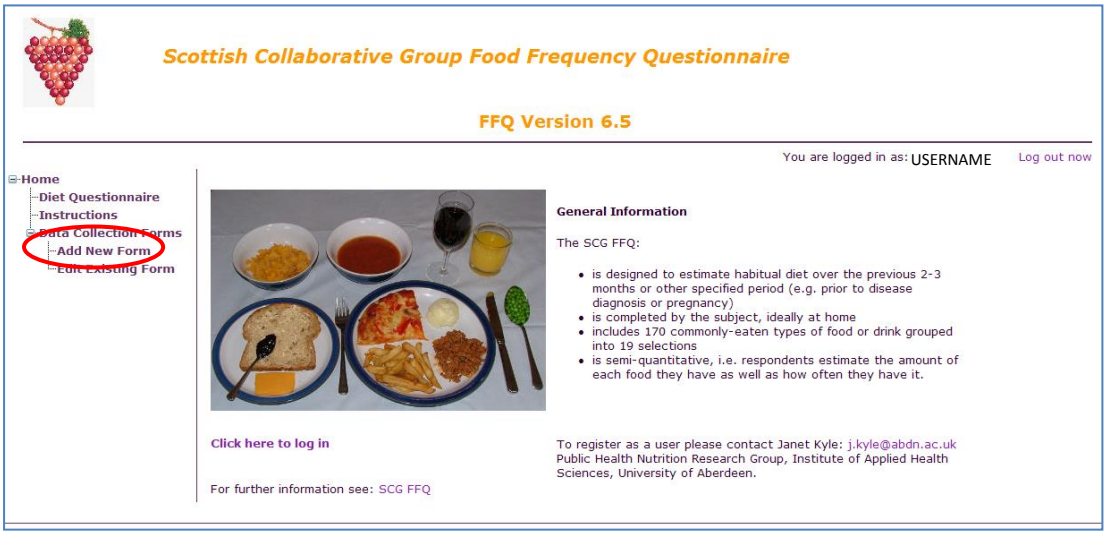
3. Clicking on web link will bring you to the window below, **click on login button** as highlighted and follow the screen instructions as follows until successfully logged in.



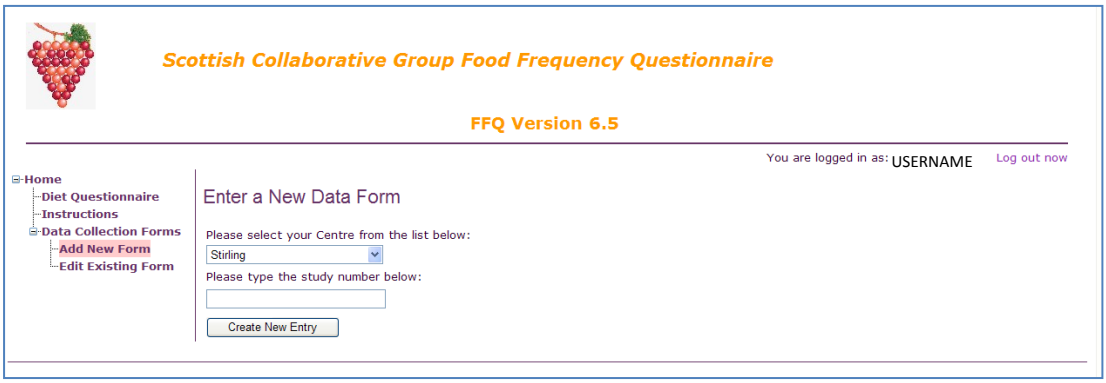






4. When entering data from previously completed and checked (see standard operating procedures 2 and 3) paper copies of the questionnaire select '**Add New Form**' from 'data collection forms' as follows.



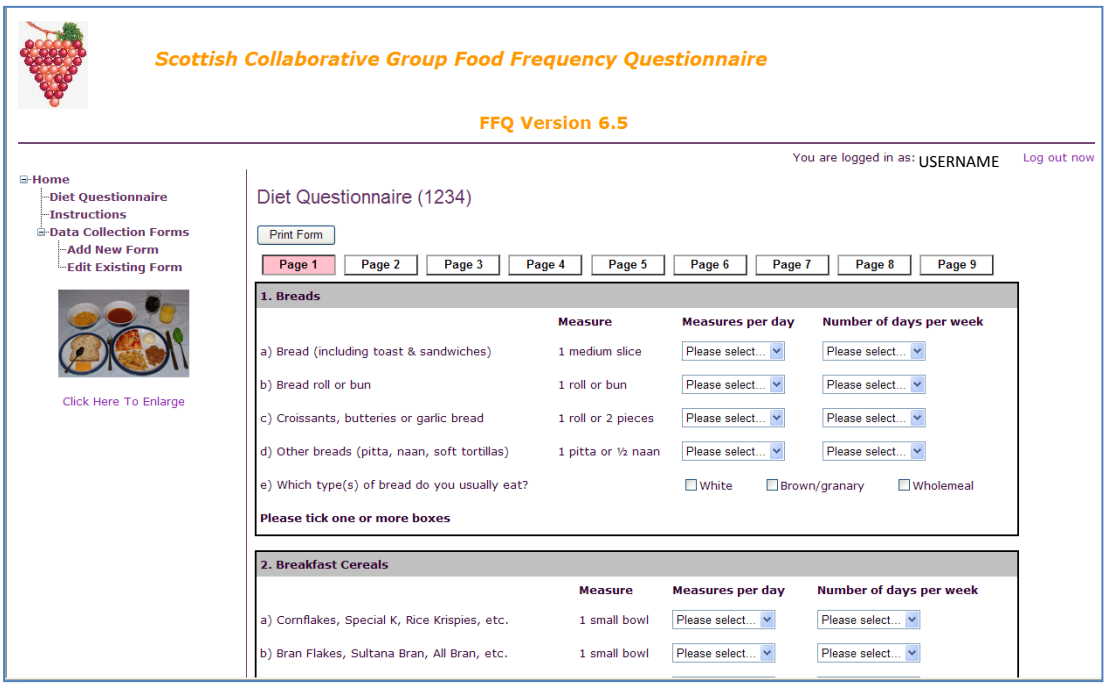

5. You will be asked '**select your centre**' this will automatically show centre name which is linked to your user name for example 'Stirling' and then to type the '**study number**' this is the unique subject/participant code or number found on the top left hand corner of the completed FFQ.

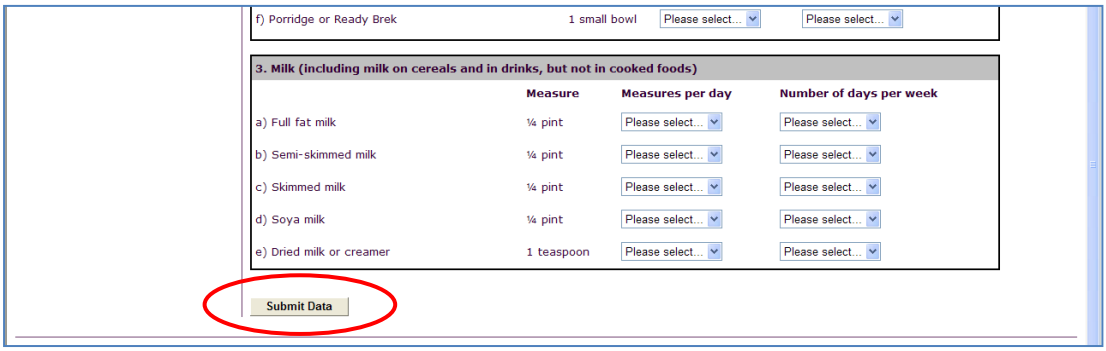



6. The subject's reported intake '**measures per day**' (measure) and '**number of days per week**' (frequency) can now be entered by selecting appropriate 'number of measures per day' and 'days per week' from the drop down boxes. The question layout of the entry package mirrors FFQ layout, therefore individual responses can be entered as reported.

USERNAME

**Please remember to save your entered data by clicking on the 'submit data' button at the end of each completed page on the website!**






7. Once the complete questionnaire has been entered, click '**add new form**' on the left hand of the screen and continue to enter the next questionnaire as described in points 4-6.

8. Please you can also edit previously entered forms by clicking the '**Edit Existing Form**' button of the left hand side of the screen and entering the appropriate study (subject/participant) number.

9. When finished entering a batch of questionnaire data and are leaving the site please remember to '**log out**'!

10. When all completed questionnaires have been entered into the web-site, we recommend double checking your data entry. Typically, a random sample of 10% of questionnaires should be double-checked by someone not involved with the original data entry and the error rate recorded.

    *If more than 2% of entries are incorrect all questionnaires should be re-entered.*

11. On completion of the double check, please notify the SCG FFQ administrator that the questionnaire data is ready for nutrient analysis.

Frimley Park Hospital NHS
NHS Foundation Trust

Aston University

Portsmouth Road
Frimley
Camberley
Surrey

## Personal Data Questionnaire

Date of Birth: ____/_____/_____ STUDY No.....

Sex: ☐ ale ☐ male

Predominant race: ☐ aucasian ☐ ive American
☐ lack ☐ ific Islander
☐ sian ☐ her, please specify _____

Height: _ _ _ cm Weight: _ _ _Kg BMI:

Occupation:

Type of Diabetes: ☐ Type1 ☐ Type2 Treatment: Insulin/Tablets/Diet

Year of Diagnosis : _ _ _ _

Current smoker: ☐ Yes ☐ No Ex-smoker? ☐ Yes ☐ No

Number of cigarettes smoked per day: _____ Numbers of years smoked: _____

Quit_____ years ago

How often do you have a drink containing alcohol?

☐ Never ☐ Once a month or less ☐ 2 to 4 times a month

☐ 2 to 3 times a week ☐ 4 or more times a week

How many standard drinks containing alcohol do you have on a typical day when drinking?

☐ None ☐ 1 or 2 ☐ 3 or 4 ☐ 5 or 6 ☐ 7 to 9 ☐ 10 or more

**Medical History**

Do you suffer from any of the following medical conditions?

☐ Heart Disease ☐ Epilepsy

☐ Cancer ☐ Asthma

☐ High Cholesterol ☐ High Blood Pressure

☐ Kidney Problems ☐ Other

**Medications YES/NO (circle)**

Details.....

.....

**Previous Ocular History YES/NO (circle)**

Details.....

**Exercise and Sleep**

How active are you?

☐ Fairly active –includes walking and exercise 1 to 2 times weekly.

☐ Moderately active –exercise 2 to 3 times weekly.

☐ Active –exercise more than 3 times per week

How many hours do you sleep nightly on average? _ _

Appendix 6 DECAN patient information sheet

Committed To Excellence | Working Together | Facing The Future

Frimley Health NHS
NHS Foundation Trust

Portsmouth
Road
Frimley
Camberley
Surrey

Aston University

PATIENT INFORMATION SHEET

**Title: A pilot study of type 1 and type 2 Diabetics examining the correlation between antioxidant levels and neuronal layer loss.**

**(DECAN study)**

## 1 Introduction

You are being invited to take part in a pilot study which is part of a PhD programme run by Aston University. This work will be carried out at Frimley Park Hospital Foundation Trust.

Before you decide it is important for you to understand why this research is being done and what it will involve.

This form contains a full explanation of the study you are being invited to take part in and a consent form that you will be asked to sign if you decide to take part.

## 2 What is the purpose of the study?

Diabetic retinopathy ( DR) is damage to the retina ( retinopathy) caused by complications of diabetes mellitus, which can eventually lead to blindness if untreated It affects up to 80% of all patients who have had diabetes for 10 years or more. The role of nutritional supplementation is of increasing interest with regard to eye diseases. Research has demonstrated the effectiveness of supplementation for age related eye disease however conclusive clinical trials on diabetes in this area are lacking. Various trials have identified positive effects of some nutrients relating to type 2 diabetics but the evidence on the beneficial effects is limited.

To explore this question further we plan to carry out a pilot study to examine the preliminary cause-effect relationship. If positive results are obtained then a larger long term prospective multi-centre interventional trial will be carried out examining the effect of supplements in these patients. Patients with type 1 and type 2 diabetes will be included to determine if poor intake of a specific nutrient called antioxidants makes the diabetic changes that can occur at the back of the eye worse in type 1 and type 2 diabetics.

We are also planning to do genetic analysis which is a study of a sample of DNA to look for mutations (changes) that may increase risk of damage to the retina. Analysis may also identify the genes involved in absorbing antioxidants and consequently reducing the risk of diabetic retinopathy.

The outcomes of this research would increase our understanding of the beneficial effects of nutrients in the development of eye disease in diabetic patients.

## 3 Why have I been chosen?

You are being asked to take part in this research study because you have diabetes.

## 4 Do I have to take part?

No, taking part in this study is voluntary. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and given time to review the information. You can have as much time as you need to read and think about this information and discuss with others (e.g. family, friends, GP) if you wish before deciding whether or not to

take part in this study. Ask us if there is anything that is not clear or if you would like more information.

If you decide not to take part, you do not have to give a reason. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care received.

## 5 What will happen to me if I take part?

If you decide to take part in this study, your involvement will last for up to 1 year.

You will be required to sign an informed consent form once all the study information has been clearly explained to you, including the risks and benefits of taking part, any procedures and tests to be performed, any questions have been answered and you have been given enough time to consider whether you wish to take part in this study. The Principle Investigator Lorraine North or your eye doctor (who is part of the research team) will be available to answer any questions you may have and will also sign the consent form. You will receive a copy of your signed consent form.

If you agree to take part in this study, after signing the informed consent form the following tests will be carried out:

### Visit Schedule

The study will begin with a visit to the eye clinic at Frimley Park Hospital. The purpose of the first visit is to find out if you meet all of the requirements to take part in this research study. The first visit will take about 1and half hours.

During the first visit, you will be asked to have the following tests and procedures:

1. A detailed history, both ophthalmic and general will be taken. This will include any eye symptoms, past ocular history, general medical history and drug history. A personal data questionnaire will be used to record demographic data,

lifestyle data, general health status, blood pressure, weight and height. This will include risk factors such as age and smoking status.

A further self-administered semi-quantitative food-frequency questionnaire (FFQ) developed and validated by the Scottish Collaborative Group will be completed when you attend and you will be given another to complete prior to your next visit.

2. An eye examination will be carried out. This includes a vision check (using log MAR vision), examination of the front of the eye, then having eye drops (tropicamide 1%, unless any contraindications) to dilate the pupils, so that the retina at the back of the eye can be examined using a slit lamp microscope.

The presence or absence of any diabetic eye disease will then be determined. Any other pathology will be noted.

3. The retina will then be photographed (using a standard fundus camera) by a technician in the eye unit and a scan using the Spectral domain OCT scan of each eye taken to measure the thickness of the retina. We will also carry out a macular pigment optical density measurement of one or both eyes.

4. Blood samples will be taken to determine the HbA1c level and investigate serum lipid levels. These will be analysed at Oxford Hospital. Blood samples may be kept for use in future research however this is optional to you. If you do not wish for your sample to be kept after this study, please make sure that you opt out on the consent form.

5. Anonymised DNA will also be taken to look at genes absorbing antioxidants and will be later analysed at Oxford Hospital.

**Follow Up Visit**

You will be asked to return for a follow-up at 6 months and 1 year later

At these visits we will repeat some of the above, so we can obtain a record of how/if your eyes have changed over the study period.

## Schedule Summary

| Assessment | Baseline ( Visit1) | Time taken for procedure ( mins) | 6 months | Time taken for procedure ( mins) | 12 months | Time taken for procedure ( mins) |
|---|---|---|---|---|---|---|
| Informed consent | X | 10 | | | | |
| Personal Data Questionnaire | X | 10 | | | | |
| Visual Acuity and dilatation | X | 5-10 | X | 5-10 | X | 5-10 |
| Food Frequency Questionnaire | X | 20 | X | AT HOME | X | AT HOME |
| Eye examination | X | 10 | X | 10 | X | 10 |
| Fundus photos | X | 5 | X | 5 | X | 5 |
| Optical coherence tomography | X | 5-10 | X | 5-10 | X | 5-10 |
| Macular pigment optical density measurement | X | 10 | X | 10 | X | 10 |
| Serum, lipids and HbA1C | X | 5 | | | X | 5 |
| Blood sample for genetic and serum analysis | X | | | | | |
| Take blood pressure measurements | X | | | | | |
| TOTAL TIME TAKEN | | UP TO 1.5 HRS | | UP TO 1 HOUR | | UP TO 1 HOUR |

## Payment

You will be reimbursed for any reasonable extra travel expenses needed to take part in this study.

There will be no other payment for participation in the study.

## 6 What do I have to do?

If you decide to take part in this study you will be asked to:

- ✓ Keep all scheduled appointments.
- ✓ Tell us at your research visit about any medications that you take, even if it is medicine you buy without a prescription or is an herbal remedy.

## 7 What are the possible disadvantages and risks of taking part?

The eye drops used in the study are used to make the pupils larger than normal allowing the investigator to view the inside of the eye more easily (the highlighted section is modifiable to reflect the primary reason for using the drug). When applied to the eye, they may sting for a few moments. The drops take about 15 to 30 minutes to work and around 6 hours to wear off, off (in some cases up to 24 hours.) The resultant large pupils will make you more sensitive to light, whilst distant and near objects may appear slightly blurred. Consequently, you shouldn't perform any activities such as driving and/ or cycling for at least 12 hours after the drops have been instilled. On a bright day, sunglasses may be advisable. It is very unlikely, but should you experience any unusual symptoms such as severe pain and/ or blood shot around the eye and cloudy vision during this period please contact the Principal Investigator, Lorraine North on 01276 526982 or your optometrist/ GP as you may be experiencing an adverse reaction to the drops.

Examination of the eyes may reveal pathology that has not previously been identified. You will be informed of any findings and your GP informed with your consent to then make an appropriate onward referral.

The tests done at each visit are standard medical tests. The most unpleasant is having blood samples taken. The risks of taking blood may include fainting, pain and/or bruising from the needle in your arm. Rarely, there may be a small blood clot or infection at the site of the needle puncture. The blood pressure cuff may also cause discomfort or bruising to the upper arm.

## 8 What are the possible benefits of taking part?

There are no personal benefits by participating in this study. However the information we gain from this study could increase our understanding of the beneficial effects of antioxidants in the development of retinopathy in diabetic patients. This may then lead to a larger study looking at supplements to prevent diabetic retinopathy which could benefit the diabetic population

## 9 Removal from the study

The Principle Investigator, eye doctor or the Sponsor may decide to take you out of the study if:

- You do not comply with the study Schedule Summary on page 4.
- You develop an ophthalmic condition that could affect the outcome

**The Sponsor, Research Ethics Committee or the Regulatory Authority decide to stop the study**

## 10 Withdrawal from the study

If you decide to withdraw from the study you should tell the Principle Investigator. If you withdraw from the study without telling the Principle Investigator, you may be contacted to confirm whether you have withdrawn or wish to continue your participation in the study. If you do withdraw from the study, no new information about you will be collected by study personnel, but with your permission we will continue to include the data and samples that have already been collected to preserve the value of the study. If you have any questions or concerns about this, it is recommended that you contact the principle investigator detailed below.

## 11 Will my taking part in this study be kept confidential?

If you join the study, the doctors, nurses and other personnel involved in this study may need access to your medical history, including your past medical records and test results, for the purposes of this study

Personal data, which may be sensitive, (e.g. date of birth) will be collected, processed and stored for research purposes in connection with this study.

All clinical information will be filed in patients notes and images stored in NHS encrypted secure servers as is routine clinical practice for clinical care. Research data and images needed for research purposes will be anonymised and stored in specific research folders on secure servers. The research assistants will be in charge of the confidential information.

Linked anonymised data will be used. Patients will be allocated a study number when initially recruited into the study.

Your legal rights are not affected by signing the informed consent form.

The Sponsor (who will control the use of the data) will take steps to ensure your personal data is protected at all times.

With your consent your family doctor (General Practitioner) will be told that you have decided to take part in this study.

## 12 Your right to access health information

You have a right to ask for access to the health information that the study doctor holds about you and to ask for changes if your health information is not correct or is incomplete, in accordance with UK law. Any request for access or changes should be made to the Principle Investigator. In specific circumstances your right of access may need to be delayed to protect the scientific integrity of the study. If this happens, access to your health information will be made available as soon as reasonably possible.

## 13 Who has reviewed this study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be given a favourable opinion by an NHS Research Ethics Committee before it goes ahead. Approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision to take part.

## 14. Complaints

Who do I contact if I have concerns about the way in which the research is conducted?

If you have any concerns about the way in which the study was conducted you should in the first instance contact the Principle Investigator (named at the end of this information sheet) who will do her best to address your concerns.

If the Principle Investigator is unable to resolve the concerns you raise, you should contact the Secretary to the Aston University Ethics Committee- Mr John Walter – on j.g.walter@aston .ac.uk or telephone 0121 204 4869

## 15 Contact names and telephone numbers for further information

For any concerns or other questions about this study please contact:

Principle Investigator: Lorraine North

Ophthalmology Clinical Trials Unit

Frimley Park Hospital Foundation Trust

Portsmouth Road

Frimley

Surrey

GU16 7UJ

01276 526982

Thank you for taking the time to read this information sheet.

Appendix 7 Example FFQ

Subject Code

Scottish Collaborative Group Food Frequency Questionnaire version 6.6

# Diet Questionnaire

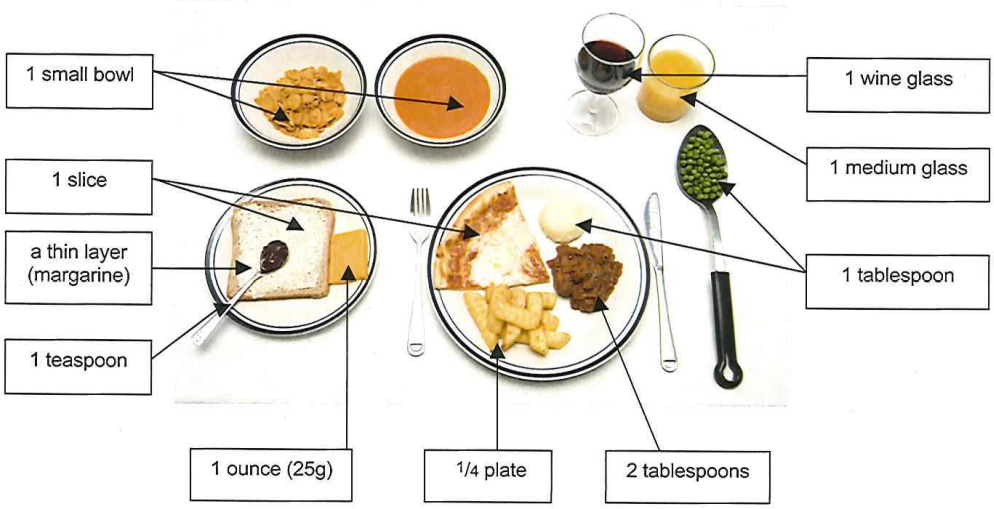


**Thank-you for agreeing to complete this questionnaire.**
**It should take about 20-30 minutes to complete.**

**Please take a few minutes to read the instructions carefully.**

We would like you to describe your usual diet over the last 2-3 months. This should include all your main meals, snacks and drinks which you had at home or away from home e.g. at work, at restaurants or cafes and with friends and family.

The questionnaire lists 170 foods and drinks. For each one a measure is given to help you estimate how much you usually have. The photograph below shows examples of some of these measures:




Please use **black or blue pen** to complete the questionnaire: do not use pencil.

## How to complete the questionnaire

For **every line** in the questionnaire, we would like you to answer two things.

- **how much** of the food you had in a day you ate the food, and
- **how many** days a week you had the food.

To estimate **how much** of the food you had, you should circle a number under 'Measures per day'. Each food is described in common measures such as slices, glasses or tablespoons as illustrated in the photograph. *Please note that the measures are designed to be quite small, so your usual portion may easily be 2 or more measures.*

To estimate **how many** days a week you had the food, you should circle a letter or number under 'Number of days per week'.

- If you had the food less than once a month, you should circle **R** (for **R**arely or never). *For these foods you do not need to fill in the number of measures per day.*
- If you had the food more than once a month but less than once a week, you should circle **M** (for **M**onth).
- If you had the food on average 1-6 days a week, you should circle 1-6 as appropriate.
- If you had the food every day, you should circle 7.

The example below shows the answers for someone who had 4 slices of bread every day, 1 apple 5 days a week, 1/2 a plate of chips (i.e. two 1/4 plates) once or twice a month but rarely or never had tomato juice:

| | Measure | Measures per day | Number of days per week |
|---|---|---|---|
| a) Bread (including toast & sandwiches) | 1 medium slice | 1 2 3 **(4)** 5+ | R M 1 2 3 4 5 6 **(7)** |
| b) Apples | 1 medium apple | **(1)** 2 3 4 5+ | R M 1 2 3 4 **(5)** 6 7 |
| c) Chips from a chip shop or restaurant | 1/4 plate | ~~1~~ **(2)** 3 4 5+ | R **(M)** 1 2 3 4 5 6 7 |
| d) Tomato juice | 1/2 medium glass | 1 2 3 4 5+ | **(R)** M 1 2 3 4 5 6 7 |

If you want to change an answer, please put a **cross** through the wrong answer and circle the new answer (see example above).

If there are any foods or drinks that you eat regularly which do not appear on the questionnaire, please list them in section 20 ('other foods and drinks').

**It is very important that you give an answer for every line.**

**If you rarely or never have a food, please make sure that you circle R.**

## 1. Breads

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Bread (including toast & sandwiches) | 1 medium slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Bread roll or bun | 1 roll or bun | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Croissants, butteries or garlic bread | 1 roll or 2 pieces | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Other breads (pitta, naan, soft tortillas) | 1 pitta or 1/2 naan | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Which type(s) of bread do you usually eat? **Please tick one or more boxes.** | | White ☐ Brown / granary ☐ Wholemeal ☐ | |

## 2. Breakfast Cereals

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Cornflakes, Special K, Rice Krispies etc. | 1 small bowl | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Bran Flakes, Sultana Bran, All Bran etc. | 1 small bowl | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Shredded Wheat, Weetabix etc. | 1 biscuit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Coco Pops, Frosties, Sugar Puffs, Crunchy Nut Cornflakes etc. | 1 small bowl | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Muesli (all types) | 1 small bowl | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| f) | Porridge or Ready Brek | 1 small bowl | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 3. Milk *(including milk on cereals and in drinks, but not in cooked foods)*

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Full fat milk | 1/4 pint | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Semi-skimmed milk | 1/4 pint | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Skimmed milk | 1/4 pint | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Soya milk | 1/4 pint | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Dried milk or creamer | 1 teaspoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 4. Cream and Yogurt

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Low fat yogurt (plain or fruit) | 1 pot (125 ml) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Full fat yogurt (e.g. Greek) | 1 pot (125 ml) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

**Please make sure you have given an answer for every line before leaving this page**

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| c) | Low calorie yogurt (plain or fruit) | 1 pot (125 ml) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Fromage frais (plain or fruit) | 1 pot (125 ml) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Cream (all types) | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 5. *Cheese*

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Full fat hard cheese (e.g. Cheddar, Gruyere, Wensleydale, Gouda) | 1 oz. (25g) or 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Medium fat cheese (e.g. Edam, Brie, Camembert, Feta, cheese spreads) | 1 oz. (25g) or 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Full fat cream cheese (e.g. Philadelphia, Boursin, Danish Blue) | 1 oz. (25g) or 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Low fat cheese (e.g. low fat cream cheese, low fat hard cheese) | 1 oz. (25g) or 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Cottage cheese (all types) | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 6. *Eggs*

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Boiled or poached eggs | 1 egg | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Fried eggs | 1 egg | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Scrambled eggs or omelette | 1 egg | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 7. ***Meats*** *(Meat substitutes e.g. Quorn or soya are listed in section 10)*

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Mince or meat sauce (e.g. bolognese) | 2 tablespoons | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Sausages (pork, beef or frankfurters) | 1 sausage | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Burgers (beef, lamb, chicken or turkey) | 1 burger | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Beef (roast, grilled, casseroled or fried) | 2 tablespoons, 2 slices or 1 steak | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Pork or lamb (roast, grilled, casseroled or fried) | 2 tablespoons, 2 slices or 1 chop | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

**Please make sure you have given an answer for every line before leaving this page**

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| f) | Chicken or turkey (roast, grilled, casseroled or fried) | 1 wing or thigh, 1/2 breast or 2 slices | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| g) | Bacon or gammon | 1 medium slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| h) | Liver, liver sausage or liver pate | 1 serving | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| i) | Haggis or black pudding | 2 tablespoons or 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| j) | Meat or chicken pies, pasties or sausage roll | 1 individual pie or 1 roll | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| k) | Cold meats (e.g. ham, corned beef, chicken roll) | 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| l) | Salami or continental sausage | 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 8. *Fish*

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Fish fingers | 1 finger | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | White fish (e.g. haddock, cod, plaice or scampi) fried or cooked in batter | 1 small fillet or 1 serving | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Grilled, poached or baked white fish | 1 small fillet | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Smoked white fish | 1 small fillet | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Fish cakes, fish pie | 1 cake or 2 tablespoons | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| f) | Fried oily fish (e.g. salmon, herring, fresh tuna or mackerel) | 1 small fillet | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| g) | Grilled, poached or baked oily fish | 1 small fillet | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| h) | Smoked oily fish (kipper, mackerel or salmon ) | 1 small fillet or 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| i) | Tinned salmon | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| j) | Tinned tuna | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| k) | Sardines, pilchards or rollmop herrings | 2 small fish or 1 large fish | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| l) | Prawns, crab etc. | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| m) | Mussels, oysters, cockles, scallops | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

**Please make sure you have given an answer for every line before leaving this page**

## 9. *Potatoes, Rice and Pasta*

| | | Measure | Measures per day | | | | | Number of days per week | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| a) | Boiled or baked potatoes | 1 medium or 1/2 large | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| b) | Mashed potatoes | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| c) | Oven chips, potato waffles or croquettes | 1/4 plate or 1 waffle | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| d) | Home-cooked chips | 1/4 plate | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| e) | Chips from a chip shop or restaurant | 1/4 plate | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| f) | Roast or fried potatoes | 1/4 plate | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| g) | White rice | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| h) | Brown rice | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| i) | Pasta (all types) or couscous | 1/4 plate | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| j) | Noodles (all types) | 1/4 plate or 1 pot | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

## 10. *Savoury foods, Soups and Sauces*

| | | Measure | Measures per day | | | | | Number of days per week | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| a) | Pizza | 1 slice or 1/2 a small pizza | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| b) | Quiche or savoury flan | 1 slice | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| c) | Baked beans | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| d) | Nut roast, nut burgers or vegetable burgers | 1 slice or burger | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| e) | Quorn products (all types) | 1 tablespoon, slice or sausage | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| f) | Soya beans, TVP, Tofu or soya meat substitute | 1 tablespoon or 1 sausage | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| g) | Other beans (kidney, butter, chick peas) | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| h) | Lentils (excluding soup) | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| i) | Soups (home-made) | 1 small bowl | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| j) | Soups (tinned/cartons) | 1 small bowl | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| k) | Soups (dried or instant) | 1 small bowl or mug | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| l) | Gravy | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

**Please make sure you have given an answer for every line before leaving this page**

| | | Measure | Measures per day | | | | | Number of days per week | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| m) | Tomato-based sauces (e.g. for pasta) | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| n) | Pesto | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| o) | Other sauces (white, cheese, curry, sweet & sour) | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| p) | Bottled sauces (e.g. ketchup) | 1/2 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| q) | Mayonnaise or salad cream | 1 teaspoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| r) | Oil & vinegar dressing | 1 teaspoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| s) | Pickled vegetables or chutneys | 1 teaspoon or 1 pickle | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| t) | Hummus | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

## 11. **Vegetables** *(including fresh, frozen and tinned vegetables)*

| | | Measure | Measures per day | | | | | Number of days per week | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| a) | Mixed vegetable dishes (e.g. stir-fry, curry or bake) | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| b) | Tinned vegetables (all kinds) | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| c) | Peas or green beans | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| d) | Carrots | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| e) | Cabbage (all kinds) | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| f) | Brussels sprouts | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| g) | Broccoli | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| h) | Spinach or spring greens | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| i) | Leeks or courgettes | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| j) | Cauliflower, swede (neeps) or turnip | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| k) | Sweetcorn | 1 tablespoon or 1 piece | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| l) | Onions | 1 tablespoon or 1/2 onion | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| m) | Tomatoes | 1/2 medium or 2 small | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| n) | Sweet peppers | 1/4 pepper | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| o) | Olives | 4 olives | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| p) | Other salad vegetables (lettuce, cucumber etc) | 2 leaves or 4 slices | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| q) | Potato salad | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| r) | Coleslaw or other veg. salads in dressing | 1 tablespoon | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

**Please make sure you have given an answer for every line before leaving this page**

## 12. *Fruit* (including fresh, cooked, frozen and tinned fruits)

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Fresh fruit salad | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Tinned fruit (all kinds) | 1 tablespoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Apples | 1 fruit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Bananas | 1 fruit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Oranges, satsumas or grapefruit | 1 small or 1/2 large fruit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| f) | Pears | 1 fruit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| g) | Peaches or nectarines | 1 fruit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| h) | Kiwi fruit | 1 fruit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| i) | Dried fruit (e.g. raisins, dates or figs) | 1 tablespoon or 1 oz (25g) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| j) | All other fruits (grapes, strawberries, melon etc) | 1 tablespoon or 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 13. *Puddings*

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Milk-based puddings (e.g. rice, semolina) | 1 small bowl | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Sponge puddings (e.g. steamed, syrup, jam) | 1 small bowl | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Gateau or cheesecake | 1 slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Fruit-based puddings (e.g. pie, tart, crumble) | 1 pie, 1 slice or 2 tablespoons | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Mousse, trifle, meringue | 2 tablespoons or 1 meringue | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| f) | Custard or other sweet sauces | 2 tablespoons | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| g) | Wrapped ice creams (Cornetto, Solero, Magnum etc.) | 1 ice cream | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| h) | Other ice cream (all flavours) | 1 scoop or small tub | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 14. *Chocolates, Sweets, Nuts and Crisps*

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Chocolate bars (e.g. Mars, Dairy Milk) | 1 bar or 2 oz. (50g) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Chocolate sweets, toffees or fudge | 2 sweets | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

**Please make sure you have given an answer for every line before leaving this page**

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| c) | Boiled sweets, mints | 2 sweets | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Fruit gums, pastilles, jellies or chewy sweets | 2 sweets | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Salted nuts (peanuts, cashews etc.) | 1 small packet or 1 oz. (25g) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| f) | Unsalted nuts | 1 small packet or 1 oz. (25g) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| g) | Crisps | 1 small bag (25g) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| h) | Reduced fat crisps | 1 small bag (25g) | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| i) | Other savoury snacks (Quavers, tortilla chips, popcorn etc.) | 1 small bag | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 15. Biscuits

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Plain (e.g. Rich Tea, digestive) | 1 biscuit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Sweet (e.g. ginger, custard creams) | 1 biscuit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Shortbread | 1 biscuit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Chocolate coated biscuits | 1 biscuit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Savoury biscuits, (crackers, crispbreads) | 1 biscuit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| f) | Oatcakes | 1 biscuit | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| g) | Cereal bars, flapjacks | 1 bar or slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

## 16. Cakes

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Plain cakes (sponge, madeira, ginger etc.) | 1 medium slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Cakes with jam, cream or icing (Victoria sponge, carrot cake etc.) | 1 medium slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Fruit cakes (all kinds) | 1 medium slice | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Pastries, doughnuts or muffins | 1 piece | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Pancakes or scones | 1 pancake or scone | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

**Please make sure you have given an answer for every line before leaving this page**

## 17. Spreads and Sugar

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Jam, honey, or marmalade | 1 teaspoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Yeast or meat extract (Marmite, Bovril etc.) | 1/2 teaspoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Peanut butter or chocolate spread | 1 teaspoon | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

d) How many teaspoons of table sugar did you use each day in drinks and on cereals or deserts? ☐☐
(If you did not use any table sugar, please enter 0).

e) Did you use any butter, margarine or other fat spread or oil on bread? Yes ☐ No ☐

If yes, please give full details of the one or two types you used most (e.g. Asda Sunflower buttery spread).
**If you did not spread any fat or oil on bread, please go straight on to question g.**

_____ Office Code ☐☐

_____ Office Code ☐☐

f) How much did you normally spread on one slice of bread? **(Please tick one answer).**
(an example of a thin layer is shown in the photograph on the front cover).

a scrape ☐ a thin layer ☐ a thick layer ☐

g) Did you use any fat or oil for home frying or cooking? Yes ☐ No ☐

If yes, please give full details of the one or two types you used most (e.g. Tesco Pure Vegetable Oil).
**If you did not use any fat or oil for home frying or cooking, please go straight on to section 18.**

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## 18. Beverages and Soft Drinks

| | | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | Tea (regular) | 1 cup or mug | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| b) | Herbal, fruit or decaffeinated tea | 1 cup or mug | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| c) | Instant coffee (regular) | 1 cup or mug | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| d) | Decaffeinated coffee | 1 cup or mug | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| e) | Filter, espresso or cappuccino coffee | 1 cup or mug | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |

**Please make sure you have given an answer for every line before leaving this page**

| | | Measure | Measures per day | | | | | Number of days per week | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| f) | Pure fruit juice (orange, apple, etc.) | 1/2 medium glass | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| g) | Tomato juice | 1/2 medium glass | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| h) | Blackcurrant squash (e.g. Ribena) | 1 medium glass | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| i) | Other fruit squash | 1 medium glass | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| j) | Diet fizzy drinks (Cola, lemonade etc.) | 1 can | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| k) | Regular fizzy drinks | 1 can | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| l) | Mineral water | 1 medium glass | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| m) | Tap water (not in other drinks) | 1 medium glass | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| n) | Hot chocolate | 1 cup or mug | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| o) | Horlicks or Ovaltine | 1 cup or mug | 1 | 2 | 3 | 4 | 5+ | R | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

## *19. Alcoholic Drinks*

Please estimate your average intake of alcohol over the last 2-3 months. If your intake varied from week to week, please try to give an overall estimate which allows for weeks with high or low intake. If you had less than one measure a week on average, please circle 0.

| | Drink | Measure | Number of measures per week | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| a) | Low alcohol lager or beer | 1/2 pint | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| b) | Dark beer (Export, bitter or stout) | 1/2 pint | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| c) | Light beer (lager or continental beers) | 1/2 pint | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| d) | White wine | 1 wine glass | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| e) | Red wine | 1 wine glass | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| f) | Sherry, port etc. | 1 sherry glass | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| g) | Spirits or liqueurs | 1 pub measure | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| h) | Alcopops (e.g. Bacardi Breezer) | 1 bottle | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |
| i) | Cider | 1 bottle or 1/2 pint | 0 | 1-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-29 | 30-39 | 40+ |

**Please make sure you have given an answer for every line before leaving this page**

## 20. Other Foods and Drinks

Please enter details of any foods or drinks which you had **more than once a week** in the last 2-3 months which you have not included in the questionnaire above. If you do not want to add any foods, please leave this section blank and go to section 21.

| | Food description | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | _____ | _____ | 1 2 3 4 5+ | 1 2 3 4 5 6 7 |
| b) | _____ | _____ | 1 2 3 4 5+ | 1 2 3 4 5 6 7 |
| c) | _____ | _____ | 1 2 3 4 5+ | 1 2 3 4 5 6 7 |
| d) | _____ | _____ | 1 2 3 4 5+ | 1 2 3 4 5 6 7 |

## 21. Vitamin, Mineral and Food Supplements

Please give details and brand name of any supplements (e.g. multivitamins, iron tablets, cod liver oil, evening primrose oil, Complan, wheatgerm, bran) which you took in the last 2-3 months.

| | Supplement type | Measure | Measures per day | Number of days per week |
|---|---|---|---|---|
| a) | _____ | _____ | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| | Brand name and details_____ | | | |
| b) | _____ | _____ | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| | Brand name and details_____ | | | |
| c) | _____ | _____ | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| | Brand name and details_____ | | | |
| d) | _____ | _____ | 1 2 3 4 5+ | R M 1 2 3 4 5 6 7 |
| | Brand name and details_____ | | | |

## 22. Other Information

Any other information or comments on your diet in the last 2-3 months

**Date of completing the questionnaire** _____

**Thank-you very much for completing this questionnaire.**

**Please return it to the investigators as requested.**