Risk factors for diabetic retinopathy blindness and its treatment

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CONTENTS

THESIS SUMMARY

Diabetes retinopathy (DR), the current fifth most common cause of blindness worldwide, is on an exponential rise.[1] Recent reports predict that the prevalence of DR will increase from an estimated 146 million to 224 million worldwide by the year 2040.[2, 3] Despite significant progress in reducing the rates of blindness, several challenges remain in its management. This thesis focuses on building knowledge in two important areas of reducing the burden of DR: 1) the disproportionate burden of DR in indigenous Australian populations and 2) the complex genetic nature of DR and its implications for risk stratification and treatment.

Two epidemiology studies were undertaken to explore the burden of DR among indigenous Australian communities. Strong associations were confirmed between DR and higher mortality, renal disease, poor DR screening rates and adherence to treatment. A randomized clinical trial was conducted to compare intravitreal bevacizumab and intravitreal dexamethasone implant in the treatment of diabetic macular oedema. Results support the preferential use of intravitreal dexamethasone implant in resource poor settings. Large genetic studies were conducted to explore three groups of genes and their association with DR. Mitochondria haplogroup, microRNA and its binding sites, and VEGF receptor genes were chosen because of the lack of studies from the literature and their proximity to the molecular pathogenesis of DR. Mitochondrial haplogroup was not shown to be associated with DR in a Caucasian population, however significant results were found for single nucleotide polymorphisms (SNP) in microRNA, its binding sites, and VEGF receptor.

Outcomes from this thesis have direct implications in improving our current approach to the treatment of DR. Epidemiology trends from indigenous communities emphasise the importance of working within existing health frameworks and community collaboration in disease prevention. New genetic findings guide the direction of future work in developing effective and targeted treatments. The results from this thesis contribute to an ongoing research initiative to improve our understanding of DR and its treatment.

DECLARATION

I certify that this thesis does not include, without acknowledgement, any material previously submitted for a degree or diploma in any university.

I certify that this thesis does not contain any material previously published or written by another person, except where referenced.

Ebony Liu 23/8/21

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THESIS OUTCOMES

Peer reviewed publications:

1. **Liu E**, Craig JE, Burdon K. Diabetic macular oedema: clinical risk factors and emerging genetic influences. ***Clinical and Experimental Optometry*** 2017; 100: 569-576.

2. **Liu E**, Estevez J, Kaidonis Get al. Long term survival rates of patients undergoing vitrectomy for diabetic retinopathy in an Australian population: a population based audit. ***Clinical and Experimental Ophthalmology*** 2019.

3. **Liu E**, Kaidonis G, Gillies MCet al. Mitochondrial haplogroups are not associated with diabetic retinopathy in a large Australian and British Caucasian sample. ***Scientific Reports*** 2019; 9: 612.

4. **Liu E**, Kaidonis G, McComish BJet al. MicroRNA-Related Genetic Variants Are Associated With Diabetic Retinopathy in Type 1 Diabetes Mellitus. ***Investigative Ophthalmology Visual Science*** 2019; 60: 3937-3942.

Conference Presentations:

1. "Genetic variants in microRNA and microRNA binding site genes associated with diabetic retinopathy" *Association for Research in Vision and Ophthalmology*, Annual Scientific Meeting 2018 (Hawaii, USA).

2. "Genetic variants in VEGF receptor genes associated with diabetic retinopathy" *Royal Australian and New Zealand College of Ophthalmologists,* Annual Scientific Meeting 2018 (Adelaide).

3. "Long term survival rates of patients undergoing vitrectomy for diabetic retinopathy in australia – a population based audit" *Royal Australian and New Zealand College of Ophthalmologists,* Annual Scientific Meeting 2018 (Adelaide).

Prizes:

1. Flinders University "3-minute thesis" competition finalist, 2017.

2. Ophthalmic Research Institute of Australia award for best paper presentation at *Royal Australian and New Zealand College of Ophthalmologists,* Annual Scientific Meeting, 2018.

ABBREVIATIONS

DR	Diabetic retinopathy
DM	Diabetes mellitus
PRD	Proliferative diabetic retinopathy
DMO	Diabetic macular oedema
ICDSS	International Clinical Disease Severity Scale
NPDR	Non-proliferative diabetic retinopathy
ETDRS	Early Treatment Diabetic Retinopathy Studies
CSMO	Clinically significant macular oedema
OCT	Optical coherence tomography
STDR	Sight threatening diabetic retinopathy
IRMA	Intra retinal microvascular abnormalities
PKC	Protein kinase C
AGE	Advanced glycation end products
RAGE	Receptors for advanced glycation end products
PAI-1	Plasminogen activator inhibitor-1
TGF	Transforming growth factor
NADPH	Nicotinamide adenine dinucleotide phosphate
DAG	Diacylglycerol
VEGF	Vascular endothelial growth factor
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
ROS	Reactive oxygen species
BRB	Blood retina barrier
HIF	Hypoxia inducible factor
DCCT	Diabetes Control and Complications Trial

HbA1c	Glycated haemoglobin
UKPDS	United Kingdom Prospective Diabetes Study
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
HR	Hazard Ratio
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
DN	Diabetic nephropathy
ACCORD	Action to Control Cardiovascular Risk in type 2 Diabetes
RCT	Randomized controlled trials
WESDR	Wisconsin epidemiologic study of diabetic retinopathy
PRP	Pan-retinal photocoagulation
BOLT	Bevacizumab or laser therapy study
DRCR	Diabetic Retinopathy Clinical Research Network
IOP	Intraocular pressure
CAOHS	Central Australian Ocular Health study
NIEHS	National Indigenous Eye Health Study
OR	Odds ratio
DCCT	Diabetes and Complications Trial
SNP	Single nucleotide polymorphisms
GWAS	Genome wide association study
WES	Whole exome sequencing
ETC	Electron transport chain
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
PIGF	Phosphatidylinositol-glycan biosynthesis class F
sFLT1	Soluble splice variant

SA	South Australia
NT	Northern Territory
HREC	Human Research Ethics Committee
SPSS	Statistical Package for Social Sciences
BCVA	Best corrected visual acuity
CRT	Central retinal thickness
GSDR	Genetic Study of Diabetic Retinopathy
RADAR	Registry of Advanced Diabetic Retinopathy
SD	Standard Deviation
MAF	Minor Allele Frequency

CHAPTER 1: GENERAL INTRODUCTION

Some parts of the literature review presented in this chapter have been published in the peer-reviewed article: Liu E, Craig JE, Burdon K. Diabetic macular oedema: clinical risk factors and emerging genetic influences. Clinical and Experimental Optometry 2017; 100: 569-576.

1.1 The rising epidemic of diabetic retinopathy

Diabetic retinopathy (DR) is the fifth most common cause of vision loss worldwide. In a recent meta-analysis of 288 population based studies for the causes of blindness from 1980 to 2014, the leading causes of moderate to severe vision loss globally were uncorrected refractive error, followed by cataract, age related macular degeneration, glaucoma and DR.[1]

The most alarming trend observed over the last few decades is the exponential increase in diabetes mellitus (DM) and therefore the inevitable increase in DR. The total number of people with DM worldwide was 135 million in 1995,[4] 422 million in 2014,[5] and projected to be 642 million by 2040.[3] The overall prevalence of any DR among those with diabetes is 34.6% according to a pooled analysis of 35 studies conducted between 1980 to 2008.[2] Therefore, in 2040 and over a period of just 26 years, the prevalence of DR will increase from an estimated 146 million to 222 million worldwide.[2, 3] This reflects global population growth, aging populations and the rise of type 2 diabetes which is associated with current epidemics of obesity, sedentary lifestyles and unhealthy diets.[5] With the increasing prevalence of diabetes mellitus, DR may become a more common cause of blindness and global priority in the future.

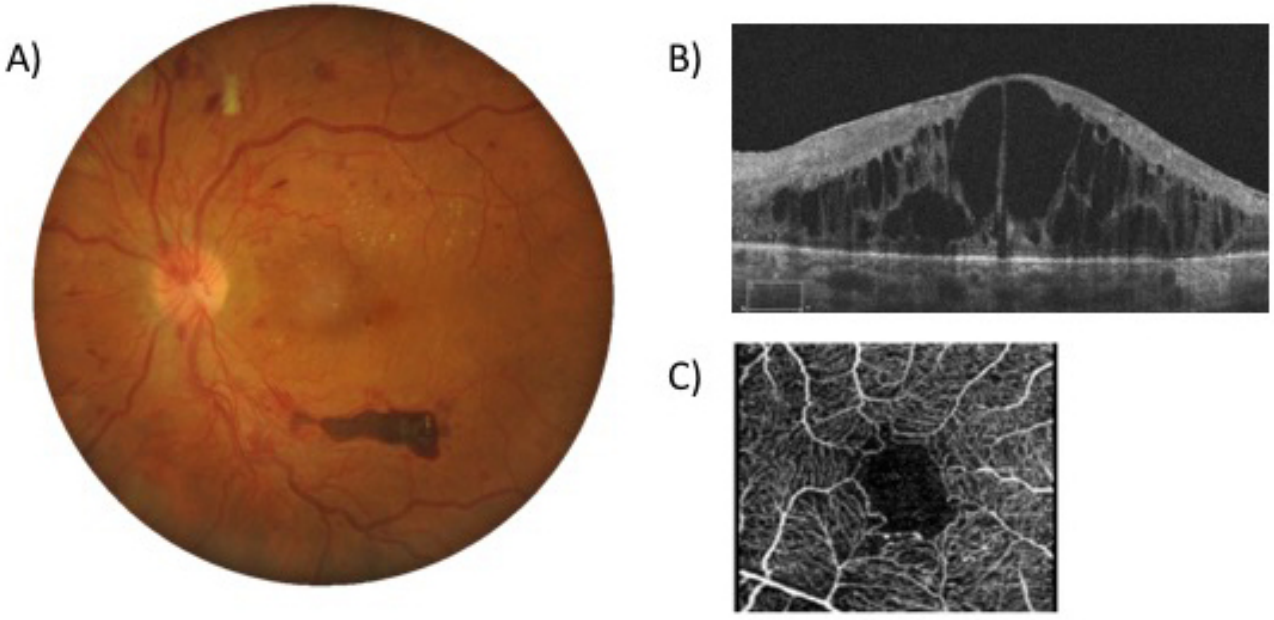
1.2 Definition and classification of diabetic retinopathy blindness

Diabetes mellitus causes blindness by damage to the retina of the eye. DM is a chronic disease defined by elevated blood glucose levels secondary to either reduced production of insulin (type 1 DM) or reduced response to insulin (type 2 DM). In both types, elevated blood sugar levels lead to toxic changes in tissue. In the retina, progressive toxic changes include micro-aneurysms, haemorrhages, leakage of lipids (exudates),

areas of ischemia (cotton wool spots) and vessel abnormalities such as venous beading, intraretinal microvascular abnormalities and neovascularisation.

There are three distinct pathologies that contribute to blindness. Proliferative diabetic retinopathy (PDR) is when neovascularization occurs in the retina because of severe tissue ischemia. New fragile vessels are prone to causing vitreous haemorrhage and fibrous retinal detachment that compromise vision. PDR usually progresses from milder changes in the retina and therefore can be predicted in advance. Diabetic macular oedema (DMO) is caused by leakage of fluid from damaged blood vessels in the macula, therefore affecting central vision. Ischaemic maculopathy causes irreversible vision loss due to extensive damage to the capillaries supplying the macula. Examples of these three entities are illustrated in Figure 1. This thesis focuses on the two reversible causes of blindness: PDR and DMO.

Figure 1.1: The three causes of blindness from diabetes



Footnote: A) Proliferative diabetic retinopathy; colour retinal photograph illustrating abnormal vessel growth and vitreous haemorrhage. B) Diabetic macular oedema; optical coherence tomography show gross intraretinal fluid at the macula. C) Ischaemic maculopathy; late phase of fluorescein angiography showing enlarged foveal avascular zone due to capillary damage.

The most commonly used criteria for DR classification is the International Clinical Disease Severity Scale (ICDSS), which is based on clinical examination alone. Retinopathy changes are classified into non-proliferative and proliferative changes. It adopts the 4:2:1 rule (as shown in Table 1.1) which is sensitive in

identifying severe non-proliferative DR (NPDR); those at up to 45% risk of developing PDR within 1 year if treatment is not instituted.[6] The ICDSS classifies DMO as being either absent or present at any level of retinopathy that could affect vision. As per the Early Treatment Diabetic Retinopathy Studies (ETDRS) trials,[7] clinically significant macular oedema (CSMO) is defined as either 1) retinal thickening within 500μm of the centre of the macula 2) hard exudates at or within 500μm of the centre of the macula if associated with thickening of the adjacent retina or 3) retinal thickening 1 disc area in size, within 1 disc diameter of the centre. With the advent of optical coherence tomography (OCT) imaging, more subtle forms of DMO, which may not be seen on clinical exam, are being diagnosed. OCT defined centre involving DMO is the presence of fluid at the fovea on OCT imaging and they are at higher risk of developing CSMO.[8] Sight threatening DR (STDR) is defined as severe NPDR, PDR or CSMO as per the ICDSS and ETDRS in this thesis. Table 1 shows the classification system which is adopted throughout the studies reported in this thesis.

Table 1.1: International Clinical Disease Severity Scale for Diabetic Retinopathy and Diabetic Macular oedema

Grade	Ophthalmoscopy Findings
Retinopathy	
No DR	No abnormalities
Minimal NPDR	Microaneurysms only
Mild to moderate NPDR	More than just microaneurysms but less than severe NPDR
Severe NPDR	Any of the following (4:2:1 rule): More than 20 intra retinal haemorrhages in each of the 4 quadrants Venous beading in at least 2 quadrants IRMA in at least 1 quadrant and no signs of PDR
PDR	One of the following: Neovascularization Vitreous or pre-retinal haemorrhage
Macular oedema	
Absent	No retinal thickening or hard exudates in the posterior pole
Present	Retinal thickening or hard exudates in the posterior pole
Clinically Significant Macular oedema	1) retinal thickening within 500μm of the centre of the macula, 2) hard exudates at or within 500μm of the centre of the macular if associated with thickening of the adjacent retina, or 3) retinal thickening 1 disc area in size, within 1 disc diameter of the centre

Abbreviations: DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; IRMA, intra retinal microvascular abnormalities. Adapted from NHMRC Guidelines for the Management of Diabetic Retinopathy 2008[9] and ETDRS[7]

1.3 The burden of diabetic retinopathy

Blindness compromises quality of life by limiting physical activity, increasing risk of social isolation and loss of independence. In a 2017 study of American adults with diabetes, Willis et al found that approximately half of those with PDR had difficulty with at least one visual function task.[10] However unlike other common causes of vision loss, the burden of those with DR extends beyond visual impairment.

Diabetes affects other parts of the body, and the severity of DR is a marker for other systemic diseases. Most notably, DR is strongly associated with other microvascular complications of diabetes such as nephropathy and neuropathy.[11-13] Diabetic neuropathy is irreversible damage to nerves, often affecting limbs, leading to reduced sensation, increased risk of tissue damage, ulceration, infection and amputation. Diabetic nephropathy (DN) is irreversible damage to the kidneys, which can lead to renal failure requiring dialysis or kidney transplant. In a prospective study of 598 people with diabetes followed for 24 months, the presence of retinopathy was predictive for nephropathy progression in both T1DM (Hazard Ratio HR 1.95, 95% confidence interval CI 1.09-3.41, $p = 0.02$) and T2DM (HR 2.87, 95%CI 1.45, 5.69, $p = 0.002$).[11] In another prospective study of similar cohort size (n = 648), retinopathy strongly predicted nephropathy (HR 5.68, $p<0.001$) and neuropathy (2.23, $p < 0.001$).[12] DR is also linked with macrovascular complications such as heart attack and stroke. In the Action to Control Cardiovascular Risk in Diabetes (ACCORD) eye study which comprised over 3000 participants, each increase in DR severity was associated with a 38% increased risk of a cardiovascular event (HR1.38, 95%CI 1.10-1.74).[14] A meta-analysis of 5 epidemiology studies showed that the presence of any DR was significantly associated with stroke (HR1.74 95% CI 1.35-2.24).[15]

All these comorbidities are significant contributors to increased mortality and morbidity of people with DR. A recent meta-analysis reported a risk ratio of 2.33 when comparing all-cause mortality rates between diabetic individuals with no DR, and diabetic individuals with DR.[15] In a large study of 2048 type 1 and type 2 diabetic individuals, diabetic complications, including retinopathy, correlated with lower quality of life scores.[16] Visual impairment is also independently associated with increased mortality. Multiple long-term epidemiology

studies in different populations have shown that best corrected visual acuity less than Snellen 6/12 leads to higher mortality[17-35] and improvement of vision leads to better survival.[19, 31]

One measure of the impact of disease is cost to the economy. The Australian Diabetes, Obesity and Lifestyles Study estimated annual costs of diabetes in excess of 10 billion Australian dollars in 2005 and this expected to double by 2033.[36] Annual public costs of treating DR in US dollars were estimated to be 2.78 to 4.38 billion in Germany in 2002,[37] 0.49 billion in the United States of America in 2002,[38] and 2.4 billion in Indonesia in 2017 (extrapolating to nearly 2% of its national budget).[39] Direct costs of DR in Australia have not been extrapolated due to low availability of public data. Costs are grossly underestimated by indirect costs to the economy from vision impairment such as loss of work capacity and community support services. As DR more commonly affects the working age population, indirect costs to society are even greater. In Australia, the indirect cost of DMO was estimated to be 2.07 billion in 2015.[40]

1.4 The pathogenesis of diabetic retinopathy blindness

The driving cause of diabetic retinopathy is hyperglycaemia, but how this causes toxic changes to the retina is complex and not fully understood. The body attempts to deal with increased glucose levels by increasing the breakdown of glucose (glycolysis) through different metabolic pathways. Unfortunately, these processes also produce oxidative stress and inflammation which have detrimental effects on the retina. The following sections will summarize current knowledge about the pathogenesis of DR.

1.4.1 Metabolic pathways

Five major biochemical pathways have been identified in the pathogenesis of DR, as illustrated in Figure 1.2: 1) over activity of the hexosamine pathway, 2) the polyol pathway flux, 3) activation of protein kinase C isoforms (PKC), 4) increased formation of advanced glycation end products (AGE), and 5) increased expression of receptors for advanced glycation end products (RAGE).[41]

In normal cells, the majority of intracellular glucose is used for glycolysis (energy production) and glycogen synthesis (energy storage), while a very small proportion is channelled through alternate pathways such as the

hexasomine biosynthetic pathway. When cells are exposed to high levels of glucose, as in diabetes, there is an increased flux of glucose through the hexasomine pathway, evident by the increase in the pathway's end products.[42] UDP-GlcNac is one such end product and can attach itself to intracellular proteins, changing protein function, in a process known as O-GlcNAcylation.[43] High glucose has been demonstrated to increase the O-GlcNAcylation of transcription factor Sp1 by 4-fold,[44] which is associated with an increase in inflammatory mediator plasminogen activator inhibitor-1 (PAI-1).[44, 45] Glucose metabolism to glucosamine via the hexasomine pathway is also associated with other inflammatory mediators such as transforming growth factor (TGF) alpha[45] and TGF beta.[46] PAI-1, TGF alpha and TGF beta are all elevated in human vitreous with DR.[47]

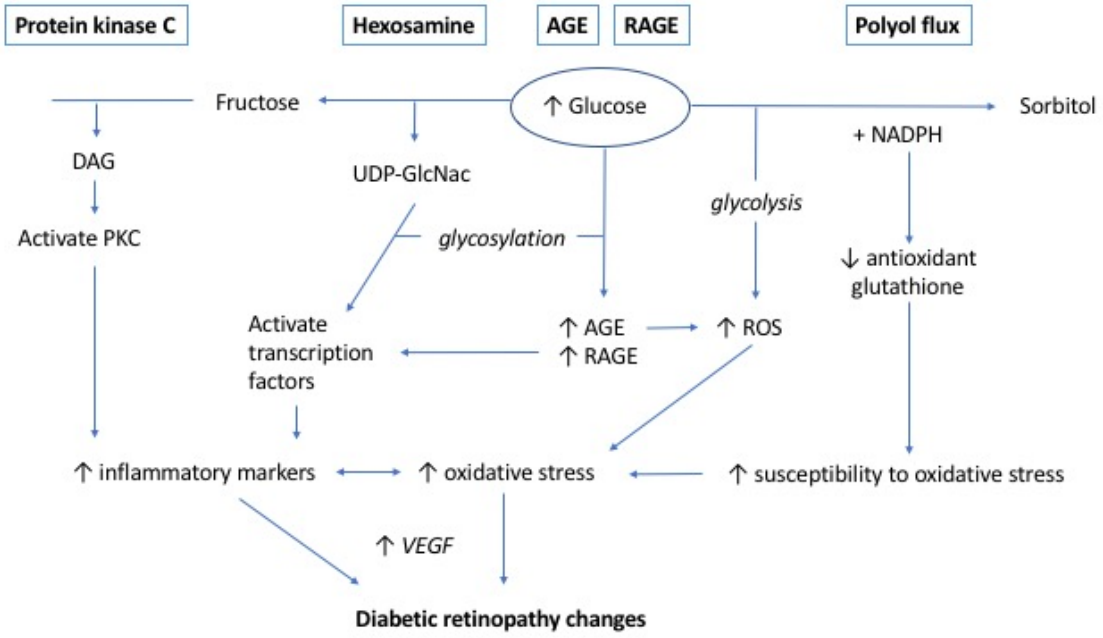
In the polyol pathway flux, excess glucose is converted to sorbitol by the enzyme aldose reductase and its cofactor nicotinamide adenine dinucleotide phosphate (NADPH). The pathway is only activated when intracellular levels of glucose are high.[48] Aldose reductase is expressed in the eye and is associated with many early changes of DR.[49] The most cited explanation for how this pathway results in tissue damage is via increased oxidative stress due to the consumption of the antioxidant glutathione.[41] NADPH is a cofactor for the production of antioxidant glutathione, therefore the polyol pathway flux inhibits the production of glutathione and increases the cell's susceptibility to oxidative stress.[50]

In the PKC pathway, glycolysis intermediate dihydroxyacetone forms diacylglycerol (DAG) during hyperglycaemia.[51] DAG greatly enhances the activity of PKC.[52, 53] Over-activity of PKC has been associated with vascular cell apoptosis in DR[54] and increased expression of inflammatory mediator vascular endothelial growth factor (VEGF).[55]

In the AGE and RAGE pathways, excess glucose binds to various proteins, forming pathological complexes (AGEs) and receptors (RAGEs) that activate transcription factors such as nuclear factor kappa light chain enhancer of activated B cells (NF-κB). This leads to an influx of inflammatory cells, cytokines, adhesion molecules and reactive oxygen species (ROS) which damage tissue.[56] Increased levels of AGEs have been

found in diabetic retina and vitreous tissues.[57, 58] RAGE is not only activated by AGE, but also by other pro-inflammatory ligands that are upregulated by hyperglycaemia.[59]

Figure 1.2: The five biochemical pathways that contribute to diabetic retinopathy



AGE: Advanced glycation end products, DAG: diacylglycerol, NADPH: Nicotinamide adenine dinucleotide phosphate, RAGE: Receptors for advanced glycation end products, ROS: reactive oxygen species, PKC: Protein Kinase C, UDP-GlcNac: Uridine diphosphate N-acetylglucosamine, VEGF: Vascular endothelial growth factor

1.4.2 Oxidative stress and inflammation

Dysregulated metabolic pathways in diabetes lead to a vicious cycle of increased oxidative stress and inflammation in the retina. Hyperglycaemia leads to increased glycolysis in mitochondria and therefore overproduction of ROS.[60] Binding of AGE to RAGE also increases ROS formation.[61] ROS are elevated in experimental models of diabetic retina[62] and human vitreous samples with DR.[63] ROS can activate epigenetic changes in the NF-κB pathway,[64] and increases the availability of DAG, a precursor to the PKC pathway,[65] both which increase inflammation in the retina.

Oxidative stress and inflammation contribute to early microscopic changes seen in DR.[66] ROS damages mitochondria essential for cell function and increases cell apoptosis.[67] PKC signalling also leads to apoptosis of vascular cells.[54] The early stages of DR are characterized by selective loss of pericytes,[68] followed by

vascular endothelial cells.[69] Microaneuryms and small haemorrhages seen in mild to moderate DR are a result of pericyte loss, disrupted tight junctions in vascular endothelium and thickening of the basement membrane.[70] This contributes to the breakdown of the blood-retina-barrier (BRB) and allows the influx of inflammatory mediators that further compromise the BRB. Processes such as tissue oedema, infiltration of immune cells, activation of microglia, upregulation of cytokines and complement have been described in both human and animal models of DR.[71] Proteomics studies of human vitreous with DR show that upregulated proteins belong to multiple inflammatory pathways.[72] Significant breakdown of the BRB causes gross oedema and leakage of proteins which are seen as hard exudates in DMO.

1.4.3 Hypoxia and angiogenesis

A second series of changes occur when eventual hypoxia from capillary dysfunction and decreased perfusion of the retinal tissue activates angiogenesis and causes PDR. During angiogenesis, new vascular networks develop from pre-existing vessels to increase blood flow to hypoxic areas. However, these pathological vessels are fragile, invade the vitreous space, are prone to haemorrhage and thus threaten vision. Deficits in oxygen delivery to the retina was first observed in diabetic rats.[73] Low oxygen increases levels of oxygen sensitive transcription factors such as hypoxia inducible factor (HIF),[74] which are responsible for activating a host of proangiogenic mediators.[75] The most well studied angiogenic factor is VEGF. In *in vitro* models, VEGF promotes angiogenesis,[76] ensures vascular endothelial cell survival[77] and increases vascular permeability.[78] VEGF is elevated in the vitreous of patients with PDR.[79] The efficacy of medications blocking VEGF has confirmed the key role of this molecule in the pathogenesis of DR.[80-82] Ongoing cell apoptosis and hypoxia ultimately lead to diabetic macula ischemia, causing irreversible vision loss.

1.5 Combating diabetic retinopathy blindness

Global efforts have succeeded, in part, to curb the growing burden of DR blindness. Prior to the availability of clinically proven treatments, the visual prognosis for PDR was poor. Studies conducted in the 1960s showed that half of those who developed PDR became legally blind at 3 to 5 years after diagnosis.[83, 84] With the discovery of photocoagulation laser treatments for PDR, the rate of vision impairment was reduced by 60%.[85]

Current evidence suggests that modifying clinical risk factors and employing effective treatments are most beneficial in the earlier stages of disease, therefore emphasizing the importance of regular screening, monitoring and timely treatment.[86]

1.5.1 Modifiable risk factors

Controlling blood sugar levels was proven to be an essential preventative measure by two landmark clinical trials in the 1980s. The Diabetes Control and Complications Trial (DCCT) was a multi-centre randomised clinical trial comprising of 1441 insulin dependent individuals with diabetes who were followed for 3 to 9 years.[87] Intensive insulin therapy (3 insulin doses daily) compared with standard therapy (one insulin dose daily) prevented the development of DR by 76% and slowed the progression of existing DR to PDR by 47%. Glycated haemoglobin (HbA1c) is a good indicator of glycaemic control over 3 months. The median of the quarterly measured HbA1c achieved was 7% in the intensive therapy group, and 9% in the standard therapy group. The United Kingdom Prospective Diabetes Study (UKPDS) was a much larger study (n=4209) which followed non-insulin dependent or type 2 individuals with diabetes for 9 years.[88] A 1% decrease in HbA1c was associated with a 31% reduction in retinopathy. These trials suggest the optimum HbA1c is less than 7% and benefits persist for 10 to 20 years.[89] A Cochrane review of 12 studies involving type 1 DM (T1DM),[90] and meta-analyses of studies involving type 2 DM (T2DM),[91] confirmed the benefit of tighter glucose control in DR risk reduction, particularly in younger patients in earlier stages of the disease. However tight glycaemic control was not always possible or beneficial due to risks of severe hypoglycaemic episodes and falls risk in older individuals.

Hypertension is another key modifiable risk factor for DR, and often coexists with type 2 diabetes. Hypertension leads to additional damage to retinal vessels by hyperperfusion, shearing forces and increased oedema formation.[92] Uncontrolled blood pressure is associated with mild retinopathy changes similar to early DR in individuals without diabetes after 15 years (Hazard Ratio (HR), 2.07; 95% confidence interval (CI), 1.51-2.83).[93] The UKPDS showed that a target blood pressure of less than 140/90 was related to a 35% reduction in the progression of DR and a 47% reduction in risk of vision loss.[94] A Cochrane review of 15

randomized controlled trials concluded that treating hypertension has a beneficial effect in reducing the occurrence of DR for up to 5 years, however there is insufficient evidence to support that it slows the progression of DR.[95]

There is less robust evidence supporting the role of altered lipid profiles and DR risk. Dyslipidaemia is characterized by high levels of low density lipoproteins, low levels of high density lipoproteins, and is associated with insulin resistance.[96] It correlates with the presence of hard exudates in the retina,[97] but the exact mechanisms on how it affects the retina are unknown. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study was a large multi-centre randomized control trial (n = 9795) which found that fenofibrate, a lipid lowering drug, reduced retinopathy progression over 5 years in type 2 diabetes (HR 0.66, 95% CI 0.47-0.94; p=0.022).[98] The Action to Control Cardiovascular Risk in type 2 Diabetes study (ACCORD) was a similar study (n = 5518) which also showed the same result (fenofibrate compared with placebo, HR 0.67, p = 0.003).[99] These findings have been criticized as retinopathy was a secondary outcome in both the FIELD and ACCORD study which originally aimed to explore the effect of fenofibrate on cardiovascular outcomes. Furthermore DR severity did not correlate with serum lipid levels. The role of dyslipidemia in DMO remains unconfirmed in a meta-analysis of prospective randomized controlled trials (RCT), despite a positive correlation found in past case-control studies.[100]

Duration of diabetes is not a modifiable risk factor, but it is strongly associated with development of DR and aids in determining the urgency of screening. Longer duration of diabetes increases the amount of time the retina is exposed to diabetes induced damage. The Wisconsin epidemiologic study of diabetic retinopathy (WESDR) is the largest population based study (n = 2366) with the longest follow up period exploring the impact of diabetes duration on retinopathy. It showed that over 80% of type 1 and type 2 diabetics developed DR after 20 years.[101, 102]

1.5.2 Clinically proven treatments

Frequent follow up with yearly dilated screening examinations, appropriate staging of DR, and prompt treatment for PDR and DMO significantly reduce the rates of blindness.[103] The first proven treatment for DR

was pan-retinal photocoagulation (PRP), which involved delivery of scattered or focal laser burns to the retina. The theory was that destroying peripheral ischaemic retina that was not important for central vision would reduce the drive for further angiogenesis. The Diabetic Retinopathy Study was a multi-centre randomised controlled trial in the 1970's (n = 1758) that showed prompt PRP compared with no treatment for PDR or severe NPDR reduced rates of vision loss within 5 years by 60%.[85] The ETDRS was another multi-centre clinical trial (n = 3711) which confirmed the efficacy of laser for PDR and severe NPDR, but also for DMO.[104] Early PRP compared with deferred PRP reduced the risk of PDR from severe NPDR (as identified with the 4:2:1 rule) within 1 year by 45%.[6] Focal laser was shown to reduce vision loss by 50% in CSMO.[7] Over the years, laser was discovered to have undesirable side effects that compromised visual outcomes such as inadvertent foveal burn, diminished visual field, and expansion of treatment scars.[105]

In the 2000s, another discovery changed the first-line management of CSMO. VEGF was discovered to be a key molecule in the pathogenesis of DR and DMO.[79] Several RCTs showed that intravitreal bevacizumab, which inhibited VEGF, resulted in significantly better visual outcomes than laser alone for CSMO, but the effect was not sustained long term.[80-82] The bevacizumab or laser therapy (BOLT) study however showed that regular follow up and a strict retreatment criteria for intravitreal bevacizumab was still superior to laser at 1 year,[106] and 2 years.[107] Different types of anti-VEGF agents have since emerged to include ranibizumab and aflibercept which work in different ways to inhibit VEGF. Ranibizumab is a monoclonal antibody fragment targeted at inhibiting the VEGF isoform VEGF-A. Bevacizumab is the whole monoclonal antibody targeted at inhibiting VEGF-A, however as it contains the Fc portion of an antibody, it may interact with other molecules. Aflibercept (Eylea) is a fusion protein which contains binding portions of VEGF receptors 1 and 2, therefore allowing it to inhibit VEGF-A and its other isoforms VEGF-B and C. It has a higher binding affinity to VEGF-A *in vitro* compared with ranibizumab and bevacizumab.[108]

The Diabetic Retinopathy Clinical Research Network (DRCR) which comprises of over 100 sites in the United States, recently published results from a large RCT that showed aflibercept yielded slightly superior visual outcomes than the other anti-VEGF agents. This effect was only shown in eyes with CSMO, with moderate

to severe baseline visual acuity (between 20/50 to 20/320), and sustained for 2 years.[109] The aflibercept group achieved a mean improvement in visual acuity of 18.1 letters, which was superior to bevacizumab (13.3 letters; $P = 0.02$), and ranibizumab (16.1 letters; $P = 0.18$). In a post-hoc area under the curve analysis, the comparison between aflibercept and ranibizumab also become significant; 58% achieved an improvement of more than 15 letters in the aflibercept group compared with ranibizumab ($p = 0.05$). The problem with all anti-VEGF agents however is that high levels of compliance are required for ongoing monthly re-treatments, a significant proportion of patients have variable responses to treatment and the costs of ongoing treatment may be prohibitive in some countries.[110] For each increase in one quality adjusted life years (defined as one year of life a person would give up in exchange for one line of vision improvement), the estimated cost of ranibizumab is $23,119 (US dollars).[111] In real world clinical practice, even in suburban areas, patients undergo less frequent monitoring and they achieve inferior visual outcomes to landmark trials.[112]

Apart from anti-VEGF agents, intravitreal steroids such as triamcinolone acetonide, have widespread anti-inflammatory effects and are effective for the treatment of DMO. It is not a first line treatment because of side effects such as cataract formation and elevated intraocular pressure (IOP). However in pseudophakic patients, it is the most cost effective option.[113] Triamcinolone acetonide plus laser was shown to be superior to laser alone, and equivalent to ranibizumab (alone or with laser).[114] In patients who have persistent or refractory DMO despite anti-VEGF therapy, intravitreal triamcinolone acetonide can be an effective alternative.[115] Other formulations include long acting implants such as Ozurdex (Allergan, Irvine, CA, United States), that release corticosteroids slowly, allowing for less frequent re-treatments. The BEVORDEX RCT showed that Ozurdex achieved similar rates of visual acuity improvement compared with Bevacizumab (41% compared with 40% achieved more than 10 ETDRS letters at 12 months), with fewer injections (2.7 compared with 8.6 mean injections over 12 months).[116] In 2014, the Food and Drug Administration approved Ozurdex for the treatment of DMO,[117] and this was followed by Pharmaceutical Benefits Scheme approval in Australia in 2017.[118]

Vitrectomy surgery (removal of the vitreous humour) continues to be important in the treatment of DR. While PDR and DMO can be initially treated with laser photocoagulation and/or anti-VEGF injections, vitrectomy

is still indicated when there is further progression of the disease such as persisting vitreous haemorrhage from proliferative vessels or scarring causing tractional retinal detachment.[119] The Diabetic Retinopathy Vitrectomy Study established the benefits of early vitrectomy surgery for vitreous haemorrhage: at 4 years follow up, 44% of patients achieved a visual acuity of 6/12 or better in the early vitrectomy group compared with 28% in the deferred vitrectomy group (vitrectomy performed after 6-12 months of persisting haemorrhage).[120]

1.6 Diabetic retinopathy in indigenous Australians

While much progress has been made to understand, and develop strategies to reduce the rates of blindness from DR, several important challenges remain. Recent epidemiology studies show a decline in sight threatening DR in developed countries, however this is not the case in developing countries and indigenous populations. A systematic review of 28 epidemiology studies reported the 4 year incidence of PDR was lower during the period 1986 to 2008 (5.4%) than the period 1975 to 1985 (39.7%) largely due to the implementation of clinically proven interventions.[121] The majority of studies were conducted in developed countries such as countries in Europe and the United States of America. A more recent systematic review in 2013 reported that in 15 out of 23 studies in developing countries and in ethnic minority groups within developed countries, the prevalence of DR was over 35%. In contrast, only 2 of 16 studies in developed countries reported a prevalence of more than 35%.[122] Public health efforts in these populations face challenges such as lack of resources, inadequate healthcare systems and low awareness of the DR epidemic.[123]

Disparities in DR are also evident between different ethnic groups. In the United States, The Salisbury Eye Evaluation Study (random population sampling of 3821 people) reported that African Americans were found to have a 4-fold increased risk of visual impairment due to DR compared with Caucasians.[124] In Australia, DR blindness is more prevalent in indigenous people compared with non-indigenous people.[125] Part one of this thesis focuses on the challenges in combating DR blindness in indigenous Australians.

1.6.1 Prevalence of diabetic retinopathy in indigenous Australians

Population based epidemiology studies have been conducted in both urban and rural locations in Australia to determine the prevalence of DR in indigenous Australians. The majority of indigenous Australians live in

rural communities,[126] and despite difficulties in conducting and interpreting studies in these locations, some general trends are observed. The Central Australian Ocular Health study (CAOHS) examined 1884 indigenous Australians living in one of 30 remote communities within Central Australia, and found the prevalence of STDR, among those with diabetes was 8.4%.[127] In Katherine (Western Australia) and remote South Australia, the prevalence of STDR was 8 to 12%.[128, 129] In non-indigenous Australians aged 40 or older in the Blue Mountains Eye Study, conducted in suburban settings, the prevalence of STDR was lower at 6%.[130] A direct comparison was made with the National Indigenous Eye Health Study (NIEHS), that examined 1738 indigenous Australians and 3098 non-indigenous Australians, sampled from 30 urban and remote areas in Australia, and found the prevalence of STDR among those with self-reported diabetes was 9.4% compared with 4.5% for indigenous and non-indigenous Australians respectively.[131] A meta-analysis of 6 indigenous Australian studies (n = 2865) and 5 non-indigenous Australian studies (n = 9801) including the above studies, confirmed these trends for PDR (4.7% vs 3.2%, p=0.001) and DMO (7.6% versus 4.9%, p = 0.008).[125]

Reasons for these differences are multifactorial and complex, involving biological, environmental and social risk factors. The following sections discuss these risk factors identified through existing literature.

1.6.2 Clinical risk factors

Diabetes is more common in indigenous Australians, and therefore the relative number of indigenous Australians with DR is higher. The NIEHS reported the prevalence of self-reported DM in indigenous Australians over the age of 40 was 37.1%.[132] The CAOHS found a higher percentage of self-reported DM over the age of 40 in remote communities (55.2%).[133] In the overall Australian population in a similar time period (2015-2016), prevalence of self-reported DM was only 6%, and ranges between 5-17% for those aged over 40 years.[134]

Clinical risk factors for the development and severity of diabetes are more prevalent in indigenous Australians. Indigenous people are at higher risk of poor nutrition and food insecurity. The most recent health and welfare report from the Australian Institute of Health and Welfare reported that indigenous babies were more than twice as likely to be of low birth weight, and children were 1.6 times more likely to be obese than non-

indigenous children.[135] Malnutrition and poor food choices play a fundamental role in the development of T2DM, obesity and insulin resistance, particularly if those factors are present from a young age.[136, 137] T2DM is the main type of diabetes in indigenous Australians and is often diagnosed in childhood and adolescence, with T1DM being extremely rare.[138] A prospective study of those aged less than 19 years old in urban New South Wales from 2001 to 2006 reported a significantly higher proportion of indigenous adolescents with T2DM than non-indigenous adolescents (Odds ratio (OR) 6.1, 95% CI 3.9-9.8, $p<0.001$).[139] Comorbidities such as obesity and hypertension are typically present by the second decade of life.[140] Early onset and longer duration of diabetes increases the risk of complications such as STDR.[141] Therefore it is not surprising to find indigenous Australians with severe forms of DR are on average 10 years younger.[142]

The shift to more 'Westernized diets' and sedentary lifestyles has significantly affected the health of indigenous people worldwide. The 'thrifty gene' theory explains that a quick insulin response was an asset to our hunter-gatherer ancestors because food shortage was common and the high insulin levels promoted fat storage which could be later mobilized in times od famine. However when diet and exercise patterns change, the change in genetics is slower, and therefore could contribute to the rapid rise of T2DM, obesity and the metabolic syndrome.[143] In addition to poor diet and exercise, indigenous Australians could be more genetically predisposed to diabetes and its complications, but there are limited studies on this subject.[144] Genetic studies have been performed in other ethnic groups. For example, Native American ancestry (defined with HapMap reference panels) was significantly associated with severe DR (PDR or severe NPDR) in a study of 944 T2DM subjects from the Los Angeles Latino Eye Study.[145] The association remained significant after adjustment for age, sex, duration of diabetes, HbA1c and systolic blood pressure (OR = 1.87, 96% CI 1.26, 2.78, $p = 0.002$).

1.6.3 Socioeconomic risk factors

Many of the clinical risk factors for diabetes and sight threatening DR in indigenous Australians are underpinned by socioeconomic disadvantage. The inverse relationship between socioeconomic standing and diabetes has been reported in various populations around the world[146] including Australia.[134] Indigenous Australians are at a higher risk of low income and poor education,[135] which contribute to inadequate

nutrition,[147] poor lifestyle choices,[148] and consequently an increased risk of T2DM. Furthermore, diabetes is a chronic disease affecting multiple body systems which requires holistic and frequent medical care to reduce risk factors and treat complications. Half of the indigenous Australian population earn the bottom 20% of gross weekly incomes and cannot afford the costs associated with diabetes.[149] The majority of indigenous Australians also live in regional and remote communities (63% compared to 28% of non-indigenous Australians) placing them at an additional disadvantage to accessing health care services.[126]

Colonization and the repression of indigenous cultures contributed significantly to the socioeconomic disadvantage in indigenous Australians.[150] Indigenous Australians were marginalized to live in sub-standard conditions that contributed to poor health, poor education and training for employment in the wider society. A disconnection from the land, different social constructs and concepts of health had significant impacts on mental health and substance abuse.[151] Racial intolerance, negative stereotyping and cross-cultural miscommunications also made it difficult for indigenous people to improve their socioeconomic standing or engage with health services that were foreign to them.[152]

Awareness of diabetic complications such as DR and adherence to treatment is poor in all populations but is worse in indigenous communities. Among indigenous Australians, only 20% of those with diabetes had an eye examination the year before, compared with 53% in the non-indigenous population.[153] The disparity in adherence to screening examinations was largest for those with mild to moderate non-proliferative disease. DR is a 'silent' disease, with no symptoms until the very late stages. Delays in diabetes diagnosis[154], DR screening and treatment[153] contribute to inadequate risk factor management and therefore faster progression and increasing severity of DR.[155]

1.6.4 Strategies to improve delivery of health services

Ineffective healthcare systems and poor resources are possibly the biggest hurdles to closing the gap in vision between indigenous and non-indigenous Australians with diabetes. The Declaration of Health and Survival from the World Health Organization outlines key strategies such as collaboration, cultural respect, autonomy

and capacity building for improving the delivery of health services to indigenous populations.[156] Intervention programs designed with these in mind appear to be most effective.[157]

A report published by the Australian Institute of Health Welfare identified key areas for improving the health of indigenous Australians include addressing physical and economic barriers, and improving cultural acceptability and appropriateness.[158] Physical and economic barriers can be addressed by providing more services locally, multi-faceted engagement, assisting with transport issues and having flexibility with setting appointments.[158] Cultural barriers can be addressed by utilizing indigenous health workers, building therapeutic and clinical relationships with community stake-holders based on trust and mutual respect, and developing services around a holistic model of health and wellbeing.[158]

1.7 The genetics of diabetic retinopathy blindness

Part two of this thesis will focus on a second challenge in combating DR blindness. DR is a known complex genetic disorder, where genetic and environmental risk factors contribute to overall disease risk. Duration of diabetes and glycaemic control were estimated to contribute only 11% of the variation in DR risk in the Diabetes Control and Complications Trial (DCCT),[159] and 10% in the WESDR study.[160] Long term studies with intensive treatment aimed at reducing multiple clinical risk factors for DR have shown conflicting results for microvascular complications.[161, 162] The phenomenon of "metabolic memory" was observed in long term follow up studies of landmark epidemiology trials such as the DCCT and UKPDS.[89, 163] Hyperglycemia in the years following onset of diabetes appeared to be 'remembered' and a higher risk of developing complications continued despite subsequent efforts to reduce glycaemic levels. Therefore, despite optimal clinical risk factor control and timely treatments, a subset of patients will still develop sight threatening disease.

Despite the success of anti-VEGF agents in the management of DMO, treatment response is variable among individuals. A post hoc analysis of the DRCR study and the BOLT study showed that there were 4 patterns of response: early and consistent, early and inconsistent, slow and variable, and no response, with the majority 60% having no response.[164, 165] While an attempt at identifying prognostic factors from landmark trials have been conducted, an accurate predictive model of patients at risk of DR and response to treatment remains to

be developed.[110] Several clinical trials are now focusing on drugs that inhibit molecular mechanisms beyond VEGF, but none have shown promising results.[166] An emerging theory is that genetic and molecular mechanisms in controlling VEGF pathways may vary between individuals. The motivation for understanding the thousands of genes potentially involved in complex genetic disorders is being able to uncover new knowledge about disease pathogenesis and develop new targets for better treatments tailored towards individuals and their families.[167, 168]

The field of genetic epidemiology has been a pivotal first step in identifying potential genes involved with disease.[169] Genetic epidemiology examines associations between genotype and phenotype, allowing us to identify and study novel pathways. There have been numerous types of studies exploring genetic risk factors for DR, each with its own merits and disadvantages, as described below.

The heritability of DR was first documented in twin studies[170, 171] and family studies.[172-177] The largest of these was the DCCT which involved T1DM participants and reported a 4.3 fold increased risk for severe DR in first degree relatives with either type 1 or type 2 diabetes.[172] The first genetic linkage studies were conducted in the 1990's. Genetic linkage identifies genes that segregate with defined phenotypes among families. The largest study examined 103 sibling pairs with DR among Pima Indians with T2DM, and they found loci on chromosome 7 and 20 were associated with diabetic nephropathy but not retinopathy.[178] Modelling inheritance patterns through linkage studies is difficult for DR because the inheritance pattern is heterogenic and environmental factors need to be accounted for. In addition, effect sizes for most variants associated with DR are small and previous linkage studies were underpowered. Other study designs have therefore been utilized in subsequent years.

In a candidate gene study, genes of interest are selected based on their mechanism of action, and frequencies of its variants are compared between cases and controls. Hundreds of candidate genes and their pathways have been studied in DR with this approach. The best studied gene is *VEGFA*, which is the target of current anti-VEGF treatments. The *VEGFA* gene is located on chromosome 6 (6p21.3) and is highly polymorphic.[179] Meta-analyses have been useful in congregating published studies to increase power and decrease error when

examining significant results found, however analyses are limited by lack of public data availability. *VEGFA* genetic variants (single nucleotide polymorphisms (SNP)) analysed via meta-analyses include rs2010963,[180-184] rs2146323,[185] rs699947,[180, 181, 183, 186, 187] rs833061,[180, 181, 187] rs3025039[180, 181] and rs1570360.[180, 181] The most recent meta-analysis published in 2016 congregated 16 candidate gene studies where cases were diabetic participants with any DR and controls were diabetic participants with no DR.[188] Five studies were of Caucasian ethnicity, while 8 were of Asian ethnicity. Both type 1 and type 2 diabetes were included. Five SNPs in the *VEGFA* gene were available for pooled analyses. The strongest result was the C allele in rs833061 (T>C) and its association with any DR: 3 studies pooled, 697 DR cases, 663 diabetic controls, OR 6.34, 95% CI 2.10–19.14, P = 0.001. The T allele of rs3025039 was also associated with DR: 6 studies pooled, 1114 cases, 1491 controls, OR 1.60, 95% CI 1.07-2.41, p = 0.02. The 3 other variants were not found to have significant associations after pooled analysis: rs699947 (C>A), rs2010963 (G > C) and rs1570360 (G>A). Stratification of diabetes type, DR severity, and adjustment for ethnicity and clinical risk factors were not performed. A few case control studies have examined the association between *VEGFA* and severity of DR. Churchill et al found −160C, rs13207351 (A>G), rs1570360 (A>G) and associated haplotypes significantly associated with PDR in T1DM and T2DM participants (45 with PDR, 61 without DR).[189] Abhary et al also found significant haplotypes in the *VEGFA* gene associated with more severe forms of DR (PDR, severe NPDR, CSMO) in a T1DM and T2DM cohort (319 with severe DR, 235 without DR).[190]

In comparison to the candidate gene study, a genome-wide association study (GWAS) is a non-hypothesis driven approach that includes the whole genome, with the advantage of discovering new disease associated genes with smaller effect sizes. The best data from GWAS for DR is a recent meta-analysis of multiple GWAS studies performed by an international consortium.[191] It included GWAS data from multi-ethnic cohorts (European, African American, Asian, Hispanic), had more stringent levels of significance (genome wide significance set at $P < 6.25 \times 10^{-9}$ for European-descent cohorts and $P < 3.75 \times 10^{-9}$ for African American or multi ethnic cohorts), and adjusted for covariates such as duration of diabetes and glycaemic control. The only result that reached genome wide significance was intronic variant rs142293996 in nuclear VCP-like (*NVL*) and its association with PDR in the European discovery cohort: 187 cases, 435 diabetic controls, OR = 2.38,

$p = 2.1 \times 10^{-9}$. However it did not remain significant in the European replication cohort ($p = = 4.10 \times 10^{-6}$). *NVL* codes for an ATPase which has various intracellular functions and is highly expressed in the retina. The most significant result for the African-American discovery cohort was rs115523882 near the *GOLIM4* gene, but did not reach genome wide significance ($p = 4.1 \times 10^{-6}$).

A cost-effective approach is whole exome sequencing (WES), where genotyping is limited to exomes, protein encoding parts of the DNA. By focusing on exomes, which take up 1% of the whole genome, it is hypothesized that this may increase the chance of finding rare mutations with higher effect sizes. Three WES have been performed for DR, using extremes of phenotypes to maximize results.[192, 193] Shtir et al defined cases as those that developed DR after 10 years of diabetes (n = 43), and controls (n = 64) that did not develop DR after 10 years.[193] Three variants reached genome wide significance through a case control design: *NME3* ($P = 1.55 \times 10^{-10}$), *LOC728688* ($P = 6.23 \times 10^{-10}$), and *FASTK* ($P = 3.21 \times 10^{-8}$), all were protective of developing any DR. Ung et al defined controls similar to Shtir et al (n = 13) but defined cases as PDR requiring surgical vitrectomy (n = 43).[192] Forty-four genes were found to be more frequent in cases compared with controls, but significance levels were not reported. The genes were not the same as the ones in Shtir's study and the cohort was of a mixed ethnicity. Cabrera et al defined cases as those who developed PDR within 15 years of diabetes (n = 6) and controls (n = 6) as those who did not develop DR after more than 25 years of diabetes. A set of genetic variants involved in angiogenesis and inflammation were found to be significant, especially COL18A, ZNF395 and PLEKHG5, which had increased mRNA expression levels in retinal cells under hyperglycaemia ($p < 0.0001$).[194] These studies were underpowered and significant results could also be false positives.

Shortcomings of genetic studies performed for DR follow the same themes as genetic studies performed for other complex diseases. Significance level of many results have been small, and no genetic region has yet reached genome-wide statistical significance with replication. Insignificant results have been attributed to small sample sizes and inadequate power. Small sample sizes also increase the frequency of false positive results and therefore lack of replication in larger studies. In addition to the inherent difficulty of recruiting

thousands of participants required for a well powered study, increasing sample size is more difficult in DR because of the many types of cases or controls that can be defined (two types of diabetes, various stages of DR). Definitions have therefore varied across previous studies, leading to challenges in combining data and replicating results. Risk genes are also likely to have small effect sizes or are rare in complex genetic diseases, making it more important to have large and well-defined cohorts.

The simple solution to these challenges is to perform larger studies with better characterized cohorts, and to collaborate with other researchers. The problem is that whole genome or exome sequencing are still very expensive. Thus, more cost effective solutions are required to study the genetics of DR.

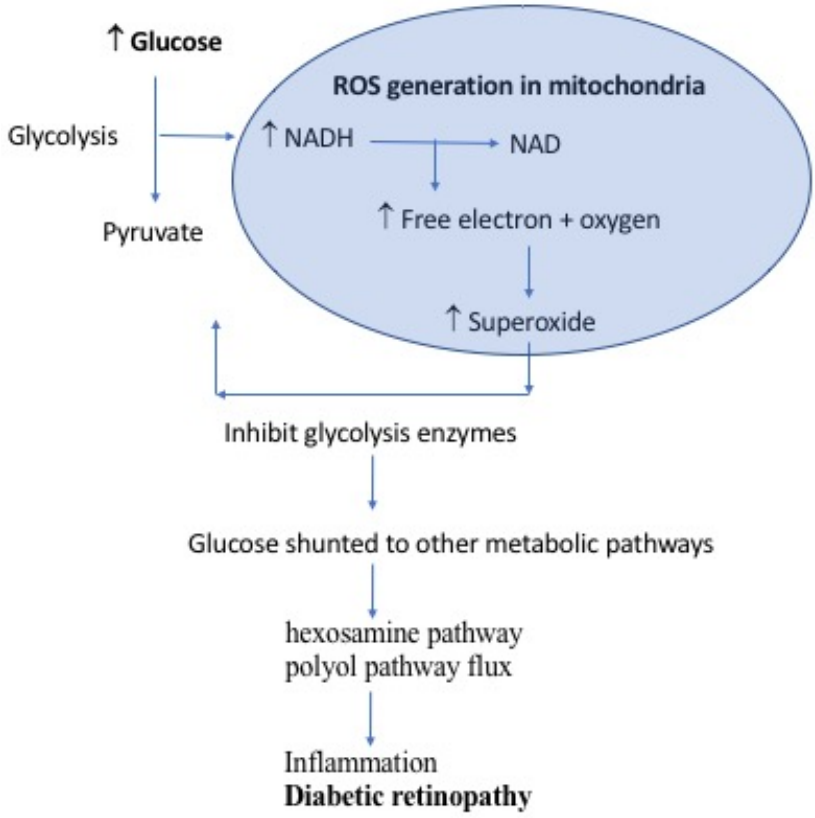
1.7.2 Choice of candidate genes for this thesis

Part 2 of this thesis aimed to study three candidate gene areas that have not been well studied previously. Three areas were chosen and their relevance to DR is described below.

1.7.3 Mitochondria

Metabolic, inflammatory and hypoxic pathways of DR necessitated a theory of a single upstream event that mediated these mechanisms. Brownlee proposed that this single upstream event was mitochondrial overproduction of ROS in response to hyperglycemia (Figure 1.3).[195] Mitochondria are vital organelles in all eukaryotic cells, being responsible for oxidative phosphorylation and ATP production. Hyperglycaemia results in excess glycolysis in cells, causing the maximal capacity of the electron transport chain in mitochondria to be reached. Electrons are instead transferred to oxygen molecules forming the ROS, superoxide.[196] Superoxide inhibits enzymes early in glycolysis, causing glycolysis intermediates to be shunted into alternative metabolic pathways that activate various inflammatory pathways.[197]

Figure 1.3 Mitochondrial overproduction of reactive oxygen species and diabetic retinopathy



Nuclear encoded mitochondrial genes that have been studied in DR include *Mn-SOD, UCP1, UCP2, Romo1* and *MT-TL1*.[198] The best studied polymorphism is Val16Ala in the superoxide dismutase gene, *Mn-SOD*. It was reported to be protective of DR in a meta-analysis of 8 studies under the dominant model (1235 cases, 1051 controls, OR=0.66, 95%CI=0.48-0.91, P<0.0001), but became less significant when ethnicity was accounted for (OR=0.64, 95%CI=0.42-0.97, P=0.04 for 5 pooled Caucasian studies).[199]

Interestingly, mitochondria possess their own DNA which is responsible for coding a limited number of essential genes with function in the electron transport chain (ETC). Mitochondrial DNA (mtDNA) is present in multiple copies in each cell. The mitochondrial genome is highly sensitive to oxidative stress and has higher rates of mutation than nuclear DNA, resulting in heterogeneous mtDNA species within the same cell. When a mutant type mtDNA exceeds a threshold level, increased expression of abnormal proteins involved in the ETC could impact on its function and therefore the metabolic pathways of DR. Sally et al showed that

expression levels of ETC proteins were abnormal in endothelial cells of diabetic mice compared with non-diabetic mice, and that it remained abnormal 6 months after the diabetic mice were treated.[200]

Abnormal mitochondria also affect cell survival. As discussed in chapter one, endothelial cell loss is an early feature of DR that contribute to breakdown of the blood retina barrier and macular oedema, as well as decreased blood supply to the retina, and proliferative changes. Tewari et al found that diabetes interfered with mtDNA's replication system. Expression levels of D-loop, an integral part of mtDNA required for successful DNA replication, was decreased in diabetic endothelial cells.[201] Subsequently, mitochondrial copy number was decreased in diabetic endothelial cells, and this has been shown to be associated with increased cell apoptosis.[202]

Only a few studies have focused on the role of hereditary mutations in mtDNA and susceptibility of DR. Given the mosaic distribution and matrilineal inheritance of mtDNA, it is difficult to determine association patterns between isolated mtDNA mutations and observed phenotypes. Instead, studies have focused on mitochondrial haplogroups, which represent the major branch points of the mitochondrial phylogenetic tree of human evolution.[203-206] Different ethnicities are defined by different haplogroups. Studies exploring mitochondrial haplogroups and DR have only been conducted in Caucasian populations. Estopinal et al reported that haplogroups H1, H2 and UK in a Caucasian sample (n=392) were associated with PDR.[204] Haplogroup H1 and H2 were risk factors for the development of PDR from NPDR, while haplogroup UK was protective against PDR. Subsequently, Bregman et al reported similar findings in a larger group from the same population (n=637), and reported further that while mitochondrial haplogroup was associated with PDR, it was not associated with DR more generally.[203] A different case control study (149 with any type of DR and 78 with no DR) found a higher prevalence of haplogroup T in those with any DR (12.1% vs 5.1%; $p = 0.046$).[207] None of these results have thus far been replicated.

1.7.4 MicroRNA and microRNA binding sites

MicroRNAs are short single stranded RNA molecules that are involved in regulating gene translation.[208] A single microRNA may bind to hundreds of genes at the 3' untranslated region of mRNA and induce mRNA

degradation or translational repression. Genetic variations in microRNA genes and their target binding sites can have significant consequences for these regulatory pathways. MicroRNAs have been implicated in a wide range of diseases including cancer, cardiovascular and neurodegenerative diseases.[209-211]

Therefore, it is not surprising to find accumulating evidence supporting the role of microRNA in DR. Differentially expressed microRNAs have been found to be associated with DR[212], as well as other microvascular diabetic complications.[213] Kovac *et al* reported 86 differentially expressed microRNA in retinal endothelial cells of streptozotocin induced diabetic rats.[212] The majority of these were NF-κB, VEGF and p53 responsive. Other studies have compared expression levels of microRNA in serum between cases and controls, using microRNA arrays.[214-216] Barutta et al found 25 differentially expressed microRNAs between 312 diabetic complication cases and 143 diabetic controls in type 1 diabetes.[217] Specifically, microRNA 126 levels were negatively associated with proliferative retinopathy (0.77 vs 1.29, $p = 0.02$). Studies profiling microRNA using microarrays have identified microRNA 21, 200b, 15a, 320a, 320b, 93, 29a and 423-5p to be significantly associated with PDR in both types of diabetes.[218-220]

Our research group studied microRNA 146a and identified functional polymorphism rs2910164 was significantly associated with DMO in T2DM.[221] Further studies are required to explore other microRNA genes and their association with DR.

1.7.5 VEGF receptors

Out of the many pharmacological agents experimented on for DR, anti-VEGF agents remain the most successful. The VEGF pathway is an essential common pathway in DR that can be inhibited, but a wide range of treatment responses necessitate an explanation. Current anti-VEGF agents focus on inhibiting VEGFA. A hypothesis to explain different treatment responses is that these agents do not inhibit VEGF's multiple isoforms and their receptors.

VEGF isoforms exert their effects by binding to three specific receptors; VEGFR1, VEGFR2 and VEGFR3. VEGFR1 binds to VEGFB and phosphatidylinositol-glycan biosynthesis class F (PIGF), is highly expressed

in retinal epithelial cells, [222] and contributes to retinal angiogenesis under pathological conditions.[223, 224] VEGFR1 also has a soluble splice variant (sFLT1) which binds strongly to VEGFA, preventing it from binding to its other receptors and therefore acts as an inert reservoir and regulator of the VEGF pathways.[225] The majority of VEGFA's intracellular effects are through VEGFR2, its primary receptor. There are differentially spliced isoforms of VEGFA with opposing intracellular effects. For example, VEGF165a promotes angiogenesis while VEGF165b inhibits angiogenesis by competitively binding to and blocking the activation of VEGFR2. The ratio of VEGFA165a and VEGFA165b protein is higher in the vitreous of individuals with PDR compared with normal eyes.[226] The role of VEGFR3 is unclear, but it binds to VEGFC, which also contributes to the angiogenesis pathways in DR. No studies have been conducted to explore whether genetic variations in these receptor genes affect DR risk. The genetics of VEGFR and the role of splicing factors are important to explore given their functional roles in disease.[227]

1.8 Thesis Aims

This thesis focuses on building knowledge in two important areas of reducing the burden of diabetic retinopathy: 1) the disproportionate burden of DR in indigenous Australian populations, and 2) the complex genetic nature of DR because of its implications on future treatment strategies.

Part one of this thesis aims to characterize the burden of DR in indigenous Australians, which would then inform improved strategies to deliver treatments. Three specific aims were sought:

1. Explore long term mortality and morbidity patterns of indigenous Australians with end stage DR. This was achieved through a population based epidemiology study based in South Australian and Central Australia. Severe renal failure was identified as an important morbidity in this cohort, and led to aim 2.
2. Explore the prevalence and severity of DR in the diabetic indigenous renal dialysis population. This was achieved through a population based study in remote and rural indigenous communities in Central Australia.

3. Explore the efficacy of a new drug for diabetic macular oedema in indigenous Australians that can be given less frequently and may alleviate adherence and access to health care issues.

Part two of this thesis aims to further explore genetic risk factors for DR by focusing on pathways that have not been well studied. Three candidate areas were chosen;

1. Confirm the association between mitochondrial haplogroups and DR risk in a Caucasian population. A replication study was conducted in a larger Caucasian population based in Australia.
2. Explore genetic variations in microRNA genes and microRNA binding sites, and their association with DR. This was achieved through a targeted genome wide approach to explore as many microRNA related variants as possible in a cost-effective way.
3. Explore genetic variations in the VEGF receptor genes and their association with DR. This was also achieved through a targeted genome wide approach.

CHAPTER 2: LONG TERM SURVIVAL RATES OF PATIENTS UNDERGOING VITRECTOMY FOR DIABETIC RETINOPATHY IN AUSTRALIA – A POPULATION BASED AUDIT

The original work presented in this chapter has been published in the peer-reviewed literature: Liu E, Estevez J, Kaidonis Get al. Long term survival rates of patients undergoing vitrectomy for diabetic retinopathy in an Australian population: a population based audit. Clin Exp Ophthalmol 2019.

2.1 Introduction

A population based audit is a useful method to characterize DR with the worst outcomes and understand disease risk patterns to tailor treatments to specific populations such as the indigenous Australian population. Our research group conducted a population based audit of end stage DR in South Australia (SA) and the Northern Territory (NT), including all rural and remote locations, stratifying our analyses between indigenous and non-indigenous individuals.[142] Diabetic vitrectomy was used as a surrogate measure of DR at its most severe form.

The audit showed that indigenous Australians with diabetes had significantly worse outcomes. They were 4.9 times more likely to require vitrectomy than non-indigenous Australians with DR. While visual outcomes after vitrectomy were similar between ethnicity groups, other diabetic complications were not. Indigenous Australians with end stage DR were more likely to have limb amputation (24.2% vs 12.4%, p=0.033), chronic renal failure (78.9% vs 50.7%, p<0.001) and be on dialysis (35.1 vs 6.5%, p<0.001), despite being younger and having a shorter duration of diabetes.

The presence of DR is a marker of advanced diabetic disease and is associated with reduced survival.[228] A recent meta-analysis reported a risk ratio of 2.33 when comparing all-cause mortality rates between diabetics with no DR and those with any DR.[15] Severity of retinopathy reflects the status of diabetes disease burden on the body and indicates the need for more urgent and comprehensive management. PDR also leads to significant visual impairment which is well-recognised to increase morbidity and mortality leading to social isolation, depression, increased risk of falls and loss of independence.[20, 24, 30, 31, 229]

Mortality and patterns of mortality are useful markers of a population's health and can guide evaluation of existing health care systems. Longitudinal mortality data is important in assessing change over time and confirming assumptions and predictions. Over the last forty years, few studies have been performed to establish the long term survival rates of those with end stage DR requiring vitrectomy. Reported survival rates at five years vary widely from 68-96% worldwide[230-235] and no studies of this type have been performed in an Australian cohort. The purpose of this follow-up study was to establish the long term survival rates of these patients, to identify risk factors and comorbidities associated with increased mortality, and to compare survival rates and risk factors between indigenous and non-indigenous Australians. Unlike other causes of vision loss, the burden of DR extends beyond vision impairment, particularly for indigenous Australians, and it is important to develop services around a holistic model of health and wellbeing.

2.2 Methods

This project has been approved by the South Australia Clinical Human Research Ethics Committee (HREC), the Southern Adelaide HREC, the Aboriginal HREC and the Central Australia HREC. It adheres to the tenets of the Declaration of Helsinki.

Patient demographics, diabetes history and diabetic complications prior to vitrectomy surgery were available from our previous audit.[142] Further details regarding data collection have been published elsewhere.[236] In brief, files of all patients identified as having had a vitreoretinal surgery during the audit period were manually examined. Data were collected retrospectively and included sex, age at time of primary vitrectomy, ethnicity (as per hospital record), type of diabetes, duration of diabetes, insulin use, pre-operative HbA1c, most recent value available prior to date of surgery), baseline best corrected visual acuity, chronic renal failure, renal failure requiring dialysis, and amputation. Legal blindness was defined as worse than best corrected visual acuity Snellen 6/60 (35 ETDRS letters equivalent) in the better eye.[237] Chronic renal failure was defined as glomerular filtration rate less than 60mL/min for more than 3 months.

Only patients who underwent their first vitrectomy for the following indications were included in this study: (i) media opacities (including recurrent or non-resorbing vitreous haemorrhage) and (ii) vitreoretinal traction,

with or without haemorrhage (including tractional retinal detachment). Diabetic patients undergoing vitrectomy for all non-diabetes related indications were not included in the study Perceived life expectancy was not an inclusion criterion for this study. Patients who were indicated for diabetic vitrectomy but could not due to poor fitness for surgery were also not included in this study.

Date of death and cause of death was obtained from the South Australian and Northern Territory Deaths Registry, using survival status on the 6th July 2018 as the primary end point. Data linkage was performed through SA NT DataLink services, using the separation principle to ensure patient confidentiality.

Statistical analyses were performed with Statistical Package for Social Sciences (SPSS) version 25.0 for Mac OS X (IBM SPSS Statistics 25.0, SPSS Inc., Armonk, NY, USA). Kaplan Meier survival curves were generated to determine survival rates from 1 to 9 years after primary vitrectomy. Mann–Whitney U and chi-square tests were used to compare demographic variables among different groups. Risk factors were explored using univariate and multivariate regression analyses (Cox proportional hazard model). P-values <0.05 were considered statistically significant. Less significant variables were removed from the model in a stepwise manner until the strongest multivariate model remained.

2.3 Results

In SA and the NT, between 1st January 2007 and 31st December 2011, 307 primary vitrectomies were performed for DR complications (media opacity or vitreoretinal traction). Demographic details and baseline clinical variables are shown in Table 3.1. The number of participants with available and missing data for each variable studied is presented in table 3.2. The mean age at the time of vitrectomy was 57 years (range 22-90). A higher percentage of participants were male (59.2%), had T2DM (77.7%) and comorbidities such as chronic renal failure (55.9%). One fifth of the cohort was indigenous Australian (n = 65, 21.2%). When extrapolating data from the National Indigenous Eye Health Survey and the Australian Bureau of Statistics, this is significantly higher than the estimated proportion of indigenous Australians with diabetes in SA and NT (1.44 (1.09 – 1.89; p = 0.01).[238, 239] Compared with non-indigenous Australian participants, indigenous Australians undergoing diabetic vitrectomy were less likely to be male (44.6% versus 65.5%, p = 0.002) and a higher

percentage had T2DM (93.8% versus 76.6%, p = 0.001). Indigenous Australians were younger at the age of primary vitrectomy (51 versus 63 years old, p<0.001), had a shorter duration of diabetes (14 versus 18 years, p<0.001), yet a higher proportion had chronic renal failure (79.6% versus 51.8%, p<0.001), renal failure on dialysis (32.2% versus 6.7%, p<0.001) and amputation (36.6% versus 12.6%, p =0.004).

Table 2.1 Demographic details and baseline clinical variables for the total cohort and the indigenous and non-indigenous groups. P values comparing variables between the indigenous and non-indigenous groups are presented.

Variable	**Total**	**Indigenous**	**Non-indigenous**	**P**
Male (n, %)*	181 (59.2)	29 (44.6)	152 (65.8)	**0.002**
Age at time of primary vitrectomy, years (mean, range)	57 (22-90)	51 (32-74)	63 (25-90)	**<0.001**
Type 2 diabetes (n, %)*	238 (77.7)	61 (93.8)	177 (76.6)	**0.001**
Diabetes duration, years (mean, range)	19 (0-53)	14 (2-29)	18 (0-45)	**<0.001**
Insulin use in type 2 diabetes (n, %)*	127 (52.2)	27 (44.3)	100 (56.5)	0.10
Pre-operative HbA1c, %(mean, range)	8.4 (5-17)	15 (32.6)	49 (21.1)	0.750
Legally blind at baseline (n, %)*	64 (22.8)	15 (24.2)	49 (23.6)	0.918
Chronic renal failure (n, %)*	157 (55.9)	47 (79.6)	110 (51.8)	**<0.001**
Renal failure requiring dialysis (n, %)*	35 (11.8)	20 (32.2)	15 (6.7)	**<0.001**
Amputation (n, %)*	41 (15.3)	15 (36.6)	26 (12.6)	**0.004**

**Percentages are calculated from the number of individuals with available data. Please refer to Table 2.2 for the numbers in each category.*

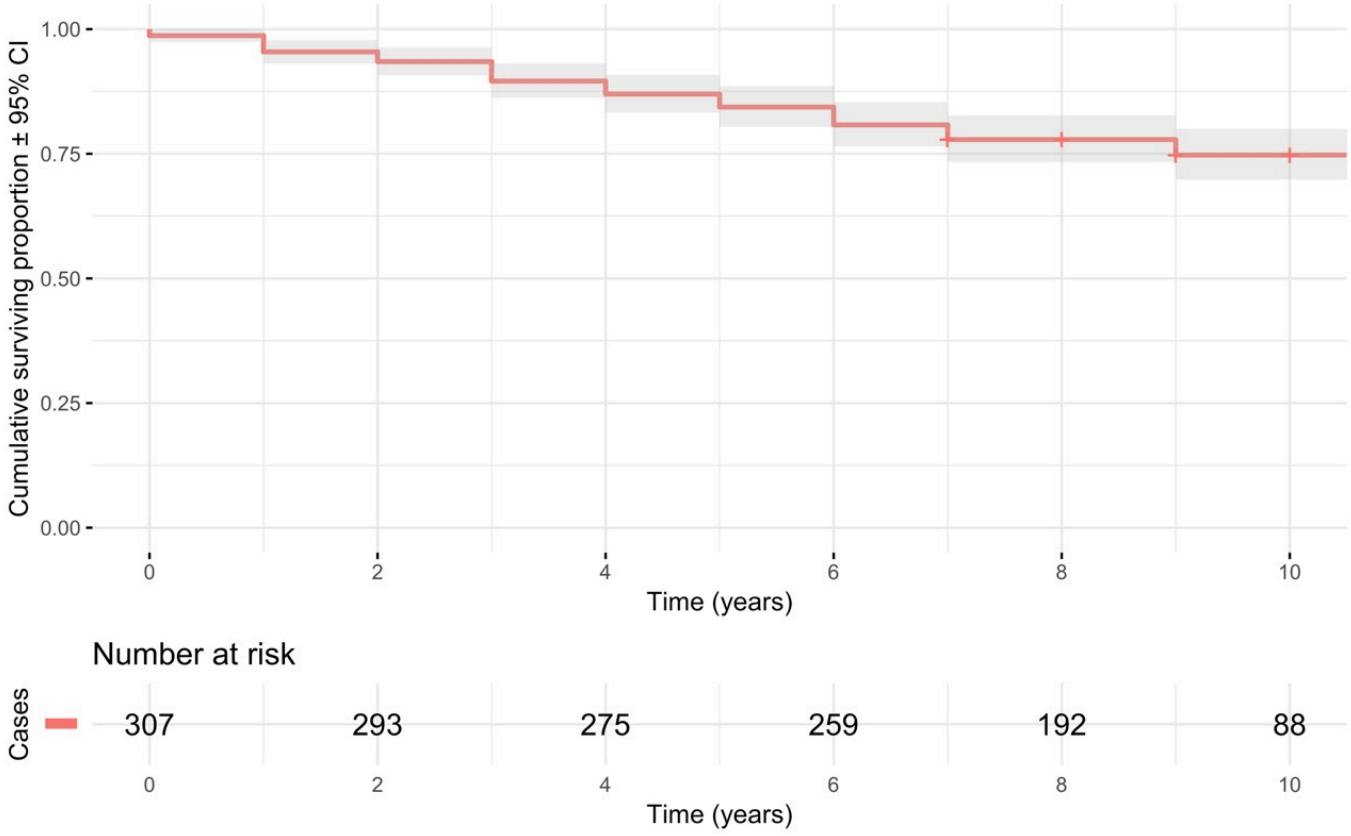
Table 2.2 Number of participants with available and missing data for each baseline variable studied

	Total n = 307		Indigenous n = 65		Non-indigenous n = 232	
Variable	Available data	Missing data	Available data	Missing data	Available data	Missing data
Ethnicity	297	10	NA	NA	NA	NA
Sex	306	1	65	0	231	1
Age at time of vitrectomy	306	1	65	0	231	1
Type of diabetes	306	1	65	0	231	1
Diabetes duration	253	54	52	13	191	41
Insulin use in type 2 diabetes	248	59	61	4	177	55
Pre-operative HbA1c	214	93	46	19	158	74
Legally blind at baseline	280	27	62	3	208	24
Chronic renal failure	281	26	59	6	212	20
Renal failure requiring dialysis	297	10	62	3	225	7
Amputation	268	39	52	13	206	26

At the primary end point, 73 out of 307 patients had a death record from the South Australian and Northern Territory Deaths Registry. Twenty out of 73 (27.4%) were indigenous Australian). The most common cause of death was cardiovascular disease (n=30), followed by sepsis (n=14), renal failure (n=11), cerebrovascular disease (n=8), cancer (n=5) and other causes (n=5).

Survival rates for 5, 7 and 9 years after primary diabetic vitrectomy were 84.4%, 77.9% and 74.7% respectively (Figure 2.1).

Figure 2.1: Kaplan-Meier plot estimating survival rates from 1 to 9 years after the primary vitrectomy



After univariate cox proportional hazard model analyses, the most significant factor associated with increased mortality was older age at the time of primary vitrectomy, (HR 1.03, 95% CI 1.02-1.05, p<0.001) and T2DM (HR 2.18, 95% CI 1.04-4.56, p=0.037) (Table 2.3). Adjusting for age, indigenous Australian ethnicity and chronic renal failure were significantly associated with increased mortality (HR 2.04, 95% CI 1.17-3.57, p=0.012 and HR 1.76, 95% CI=1.07-2.89, p=0.026, respectively).

Table 2.3 Cox proportional hazard model analyses of available baseline clinical variables and their association with mortality

Variable	Univariate analysis		Adjusting for age at time of vitrectomy	
	HR, 95% CI	P value	HR, 95% CI	P value
Male	0.82 (0.52-1.30)	0.393	0.81 0.51-1.28	0.362
Age at time of primary vitrectomy	1.03 (1.02-1.05)	**<0.001**	NA	NA
Indigenous Australian ethnicity	1.42 (0.85-2.38)	0.186	2.04 (1.17-3.57)	**0.012**
Type 2 diabetes	2.18 (1.04-4.56)	**0.037**	1.34 0.61-2.95	0.462
Diabetes duration	1.02 (0.99-1.04)	0.263	1.00 0.98-1.03	0.618
Insulin use in type 2 diabetes	1.18 (0.72-1.92)	0.509	1.19 0.73-1.94	0.490
Pre-operative HbA1c <7%	0.73 (0.41-1.29)	0.276	0.82 (0.46-1.45)	0.490
Legally blind at baseline	1.34 0.78-2.30	0.289	1.51 (0.88-2.62)	0.137
Chronic renal failure	1.54 0.95-2.53	0.083	1.76 (1.07-2.89)	**0.026**
Renal failure requiring dialysis	1.78 0.98-3.25	0.06	2.32 (1.25-4.32)	**0.008**
Amputation	0.86 0.46-1.60	0.626	1.17 0.63-2.18	0.624

Patients with chronic renal failure requiring dialysis had an even higher risk of death (HR 2.32, 95% CI 1.25-4.32, p=0.008). T2DM was not associated with mortality after adjustment for age. Duration of diabetes, insulin use in T2DM, pre-operative HbA1c and amputation were not associated with mortality without and with after adjustment for age.

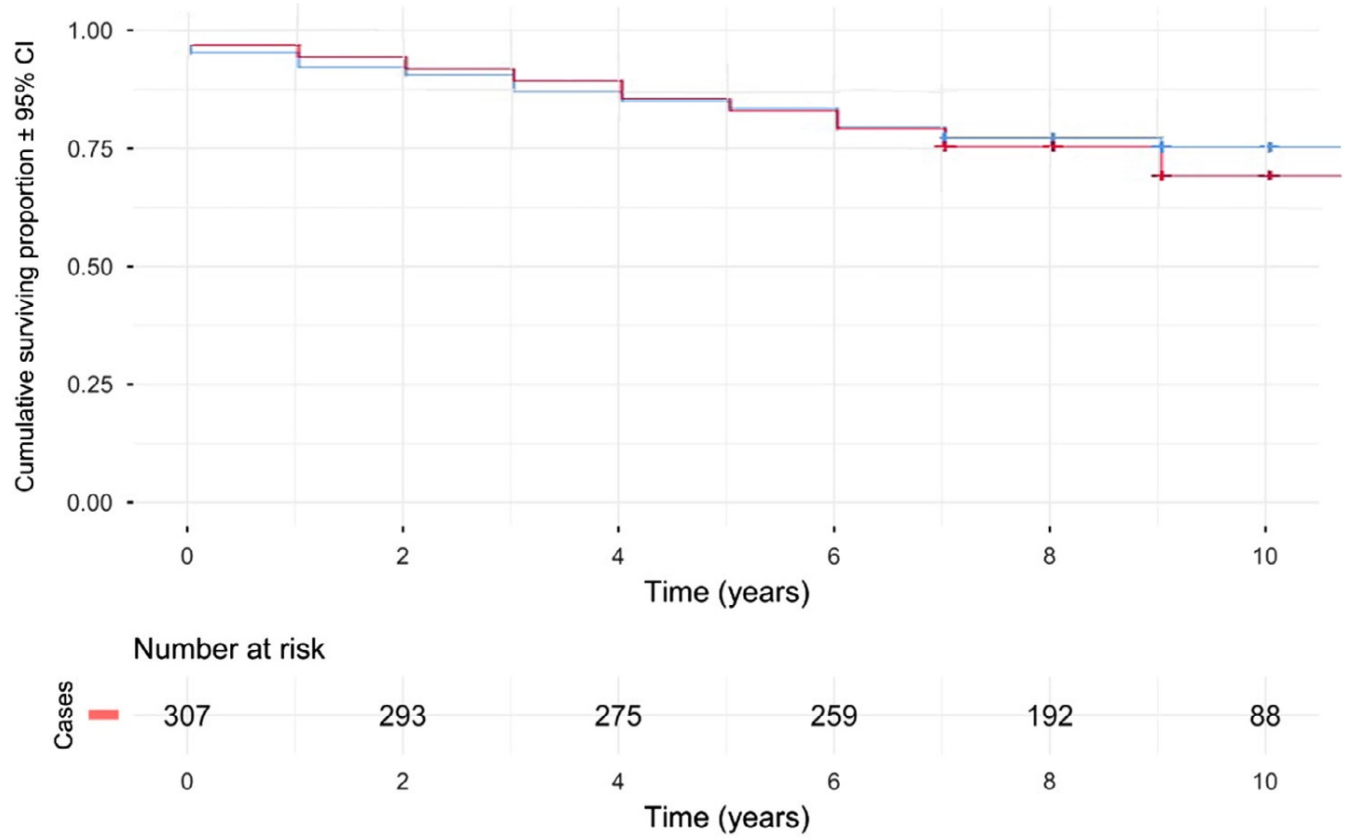
Five variables were included in the multivariate model based on level of significance from the univariate analyses: age at time of primary vitrectomy, indigenous Australian ethnicity, T2DM, chronic renal failure and renal failure requiring dialysis. The strongest model had two variables: age at time of primary vitrectomy and renal failure requiring dialysis with an overall chi-square value of 17.6, p<0.001 (Table 2.4).

Table 2.4 Best fitting multivariate cox proportional hazard model for baseline variables and their association with mortality

Variables in model	HR, 95% CI	P value
Age at time of primary vitrectomy	2.32 (1.25-4.32)	**0.008**
Renal failure requiring dialysis	1.04 (1.02-1.05)	**<0.001**
Overall model chi-square = 17.6, p**<0.001**		

As the indigenous Australian cohort in this study are significantly younger and have a shorter duration of diabetes than non-indigenous Australians (Table 2), sub-analyses between the two ethnicities were done for comparison. Five, seven and nine year survival rates after primary vitrectomy for indigenous Australians (n=65) were 83.1%, 73.8% and 66.2% respectively. Five, seven and nine year survival rates for non-indigenous Australians (n= 242) were 85.3%, 79.3% and 77.3% respectively. This was not statistically significant (Figure 2.2, Kaplan-Meier curves, log rank test, chi-square 1.82, p=0.177). However, as shown in Table 2.3, ethnicity became significantly associated with long term mortality after adjustment for age (HR 2.04, 95% CI 1.17-3.57, p=0.012).

Figure 2.2 Kaplan-Meier plot estimating survival rates from 1 to 9 years after the primary vitrectomy, shown separately for indigenous and non-indigenous Australians



Legend: Red line illustrates survival rate of indigenous Australians. Blue line illustrates survival rate of non-indigenous Australians. Log rank test to compare the two groups: chi-square =1.82, p=0.177.

No baseline variables were found to be significantly associated with mortality in the indigenous Australian group after univariate and multivariate analysis, and this could be because of inadequate power (n=65).

However, in the non-indigenous Australian group (n=242), older age at time of primary vitrectomy (HR 1.05, 95% CI 1.02-1.07, p<0.001) and diabetes duration (HR 1.04, 95% CI 1.01-1.07, p=0.022) were associated with increased mortality (Table 2.5).

After adjusting for age, the only significant variables associated with mortality were found to be legal blindness at baseline (HR 1.92 95% CI 1.02-3.65, p=0.045) and chronic renal failure (HR 1.81 95% CI 1.02-3.21, p=0.044) (Table 2.5). However, the strongest multivariate cox proportional model included the variables, age at time of primary vitrectomy, type of diabetes and duration of diabetes, with an overall chi-square value of 19.96, p<0.001 (Table 2.6).

Table 2.5 Cox proportional hazard model analyses of available baseline clinical variables and their associations with mortality: non-indigenous Australians

Variable	Univariate		Adjusting for age at time of primary vitrectomy	
Non-indigenous (n = 242)	HR, 95% CI	P value	HR, 95% CI	P value
Male	0.71 (0.41-1.24)	0.231	0.78 (0.44-1.38)	0.396
Age at time of primary vitrectomy	1.05 (1.02-1.07)	**<0.001**	NA	NA
Type 2 diabetes	2.04 (0.92-4.53)	0.080	0.88 (0.36-2.14)	0.778
Diabetes duration	1.04 (1.01-1.07)	**0.022**	1.03 (0.99-1.06)	0.103
Insulin use in type 2 diabetes	1.58 (0.85-2.95)	0.149	1.65 (0.88-3.07)	0.117
Pre-operative HbA1c <7%	0.94 (0.47-1.89)	0.860	0.90 (0.45-1.82)	0.771
Legally blind at baseline	1.64 (0.87-3.10)	0.126	1.92 (1.02-3.65)	**0.045**
Chronic renal failure	1.59 (0.90-2.82)	0.110	1.81 (1.02-3.21)	**0.044**
Renal failure requiring dialysis	1.63 (0.65-4.10)	0.301	2.01 (0.79-5.10)	0.142
Amputation	0.89 (0.38-2.10)	0.799	0.83 (0.35-1.95)	0.669

Table 2.6 Best fitting multivariate cox proportional hazard model and their associations with mortality: non-indigenous Australians

Variables in model	HR, 95% CI	P value
Age at time of primary vitrectomy	2.32 (1.25-4.32)	**0.008**
Type 2 diabetes	3.99 (1.07-14.88)	**0.039**
Duration of diabetes	1.05 (1.01-1.08)	**0.035**
Overall model chi-square = 19.96, p<0.001		

2.4 Discussion

This is the first study to explore mortality rates after diabetic vitrectomy in an Australian population. We found the 5, 7 and 9 year survival rates after primary vitrectomy performed for diabetic complications in SA and the NT were 84.4%, 77.9% and 74.7%, respectively.

This is similar to rates found in other studies, but it is difficult to make direct comparisons because of different study populations, sampling methods, time-frames and follow up periods. The most recent published study analysed 182 diabetic eyes at a single hospital in New Zealand that underwent vitrectomy for vitreous haemorrhage and/or tractional retinal detachment, and found a 5 year survival rate of 70.1%.[235] They also found renal failure on dialysis, creatinine levels (indicative of renal function), age and non-European ethnicity to be significantly associated with increased mortality. The New Zealand study included 49.5% patients of Maori descent, while our sample comprised of 21.2% indigenous patients. Indigenous populations worldwide have lower life expectancy[240] and this may explain the lower survival rate found in the New Zealand study when compared with our study. Despite earlier indications for diabetic vitrectomy[241] and improved diabetes management,[242] mortality rates have remained similar over the decades. Older studies conducted between 1970's to early 2000's report 5 year survival rates that range widely from 68-96% in countries such as Japan, Germany, Finland and the United Kingdom.[230-235]

The Australian population that we have studied is unique in that a large proportion (21.2%) is of indigenous Australian ethnicity. This is significantly higher than the estimated proportion of indigenous Australians with diabetes in SA and NT (OR 1.44, CI 1.09 – 1.89, p = 0.01).[238, 239] Trends in our study are similar to other population studies of indigenous Australians with diabetes. Indigenous Australians are on average 10 years younger than non-indigenous Australians when diagnosed with T2DM[131] and at the onset of primary diabetic vitrectomy.[236] They are more likely to have T2DM, have a shorter duration of diabetes, a higher risk of developing severe complications, and this is not necessarily associated with poorer glycaemic control.[236] The reasons for earlier onset of diabetes are not well understood but could be attributed to more prevalent gestational diabetes and intrauterine risks, genetic predisposition, obesity, physical inactivity, nutrition and

associated socioeconomic factors.[243] Earlier onset of T2DM is an independent risk factor for increased prevalence and severity of DR.[141]

These differences may skew our analyses. From our unadjusted results, there appears to be no difference in survival between indigenous and non-indigenous Australians requiring diabetic vitrectomy. However after adjustment for age, the risk of death after vitrectomy over the next 5 to 9 years is twice as high for indigenous Australians (HR 2.04, 95% CI 1.17-3.57, p=0.012). A similar pattern was also reported for the gap in survival between indigenous and non-indigenous Australians commencing renal replacement therapy before and after adjustment of age and other comorbidities.[244]

The differences in baseline characteristics between the indigenous and non-indigenous cohorts make interpretation of other mortality risk factors difficult. While our study did not have enough power to separately analyse the indigenous Australian cohort, by comparing the overall cohort (including indigenous Australians) with the non-indigenous cohort, a few conclusions can be extrapolated.

Chronic renal failure and renal failure requiring dialysis were the most significant variables predicting mortality in the overall cohort. Renal failure requiring dialysis, representing end stage kidney disease, lost significance in the non-indigenous Australian cohort. This suggests that renal failure is a more important risk factor of mortality in indigenous Australians. In the Central Australian Ocular Health Study, the presence of DR among diabetic indigenous Australians, increased the risk of death from renal disease by almost 3 fold.[245] End stage renal disease is more than six fold more prevalent in indigenous Australians compared with non-indigenous Australians,[246] and in our study population, renal failure requiring dialysis was the most significantly different baseline variable when comparing indigenous and non-indigenous Australians (23.1% versus 10.7%, p<0.001). In other studies, renal failure is the most common significant predictor of mortality following diabetic vitrectomy.[230, 232, 234, 235] Helbig et al reported a hazard ratio of 1.42 (p=0.044) for a creatinine level >150mmol[230] and Kim et al reported a hazard ratio of 4.2 (p<0.001) for renal failure requiring dialysis.[235] The link between renal failure and mortality is unsurprising because chronic renal failure is known

to be associated with a high risk of cardiovascular related death[247] and this is the most common cause of death following diabetic vitrectomy.

Our data suggest that T2DM, longer duration of diabetes and legal blindness at baseline also contribute to mortality risk, although to a lesser degree than renal failure. These associations only reached statistical significance in non-indigenous Australians. Type of diabetes and duration of diabetes were not strongly associated with mortality in the overall cohort, however once indigenous Australians were removed from the analyses, these variables became significant and were selected for the multivariate model. In other studies, type and duration of diabetes were also important predictors of mortality but were not the most significant.[30] T2DM and longer duration of diabetes are associated with increasing age, and therefore increased mortality. Longer duration of diabetes increases risk of microvascular and macrovascular complications of diabetes such as chronic renal disease and cardiovascular disease. Interestingly, legal blindness at time of vitrectomy became significant in the non-indigenous population of our study, but lost significance after multivariate analyses. Poor vision is associated with social, functional and medical decline,[30] and poor visual acuity has been previously independently linked to mortality.[248]

The overall strengths of this study include the study design which allowed us to accurately capture the whole target population. Accurate data that includes rural and remote communities in SA and the NT are often difficult to obtain, and therefore a challenge when conducting population based studies. Vitrectomies in SA and the NT are only performed in two tertiary centre hospitals and a small number of private hospitals and all of these were audited between 2007 and 2011. The study had a long follow up time, of up to 9 years and it is the first study to be conducted in an Australian population.

There are some limitations of this study. It is possible that not all deaths were captured as participants may have moved interstate. Only deaths recorded in SA and the NT were captured through our methodology. Death data are also more likely to be underestimated in indigenous Australians due to under-identification.[249] Patients who were unable to have a vitrectomy (due to poor fitness for surgery, declined surgery or socioeconomically disadvantaged) would not be included in this study and this could further underestimate mortality rates. Pars

plana vitrectomy was performed by a small number of surgeons in SA, and we have made the assumption that there were no major differences in surgical methods during the five year period of the audit. We were also not able to accurately explore all prognostic variables. HbA1c measurement available to us was a single measurement prior to surgery, and this may not accurately reflect the patient's overall glycaemic control over time. Data on prior heart disease or neuropathy, which have been shown to be associated with mortality in other similar studies,[232, 234] were also not available. Separate analyses of the indigenous Australian group did not yield significant results, possibly due to inadequate power.

2.5 Conclusion

Long term mortality rates after primary diabetic vitrectomy in SA and the NT are similar to other populations around the world.[230-235] A substantial proportion of those undergoing diabetic vitrectomy in SA and the NT are indigenous Australians. The risk of death over the next 5 to 9 years is twice as high for indigenous Australians compared with non-indigenous Australians after age adjustment. It is important to recognise chronic renal failure as a significant comorbidity contributing to mortality, particularly in indigenous Australians. Our data suggest that ophthalmologists managing indigenous patients with severe diabetic retinopathy should refer patients for investigation and management of potentially co-existing renal failure and vice versa. In non-indigenous Australians, T2DM, longer duration of diabetes and being legally blind at baseline are additional risk factors for increased mortality. Patients with these risk factors should be referred to a physician for better management of their diabetes and cardiovascular health. This information can guide allocation of future resources to improve the prognosis of these high risk groups, and assist in closing the gap in mortality between indigenous and non-indigenous Australians.

CHAPTER 3: PREVALENCE AND SEVERITY OF DIABETIC RETINOPATHY IN INDIGENOUS AUSTRALIANS WITH END STAGE RENAL DISEASE

3.1 Introduction

DR and diabetic nephropathy (DN) are common microvascular complications in both T1DM and T2DM.[250, 251] They share common biological risk factors such as duration of diabetes and glycaemic control, suggesting common pathogenic mechanisms.[252] DR and DN tend to occur together and this has been demonstrated in large epidemiology studies worldwide.[253-256]

DN is a significant problem among indigenous Australians. National data from the Australian Institute of Health and Welfare reported that the incidence of end stage renal disease was five times higher in indigenous Australians when compared with non-indigenous Australians during 1997 to 2013.[257] The largest renal dialysis unit in the Southern Hemisphere is located in Central Australia, where the majority of patients are indigenous with diabetes as the primary cause of renal failure. The Australian and New Zealand Dialysis and Transplant registry reported in 2000 that out of 53 people commencing on renal replacement therapy in the northern territory, 43 (81%) were indigenous Australian.[258] Indigenous Australians living in remote regions are less likely to access medical care, and have higher mortality rates, and therefore this may underestimate reported incidence rates.

The association between retinopathy and nephropathy is strong in indigenous Australian communities. As discussed in chapter 2, our population based audit showed that indigenous Australians in Central Australia and South Australia with end stage DR were significantly more likely to have end stage renal disease and have higher mortality rates than non-indigenous Australians. It is unknown whether the inverse is true; whether high rates of DR exist in indigenous Australians with end stage DN. We therefore determined this by conducting a cross-sectional population based study in Central Australia to screen for DR in patients in dialysis units. Should DR be more prevalent and severe among the renal dialysis population, then a targeted screening strategy for this population could be a cost-effective way to reduce vision threatening disease.

3.2 Methods

3.2.1 Ethics statement

The research team in Alice Springs consulted the Directors of Western Desert Dialysis, an Aboriginal corporation that provides dialysis and social support in remote Aboriginal communities, during the early stages of the project. With their support, this project was approved by the Central Australian Human Research Ethics Committee. It adheres to the tenets of the Declaration of Helsinki. The aims of the study were explained with an interpreter, and written informed consent was obtained from each participant before enrolment in the study.

3.2.2 Recruitment and data collection

Suitable participants were identified by clinical staff at Flynn Drive Renal Unit and Alice Springs Renal Unit. We included all Indigenous Australians, 18 years and older, in Central Australia who received dialysis for end stage renal disease from diabetes. Recruitment began in November 2018 and ceased when all suitable participants at each site had been approached (February 2019). We excluded any participants unable to give consent, who had cognitive difficulties and were unable to obtain retinopathy grading (due to opaque media such as cataract or other comorbid retinal pathologies).

Potential participants were then approached by members of the research team and interpreters from the Aboriginal Interpreter Services in Alice Springs to obtain written consent, conduct questionnaires and perform an eye examination. In consultation with the renal consumer group, Central Australian Renal Voice, five languages were represented: Pintupi/Luritja, Warlpiri, Pitjantjatjara, Arrernte and Alywarra. Demographic and clinical information were collected through patient questionnaires and medical records. Best corrected visual acuity was measured with a Snellen Tumbling E chart. Intraocular pressure was measured with a portable hand held tonometer. Participants were dilated with 1% Tropicamide & 2.5% Phenylephrine in each eye. A minimum of one macula centred photograph of each eye was taken with the Canon Digital Non-Mydriatic Retinal Camera. Photographs were assessed for quality, and the best quality image was selected for retinopathy grading. Table 3.1 shows how photograph quality was assessed.

Table 3.1 Criteria for grading of photograph quality used in the study for diabetic retinopathy

Quality of photograph	Criteria
Good	Macula centred, blood vessels and micro aneurysms are clearly identifiable, no or minimal shadowing across the central part of the image
Moderate	Clarity or illumination decreased in quality
Poor	Difficult to grade with certainty
Ungradable	Obscuration of most or all of the available image

Grading of retinopathy photographs were undertaken by consultant ophthalmologists, as per the ICDSS and use of ETDRS standard photographs. One eye per patient was included in the analysis. The worst grading of the two eyes was used as the final grading.

3.2.3 Data analyses

De-identified data was transcribed to and analysed using SPSS version 25.0 for Mac OS X (IBM SPSS Statistics 25.0, SPSS Inc., Armonk, NY, USA. The crude rate of DR, segregated by DR severity was determined. Best corrected visual acuity was converted from Snellen fractions to ETDRS letters for statistical analyses. Results are presented as proportions or means and standard deviations unless otherwise specified.

3.3 Results

A total of 103 participants were recruited from Flynn Drive Renal Unit (n= 45) and Alice Springs Renal Unit (n = 58). Table 3.2 summarizes the characteristics of the cohort. The mean age of the group was 54 years, with the youngest participant having dialysis at age 30. A large majority were female (n = 72, 69.9%). All participants had T2DM, with an average HbA1c of 7.09% (4.9-16) taken from the 3 most recent measurements. It was difficult to determine the duration of diabetes due to missing data, but the average duration of dialysis was 3.14 years (0-13). Hypertension was a common comorbidity with 92.2% of the cohort affected. Ischaemic heart disease was the most common macrovascular diabetic complication (40.8%), followed by peripheral vascular disease (28.6%) and cerebral vascular disease (n = 7.8%).

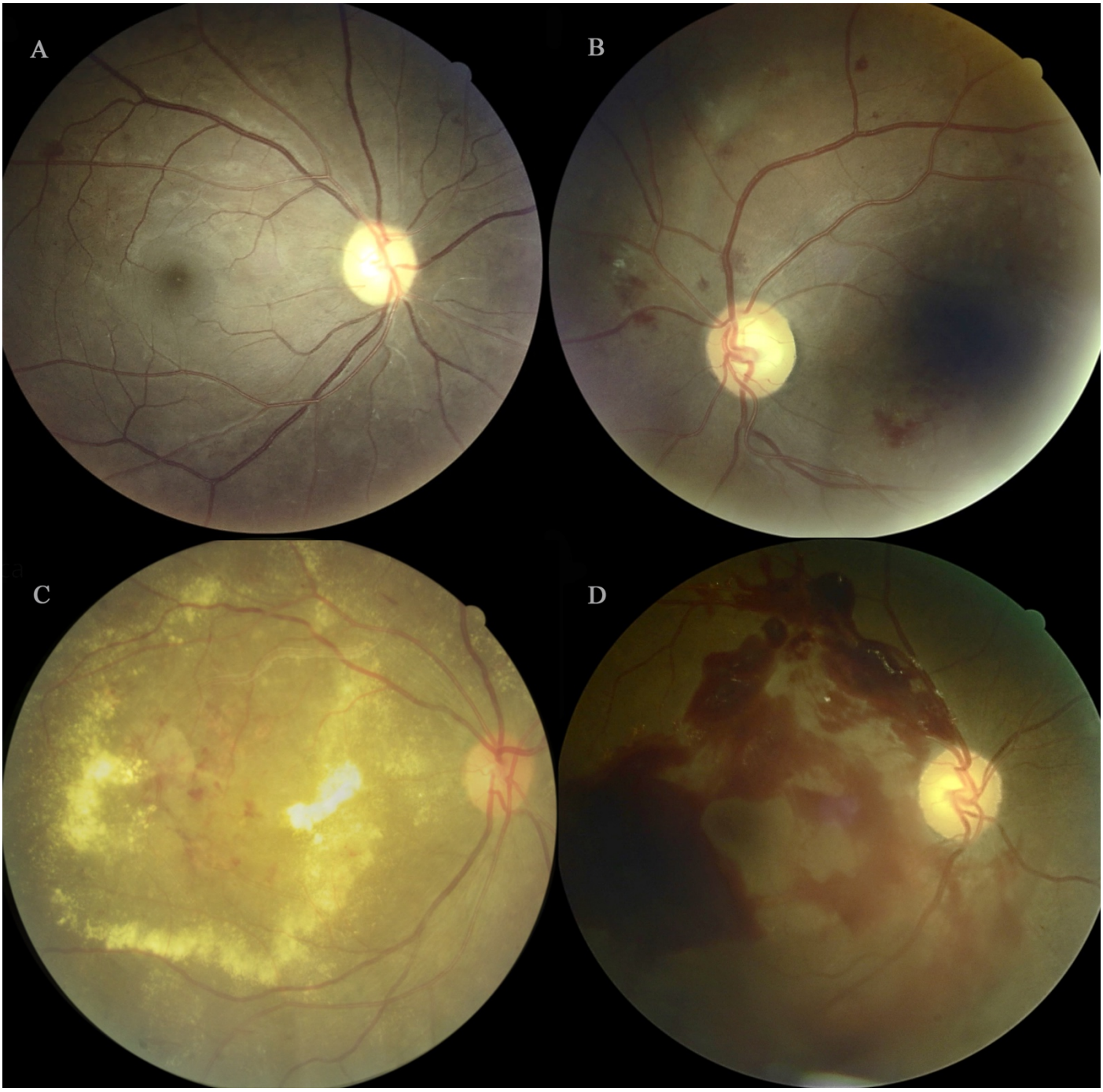
Table 3.2 Demographic and clinical characteristics of study cohort

Demographics		N
Age (years)	54 (30-81)	103
Sex (female)	73 (69.9%)	103
Diabetes history		
Type 2 diabetes mellitus	103 (100%)	103
Duration of diabetes (years)	17.71 (1-38)	21
Average of 3 most recent HbA1c (%)	7.09 (4.9-16)	102
Duration of dialysis (years)	3.14 (0-13)	100
Comorbidities and other complications		
Hypertension	94 (92.2%)	102
Hypercholesterolemia	21 (37.5%)	56
Ischaemic heart disease	42 (40.8%)	103
Cerebral vascular disease	8 (7.8%)	102
Peripheral Vascular disease	28 (28.6%)	98

Mean and range are presented for continuous variables. Number and proportion are presented for categorical variables. The number of participants with available data are presented in column labelled "N".

Out of the 103 participants (206 eyes, 206 retinal photographs taken), 4 photographs were ungradeable and 14 photographs were poor quality due to poor view, dense cataract, phthisical or prosthetic eyes. The remaining 188 photographs were of adequate quality to be graded. Using the worst eye as the final DR grade, 102 out of 103 participants were able to be assigned a grade. Twelve participants (11.8%) had no DR, 53 (52%) had mild NPDR, 25 (24.5%) had moderate NPDR, 8 (7.8%) had severe NPDR and 4 (3.9%) had PDR. Presence of CSMO was not recorded as it cannot be accurately determined from photographs. Examples of photographs from the study in each grade is shown in Figure 3.1.

Figure 3.1 Examples of photographs from the study showing each diabetic retinopathy grading



Legend: A) Mild NPDR; micro aneurysms and few dot haemorrhages. B) Moderate NPDR; more dot and blot haemorrhages C) Severe NPDR and diabetic macular oedema; 2 quadrants of venous beading. D) PDR; vitreous haemorrhage from likely proliferative vessels.

Sixty-eight (66%) had an eye exam within the last 12 months by either an ophthalmologist or an optometrist. Thirty-four (33%) could be identified as having previous CSMO in one or both eyes. Thirty-three (32%) had received an anti-VEGF injection for treatment of CSMO. Forty-seven (45.6%) had been previously treated with laser for either previous DMO or PDR (focal, grid or pan photocoagulation). Seven (6.8%) had undergone vitrectomy for diabetic vitreous haemorrhage from PDR. Of those who had previously received treatment for PDR (pan coagulation laser or vitrectomy), one participant was found to have reoccurring PDR in the same eye and was therefore promptly referred for retreatment. A large proportion of those who had not been screened nor treated in the preceding 12 months had more advanced forms of DR; moderate NPDR (n = 17, 68%), severe NPDR (n = 2, 25%) and PDR (n = 2, 50%).

Best corrected visual acuity in the better performing eye was analysed as it best corresponds to functional capacity. The mean and median BCVA were 70 ETDRS letters (range 0-85) and 75 ETDRS letters, respectively. Twenty-nine (28%) participants did not meet Australian driving standards (worse than 6/12 Snellen or 70 ETDRS letters). Table 3.3 summarises these ocular findings.

Table 3.3 Ocular health of study cohort

DR grading (n = 102)	
No DR	12 (11.8%)
Any DR	90 (88.2%)
Mild NPDR	53 (52%)
Moderate NPDR	25 (24.5%)
Severe NPDR	8 (7.8%)
PDR	4 (3.9%)
Previous treatment for DR	
Screening within the last 12 months	68 (66%)
Laser for DMO or PDR	47 (45.6%)
Anti-VEGF injection for CSMO	33 (32%)
Vitrectomy	7 (6.8%)
Functional capacity	
BCVA(best eye, ETDRS letters)	70 (0-85)
Not meeting driving standards (worse than 6/12 or 70 ETDRS letters)	29 (28%)

A comparison of studied characteristics between those who had no DR and those who had DR showed no significant differences in demographics, comorbidities or other complications. The only significant differences were duration of dialysis. Within the dialysis population, those with DR had longer duration of dialysis (3.42 vs 1.18 years, $p = 0.032$).

An additional co-benefit in conducting this research was that we could educate participants and their communities the importance of DR screening. At time of screening, participants were shown their retinal photographs and given direct feedback regarding their eye health. Retinopathy requiring treatment was referred to and managed by the eye clinic at Alice Springs Hospital. Results were presented to the Directors of Western Desert Dialysis and consumer group, Central Australia Renal Voice, and current work with this group is ensuring that the findings are explained in a context and linguistically appropriate way, and relayed back to remote Aboriginal communities in the region. A presentation was given to the stakeholders involved in the regional eye service (the Central Australian and Barkly Integrated Eye Health Service Committee) which included representatives of the various Aboriginal Medical Services (Congress), the Fred Hollows Foundation, department of Health and the University of Melbourne Indigenous eye health unit.

3.4 Discussion

Surprisingly, few studies have recorded the prevalence of DR in diabetic renal dialysis patients. Among 117 Chinese diabetic patients undergoing peritoneal dialysis, 69% (n=81) were found to have DR on fundoscopic examination but the severity of DR was not examined.[259] In another study, among 252 diabetic patients undergoing haemodialysis, 45% (n=113) had any DR.[260] The largest study involved 19 diabetic clinics in Italy. Among 258 European T2DM patients with an estimated glomerular filtration rate of less than 30 (meeting dialysis criteria), 47% (n=120) had DR on dilated fundoscopic exam, of which 29% (n=72) had advanced DR (defined as severe NPDR, PDR or maculopathy).[261] Compared to other dialysis populations, our cohort had much higher rates of any DR (88.2%) and advanced DR (38%). No study has determined rates of CSMO or DMO among diabetic dialysis patients. Studies have instead explored whether dialysis affects macula leakage and macula thickness pre and post commencing dialysis.[262, 263] These studies are small and do not confirm any positive associations.

Rates of DR in this study were much higher than that of the general indigenous Australian communities when compared to data from the Central Australia Ocular Health Study; 88.2% vs 22.2% for any DR, 38% vs 7% for advanced DR.[127] As expected, those with severe renal disease also had worse retinopathy. Loose

comparisons between non-indigenous populations (of different studies) show that rates of DR in dialysis populations versus general diabetic population were 47% vs 28.5% for any DR and 29% vs 4.5% for advanced DR.[131, 261] Previous epidemiology studies have identified that indigenous Australians have significantly worse retinopathy than non-indigenous Australians, despite having similar rates of any DR.[125] Our data supports that both mild and severe cases of retinopathy are likely to be concentrated among those with severe DN in indigenous Australian populations, thus making it an opportunistic population for screening and intervention.

We identified 66% of this cohort had their eyes screened for DR within the last 12 months. This is towards the lower end of reported compliance worldwide (61 to 89%),[264] but is encouragingly higher than the 20% adherence rate of the general indigenous diabetic population as reported in the National Eye Health Survey.[153] This study was not designed to explore underlying reasons for non-compliance, however some important reasons were identified through conversations with participants. Very few were aware of the link between diet, exercise, diabetes and its complications, particularly for participants from more remote communities. Good vision in one eye discouraged people from seeking help, until both eyes were affected. Poor health was attributed to cultural reasons such as 'bad spirits' or punishment for wrong doing, thus seeking for medical help was not seen as a priority.

An interesting observation was the higher proportion of females in the indigenous renal dialysis cohort. Similarly, the indigenous cohort with end stage diabetic retinopathy in chapter 2 also had a significantly lower proportion of males when compared with the non-indigenous cohort (Table 2.1). The Australian and New Zealand Dialysis and Transplant registry reported an incidence rate of 513 per million population for indigenous females receiving dialysis compared with 406 per million population for indigenous men receiving dialysis.[265] Indigenous males have a higher mortality rate than females and this could explain the lower proportion of males with end stage diabetic complications. ABS data from 2008 to 2012, predict male life expectancy is lower than females; 68 years compared with 63 years respectively, for indigenous Australians living in the Northern Territory.[266] The Central Australian Ocular Health Study showed that indigenous females were 17% less likely to die in the 10 year follow up period from 2009 to 2013.[267] Female mortality is

generally lower than males worldwide for a number of reasons including biological, social and behavioural factors. Oestrogen has a protective effect on cardiovascular disease, and may be protective in other diseases.[268] Females are more willing to report health problems and use health services.[269] Men are more prone to high risk behaviours in certain age groups and this increases overall male mortality.[270] Additional risk factors for the higher proportion of females on dialysis in the indigenous population include higher rates of albuminuria (an important predictor of progressive kidney disease),[271] increased rates of renal comorbidities such as post streptococcal glomerulonephritis during childhood, and being genetically predisposed to lower nephron numbers.[271-273] Socioeconomic vulnerabilities such as limited access to education, employment opportunities, increased carer responsibilities and exposure to violence, particularly in remote communities, are postulated to also contribute to the gender disparity in the indigenous dialysis population.[265]

The desired outcome of DR screening is identification of those who require direct treatment and those who should be screened more frequently and have their risk factors optimised. Current treatments for DR are only available for advanced DR (severe NPDR, PDR or DMO). We identified 12 (11.8%) of the cohort had severe NPDR or PDR which were promptly referred for treatment. Four of these (33%) had not been screened or treated in the preceding 12 months. Most of those identified with advanced DR had milder forms of DR previously. One participant had a reoccurrence of PDR despite previous laser treatment. This study did not screen for DMO, as we did not have a portable slit lamp or OCT machine. All participants with any form of DR (which were most of the cohort) were given advice on the importance of regular eye screening and optimizing their glycaemic control. With their consent, their health information from the study was forwarded to their renal and primary care physician for ongoing management.

The existing dialysis units in Alice Springs could provide a great framework for targeted DR screening to improve current screening rates. Patients require dialysis three times a week, and the majority are transported to either of the two units located in Alice Springs. Provision of dialysis, transport arrangements between remote communities, interpreters and community workers to provide cultural support is already maintained by organizations such as the Central Australia Renal Voice, various Aboriginal Medical Services in the

community and the public hospital. Systems thinking and innovative evaluation are key elements to consider when improving health care in resource poor settings.[274] Results of this research have been disseminated to hospital staff via departmental meetings and education opportunities.

Weaknesses of this study were related to difficulties in data acquisition. While we recruited all diabetic patients on dialysis in the two main units in Alice Springs, we were unable to recruit those who received dialysis from the 18 mobile dialysis units coordinated by the organisation Purple House. The most accurate clinical information was sought from participants, clinicians and hospital records, but some data was missing. For example, duration of diabetes was extremely difficult to determine, as was renal biopsy to confirm a diabetic cause of renal failure. Tumbling E Snellen charts were used to accommodate illiteracy, and best corrected visual acuity was converted to ETDRS letters for statistical purposes, thus could affect the accuracy of vision when comparing to other studies. Patients could not be examined at the slit lamp and grading of retinopathy was performed via photographs of various quality. In many cases, only one macula centred photo was obtained. One macula centred photo covers 60% of the retina imaged in a seven-field 30 degrees photo from the ETDRS. Two photos, one macula and one disc centred, covers 80% and is therefore more accurate in capturing moderate to severe retinopathy.[275, 276] Therefore this study likely underestimated the severity of DR in this population. Recent previous DR grading on slit lamp were not always performed and thus could not be used for comparison. OCT imaging was not available to assess and study the presence of DMO among this population.

Strengths of this study are as follows. This was a population based study, with minimal sampling as all diabetic participants at the two sites were recruited. The study is the first of its kind in determining rates of DR among indigenous Australians undergoing dialysis for DN. Our experience with conducting this study also confirmed the importance of key strategies in implementing health changes in indigenous populations.[156] An early collaboration with stake holders of the community was essential to establish and complete the study, as well as discuss how the information could be advocated to benefit the communities. Engagement with interpreters

and direct feedback to participants has raised awareness and helped educate participants about the importance of DR screening.

3.5 Conclusion

In this population based study, we identified the rates of any DR and advanced DR were 88.2% and 38%, respectively among indigenous Australians in Central Australia with end stage DN requiring dialysis. Adherence to previous eye screening as per recommended guidelines was low at 66%. Our data supports that both mild and severe cases of retinopathy are likely to be concentrated among diabetic dialysis patients, thus making it an opportunistic population for screening and intervention in resource poor settings. It is hoped that our experiences with the indigenous communities in conducting this study may lead to improvements in the current service provisions for DR screening and treatments in Alice Springs, and other resource poor and indigenous communities around the world.

CHAPTER 4: DEXAMETHASONE INTRAVITREAL IMPLANT TO PREVENT BLINDNESS FROM DIABETIC MACULOPATHY IN INDIGENOUS AUSTRALIANS – A RANDOMISED CONTROLLED TRIAL

4.1 Introduction

Diabetic macular oedema is more prevalent in indigenous Australians than non-indigenous Australians. From the previous chapter, the prevalence of DMO was at least 30% among indigenous Australians in Central Australia with end stage renal failure. A meta-analysis of 6 indigenous Australian studies (n = 2865) and 5 non-indigenous Australian studies (n = 9801) found that the estimated prevalence of DMO among those with diabetes was 7.6% versus 4.9% respectively ($\chi^2 = 6.67$, P = 0.01).[125] In addition, the prevalence of diabetes is eight times more common, meaning that even greater numbers of indigenous Australians develop DMO.[133, 238] Therefore a strategy needs to be developed in order to reduce this disparity.

Multiple reasons can explain this trend. DMO is more common in T2DM, and the majority of Indigenous Australians develop T2DM, with T1DM being extremely rare.[277] Earlier onset of diabetes is well documented [139, 278, 279] and increases the risk of sight threatening disease.[141] Delays in diabetes diagnosis[154], DR screening and treatment[153] remain significant problems, contributing to inadequate risk factor management, faster progression and increasing severity of DR.[155]

Approximately 63% of Indigenous Australians live in regional and remote communities, compared to 28% of non-indigenous Australians, placing them at a disadvantage to accessing frequent medical care.[126] In addition to extreme travel distances, low income, competing priorities, communication barriers and poor understanding of health, result in frequent non-attendance for screening, prevention and treatment of medical conditions.[280] Unfortunately, the best current standard of care for DMO is regular monthly intraocular injections of an anti-VEGF agent such as bevacizumab. Over the last twenty years, multiple clinical trials have shown that monthly intravitreal injections of ranibizumab or bevacizumab is superior to laser treatment in improving vision but only when a high level of compliance is achieved.[80-82] In real world clinical practice, even in suburban areas, patients undergo less frequent monitoring and achieve inferior visual outcomes to landmark trials.[112]

It is imperative to try and tailor health systems to a population's specific needs. Central Australia is home to approximately 35,000 people, one-third of whom are Indigenous, living in over 30 very remote communities which are 200 to 400km away from hospital facilities in Alice Springs. Ophthalmology services are provided from one hospital and a remote clinic is only available once or twice a year in small communities because of staff and resource limitations.[281] Monthly anti-VEGF regimens are therefore impractical in populations where compliance and access to ophthalmology services remain significant issues.

In 2014, the Food and Drug Administration approved a new drug, intravitreal dexamethasone implant (Ozurdex, Allergan, Irvine, CA, United States), for the treatment of DMO[117] that was followed by Pharmaceutical Benefits Scheme approval in Australia.[118] The biodegradable intravitreal implant slowly releases dexamethasone; a highly potent steroid which has anti-inflammatory effects.[282] Intravitreal concentrations peak within 3 months and can be sustained for up to 6 months post injection.[283] The BEVORDEX RCT showed that dexamethasone implant achieved similar rates of visual acuity improvement compared with bevacizumab; 41% compared to 40% participants achieved more than 10 ETDRS letters at 12 months with fewer injections (2.7 compared to 8.6 mean injections over 12 months).[116] Increased rates of cataract and elevated IOP are the main adverse effects of intravitreal corticosteroid treatments.[284] Progression of DMO after cataract surgery is also frequently observed, particularly in patients with pre-existing DR and DMO.[285] Therefore use of dexamethasone implant is best suited for pseudophakic patients or patients who are scheduled to have cataract surgery.

We developed a hypothesis that intravitreal dexamethasone implant is likely to have greater feasibility in treating DMO in rural and remote indigenous Australian communities compared with conventional anti-VEGF agents. Treatment limited to 3 to 6 monthly rather than monthly will likely offset issues with poor access to health care and low patient compliance. No clinical trial has explored the efficacy and safety of using dexamethasone implant for DMO in remote indigenous Australian communities. Therefore, we conducted a RCT to compare intravitreal dexamethasone implant to intravitreal bevacizumab for the treatment and prevention of DMO after cataract surgery in Central Australia. If a practical regimen is found to work at least

as well as the existing combination bevacizumab and laser treatment, then it may have implications for managing DMO in remote Australia, as well as remote communities in other parts of the world where regular and frequent attendance is not possible.

4.2 Methods

4.2.1 Ethics statement

This project was approved by the Central Australian human research ethics committee. It adheres to the tenets of the Declaration of Helsinki. The aims of the study were explained, with an interpreter whenever necessary, and written informed consent was obtained from each participant before study enrolment.

4.2.2 Patient enrollment

This study was designed in 2014 and patient recruitment began in August 2015. It was a single site and single surgeon study. Patients were recruited from Central Australia, who were being treated by the Central Australian & Barkly Integrated Eye Health Service. This encompasses more than 30 surrounding remote communities. The inclusion criteria were: adult indigenous Australians with significant lens opacity (more than grade 3 for any type of cataract as per the Lens Opacity Classification System III[286]) scheduled for cataract surgery, having any active DMO (diagnosed on clinical exam or OCT) or at risk of DMO (previous DMO, more severe than moderate NPDR) as assessed on clinical examination at the time of enrolment. The exclusion criteria included: 1) prior intervention in the affected eye (intravitreal anti-VEGF injections within the last 6 weeks, laser treatment within the last 3 months, or intravitreal triamcinolone injections within the last 6 months), 2) history of open-angle glaucoma, steroid induced IOP elevation requiring treatment or IOP $\geq$25, and 3) eyes with concurrent ocular pathology other than DMO and cataract causing visual loss.

4.2.3 Sample size

Sample size calculations were difficult to determine as there was little data to base them on. A non-inferiority design was initially sought, based on data from patients with DR who were treated with intravitreal bevacizumab at the time of cataract surgery.[287] Takamura et al showed that those with intravitreal bevacizumab gained a mean of 25 ETDRS letters at 3 months post operatively, compared with a mean of 17 ETDRS letters

in those who had no adjuvant treatment. Thus, a tentative 15 ETDRS letter limit was set, which resulted in a sample size calculation of 40 participants (20 participants in each arm) to allow for 80% power and 5% significance level.[288, 289]

4.2.4 Treatment assignment

The two treatment arms were intravitreal dexamethasone implant (0.5mg) and intravitreal bevacizumab (1.25mg/0.5mL) given at the time of cataract surgery. Randomisation was performed via concealed envelopes containing the treatment allocation, which were randomly numbered from 1 to 40 by a statistician not involved in the study. The envelopes were allocated in a sequential manner and opened after participant consent was gained. The treating ophthalmologist and nursing staff were not blinded to the treatment allocation due to limitations in staff in remote clinic locations.

4.2.5 Data collection

At study enrollment and prior to cataract surgery, baseline clinical information collected included best corrected visual acuity (BCVA), IOP, DR grading and central retinal thickness (CRT) measured on optical coherence tomography (OCT) for the eye to be operated on. The same Zeiss Cirrus Photo 800 machine was used for all patients. BCVA was measured with tumbling E acuity charts as most participants could not read English letters. Acuity was initially measured as a Snellen fraction, but was then converted to logMAR acuity for statistical analyses and comparison to ETDRS trials.[290] IOP was measured using Icare tonometer (Tiolat Oy, Helsinki, Finland). DR grade was determined by an ophthalmologist on dilated fundoscopic examination.

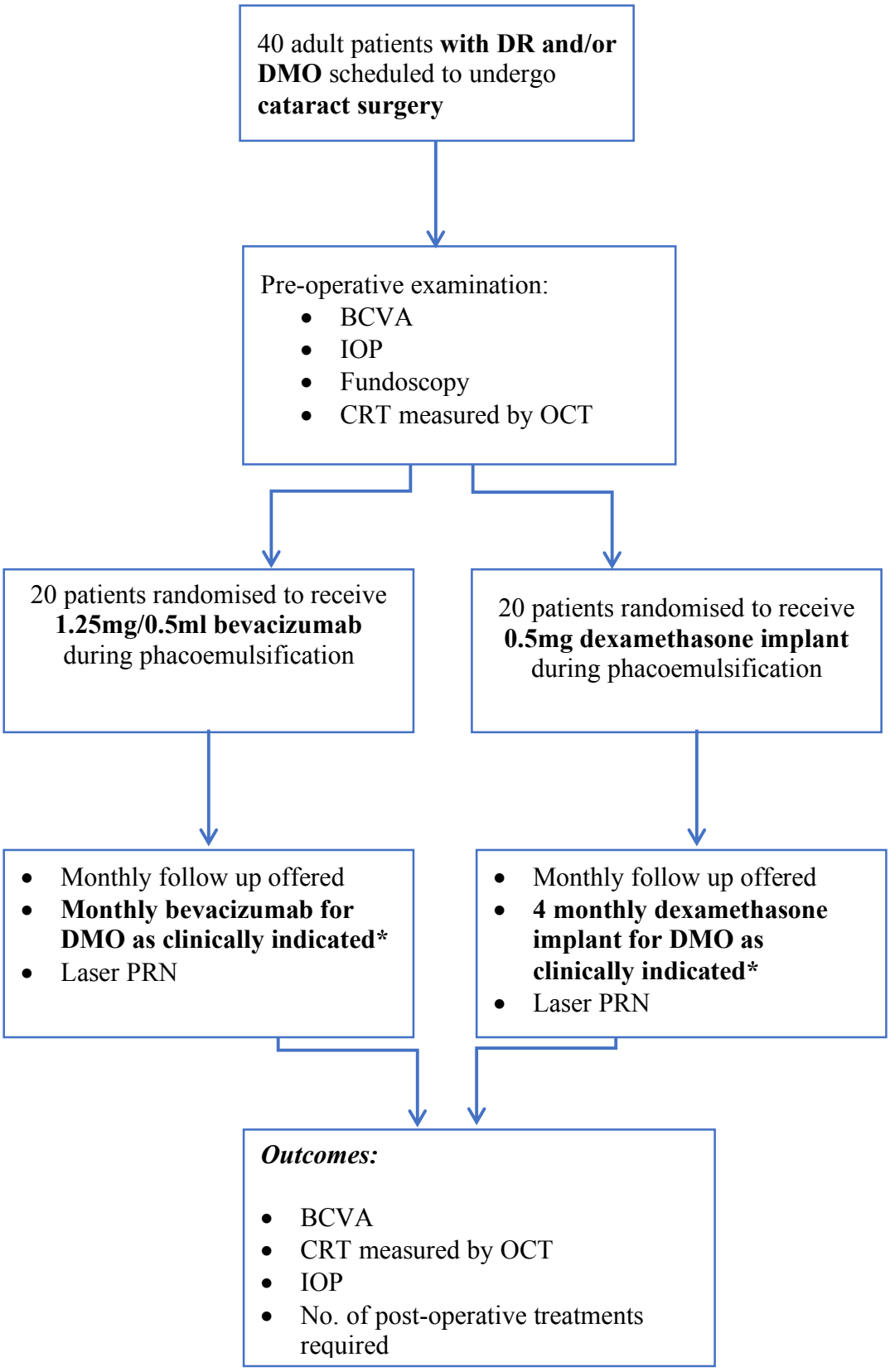
Patients were offered monthly outpatient clinic review and ideal standard of care for a follow up period of 12 months after cataract surgery. We worked closely with an indigenous liaison officer to communicate with individuals and their communities about follow up visits. At each visit, they underwent BCVA and IOP measurement, dilated fundoscopic exam, OCT imaging and consideration for retreatment. Administration of further intravitreal injections or laser treatment post-operatively was at the discretion of the treating

ophthalmologist and based on the presence of DMO on clinical examination and OCT imaging. The minimum time between post-operative intravitreal bevacizumab was set at 1 month and between post-operative intravitreal dexamethasone implant at 4 months.

4.2.6 Outcome measurements

The primary outcome was change in BCVA at 3, 6 and 12 months; where an expected gain in BCVA would translate to adequate treatment response and improved vision. Secondary outcome measurements included the following: 1) change in CRT measured by OCT, where a decrease in CRT would reflect resolving DMO; (2) number of intravitreal injections required post-operatively; (3) amount of additional laser treatment required; and (4) adverse events. Significant elevation of IOP was defined as more than 5mmHg compared with baseline. Figure 4.1 illustrates and summarizes the study design and protocol.

Figure 4.1: Study Protocol



**Re-treatment criteria: DMO affecting or threatening BCVA.*

Abbreviations: DR, diabetic retinopathy; DMO, Diabetic Macular Oedema; BCVA, Best corrected visual acuity; IOP, intraocular pressure; CRT, central retinal thickness; OCT, Optical coherence tomography; PRN, as needed.

4.2.7 Statistical analysis

Statistical analyses were performed with SPSS version 20.0 for Mac OS X (IBM SPSS Statistics 20.0, SPSS Inc., Armonk, NY, USA). Mann–Whitney U-tests and chi-squared tests were used to compare differences in continuous and categorical variables between the two treatment groups. Outcome measures, where appropriate, were assessed with logistic regression adjusting for age, sex and baseline BCVA.

4.3 Preliminary results

4.3.1 Baseline characteristics of the study

Patient recruitment began in August 2015, and to date, we have enrolled 30 eyes from 29 patients, of which 13 were randomised to receive bevacizumab and 17 received dexamethasone implant. One patient was removed because of developing vitreous haemorrhage and requiring vitrectomy. One patient had both eyes eligible for study recruitment. Eighteen patients (n = 19 eyes) have completed their 12 month follow up period.

Table 4.1 shows that the baseline characteristics of the two treatment groups at time of cataract surgery were similar.

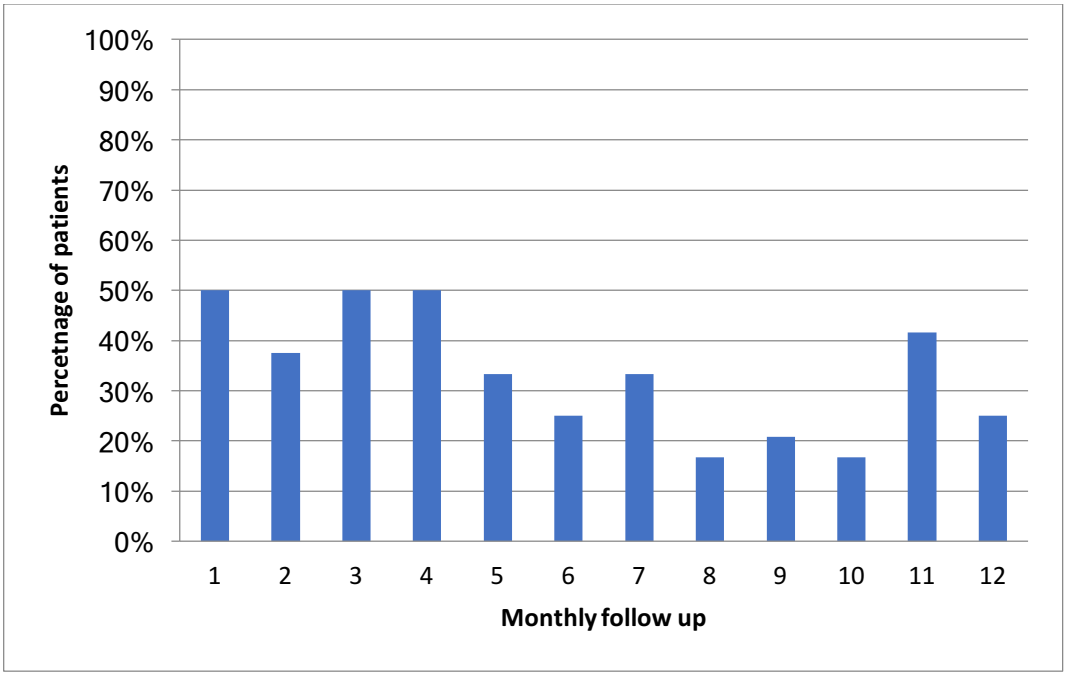
Table 4.1: Comparison of baseline characteristics between treatment groups

Characteristic	Bevacizumab		Dexamethasone implant		P value
	Mean, SD	n	Mean, SD	n	
Age (years)	58.0±5.6	13	52.5±17.5	17	0.390
Female (%)	76.9%	13	76.5%	17	0.977
BCVA (ETDRS letters)	45.4±24.0	12	42.9±33.7	12	0.726
CRT (micrometre)	230.9±42.8	8	319.0±123.9	9	0.163
IOP (mmHg)	9.5±3.0	10	10.3±2.5	13	0.238
Duration of diabetes (years)	15.3±7.3	6	15.5±6.5	13	0.720

Data presented are for those with available data; numbers are shown in the columns labelled 'n'. Data are mean and standard deviation unless otherwise indicated. Abbreviations: SD, standard deviation; BCVA, Best corrected visual acuity; CRT, central retinal thickness; IOP, intraocular pressure.

Non-attendance rates were high. Out of the 19 eyes that were followed up for 12 months, attendance rates were less than 50% at each visit (Figure 4.2).

Figure 4.2 Attendance rates at each monthly follow up after cataract surgery



4.3.1 Primary outcomes

High non-attendance rates and incomplete 12 month follow up for some participants led to missing data and difficulties in interpreting outcomes. All data were included to maximize preliminary analyses. Outcomes were stratified into groups for more meaningful analyses: 1 to 3 months, 4 to 6 months, 7 to 9 months and 10-12 months post cataract surgery and the first adjuvant treatment. No results were statistically significant, however some general trends were observed. There was improvement and maintenance of BCVA within 12 months compared with baseline in both treatment arms (Figure 4.2).

Figure 4.2 Best corrected visual acuity after cataract surgery and the first adjunct treatment. Mean and 95% confidence intervals are shown.

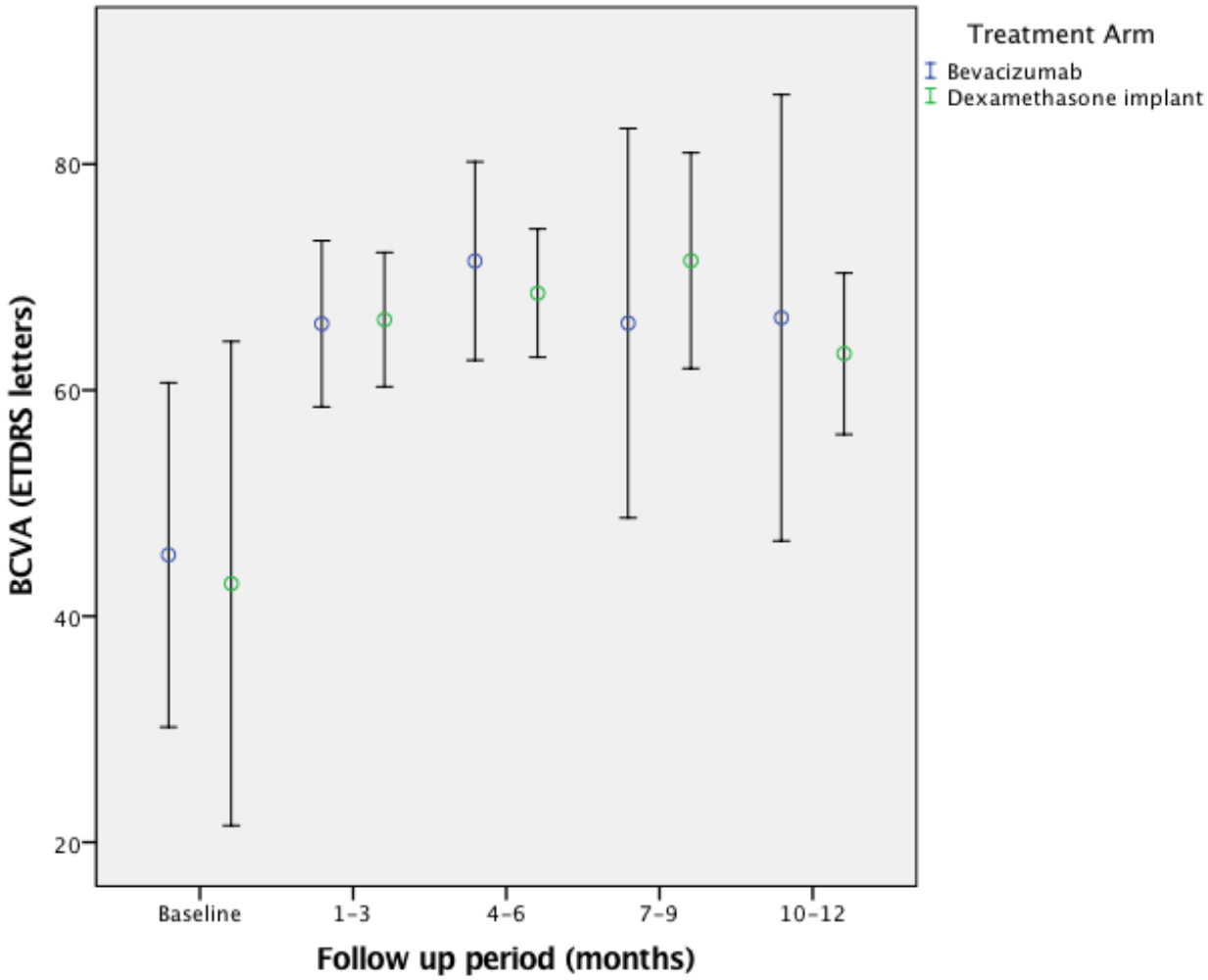


Table 4.2 shows that eyes in the dexamethasone implant group had a larger BCVA gain from baseline for all follow up times except at 7 to 9 months post cataract surgery.

Table 4.2 Mean gain in BCVA (ETDRS letters) at various time points after cataract surgery and the first adjuvant bevacizumab or dexamethasone implant

Months after cataract surgery and the first adjuvant treatment	Bevacizumab		Dexamethasone implant		P value (Mann-Whitney U test)
	Mean, SD	n	Mean, SD	n	
1 to 3 months	23.9±28.3	9	27.0±30.0	18	0.587
4 to 6 months	15.7±14.9	7	21.4±34.6	15	0.944
7 to 9 months	12.0±27.0	5	6.3 ±18.5	4	0.806
10 to 12 months	10.0±30.1	7	16.0±36.0	10	0.625

Data presented are for those with available data; numbers are shown in the columns labelled 'n'. Failure to attend follow up and incomplete 12 month follow up period were the reasons for missing data. Abbreviations: SD, standard deviation

At 1 to 3 months post cataract surgery, the proportion of eyes gaining more than 15 ETDRS letters was 55.6% vs 61.1% for the bevacizumab and dexamethasone implant groups respectively (p = 0.537), and 57.1% vs 60.0% at 4 to 6 months (p = 0.996) after adjusting for age, sex, duration of diabetes and baseline BCVA.

4.3.2 Secondary outcomes

A mean reduction in CRT was achieved for the dexamethasone implant treatment group at all follow up periods except for at 7 to 9 months where there was not enough data to determine a mean (n =1). In the bevacizumab group, a mean increase in CRT occurred at all follow up periods. (Table 4.3) Standard deviations were large, reflecting that some participants did not attend regular follow up and receive regular treatment, leading to various responses to treatment and reoccurrence of DMO.

Table 4.3 Mean change in central macular thickness

Months after cataract surgery	Bevacizumab		Dexamethasone implant		P value (Mann-Whitney U)
	Mean, SD	n	Mean, SD	n	
1 to 3 months	18±136	4	-65±214	6	0.522
4 to 6 months	39±161	5	-59±148	8	0.242
7 to 9 months	160±51	4	NA	1	0.480
10 to 12 months	51±108	5	-24±121	3	0.297

Data presented are for those with available data; numbers are shown in the columns labelled 'n'. Abbreviations: SD, standard deviation

The mean number of re-treatments and additional laser given over 12 months was less than one in both treatment groups (Table 4.3). Two patients in each treatment group were clinically indicated for re-treatment but refused. No participants in the dexamethasone implant group had an increase in intraocular pressure over 5mm Hg after treatment.

Table 4.4 Number of re-treatments, additional laser and raised intraocular pressure within 10 to 12 months post cataract surgery and adjunct treatment

Secondary outcome	Bevacizumab n = 7	Dexamethasone implant n = 12	P value
Number of re-treatments (mean, range, SD)	0.78 (0-5, 1.64)	0.63 (0-3, 0.87)	0.289
Number of additional laser treatments (mean, range, SD)	0.2 (0-1, 0.42)	0.13 (0-1, 0.34)	0.613
Raised intraocular pressure >5mmHg from baseline (n, %)	0 (0)	0 (0)	NA

Abbreviations: SD; standard deviation, n; number.

4.4 Discussion

Our experiences in conducting this trial revealed some unexpected trends in diabetic maculopathy among Indigenous Australians living in Central Australia. Firstly, there were surprisingly few participants suitable for trial inclusion, and hence recruitment of participants is still ongoing to reach a target sample size of 40. Many participants with recent interventions for DR had to be excluded as per our inclusion criteria to minimize confounders (intravitreal anti-VEGF injections within the last 6 weeks, laser within the last 3 months, or intravitreal triamcinolone within the last 6 months), reflecting the large burden of disease in Central Australia and adequate treatment of those who present to health services. DR is rapidly becoming the leading cause of vision loss in this population. Among indigenous Australians in Central Australia with visual impairment, 48.8% is due to DR.[228] Other causes of vision loss such as trachoma are declining.[291]

Extreme difficulties in ensuring frequent follow up and re-treatment are clear from our preliminary results. Despite working closely with indigenous liaison health workers, we could only ensure a follow up rate of approximately 50% which gradually decreased to 25% at 12 months after the initial cataract surgery. This is

considerably lower than other published real world studies on adherence to anti-VEGF treatments for DMO. In a French study of 72 eyes with DMO treated with ranibizumab, the 12 month follow up rate was 75%.[292] Low adherence to treatment is an issue for all diabetic populations. Diabetic patients typically have multiple appointments to medical specialities and are on several treatment regimes. Real world studies report adherence to oral hypoglycaemic agents is also low and variable, ranging from 36 to 93%.[293, 294] In a prospective observational study of 136 Caucasian patients with DMO and 109 patients with AMD, both requiring regular anti-VEGF injections, 46% of those with DMO missed an appointment compared with 22% of those with AMD. [295] A strong correlation was found between missed appointments and worse visual acuity ($p = 0.017$). Reasons for missed appointments identified during questionnaires were other illness, personal reasons and 'no explanation." No explanation was more frequently given by DMO patients compared with AMD patients. The increased burden of disease and 'treatment fatigue' is a likely contributing factor. We noticed specific socioeconomic and cultural barriers to follow up in Central Australia such as long travel distances between remote communities and Alice Springs Hospital, personal reasons such as deaths in the communities, and different concepts of illness as a "punishment that cannot be fixed."

Although no significant results were found in our small preliminary dataset, some general trends favouring the use of dexamethasone implants were observed. Dexamethasone implants resulted in a greater positive change in BCVA, correlating with a greater decrease in CRT achieved with fewer retreatments and additional laser within the 12 month follow up period. No published RCT of a similar design has been conducted to compare dexamethasone implants with bevacizumab in preventing DMO post cataract surgery. The two closest trials are the DiMECAT study (comparing another steroid, intravitreal triamcinolone, with bevacizumab in preventing DMO post cataract surgery)[296] and the BEVORDEX study (comparing intravitreal dexamethasone with bevacizumab for DMO but in the absence of cataract surgery).[116] Intravitreal triamcinolone was shown to have similar visual and anatomical outcomes as intravitreal dexamethasone implants for the treatment of a similar condition (macular oedema secondary to uveitis) and thus can be extrapolated for comparisons.[297]

Our preliminary results showed that the dexamethasone had greater visual gains at 4 to 6 months (mean gain in 21.4 ETDRS letters) compared with the bevacizumab group (mean gain in 15.7 ETDRS letters, p = 0.94). Vision is the most relevant primary outcome measure, but interpretations are limited by ceiling and floor effects. A better baseline vision is associated with smaller visual gains but a greater likelihood of achieving a good final vision, and vice versa for poorer baseline vision.[298] In this study, although insignificant, the dexamethasone group had a slightly worse mean baseline vision than the bevacizumab group (42.9 compared with 45.4 ETDRS letters, p = 0.73), and perhaps this is reflected in greater visual gains at 4 to 6 months. In the DiMECAT and BEVORDEX RCTs, no significant differences were also found in mean ETDRS letter gain between steroid and bevacizumab treatment for DMO. In real life observational studies, dexamethasone implants achieved superior visual gains.[299] This could be explained by the fact that less anti-VEGF injections are administered in real life compared with trials because of lower adherence to treatment.

Preliminary data in this study also showed a trend in the dexamethasone group achieving superior anatomical outcomes compared with bevacizumab (mean CMT change -58 versus +39, p = 0.24). The DiMECAT and BEVORDEX study also showed superior anatomical outcomes for steroid treatment (DiMECAT study; mean CMT change -51.5μm versus +15.6μm, p = 0.04, BEVORDEX study; mean CMT change -186μm versus -122μm, p = 0.015). Unfortunately change in CMT has a poor and highly variable correlation with visual acuity, and thus can only be used as an adjunct measurement.[300]

Frequency of additional treatments are useful and more clinically relevant adjunct outcome measurements, in the context of the limitations of vision and CMT as outcome measurements. In this study, dexamethasone implants had slightly lower mean number of retreatments (0.63 compared with 0.78, p = 0.29) and adjunct laser (0.13 compared with 0.2, p = 0.61). Similarly, the DiMECAT and BEVORDEX study also showed fewer retreatments for the steroid group to achieve non-inferior visual outcomes (DiMECAT study; 24% versus 43% requiring retreatment, p = 0.009, BEVORDEX study; mean number of retreatments 2.7 versus 8.6 over 12 months).

Interestingly, short term outcomes of this study are comparable to other populations where re-treatments were infrequent. For example, in our study, the mean change in BCVA at 1 to 3 months post cataract surgery was 23.9 ETDRS letters for adjunct bevacizumab and 27 ETDRS letters for dexamethasone implant. In a Japanese study where no retreatments were given, the mean BCVA change at 1 and 3 months post operatively for adjuvant bevacizumab were 15 and 25 ETDRS letters respectively.[287] Similarly in a case series from India, the mean BCVA change at 1 month and 4 months for dexamethasone implant were 25 and 15 ETDRS letters respectively.[301] These comparisons suggest similar prognoses in all populations if the same level of care was received.

No adverse events were reported with the use of dexamethasone implants in this trial. There were no increases in IOP greater than 5mm Hg from baseline for those who attended follow up. However, it is unknown whether those who did not attend follow up had any clinically significant increases in IOP. Adjunct measures of ocular hypertension and evidence of glaucoma such as visual field testing and OCT imaging of the retinal nerve fibre layer may be helpful in future studies. Reassuringly, the use of dexamethasone implants have an excellent safety profile among pseudophakic eyes. Ocular hypertension and glaucoma are less common in indigenous Australians.[302] Detected ocular hypertension can be safely managed with pressure lowering drops.[116]

A significant weakness of this study was the determination of sample size. There were no previous studies on DMO in the context of cataract surgery and adjunct therapy involving dexamethasone implants. Thus, sample size was determined on expected visual outcomes of patients with DR undergoing cataract surgery with intravitreal bevacizumab, compared with no adjunct treatment. A non-inferiority limit of 15 ETDRS letters was used to determine a sample size of 40.[287] In landmark clinical trials such as Protocol S and T, comparing anti-VEGF and laser treatments for DMO outside the context of cataract surgery, the non-inferiority limit was much smaller at 5 ETDRS letters. If a more meaningful 5 ETDRS letter limit was applied to this study design, an unfeasible sample size of 212 would be required to detect a significant difference, at 5% significance level and 80% power.[289]

Subsequent RCTs comparing steroid with bevacizumab treatment for DMO have used a superiority design. The BEVORDEX study used proportion of eyes in which BCVA improved by 10 letters or more as their primary outcome, resulting in a sample size of 80.[116] The DiMECAT study which compared intravitreal bevacizumab with intravitreal triamcinolone for the management of DMO in the context of cataract surgery, used mean change in BCVA as their primary outcome, resulting in a sample size of 92. The DiMECAT study is the closest study to base our sample size recalculations. The POINT study showed that intravitreal triamcinolone and intravitreal dexamethasone implant achieved similar anatomical outcomes at 8 weeks after treatment for macular oedema secondary to a different cause (uveitis).[297] Extrapolating from above, if a superiority design was used with the primary outcome as mean change in BCVA, a sample size of 50 (25 in each arm) would be sufficient in this study to detect a difference, with 5% significance level and 80% power.[289]

The obvious limitation in interpreting current collected data is the low number of participants in each analysis due to incomplete sample size and missing data. Grouping data from different follow up times leads to doubling up on the few participants who did attend follow up every month, therefore over exaggerating the cumulative outcome measures presented. We anticipate completing recruitment by the end of 2022. Strategies to increase sample size and account for missing data include expanding recruitment in other Australian states. It is unknown what the screen failure rate was as this data was not prospectively collected. While RCTs have shown anti-VEGF treatments are superior to laser treatment,[107] laser may have been preferentially chosen by clinicians for suitable non-compliant patients. A retrospective study to examine laser, anti-VEGF and dexamethasone implants for the treatment for DMO in this population would provide greater clarification.

4.5 Conclusion

DMO continues to be a significant cause of visual impairment among indigenous Australians living in Central Australia. Follow up rates for the management of DMO post cataract surgery is extremely low. Our preliminary results suggest dexamethasone implants achieve superior visual and anatomical outcomes with fewer retreatments than bevacizumab in this population where adherence to treatment is poor. No adverse effects were experienced with the use of dexamethasone implants in indigenous Australians. Completion of

this trial and expansion into other sites could confirm our preliminary results, with the potential to change current management strategies in Central Australia and other remote regions worldwide. These early results highlight the ongoing need to address socioeconomic and cultural barriers in the treatment of DR in remote indigenous Australian communities.

CHAPTER 5: A TARGETED GENOME WIDE APPROACH TO FIND MICRORNA RELATED GENES ASSOCIATED WITH DIABETIC RETINOPATHY

Some of the original work presented in this chapter has been published in the peer-reviewed literature: Liu E, Kaidonis G, McComish BJet al. MicroRNA-Related Genetic Variants Are Associated With Diabetic Retinopathy in Type 1 Diabetes Mellitus. Invest Ophthalmol Vis Sci 2019; 60: 3937-3942.

5.1 Introduction

Our research group established two large national registries in Australia to study genetic variants associated with DR: the Genetic Study of Diabetic Retinopathy (GSDR) and the Registry of Advanced Diabetic Retinopathy (RADAR). GSDR was established in 2006, with the aim to build a repository of DNA samples from well characterised T1DM and T2DM participants with and without DR. RADAR was established in 2012 as an online and phone application recruitment method to assist with recruitment from remote and interstate locations. Collaborations were sought with various hospitals around Australia and Moorfields Eye Hospital in the United Kingdom. These registries aim to support high quality genetic studies by facilitating collection of data and specimens from large cohorts, with strict classification of DR phenotypes, and excellent characterisation of clinical risk factors to allow correction in genetic analyses.

5.1.1 Previous findings

Multiple candidate gene studies and two pilot GWAS have been conducted with participants of the GSDR and RADAR. A pilot GWAS was conducted in 2015 on over 1000 participants and found a significant association between rs9896052 located on chromosome 17 and sight threatening DR.[303] The association was found in a T2DM Caucasian discovery cohort, and then replicated in 3 independent cohorts (a T1DM Caucasian cohort, a T2DM Caucasian cohort and a T2DM Indian cohort). While these remain the first and only published result from a GWAS focusing on DR that has replicated, no further replication has been found in subsequent studies. Genome wide significance was reached in the meta-analysis combining the discovery and replication cohorts ($p = 4.15 \times 10^{-8}$). The most promising candidate gene near rs9896052 is *GRB2,* which is involved in insulin

and VEGF signalling.[304, 305] Immunohistochemistry for GRB2 identified the protein was abundant in all layers of the normal human retina.[303] After Müller cell ablation in mice which mimics blood retinal barrier breakdown in DR, GRB2 was found to be elevated.[303] A second GWAS was conducted on the T2DM cohort; 270 DMO cases and 176 PDR cases compared with 435 non-retinopathy diabetic controls.[306] While no SNP reached genome wide significance, the top ranked SNP for DMO and PDR were rs1990145 ($p = 4.10 \times 10\text{-}6$, OR = 2.02 95%CI [1.50, 2.72]) and rs918519 ($p = 3.87 \times 10\text{-}6$, OR = 0.35 95%CI [0.22, 0.54]) respectively. Rs1990145 is in an intron of the mitochondrial ribosomal protein L19. Rs918519 is in a non-coding RNA (*LOC285626*) located near the *L12B* gene. Both mitochondrial ribosomal protein L19 and L12B are expressed in the retina (The Ocular Tissue Database),[307] but their potential roles in DR are currently unknown.

Previous candidate gene studies from our group have focused on hypoxic and inflammatory pathways involved in DR. As described in chapter 1, hyperglycaemia activates five metabolic pathways, which contribute to increased oxidative stress and inflammation in DR. Significant findings include genetic variants in microRNA146a and the VEGF gene family. MicroRNA146a was studied given its role in regulating NF-κB,[308] a key transcription factor that is activated by oxidative stress in DR.[309] The VEGF gene family was studied as they are key growth factors induced in response to ischaemia and regulate angiogenesis in proliferative DR.

Polymorphism rs2910164 in microRNA 146a has been reported to be associated with gastrointestinal cancers and autoimmune diseases in case control genetic studies.[310, 311] Expression analysis and cell models of thyroid cancer suggested that rs2910164 regulated microRNA 146a expression.[312] Our study showed this SNP was significantly associated with DMO in T2DM but not T1DM after adjusting for clinical risk factors and multiple hypothesis testing (895 controls, 856 cases, additive model, OR, 1.25; CI, 1.03-1.53; P = 0.025).[221]

Earlier work with 281 controls and 215 sight threatening DR cases studied the VEGFA gene to determine whether it was associated with DR in the Australian Caucasian population. Fifteen common SNPs which comprehensively covered the gene were identified and genotyped. Rs3025021 and rs10434 were protective of sight threatening DR in T2DM (OR 0.40, p =0.002 and OR 0.99, p=0.002 respectively).[190] The same approach

was utilised to study other isoforms of VEGF in a much larger cohort from RADAR combined with GSDR. Most notably, significant associations were found between any DR and common tagging SNPs in the *VEGFC* gene: rs17697419 (1919 controls, 980 cases, OR 0.67, 95% CI 0.52-0.85, P = 0.001), rs17697515 (OR, 0.62, 95% CI 0.47-0.81, P=0.001) and rs2333526 (OR, 0.69, 95% CI 0.54-0.90, P= 0.005). Polymorphism rs17697515 was also specifically associated with DMO in those with T2DM (1919 controls, 909 cases, OR, 0.53, 95% CI 0.35-0.82, P =0.004).[313] VEGF-C is part of the VEGF family and contributes to retinal angiogenesis and increased blood retinal barrier permeability. VEGF-C levels are increased in the vitreous of PDR cases.[314] This could be independent of VEGF-A signalling as VEGF-C has been found to be increased when VEGF-A is inhibited by bevacizumab.[315]

5.1.2 A targeted genome wide approach

It is difficult to comprehensively study genetic variants in microRNA and their bindings sites. Even though microRNA sequences are short with few polymorphisms, there are over 2000 known microRNA. A single microRNA may bind to hundreds of genes to regulate their expression. Whole genome sequencing, while decreasing in cost over time, are still expensive endeavours.

Additional participants (n =911) have been recruited to our registries since the pilot GWAS. A two stage design was undertaken to explore microRNA related variants and their association with DR: a proportion of samples were already genotyped for a large number of markers (the discovery GWAS cohort), followed by the remaining samples being genotyped for markers of interest (the replication cohort with the additional participants).[316] This approach took advantage of genotyping already performed and was therefore cost effective. A meta-analysis of the two cohorts also provided additional insights into potentially significant associations identified through the pilot GWAS.

5.2 Methods

5.2.1 Recruitment method and data collection

Methods on recruitment of participants and data collection for the GSDR and RADAR have been published,[317] but are also described in detail below. Both studies were approved by HRECs in Australia (Southern Adelaide

Clinical HREC, Royal Adelaide Hospital HREC, The Queen Elizabeth Hospital (Adelaide) HREC, Royal Melbourne Hospital HREC, Royal Victorian Eye and Ear Hospital HREC, St. Vincent's Hospital (Melbourne) HREC, South Eastern Sydney Illawarra HREC, Tasmania Health and Medical HREC, Canberra Hospital HREC) and the NHS Health Research Authority in London. It adheres to the tenets of the Declaration of Helsinki. Written informed consent was obtained from each participant before study enrolment.

Multiple recruitment centres included the following Australian hospitals; Flinders Medical Centre, The Repatriation General Hospital, The Royal Adelaide Hospital, The Queen Elizabeth Hospital, The Royal Melbourne Hospital, Royal Victorian Eye and Ear Hospital, St. Vincent's Hospital, Sydney Eye Hospital, Canberra Hospital, Royal Hobart Hospital, and from the United Kingdom; The National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology.

Eligible participants were actively recruited from ophthalmology, diabetes and renal clinics, with the following inclusion criteria: T1DM or T2DM having received at least 5 years of medical treatment for diabetes (oral hypoglycemic agents or insulin) prior to enrolment, and must be over 18 years of age. All participants underwent a questionnaire and venous blood sample collection for DNA analysis. Clinical information was collected from case notes and electronic records, including HbA1c, renal and lipid measures, medications and the presence of hypertension and non-ocular diabetic complications. HbA1c was recorded as the average of the three most recent, available measurements or of three measurements immediately prior to a diagnosis of PDR. Hypertension was defined as systolic and diastolic blood pressure greater than 140 and 90 mmHg respectively or pharmacological treatment for hypertension. DR grading (defined as the worst ever grading) and the presence of DMO were determined from documented dilated fundus exams performed by an ophthalmologist. DR grading was defined by the International Clinical DR Severity Scale as described in chapter one.[318] CSMO was defined according to the ETDRS protocol.[14] Sight threatening DR was defined as severe NPDR, PDR or CSMO.

For each participant, 8 mL of blood was collected in EDTA blood collection tubes and underwent DNA extraction using the QIAamp Blood DNA Maxi Kits (Qiagen, Venlo, The Netherlands).

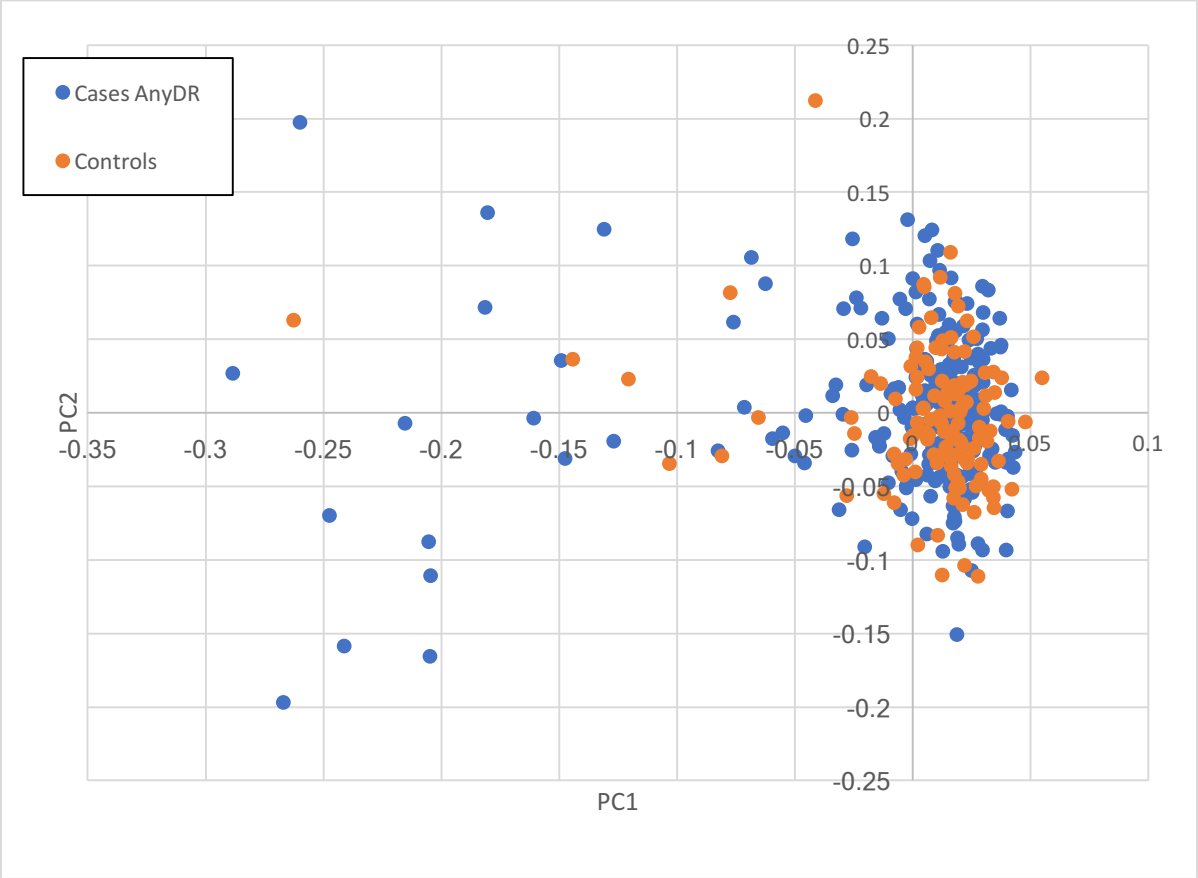
5.2.2 Power calculations

Power calculations were determined from an online calculator derived from Skol et al's paper on joint two-stage genetic analyses.[316] For the combined T1DM cohort (228 controls, 332 any DR cases), assuming a case prevalence of 7%, the study had 80% power to detect a disease allele with frequency of at least 10% and a relative risk of 2.6 within a multiplicative model and significance set at genome wide level ($p = 5 \times 10^{-8}$). For the T2DM cohort (716 controls, 916 any DR), assuming the same disease prevalence, model and significance level, the study had 80% power to detect a disease allele with frequency of at least 10% and a relative risk of 1.8.

5.2.2 Stage 1 *in silico* analyses

In our previous published GWAS, genotyping of 354 T1DM patients and 1024 T2DM patients have already been conducted on the OmniExpress Array (Illumina).[303] Bennet McComish from the Menzies Research Institute (University of Tasmania) conducted the imputation of SNP data (related to our genes of interest) from previous genotyped data and the steps he undertook are described below. Following data cleaning, principal components were calculated using EIGENSTRAT and individuals removed if they were further than six standard deviations (SD) from the mean of any principal component, limiting the analysis to participants of predominantly European descent. Additional outliers were removed based on visualising the plot of PC1 vs PC2 (Figure 5.1). Relatedness was calculated using PLINK and one of each pair with pi-hat > 0.2 were removed. Only individuals with complete clinical information were included in the analyses. After quality control, a total of 325 T1DM samples and 956 T2DM samples remained.

Figure 5.1: The first two principle components by case/control status for any type of DR. Individuals with PC1 <-0.1 were excluded from the analysis as they appear to be distant from the main cluster

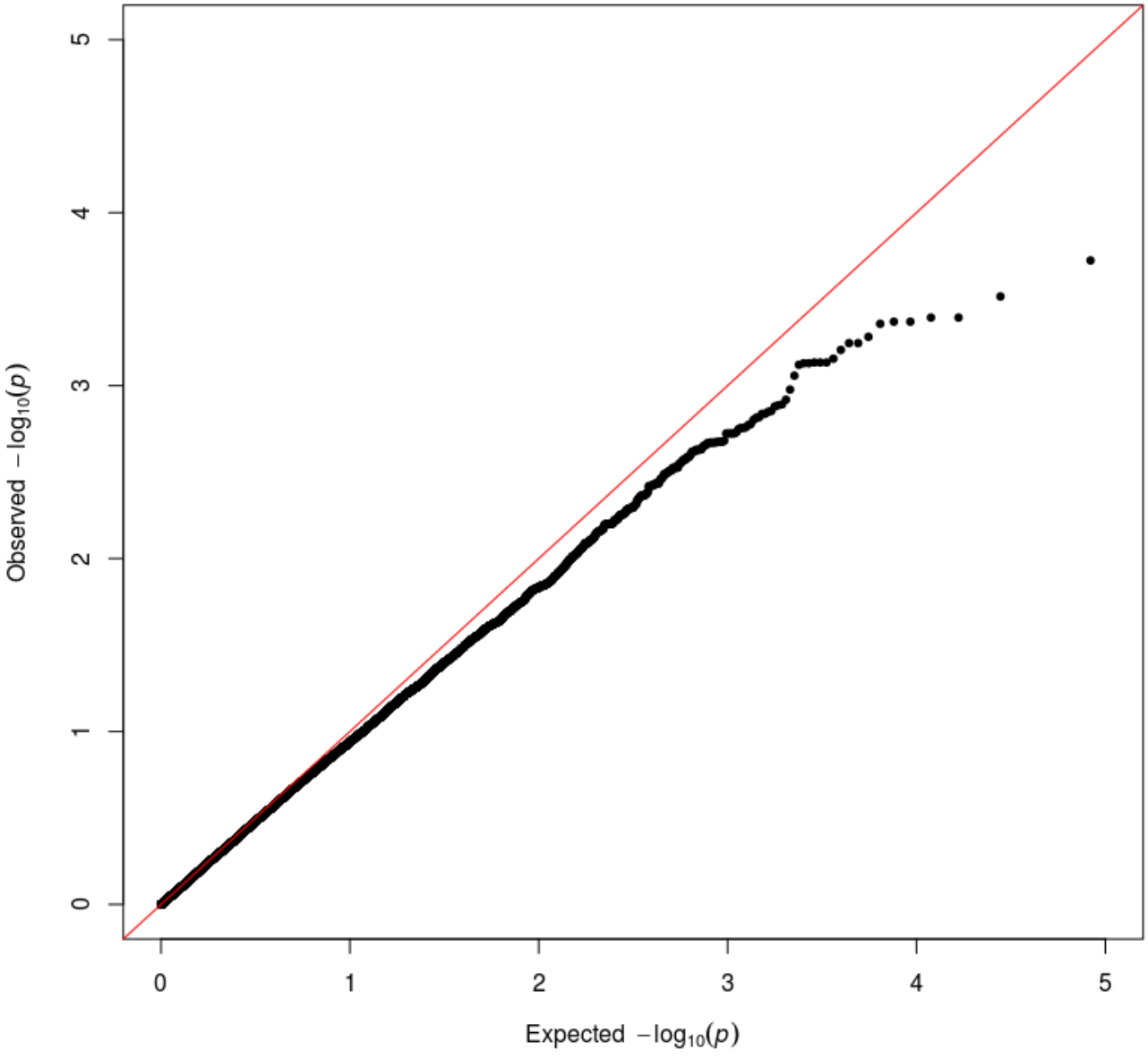


The autosomal genotype data were phased using Eagle (version 2.3.5)[319] and genotypes were then imputed on the basis of the 1000 Genomes Project reference panel (EUR reference, Phase III, version 5)[320] using Minimac3 (version 2.0.1).[321] Indels, SNPs within 5bp of an indel, rare variants (minor allele frequency, MAF < 0.01), and variants with poor imputation quality (R^2 < 0.8) were removed. Known microRNA genes and their binding sites were downloaded from the miRNASNP and PolymiRTS (version 2) databases (accessed 31/08/2017). These SNPs were assessed for association with DR in the imputed GWAS data, if they had been successfully imputed.

The most likely genotype was used for the association analyses with the imputed SNPs. Association tests with DR phenotypes were performed in PLINK (version 1.07). T1DM and T2DM samples were analysed separately because of different underlying aetiologies and risk factor profile. Controls were defined as those with diabetes but no DR. Analysis for association with DR phenotypes was repeated for different definitions

of cases: 1) any DR, 2) PDR, 3) sight threatening DR (severe NPDR, PDR and CSMO). Chi-square tests were used for univariate analysis and binary logistic regression for multivariate analysis which incorporated age, sex, duration of diabetes, HbA1c and hypertension. QQ plot of the p values in the association study for any DR compared with no DR in the GWAS discovery cohort is shown in the Figure 5.2. The plot comprises of all the samples in the GWAS discovery cohort and therefore reflects all the analyses done. It shows that the observed p values follow a normal distribution, has minimal bias and does not show inflation.

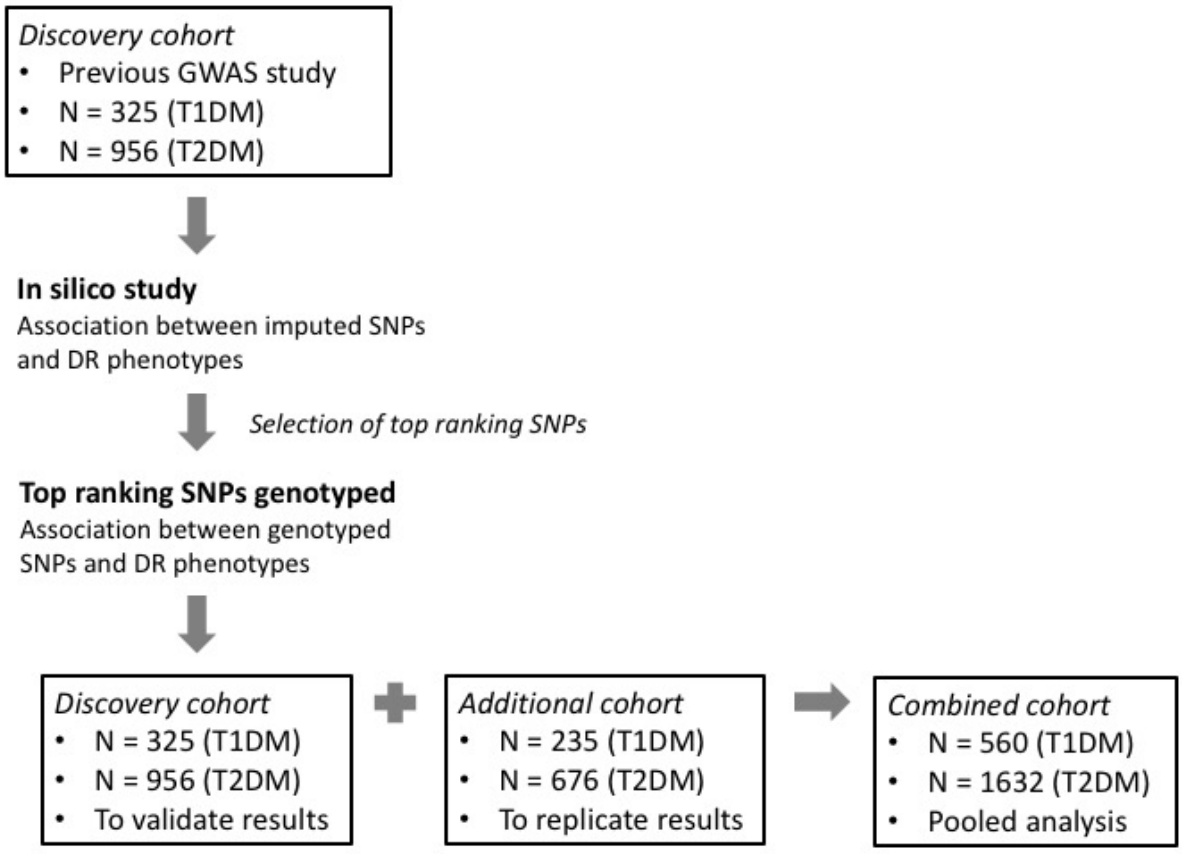
Figure 5.2: QQ plot of p values in the association study for any DR compared with no DR in the GWAS discovery cohort



5.2.3 Stage 2 genotyping and combined analyses

Top ranking SNPs were selected for genotyping in stage 2 to confirm their association with DR (T2DM n=1632, T1DM n= 560). SNPs were chosen based on minor allele frequencies (greater than 1% in the imputed data) and strength of association (odds ratios and p value). Genotyping was performed through the Australian Genome Research Facility (AGRF), using the Agena Bioscience MassARRAY platform. The same statistical analysis as described above was performed. The genotyped SNPs were separately analysed in the original GWAS sample, the additional samples and the final combined sample to check the reproducibility and strength of results. Figure 5.3 summarizes the overall study design.

Figure 5.3 Study Design



5.3 Results; demographic details of study cohort

There were 560 type 1 diabetic participants in the total study population, comprising of 325 in the discovery cohort and 235 additional participants. For the type 2 diabetic cohort, there were 1632 in the total population, comprising of 956 in the discovery cohort and 676 additional participants. Demographic details of both the T1DM and T2DM groups are shown in Table 6.1 for no DR and any DR, and Table 6.2 for PDR, DMO and sight threatening DR. The numbers of cases and controls at each stage of analyses are shown in Table 5.3. As expected, sex, age, diabetes duration, HbA1c and hypertension were significantly different between those with DR and those without DR. Therefore these clinical risk factors were corrected for in subsequent genetic analyses.

Table 5.1 Demographics of total type 1 and type 2 diabetic study population stratified by no DR and Any DR

	Type 1 diabetes			Type 2 diabetes		
Demographic	No DR	Any DR	P-value	No DR	Any DR	P-value
n	228	332	NA	716	916	NA
Female (n, %)	99 (43.8)	178 (47.3)	0.440	350 (49.6)	363 (39.2)	**<0.0001**
Age, yrs (median, range)	34 (17-83)	47 (18-88)	**<0.0001**	66 (27-95)	68 (27-94)	**<0.0001**
Diabetes duration, yrs (median, range)	13 (5-60)	28 (5-70)	**<0.0001**	11.5 (5-60)	18 (5-61)	**<0.0001**
HbA1c % (median, range)	8.06 (5-15)	8.6 (5-15)	**<0.0001**	7.3 (2-22)	8 (4-15)	**<0.0001**
Hypertension (n, %)	53 (23.5)	225 (59.8)	**<0.0001**	572 (81.1)	823 (88.9)	**<0.0001**

Abbreviations: DR diabetic retinopathy, HbAc1 glycosylated haemoglobin. P values presented for demographic variables are related to comparisons made between no DR and any DR

Table 5.2 Demographic details of total type 1 and type 2 diabetic study population stratified between no DR, PDR, DMO and Sight threatening DR

	No DR	PDR	P-value	DMO	P-value	Sight threatening DR*	P-value
Type 1 diabetes							
n	228	181		89		223	
Female (n, %)	99 (43.8)	85 (43.8)	0.612	43 (48.3)	0.904	108 (45.8)	0.943
Age, yrs (median, range)	34 (17-83)	48 (21-85)	**<0.0001**	52 (18-83)	**<0.0001**	49 (21-88)	**<0.0001**
Diabetes duration, yrs (median, range)	13 (5-60)	32 (8-70)	**<0.0001**	23 (2-50)	**<0.0001**	31 (5-70)	**<0.0001**
HbA1c % (median, range)	8.06 (5-15)	8.8 (5-15)	**<0.0001**	8.2 (5-14)	0.055	8.7 (5-15)	**<0.0001**
Hypertension (n, %)	53 (23.5)	136 (70.1)	**<0.0001**	47 (52.8)	**<0.0001**	155 (65.7)	**<0.0001**
Type 2 diabetes							
n	716	260		484		523	
Female (n, %)	350 (49.6)	96 (36.0)	**2.85×10^{-4}**	184 (42.5)	**0.005**	226 (40.4)	**0.002**
Age, yrs (median, range)	69 (27-95)	63 (47-87)	**<0.0001**	67 (33-95)	0.485	65 (35-90)	**<0.0001**
Diabetes duration, yrs (median, range)	11.5 (5-60)	20 (5-55)	**<0.0001**	18 (5-48)	**<0.0001**	19 (5-55)	**<0.0001**
HbA1c % (median, range)	7.3 (2-22)	8.4 (4-15)	**<0.0001**	8.1 (5-15)	**<0.0001**	8.2 (4-15)	**<0.0001**
Hypertension (n, %)	572 (81.1)	237 (88.8)	**2.85×10^{-4}**	377 (87.1)	**0.104**	495 (88.4)	**0.013**

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, HbAc1 glycosylated haemoglobin. Sight threatening DR includes severe NPDR, PDR and CSMO

Table 5.3 Number of diabetic individuals in each stage of analysis by phenotype included in logistic regression analysis

	Type 1 diabetes			Type 2 diabetes		
	Discovery cohort	Replication cohort	Total	Discovery cohort	Replication cohort	Total
No DR	123	105	228	424	292	716
Any DR	202	130	332	532	384	916
DMO	48	41	89	261	223	484
PDR	125	56	181	165	95	260
Sight threatening DR	155	68	223	330	193	523

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, Sight threatening DR includes severe NPDR, PDR and CSMO

5.4 Results: Type 1 diabetes

In the discovery cohort, 2420 SNPs in microRNA genes and 401,000 SNPs in microRNA binding sites of target genes were retrieved from the imputed SNP data (n = 325 type 1 diabetes patients). After quality control, 213 microRNA variants and 41,578 microRNA binding site variants remained. Nominal associations between these microRNA SNPs and DR phenotypes ($p < 0.05$) are shown in Appendix 1. Nominal associations between microRNA binding site SNPs and DR phenotypes ($p < 0.05$) are shown in Appendix 2. No SNPs reached significance after multiple hypothesis testing (41,791 variants, 4 phenotype outcomes, $p < 3.00 \times 10^{-7}$). Therefore the top SNPs across the phenotypes were selected for genotyping (Table 5.4.1). No additional SNPs in the DMO group reached the significance criteria for further genotyping, and this is likely because the numbers are very small (n = 48). Only three (one in a microRNA gene and two in binding sites) were successfully genotyped in replication population: rs10061133 (MIR449), rs9501255 (HLA-DPB1) and rs1049835 (GPM6A).

Table 5.4.1 SNPs in microRNA related genes selected from imputed GWAS data for type 1 diabetes mellitus cohort

Gene	SNP	OR	95% CI (upper)	95% CI (lower)	P	Cases	Genotyped successfully
MIR449	rs10061133	0.36	0.74	0.17	**5.3×10^{-3}**	Sight threatening DR	Yes
GPM6A	rs1049835	2.32	3.44	1.57	**2.4×10^{-5}**	Sight threatening DR	Yes
HLA-DPB1	rs9501255	0.13	0.36	0.05	**6.1×10^{-5}**	Any DR	Yes
MIR4302	rs11048315	0.34	0.69	0.17	**2.6×10^{-3}**	Any DR	No
MIR4433B	rs12473206	0.55	0.85	0.35	**7.6×10^{-3}**	PDR	No
MIR4803	rs3112399	1.78	2.73	1.17	**7.6×10^{-3}**	Sight threatening DR	No
MIR5689	rs9295535	0.50	0.84	0.29	**9.3×10^{-3}**	Sight threatening DR	No
TRIM25	rs205500	0.26	0.50	0.14	**5.2×10^{-5}**	PDR	No

Abbreviations: SNP; single nucleotide polymorphism, OR; odds ratio, CI; confidence interval, DR; diabetic retinopathy, PDR; proliferative diabetic retinopathy. Sight threatening DR defined as severe NPDR, PDR and CSMO.

Two of the three SNPs were associated with a DR phenotype after multivariable analysis (adjusting for age, sex, duration of diabetes, HbA1c and hypertension) of the genotyped data in the original discovery cohort (Table 5.4.2). The lack of validation of rs9501255 in HLA-DPB1 in association with all DR phenotypes suggests the imputation of this SNP was of poor quality, as is often seen with HLA SNPs.[322] Of the two remaining SNPs associated with sight threatening DR in the discovery cohort, neither were associated with sight threatening DR ($p > 0.05$) in the additional samples genotyped ($n = 235$). When all samples were combined, rs10061133 (MIR449b) and rs1049835 (GPM6A) were associated with sight threatening DR. Rs1006113 (MIR449b) in particular, became more significantly associated with sight threatening DR as the sample size increased ($p = 3.68 \times 10^{-4}$) and

showed consistent odds ratios between the discovery and the additional samples. This SNP was protective against sight threatening DR with an OR of 0.31-0.37 across the three analyses. The association between rs1049835 (GPM6A) and sight threatening DR became weaker in the combined analysis, indicating that it is not a true result.

The two associated SNPs (rs10061133 and rs1049835) were then analysed for association with DMO and subtypes of sight threatening DR (PDR and CSMO) in the final combined sample. Rs10061133 in MIR449b was strongly associated with a decreased risk of PDR (OR = 0.30, 95% CI 0.15-0.61, p = 8.12×10^{-4}) but not the other phenotypes.

Table 5.4.2: Imputation, validation, replication and meta-analyses of association results between significant SNPs and DR phenotypes in type 1 diabetes

			Discovery - imputed		Discovery - genotyped		Additional genotyped		All samples combined	
SNP Gene	Minor Allele	DR phenotype	MAF* cases vs controls (n)	OR 95% CI P	MAF* cases vs controls (n)	OR 95% CI P	MAF* cases vs controls (n)	OR 95% CI P	MAF* cases vs controls (n)	OR 95% CI P
rs10061133 MIR449B	G	Sight threatening DR	0.08 vs 0.14 (155, 123)	0.36 (0.17-0.74) **5.32×10^{-3}**	0.07 vs 0.14 (155, 123)	0.31 (0.15-0.65) **1.63×10^{-3}**	0.08 vs 0.10 (68, 105)	0.37 (0.10-1.36) 0.13	0.08 vs 0.13 (223, 228)	0.32 (0.17-0.60) **3.68×10^{-4}**
rs1049835 GPM6A	G	Sight threatening DR	0.42 vs 0.27 (155, 123)	2.32 (1.57 - 3.44) **2.40×10^{-5}**	0.42 vs 0.27 (155, 123)	2.17 (1.38-3.43) **8.63×10^{-4}**	0.27 vs 0.36 (68, 105)	1.15 (0.59-2.25 0.68	0.37 vs 0.31 (223, 228)	1.72 (1.20-2.48) **3.52×10^{-3}**
rs9501255 HLA-DPB1	T	Any DR	0.02 vs 0.07 (202, 123)	0.13 (0.05 - 0.36) **6.10×10^{-5}**	0.02 vs 0.08 (202, 123)	0.26 (0.22-0.86) 0.017	0.03 vs 0.02 (139, 105)	1.77 (0.39-8.07) 0.46	0.02 vs 0.05 (332, 228)	0.42 (0.17-1.00) 0.050

Abbreviations: n sample size, SNP single nucleotide polymorphism, DR diabetic retinopathy, MAF minor allele frequency, OR odds ratio, CI confidence interval. Sight threatening DR defined as severe NPDR, PDR and CSMO. All analyses are adjusted for age, sex, duration of diabetes, HbA1c and hypertension. Sample sizes for each analyses are listed in Table 6.3.

5.5 Results: type 2 diabetes

In the discovery cohort (n= 956 T2DM participants), the same imputed SNPs (213 microRNA variants and 41578 microRNA binding site variants) were analysed with all DR phenotypes. Nominal associations between these SNPs and DR phenotypes ($p < 0.05$) and top ranking SNPs selected for genotyping are shown in Appendix 3 and Appendix 4 for microRNA and microRNA binding sites respectively. Eight top ranking SNPs were in microRNA genes and 13 SNPs were in microRNA binding sites. However only 1 SNP in microRNA genes and 8 SNPs residing in microRNA binding sites were successfully genotyped (Table 5.5.1).

Table 5.5.1 SNPs in microRNA related genes selected from imputed GWAS data for type 2 diabetes mellitus cohort

Gene	SNP	OR	95% CI (upper)	95% CI (lower)	P	Cases	Genotyped successfully
MIR345	rs72631832	3.90	1.68	9.03	**1.50×10^{-3}**	Sight threatening DR	Yes
SEPT05	rs448203	1.68	1.32	2.14	**2.32×10^{-5}**	Sight threatening DR	Yes
OR2B11	rs10802501	1.88	1.40	2.51	**2.38×10^{-5}**	Any DR	Yes
ADAMTS5	rs1444269	0.55	0.42	0.73	**3.00×10^{-5}**	Sight threatening DR	Yes
PCDH7	rs11567	0.26	0.14	0.50	**3.75×10^{-5}**	DMO	Yes
ADAMTS5,	rs11700721	0.5548	0.4167	0.7388	**5.53×10^{-5}**	Sight threatening DR	Yes
CD209	rs11465396	0.38	0.23	0.61	**7.80×10^{-5}**	Any DR	Yes
DDX56	rs2289050	5.45	2.34	12.70	**8.40×10^{-5}**	Any DR	Yes
AFAP1	rs2285768	0.42	0.27	0.65	**9.32×10^{-5}**	Sight threatening DR	Yes
MIR2278	rs356125	0.48	0.30	0.78	**2.99×10^{-3}**	Any DR	No

MIR1229	rs2291418	0.17	0.05	0.58	**4.96 x10^{-3}**	PDR	No
MIR559	rs58450758	0.61	0.43	0.86	**4.99 x10^{-3}**	Any DR	No
MIR585	rs62376935	1.88	1.20	2.94	**5.56 x10^{-3}**	Any DR	No
MIR4698	rs832733	1.48	1.13	1.94	**4.99 x10^{-3}**	PDR	No
MIR4762	rs60308683	0.60	0.41	0.87	**7.89 x10^{-3}**	Sight threatening DR	No
MIR4762	rs60308683	0.60	0.41	0.87	789 **x10^{-3}**	DMO	No
SUCLG2	rs3206428	2.11	1.50	2.97	**2.02 x10^{-5}**	Any DR	No
TTC19	rs118174899	7.66	2.87	20.41	**4.69 x10^{-5}**	PDR	No
CPM	rs1196278	9.83	3.21	30.12	**6.29 x10^{-5}**	Any DR	No
TTC37	rs1062083	1.76	1.33	2.34	**8.39 x10^{-5}**	Any DR	No
BDH1	rs8034	0.20	0.09	0.44	**8.50 x10^{-5}**	Any DR	No

Abbreviations: SNP; single nucleotide polymorphism, OR; odds ratio, CI; confidence interval, DR; diabetic retinopathy, PDR; proliferative diabetic retinopathy, DMO; diabetic macular oedema. Sight threatening DR defined as severe NPDR, PDR and CSMO.

Minor allele frequencies in cases and controls, odds ratios and p values were similar between analyses with imputed SNPs and the successfully genotyped 9 SNPs. Unfortunately, none of these associations were replicated in the additional samples genotyped, nor confirmed in the combined samples (Table 5.5.2). Inconsistent data from wide range of 95% confidence intervals, flipped odds ratios from similar minor allele frequencies (MAF) between cases and controls, or different MAFs between discovery and replication cohorts probably reflect false positive results. The only result that remains consistent across discovery, replication and meta-analysis is rs72631832 in MIR345 and its association with PDR, DMO and sight threatening DR. However the result does not reach significance after multiple hypothesis testing.

Table 5.5.2 Imputation, validation, replication and meta-analyses of association results between significant SNPs and DR phenotypes in type 2 diabetes

			GWAS - imputation		GWAS - genotyped		Additional genotyped		Combined - genotyped	
SNP Gene	Minor Allele	DR case phenotype	MAF* cases vs controls (%) (n)	OR 95% CI P	MAF* cases vs controls (%) (n)	OR 95% CI p	MAF* cases vs controls (%) (n)	OR 95% CI p	MAF* cases vs controls (%) (n)	OR 95% CI p
rs72631832 MIR345	T	PDR	2.73 vs 1.42 (165, 424)	4.47 (1.54-12.96) **5.9×10^{-3}**	2.37 vs 1.47 (165, 424)	2.49 (0.88-7.00) 0.084	3.76 vs 2.56 (95, 292)	1.56 (0.52-4.65) 0.424	2.86 vs 1.90 (260, 716)	1.77 (0.86-3.66) 0.124
		DMO	2.49 vs 1.45 (261, 424)	3.97 (1.55-10.19) **4.08×10^{-3}**	2.91 vs 1.47 (261, 424)	2.40 (0.97-5.95) 0.059	3.67 vs 2.58 (223, 292)	1.36 (0.64-2.91) 0.427	3.30 vs 1.87 (484, 716)	1.75 (0.99-3.08) 0.054
		Sight threatening DR	2.58 vs 1.29 (330, 424)	3.90 (1.68-9.03) **1.50×10^{-3}**	2.48 vs 1.47 (330, 424)	2.14 (0.95-4.79) 0.065	4.71 vs 2.56 (193, 292)	1.92 (0.89-4.10) 0.094	3.25 vs 1.90 (523, 716)	1.83 (1.06-3.16) 0.031
rs10802501 NLRP3	A	Any DR	18.61 vs 13.44 (532, 424)	1.88 (1.4-2.51) **2.38×10^{-5}**	17.82 vs 13.59 (532, 424)	1.57 (1.19-2.07) **1.29×10^{-3}**	15.7 vs 16.7 (384, 292)	0.89 (0.65-1.22) 0.483	16.94 vs 14.9 (916, 716)	1.22 (0.99-1.50) 0.060

			GWAS - imputation		GWAS - genotyped		Additional genotyped		Combined - genotyped	
SNP Gene	Minor Allele	DR phenotype	MAF* cases vs controls (%) (n)	OR 95% CI P	MAF* cases vs controls (%) (n)	OR 95% CI p	MAF* cases vs controls (%) (n)	OR 95% CI p	MAF* cases vs controls (%) (n)	OR 95% CI p
rs11465396 CD209	G	Any DR	3.76 vs 7.43 (532, 424)	0.38 (0.23-0.61) **7.80×10^{-5}**	3.93 vs 7.07 (532, 424)	0.46 (1.29-0.72) **7.27×10^{-4}**	6.02 vs 3.99 (384, 292)	1.56 (0.90-2.72) 0.116	4.78 vs 5.78 (916, 716)	0.75 (0.53-1.06) 0.103
rs2289050 DDX56	A	Any DR	3.38 vs 1.06 (532, 424)	5.45 (2.34-12.7) **8.40×10^{-5}**	2.65 vs 1.0 (532, 424)	2.63 (1.19-5.84) **0.017**	3.07 vs 2.11 (384, 292)	1.11 (0.52-2.33) 0.794	2.82 vs 1.46 (916, 716)	1.90 (1.08-3.35) **0.026**
rs448203 SEPT05	C	Sight threatening DR	48.64 vs 38.54 (330, 424)	1.68 (1.32-2.14) **2.32×10^{-5}**	46.94 vs 38.97 (330, 424)	1.51 (0.12-1.91) **4.92×10^{-4}**	43.19 vs 43.59 (193, 292)	0.87 (0.66-1.15) 0.328	45.64 vs 40.80 (523, 716)	1.21 (1.02-1.44) **0.032**
rs1444269 ADAMTS5	G	Sight threatening DR	20.45 vs 28.66 (330, 424)	0.55 (0.42-0.73) **3.00×10^{-5}**	22.38 vs 28.20 (330, 424)	0.68 (0.52-0.88) **4.03×10^{-3}**	26.84 vs 26.69 (193, 292)	1.08 (0.79-1.47) 0.649	23.91 vs 27.60 (523, 716)	0.79 (0.65-0.97) **0.021**

			GWAS - imputation		GWAS - genotyped		Additional genotyped		Combined - genotyped	
SNP Gene	Minor Allele	DR case phenotype	MAF* cases vs controls (%) (n)	OR 95% CI P	MAF* cases vs controls (%) (n)	OR 95% CI p	MAF* cases vs controls (%) (n)	OR 95% CI p	MAF* cases vs controls (%) (n)	OR 95% CI p
rs11700721 ADAMTS5	T	Sight threatening DR	18.03 vs 25.99 (330, 424)	0.55 (0.42-0.74) **5.53×10^{-5}**	19.63 vs 25.33 (330, 424)	0.69 (0.52-0.91) **8.22×10^{-3}**	23.63 vs 23.10 (193, 292)	1.09 (0.77-1.53) 0.623	21.0 vs 24.40 (523, 716)	0.79 (0.64-0.98) **0.033**
rs2285768 AFAP1	A	Sight threatening DR	5.91 vs 10.57 (330, 424)	0.42 (0.27-0.65) **9.32×10^{-5}**	6.06 vs 9.81 (330, 424)	0.46 (0.30-0.72) **5.67×10^{-4}**	13.61 vs 12.58 (193, 292)	1.24 (0.83-1.86) 0.296	8.66 vs10.91 (523, 716)	0.78 (0.58-1.04) 0.089
rs11567 PCDH7	T	DMO	4.22 vs 8.49 (261, 424)	0.26 (0.14-0.5) **3.75×10^{-5}**	3.88 vs 9.79 (261, 424)	0.20 (0.10-0.38) **1.93×10^{-6}**	8.03 vs 4.61 (223, 292)	1.96 (1.16-3.32) **0.011**	6.01 vs 7.91 (484, 716)	0.76 (0.54-1.08) 0.133
		Sight threatening DR	4.85 vs 8.99 (330, 424)	0.35 (0.21-0.59) **6.69×10^{-5}**	4.96 vs 9.78 (330, 424)	0.29 (0.18-0.48) **1.22×10^{-6}**	7.07 vs 5.93 (193, 292)	1.54 (0.91-2.61) 0.110	5.69 vs 8.26 (523, 716)	0.63 (0.44-0.89) **8.53×10^{-3}**

Abbreviations: n sample size, SNP single nucleotide polymorphism, DR diabetic retinopathy, MAF minor allele frequency, OR odds ratio, CI confidence interval. Sight threatening DR defined as severe NPDR, PDR and CSMO. All analyses are adjusted for age, sex, duration of diabetes, HbA1c and hypertension. Sample sizes for each analyses are listed in Table 5.3.

We also analysed the genotyped SNPs with DR phenotypes, other than the one they were selected for. The most consistent results were found for rs11700721 and rs1444269 in *ADAMTS5* and their association with PDR. Table 6.5.3 shows the ORs and p values for these two SNPs across the 4 analysed groups. Rs11700721 and rs1444269 in ADAMTS5 were both protective of PDR: OR 0.63, $p = 0.0023$ and OR 0.65, $p = 0.0018$ respectively in the combined sample group. Of note, significance increased as the cohort became larger in size and the associations survive multiple hypothesis correction ($p < 0.0055$ for 9 SNPs tested).

Table 5.5.3 Associations between rs11700721 and rs1444269 in ADAMTS5 and PDR across the 4 analysed groups

			Discovery - imputed		Discovery - genotyped		Additional genotyped		All samples combined	
SNP Gene	Minor Allele	DR case phenotype	MAF* cases vs controls	OR 95% CI P	MAF* cases vs controls	OR 95% CI P	MAF* cases vs controls	OR 95% CI P	MAF* cases vs controls	OR 95% CI P
rs11700721 ADAMTS5	T	PDR	18.03 vs 25.99	0.64 0.43-0.94 **0.022**	17.90 vs 25.33	0.64 0.43-0.93 **0.02**	15.73 vs 23.10	0.63 0.33-0.99 **0.046**	17.13 vs 24.44	0.63 0.47-0.85 **0.0023**
rs1444269 ADAMTS5	G	PDR	20.45 vs 28.66	0.65 0.45-0.94 **0.023**	21.60 vs 28.20	0.65 0.46-0.92 **0.014**	18.48 vs 26.69	0.59 0.36-0.96 **0.034**	20.50 vs 27.60	0.65 0.50-0.85 **0.0018**

Abbreviations: SNP single nucleotide polymorphism, DR diabetic retinopathy, PDR proliferative diabetic retinopathy, MAF minor allele frequency, OR odds ratio, CI confidence interval. All analyses are adjusted for age, sex, duration of diabetes, HbA1c and hypertension. Sample sizes for each analyses are listed in Table 6.3.

5.6 Discussion

In our discovery cohort, no SNPs reached significance after multiple hypothesis testing ($p < 3.00x10^{-7}$). Therefore we undertook a two stage design, selecting the top ranking SNP for evaluation in the second cohort and a combined analysis of the two groups. We found that rs10061133 in MIR449b was most consistently associated with sight threatening DR and PDR. The minor allele G was protective against sight threatening DR and PDR after adjustment for covariates and was confirmed with greater significance in the larger sample. A large proportion of the sight threatening DR group consisted of PDR samples (181 out of 223), and so this association is likely to be more important in conferring PDR risk, rather than CSMO. Rs10061133 in MIR449b has been reported to be associated with other diseases such as oesophageal cancer[323], thyroid cancer[324], premature ovarian insufficiency[325] and recurrent pregnancy loss.[326] This is the first study to report an association between rs10061133, microRNA 449b, and DR in type 1 diabetes.

MicroRNA 449b is part of a family of microRNAs with similar sequences and secondary structures including microRNA 449a, 449c and 38. They are all involved in cell proliferation, tumorigenesis and angiogenesis, and are importantly hypoxia regulated. MicroRNA 449a and 449b have been found in retinal tissues of mice.[327, 328] In an oxygen induced DR mouse model, microRNA 449a was significantly down regulated, which is in keeping with a protective role against DR.[328] MicroRNA 449b has not been reported in human vitreous, however the studies investigating vitreous microRNAs are few and a small number of specimens (less than 5 PDR samples) have been analysed.[218-220] The angiogenesis pathway that microRNA 449b regulates is the E2F pathway.[329] E2F is a transcription factor essential for cell proliferation. E2F increases the levels of microRNA449b which then inhibits E2F in a negative feedback loop. Activation of E2F regulates the expression of other genes involved in angiogenesis such as VEGF and PIGF, and breakdown of the blood retinal barrier such as E-cadherin; hence there is good biological plausibility for the involvement of microRNA 449b in DR susceptability.[330, 331] Another downstream target of microRNA 449a/b is SERPINE1, also known as

plasminogen activator inhibitor-1 (PAI-1). Under hypoxic conditions, downregulation of microRNA 449a/b increases expression levels of PAI-1.[332] The main role of PAI-1 is to inhibit fibrinolysis, but it has also been shown to play an important role in facilitating retinal angiogenesis.[333] PAI-1 levels are increased in the serum of DR patients compared with diabetic controls[334] and polymorphisms of the PAI-1 gene have been previously linked with DR.[335]

Polymorphisms of microRNA genes can affect either the seed region (binding site) or the processing of precursor microRNA to mature microRNA and therefore the overall production and function of the molecule. Evidence supporting this has largely come from bioinformatics and statistical analyses.[336] Rs10061133 is a single nucleotide polymorphism, where the replacement of G over A in the MIR449b gene is predicted to alter a Dicer cleavage site according to the miRvar database.[337] Dicer is the enzyme that cleaves the precursor microRNA to the mature form. Therefore rs10061133 could have functional consequences on microRNA449b through this mechanism. Further functional studies are required to explore this hypothesis.

Despite our intention to comprehensively study all known microRNA variants, some microRNAs could not be studied. We attempted to study 2420 known microRNA genes variants and 401,000 microRNA binding site variants, but only 213 and 41,578 SNPs respectively were imputed with good quality. While we had many type 1 diabetic participants from our registry, after stratifying the group into different phenotypes, the numbers for analyses were much smaller. This clearly limits the scope of the current study. Analyses with grouped phenotypes such as 'any DR' and 'sight threatening DR' failed to reveal significant results. Future work should focus on directly genotyping microRNA SNPs in a suitably powered cohort, with replication in independent cohorts. Some of the top ranked SNPs in microRNA genes also failed genotyping in the replication dataset. It is possible that as microRNAs form secondary structures, the DNA template could have also formed secondary structures during the PCR stage and potentially inhibited primer binding.[338] Analysis of miRNAs in biological tissues (such as plasma/serum) of patients with type 1 diabetes could strengthen this study, however as the genetic

association results do not reach significance after multiple hypotheses testing ($p < 3.00x10^{-7}$), the results should be replicated by independent studies before resources are directed to functional assays. Interpretation of genetic associations is also complicated by the clinical picture of DR. Age and duration of diabetes are significant risk factors for worsening DR, resulting in the selection of a younger and healthier control group. We have corrected for hypertension, given its strong association with DR, but did not adjust for other comorbidities such as hyperlipidaemia or macrovascular complications (e.g. heart disease). We tried adjusting for nephropathy but no difference in results were achieved. Strengths of this study include sample population from multiple sites, rigour of DR status characterisation and categorisation, analyses with adjustments for known clinical risk factors and meta-analyses after a two stage design.

A disappointing number of positive associations were found with the discovery cohort but the majority did not replicate in the replication cohort or meta-analyses of the discovery and replication cohorts.. Inconsistent findings are common in genetic epidemiology studies and a number of reasons have been identified.[339] Selection bias, miscalculation of genotype or phenotype errors and confounding publication biases are unlikely for this study. Participants were sampled from a variety of sites. Significantly different clinical variables between cases and controls were adjusted for in the analyses, and only one ethnicity (Caucasian) was studied. The same clinical criteria for DR grading was utilised across sites and determined by trained ophthalmologists. The worst ever grading was used and not the most current grading. Genotyping error rates were below 5%.

Possible reasons for lack of replication could be that none of the SNPs in the discovery cohort were significant (at genome wide significance) or imputations could have been poor quality. Less stringent significance levels applied in preparation for stage 2 of the study increases the likelihood of false positives. T2DM is also a more heterogeneous disease and not accounting for these other phenotypic differences could have contributed to inconsistent results. Other possibilities include true variations in effect size between an allele and a disease. Genotyped variants could be in linkage equilibrium

with the disease causing variant that was not imputed or genotyped. The disease causing variant could be rare and subject to different selection by chance and inadequate sample sizes, causing different results in the replication cohort. Environmental factors and other epigenetic mechanisms may also modify these associations. Many of these variants appear to have small effect sizes and therefore the study design did not allow enough power to detect them.

Nevertheless, by expanding our analyses beyond the original design, we found that rs11700721 and rs1444269 in the microRNA binding sequences of the gene *ADAMTS5* were associated with PDR in T2DM. More importantly, in combined analyses, the association survives multiple hypotheses testing and could represent a true result. Rs11700721 and rs1444269 in the *ADAMTS5* gene have not been previously reported to be associated with DR. Using the PolymiRTS (version 2) online database, we searched for how rs11700721 and rs1444269 in the *ADAMTS5* gene might affected microRNA binding.[340] Bioinformatics predict that rs1444269 (G>A) disrupts an already existing conserved microRNA binding site for microRNA 3152-3p, 4694-5p and 6730-5p. Rs11700721 (C>T) also disrupts an already existing conserved microRNA binding site for microRNA 4536-5p and creates a new microRNA binding site for microRNA 3677-3p. There is little information about these microRNAs in the literature. MicroRNA 3152-3p, 4694-5p, 4536-5p were discovered in human cancer tissue by next generation sequencing, suggesting a potential role in cell proliferation.[341, 342] How the altered binding of these microRNA may affect *ADAMTS5* gene expression is unknown.

ADAMTS5 is a member of the ADAMTS family; a group of proteins involved in embryonic development and angiogenesis. ADAMTS5 has been found in retinal epithelial cells (ARPE-19) and was shown to be upregulated after exposure to TNF alpha.[343] TNF alpha is known to play a key role in neovascularisation in retinal pathologies.[344] In ocular diseases, ADAMTS5 was found to be significantly upregulated in age related macular degeneration, a disease similar to DR which also involves hypoxic and inflammatory pathways.[345]

5.7 Conclusion

Through a targeted genome-wide approach, this study has identified novel microRNA and microRNA binding site genetic variants associated with DR phenotypes in a large Caucasian population of type 1 and type 2 diabetic participants. Rs10061133 in MIR449b was found to be consistently associated with sight threatening DR and PDR in T1DM, when compared with diabetic controls (no DR). Bioinformatics predict rs10061133 has functional consequences on microRNA449b and therefore can affect regulation of inflammatory pathways in DR. Rs11700721 and rs1444269 in the microRNA binding sequences of the gene *ADAMTS5* were associated with PDR in T2DM when compared with diabetic controls (no DR). These SNPs affect the binding of 5 known microRNAs that could regulate the expression of *ADAMTS5;* a protein involved in the TNF-alpha induced NF-kb signalling pathways of DR. While further independent replication is desirable, these results can guide future studies on genetic risk profiling.

CHAPTER 6: MITOCHONDRIAL HAPLOGROUPS AND THEIR ASSOCIATION WITH DIABETIC RETINOPATHY

The original work presented in this chapter has been published in the peer-reviewed literature: Liu E, Kaidonis G, Gillies MCet al. Mitochondrial haplogroups are not associated with diabetic retinopathy in a large Australian and British Caucasian sample. Sci Rep 2019; 9: 612.

6.1 Introduction

Mitochondrial haplogroups H1, H2 and UK have been previously reported to be associated with PDR in Caucasian patients with diabetes.[204] We aimed to replicate this finding with a larger sample and expand the analysis to include different severities of DR, and DMO.

6.2 Methods

We utilised data from the GSDR and RADAR. Caucasian participants (self-identifying as of European descent) and only those with complete clinical risk factor information (sex, age, duration of diabetes, HbA1c, hypertension) were included in this study.

Genotyping was performed through the Australian Genome Research Facility, using the Agena Bioscience MassARRAY platform. We utilised the same panel of 22 mtDNA SNPs designed by Estopinal et al in previous studies to determine mitochondrial haplogroups (Table 5.1).[204] Haplogrep software was used to facilitate haplogroup identification.[346] Samples identified as non-Caucasian after haplogroup determination were removed.

Statistical analysis was performed with Statistical Package for Social Sciences versions 23.0 (For Windows; IBM Corp, Armonk, NY). Chi Square tests were performed to analyse the association of haplogroup type with various DR phenotypes such as any DR, any NPDR, PDR, DMO and CSMO. Logistic regression was used to adjust for covariates age, sex, type of diabetes, duration of diabetes, HbA1c and presence of hypertension. Statistical significance was set at $p<0.05$. Further analysis was performed by stratifying the analysis into T1DM and T2DM, and the major European

haplogroups (H1 and H2, UK). De-identified datasets generated during and/or analysed for this study have been made available at Research Data Australia: https://doi.org/10.25957/5c060cbdb9162

Table 6.1 List of 22 SNPs genotyped for haplogroup determination[204]

SNP	rCRS position	Haplogroup
rs2001030	1438	H2
rs28358576	1811	U
rs3928306	3010	J1, H1
rs2854131	3197	U5
rs2854134	3594	L0-2
rs28357980	4917	T
rs3021088	5460	W
rs41347846	10034	I
rs28358275	10238	I
rs2857284	10873	N
rs2853493	11467	U
rs3088053	11812	T2
rs28359168	11947	W
rs2853498	12308	U
rs2853499	12372	U
rs3926883	12633	T1
rs2854122	12705	R
rs2853503	13617	U5
rs28359178	13708	J
rs3135030	14470	X
rs28357681	14798	UK, J1c
rs41518645	15257	J2

Abbreviations: SNP Single nucleotide polymorphism, rCRS Cambridge reference position.

6.3 Results

6.3.1 Participant demographics

Patient demographics for the entire cohort (n=2935) stratified by DR phenotype are presented in Table 6.2.1 Chi square tests and Mann-Whitney U tests were used to compare demographic variables between the different phenotype groups, with p values presented in Table 6.2.2. Duration of diabetes, HbA1c and hypertension were associated with all subtypes of DR and DMO. Diabetes duration and HbA1c significantly increased from no DR to all DR phenotypes ($p<0.0001$, Mann-Whitney U test) and similarly from NPDR to PDR. Type of diabetes was also a significant variable affecting DR phenotypes PDR, any DMO and CSMO. Participants with PDR were younger than those with NPDR (median 59 versus 65 years, $p<0.0001$).

After separating the samples per diabetes type, the majority were T2DM participants (n =2265) compared with T1DM participants (n= 670). As we also separately analysed the cohort via diabetes type, the demographics of the T1DM and T2DM groups are given in Tables 6.3A and 6.4A respectively, with p values presented in tables 6.3B and 6.4B. Similar to the entire cohort, duration of diabetes and HbA1c were significantly increased from no DR to all DR phenotypes ($p<0.01$, Mann-Whitney U test) and from NPDR to PDR in both types of diabetes.

Table 6.2A Demographics of study population stratified by diabetic retinopathy phenotype

Demographic	Total	No DR	Any DR	Any NPDR	PDR	Any DMO	CSMO	Sight threatening
n	2935	1124	1811	1161	650	936	643	1278
Female; n (%)	1309 (44.6)	521 (46.5)	788 (43.7)	514 (44.4)	274 (42.4)	420 (45.5)	289 (45.2)	558 (43.9)
Age in years; median (range)	61 (17-95)	65 (17-95)	63 (18-95)	65 (18-95)	59 (21-90)	65 (21-92)	65 (26-92)	63 (21-92)
Diabetes duration in years; median (range)	18 (5-70)	12 (5-67)	20 (5-70)	18 (5-65)	23 (5-70)	19 (5-64)	19 (5-59)	20 (5-70)
Type 1 diabetes; n (%)	670 (22.8)	239 (21.5)	431 (24.1)	208 (18.2)	223 (34.6)	148 (16.0)	100 (15.7)	297 (23.5)
HbA1c %; median (range)	8.07 (2-22)	7.40 (2-22)	8.10 (4-15)	7.90 (5-15)	8.50 (4-15)	8.20 (4-15)	8.10 (5-15)	8.25 (4-15)
Hypertension; n (%)	1935 (65.9)	695 (61.8)	1240 (68.5)	782 (67.4)	458 (70.5)	642 (68.6)	471 (73.3)	874 (68.4)

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, HbAc1 glycosylated haemoglobin. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.2B P values of demographic variables compared between the DR phenotype groups

	No DR vs any DR	No DR vs any NPDR	No DR vs PDR	No DR vs DMO	No DR vs CSMO	No DR vs Sight threatening DR
Sex	0.077	0.333	0.102	0.563	0.619	0.216
Age	0.103	0.190	**<0.0001**	0.761	0.848	**0.041**
Diabetes duration	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Diabetes type	0.103	0.051	**<0.0001**	**$1.79x10^{-3}$**	**$3.79x10^{-3}$**	0.258
HbA1c	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Hypertension	**$3.74\ x10^{-4}$**	**$2.91x10^{-3}$**	**$1.18x10^{-3}$**	**$2.36x10^{-3}$**	**<0.0001**	**$1.37x10^{-3}$**

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, HbAc1 glycosylated haemoglobin. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.3A Demographics of type 1 diabetes group

Demographic	No DR	Any DR	Any NPDR	PDR	Any DMO	CSMO	Sight threatening DR
n	239	431	208	223	148	100	297
Female (n, %)	106 (44.5)	199 (49.3)	95 (45.7)	104 (46.8)	80 (54.1)	55 (55.0)	145 (49.0)
Age, years (median, range)	31 (17-83)	48 (18-88)	48 (18-88)	48 (21-85)	52 (21-88)	52 (26-85)	49 (21-88)
Diabetes duration, years (median, range)	13 (5-60)	27 (6-70)	23 (6-65)	31 (8-70)	26 (7-64)	27 (8-59)	29 (7-70)
HbA1c % (median, range)	7.8 (5-15)	8.4 (5-14)	8.2 (5-14)	8.7 (5-15)	8.0 (5-15)	8.6 (5-14)	8.6 (5-14)
Hypertension (n, %)	58 (24.3)	239 (55.5)	95 (45.7)	144 (64.6)	86 (58.1)	59 (59.0)	179 (60.3)

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, HbAc1 glycosylated haemoglobin. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.3B P values of demographic variables compared between the DR phenotype groups in type 1 diabetes

	No DR vs any DR	No DR vs any NPDR	No DR vs PDR	No DR vs DMO	No DR vs CSMO	No DR vs Sight threatening DR
Female	0.258	0.129	0.640	0.075	0.095	0.337
Age	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Diabetes duration	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
HbA1c	**<0.0001**	**0.008**	**<0.0001**	**<0.0001**	**0.007**	**<0.0001**
Hypertension	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, HbAc1 glycosylated haemoglobin. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.4A Demographics of type 2 diabetes group

Demographic	No DR	Any DR	Any NPDR	PDR	Any DMO	CSMO	Sight threatening DR
n	906	1359	938	421	775	535	966
Female (n, %)	408 (46.9)	565 (41.7)	395 (42.2)	170 (40.5)	332 (43.0)	230 (43.2)	405 (42.1)
Age, years (median, range)	68 (24-95)	66 (27-95)	67 (27-95)	63 (31-90)	65 (31-92)	65 (31-92)	65 (31-92)
Diabetes duration, years (median, range)	11 (5-67)	17 (5-58)	17 (5-58)	20 (5-55)	18 (5-58)	18 (5-58)	18 (5-58)
HbA1c % (median, range)	7.3 (2-22)	7.9 (4-15)	7.8 (5-15)	8.4 (4-15)	8.1 (4-15)	8.1 (5-15)	8.1 (4-15)
Hypertension (n, %)	636 (77.5)	995 (77.8)	684 (78.0)	311 (77.4)	553 (75.6)	409 (80.0)	691 (76.0)

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, HbAc1 glycosylated haemoglobin. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.4B P values of demographic variables compared between the DR phenotype groups in type 2 diabetes

Comparisons (n)	No DR vs any DR (906, 1359)	No DR vs any NPDR (906, 938)	No DR vs PDR (906, 421)	No DR vs DMO (906, 775)	No DR vs CSMO (906, 535)	No DR vs Sight threatening DR (906, 966)
Female	**0.016**	**0.047**	**0.032**	0.123	0.185	**0.038**
Age	**<0.0001**	**0.047**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Diabetes duration	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
HbA1c	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Hypertension	0.872	0.816	1.00	0.400	0.274	0.494

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema, HbAc1 glycosylated haemoglobin. Sight threatening DR includes severe NPDR, PDR and CSMO.

6.3.2 Mitochondria haplogroup and DR phenotype

A total of 7 European mitochondrial haplogroups were identified in our Caucasian sample. The most common were haplogroup H1 and H2 (analysed collectively) and UK at 50.8% and 22.5% respectively. Other types included JT (12.4%), R (7.1%), I (4.2%), W (2.0%) and X (1.0%)) (Table 5.5). One SNP (rs3088053, rCRS position 11812) failed genotyping and therefore Haplogroup T2 could not be identified in our samples. As T2 is a subtype of J, we have therefore combined haplogroups J, T1 (and T2) in our analyses.

Table 6.5 Haplogroup frequencies in this study

Haplogroup	n (%)
H	1483 (50.5)
UK	667 (22.7)
JT	371 (12.6)
R	208 (7.1)
I	122 (4.2)
W	57 (1.9)
X	26 (0.9)

We found the percentages of the three most common haplotype groups (H1 and H2, UK and JT) were distributed similarly in each of the different phenotype groups, and that any differences when compared with no DR controls were not statistically significant after performing Chi Square association tests (Table 6.6). We also found no significant associations when haplogroups were compared between NPDR and PDR. There were no significant differences when all 7 haplogroups were analysed separately instead of grouping less common haplogroups into one category.

Table 6.6 Haplogroup distribution (H, UK, JT, Other) per DR phenotype

	Proportion (%)				P value
Haplogroup	**H**	**UK**	**JT**	**I, R,W,X**	
No DR	50.7	22.5	12.2	14.6	NA
Any DR	50.4	22.9	12.9	13.8	0.885
NPDR	50.9	22.9	12.4	13.8	0.954
PDR	49.5	22.8	13.8	13.8	0.760
DMO	51.0	23.3	12.5	13.2	0.830
CSMO	49.6	23.3	13.2	13.8	0.867
Sight threatening DR	50.8	23.2	12.9	13.1	0.721
NPDR	50.4	22.9	12.8	13.9	0.793

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema. Sight threatening DR includes severe NPDR, PDR and CSMO.

P values are derived from chi square tests. All comparisons are made to the phenotype 'no DR' except for the last row where comparisons are made to the phenotype 'PDR'.

Binary logistic regression show that haplogroups H1 and H2, and UK were not associated with any DR phenotypes in either T1DM or T2DM after adjustment for sex, age, diabetes duration, HbA1c and hypertension (Table 6.7 and 6.8 and Table 6.9 and 6.10 respectively). Gender was not associated with any DR phenotypes. Age was significantly associated with more DR phenotypes in T2DM than T1DM. After logistic regression, diabetes duration and HbA1c remained significant risk factors for DR in both T1DM and T2DM, while hypertension only remained significant in T1DM.

The next most common haplogroups (JT and K separately from UK) were analysed separately (frequencies 12.4% and 7.8% respectively). Significant results were: haplogroup K was nominally associated with any DR (135 cases, 98 controls, OR 0.49, 96% CI 0.24-1.00, p=0.05) and NPDR (85 cases, 98 controls, OR 0.31, 95% CI 0.13-0.78, p=0.012). JT was nominally associated with NPDR (144 cases, 137 controls, OR 2.20, 95%CI 1.09-4.43, p=0.027) and CSMO (85 cases, 137 controls, OR 2.06, 95% CI 1.16-8.08, p=0.024) These results

should be treated with caution as the numbers are small and the association does not survive correction for multiple hypothesis testing.

Table 6.7 Association of haplogroup H (H1 and H2) and other variables with DR phenotypes in type 1 diabetes

DR phenotype (n)	Any DR (431)	NPDR (208)	PDR (223)	DMO (148)	CSMO (100)	Sight threatening DR (297)	PDR (compared with NPDR) (223, 208)
Haplogroup H1 and H2	0.90 (0.58-1.40) P=0.652	0.92 (0.57-1.48) P=0.718	0.95 (0.53-1.69) P=0.848	0.95 (0.52-1.77) P=0.881	0.79 (0.38-1.63) P=0.524	0.97 (0.58-1.63) P=0.918	0.98 (0.62-1.55) P=0.924
Sex (female)	1.34 (0.86-2.09) P=0.199	1.30 (0.80-2.10) P=0.294	1.44 (0.79-2.62) P=0.231	1.43 (0.77-2.64) P=0.255	1.54 (0.78-3.17) P=0.243	1.43 (0.85-2.41) P=0.182	0.84 (0.53-1.33) P=0.453
Age	1.02 (1.0-1.03) P=0.063	1.02 (1.0-1.04) **P=0.011**	0.99 (0.96-1.01) P=0.347	1.04 (1.02-1.06) **P=3.81x10^{-3}**	1.03 (1.01-1.06) **P=0.007**	1.02(1.0-1.04) P=0.104	0.97 (0.95-0.99) **P=0.001**
Diabetes duration	1.13 (1.10-1.16) **P<0.0001**	1.10 (1.07-1.13) **P<0.0001**	1.14 (1.11-1.19) **P<0.0001**	1.08 (1.04-1.11) **P<0.0001**	1.08 (1.04-1.12) **P<0.0001**	1.13 (1.10-1.17) **P<0.0001**	1.08 (1.06-1.11) **P<0.0001**
HbA1c	1.46 (1.26-1.70) **P<0.0001**	1.38 (1.18-1.63) **P<0.0001**	1.54 (1.28-1.86) **P<0.0001**	1.57 (1.30-1.91) **P<0.0001**	1.49 (1.19-1.87) **P<0.0001**	1.52 (1.28-1.79) **P<0.0001**	1.25 (1.07-1.46) **P=0.004**
Hypertension	2.19 (1.30-3.68) **P<0.0001**	1.61 (0.90-2.87) P=0.108	4.34 (2.24-8.41) **P<0.0001**	2.60 (1.34-5.07) **P=4.85x10^{-3}**	2.61 (1.19-5.73) **P=0.017**	2.82 (1.57-5.05) **P=0.001**	2.21 (1.31-3.73) **P=0.003**

Values presented are odds ratio, 95% confidence interval and p values. Binary logistic regression was performed, adjusting for other variables listed in the table. DR phenotype listed was compared against controls, defined as 'no DR', except for the last column where PDR was compared with NPDR.

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.8 Association of haplogroup UK and other variables with DR phenotypes in type 1 diabetes

DR phenotype (n)	Any DR (431)	NPDR (208)	PDR (223)	DMO (148)	CSMO (100)	Sight threatening DR (297)	PDR (compared with NPDR) (223, 208)
Haplogroup UK	0.80 (0.49-1.32) P=0.391	0.68 (0.39-1.21) P=0.191	(0.53-1.91) P=0.989	0.65 (0.31-1.36) P=0.255	0.50 (0.21-1.24) P=0.136	0.88 (0.49-1.58) P=0.674	1.60 (0.93-2.75) P=0.087
Sex (female)	1.34 (0.86-2.09) P=0.201	1.28 (0.79-2.08) P=0.311	1.44 (0.79-2.62) P=0.231	1.46 (0.79-2.70) P=0.231	1.53 (0.74-3.16) P=0.251	1.43 (0.85-2.42) P=0.177	0.84 (0.53-1.34) P=0.457
Age	1.02 (1.0-1.03) P=0.064	1.02 (1.00-1.04) **P=0.011**	0.99 (0.96-1.01) P=0.351	1.04 (1.02-1.06) **P=4.17x10^{-4}**	1.04 (1.00-1.06) **P=0.007**	1.02 (1.00-1.04) P=0.108	0.97 (0.95-0.99) **P=0.001**
Diabetes duration	1.13 (1.09-1.16) **P<0.0001**	1.10 (1.06-1.13) **P<0.0001**	1.14 (1.11-1.19) **P<0.0001**	1.08 (1.04-1.11) **P<0.0001**	1.08 (1.04-1.12) **P<0.0001**	1.13 (1.10-1.17) **P<0.0001**	1.08 (1.06-1.11) **P<0.0001**
HbA1c	1.46 (1.26-1.70) **P<0.0001**	1.39 (1.18-1.63) **P<0.0001**	1.54 (1.28-1.86) **P<0.0001**	1.56 (1.28-1.89) **P<0.0001**	1.47 (1.17-1.85) **P=0.001**	1.51 (1.28-1.79) **P<0.0001**	1.25 (1.07-1.46) **P=0.004**
Hypertension	2.22 (1.32-3.75) **P=0.003**	1.67 (0.93-2.98) P=0.086	4.34 (2.23-8.42) **P<0.0001**	2.75 (1.40-5.41) **P=0.003**	2.86 (1.28-6.40) **P=0.011**	2.85 (1.58-5.13) **P=4.80x10^{-4}**	2.27 (1.35-3.84) **P=0.002**

Values presented are odds ratio, 95% confidence interval and p values. Binary logistic regression was performed, adjusting for other variables listed in the table. DR phenotype listed was compared against controls, defined as 'no DR', except for the last column where PDR was compared with NPDR.

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.9 Association of haplogroup H (H1 and H2) and other variables with DR phenotypes in type 2 diabetes

DR Phenotype (n)	Any DR (1359)	NPDR (938)	PDR (421)	DMO (775)	CSMO (535)	Sight threatening DR (966)	PDR (compared with NPDR) (421, 938)
Haplogroup H	1.03 (0.83-1.28) P=0.777	1.07 (0.85-1.34) P=0.571	0.92 (0.66-1.27) P=0.612	1.04 (0.80-1.34) P=0.780	0.91 (0.69-1.21) P=0.521	1.04 (0.81-1.32) P=0.763	0.93 (0.69-1.23) P=0.598
Sex (female)	0.75 (0.60-0.93) **P=0.008**	0.76 (0.60-0.96) **P=0.019**	0.70 (0.50-0.97) **P=0.033**	0.87 (0.67-1.12) P=0.270	0.87 (0.65-1.15) P=0.328	0.79 (0.63-1.01) P=0.064	0.95 (0.70-1.27) P=0.711
Age	0.98 (0.97-0.99) **P<0.0001**	0.99 (0.98-1.0) **P=0.016**	0.96 (0.94-0.97) **P<0.0001**	0.98 (0.97-0.99) **P=4.81x10^{-4}**	0.98 (0.97-0.99) **P=0.002**	0.97 (0.96-0.99) **P<0.0001**	0.97 (0.96-0.98) **P<0.0001**
Diabetes duration	1.09 (1.08-1.11) **P<0.0001**	1.08 (1.07-1.10) **P<0.0001**	1.12 (1.10-1.15) **P<0.0001**	1.10 (1.08-1.12) **P<0.0001**	1.10 (1.08-1.12) **P<0.0001**	1.11 (1.09-1.12) **P<0.0001**	1.04 (1.02-1.06) **P<0.0001**
HbA1c	1.37 (1.27-1.48) **P<0.0001**	1.33 (1.22-1.44) **P<0.0001**	1.50 (1.34-1.67) **P<0.0001**	1.45 (1.32-1.58) **P<0.0001**	1.44 (1.30-1.60) **P<0.0001**	1.45 (1.33-1.58) **P<0.0001**	1.15 (1.06-1.26) **P=0.001**
Hypertension	0.96 (0.74-1.24) P=0.742	0.94 (0.71-1.25) P=0.681	0.96 (0.65-1.43) P=0.846	0.77 (0.57-1.04) P=0.092	1.06 (0.74-1.51) P=0.758	0.83 (0.62-1.11) P=0.208	1.01 (0.71-1.44) P=0.943

Values presented are odds ratio, 95% confidence interval and p values. Binary logistic regression was performed, adjusting for other variables listed in the table. DR phenotype listed was compared against controls, defined as 'no DR', except for the last column where PDR was compared with NPDR.

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema. Sight threatening DR includes severe NPDR, PDR and CSMO.

Table 6.10 Association of haplogroup UK and other variables with DR phenotypes in type 2 diabetes

DR Phenotype (n)	Any DR (1359)	NPDR (938)	PDR (421)	DMO (775)	CSMO (535)	Sight threatening DR (966)	PDR (compared with NPDR) (421, 938)
Haplogroup UK	(0.77-1.29) P=0.981	0.98 (0.74-1.29) P=0.863	1.13 (0.76-1.67) P=0.541	1.08 (0.80-1.46) P=0.630	1.13 (0.81-1.59) P=0.464	1.03 (0.77-1.38) P=0.849	1.07 (0.75-1.51) P=0.714
Sex (female)	0.75 (0.60-0.93) **P=0.008**	0.76 (0.60-0.95) **P=0.018**	0.70 (0.50-0.97) **P=0.034**	0.87 (0.67-1.12) P = 0.267	0.87 (0.66-1.16) P=0.348	0.79 (0.62-1.01) P=0.063	0.95 (0.70-1.27) P=0.712
Age	0.98 (0.97-0.99) **P<0.0001**	0.99 (0.98-1.0) **P=0.016**	0.96 (0.94-0.97) **P<0.0001**	0.98 (0.97-0.99) **P= 4.73×10^{-4}**	0.98 (0.97-0.99) **P=0.002**	0.97 (0.96-0.99) **P<0.0001**	0.97 (0.96-0.98) **P<0.0001**
Diabetes duration	1.09 (1.08-1.11) **P<0.0001**	1.08 (1.07-1.10) **P<0.0001**	1.12 (1.10-1.15) **P<0.0001**	1.10 (1.08-1.12) **P<0.0001**	1.10 (1.08-1.12) **P<0.0001**	1.11 (1.09-1.12) **P<0.0001**	1.04 (1.02-1.06) **P<0.0001**
HbA1c	1.37 (1.27-1.48) **P<0.0001**	1.33 (1.22-1.44) **P<0.0001**	1.50 (1.34-1.67) **P<0.0001**	1.45 (1.32-1.58) **P<0.0001**	1.44 (1.30-1.60) **P<0.0001**	1.45 (1.33-1.58) **P<0.0001**	1.15 (1.06-1.26) **P=0.001**
Hypertension	0.96 (0.74-1.25) P=0.746	0.95 (0.72-1.25) P=0.695	0.96 (0.64-1.43) P=0.839	0.77 (0.57-1.04) P=0.091	1.05 (0.74-1.50) P=0.772	0.83 (0.62-1.11) P=0.207	1.01 (0.71-1.44) P=0.953

Values presented are odds ratio, 95% confidence interval and p values. Binary logistic regression was performed, adjusting for other variables listed in the table. DR phenotype listed was compared against controls, defined as 'no DR', except for the last column where PDR was compared with NPDR.

Abbreviations: DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, DMO diabetic macular oedema, CSMO clinically significant macular oedema. Sight threatening DR includes severe NPDR, PDR and CSMO.

6.4 Discussion

In our larger Caucasian sample, unlike earlier smaller studies, we found no significant associations between mitochondrial haplogroups and the presence of any DR, DMO, nor more severe phenotypes such as PDR, CSMO or sight-threatening DR (severe NPDR, CSMO or PDR). This was true for analysis as a group or when stratified for T1DM, despite following the same methods as the previous studies which found positive associations.

Estopinal *et al* first demonstrated that haplogroups H1 and H2 (analysed collectively) and UK were associated with PDR when compared with NPDR in an American Caucasian sample (n=197 NPDR, 195 PDR).[204] Haplogroup H1 or H2 was a risk factor, while haplogroup UK was protective. Bregman *et al* expanded from this initial study with 513 additional diabetic controls from the same databases (Vanderbilt Eye Institute and Vanderbilt University).[203] They found that haplogroup H1 and H2, and UK were not associated with any incident DR compared with no DR. Using the same cohort, Mitchell et al found duration of diabetes and HbA1c was significantly associated with PDR in patients with haplogroups H1 and H2, but not UK, suggesting that mitochondrial haplogroups modify these clinical risk factors for the development of PDR in T2DM.[206] In a different study, Kofler et al reported haplogroup T was significantly associated with any DR compared with no DR (12.1% vs 5.1%; p = 0.046).[207]

Inconsistent results are common in haplogroup association studies in all areas. For example Crispim *et al* reported haplogroup cluster J/T was significantly associated with insulin resistance in a Caucasian Brazilian population,[347] but this was refuted by two other studies of Caucasian samples.[348, 349] Challenges in interpreting mitochondrial association studies include differences in study design, case and control definitions, statistical analysis, population stratification, inadequate power and lack of replication.[350]

Different results could be due to different populations and study design, however in examining the demographics and distribution of the haplogroups, our study cohort appears to be similar to the cohort

from the Vanderbilt Eye Institute and Vanderbilt University. Both cohorts consist of Caucasian patients of European descent. We used the same criteria for selection of retinopathy cases and controls and the same statistical analyses. The most common haplogroups were H1 and H2, and UK; 73.3% in our study, compared with 68% in Bregman et al's study conducted on the cohort from the Vanderbilt Eye Institute and University. As expected in both studies, age, diabetes duration, type of diabetes and HbA1c were strongly associated with increasing severity of DR.

An important reason why our conclusions are different is because our study consisted of a much larger population; 1124 diabetic retinopathy controls (no DR), 1161 NPDR cases and 650 PDR cases. Therefore our study has increased statistical power to identify any true associations. We were unable to replicate previously reported associations, suggesting that these previous association may be false. Smaller studies and sub analyses of phenotype groups lead to a higher risk of type 1 errors.[351] Our larger study size allowed us to analyse other phenotypes such as DMO and CSMO, as well as to separately analyse less common haplogroups such as JT, and K separate from UK. The only statistically significant results we found were haplogroup K was nominally associated with any DR, and haplogroup JT was nominally associated with NPDR and CSMO. As the numbers were small in these comparisons and the result does not survive multiple hypothesis testing, this is likely to be a type 1 error. Haplogroup K was not a common haplogroup in the previous two studies and is not implicated in diabetes and other associated diseases. Kofler et al reported haplogroup T was significantly associated with any DR but this study also had a much smaller sample size (149 with any DR and 78 with no DR).[207] As noted in our results, we were unable to separately analyse haplogroup T due to genotyping failure, and so direct comparison to Kofler et al's study could not be made.

In addition to study size, strengths of this study include the inclusion of both T1DM and T2DM subjects from multiple sites, rigour of retinopathy status characterisation, wide range of levels of DR

and use of the same haplotyping methods and statistical analyses as previous studies so comparisons could be made.

The haplotyping method we utilised from previous studies has a major limitation. SNPs chosen to represent the H haplogroup (rCRS position 3010 and 1438) only identify haplogroups H1 and H2. Therefore 7 other major subtypes of haplogroup H were not analysed. One SNP completely failed genotyping (rCRS 11812, determination of haplogroup T2). Another 4 SNPs had a 2% failure rate, and this could have contributed to the percentage of samples with haplogroup R (a major clade consisting of H, J, T, and UK). We chose our Caucasian sample based on participants self-identifying as Caucasian, but a small number had non-Caucasian haplogroups (for example haplogroup A, B, C, L, M, N and Q). Some of the 22 SNPs chosen for haplogroup determination are also found in other ethnic populations (for example rCRS position 3197 determines U5 but also L3e3 which is found in Asian populations). Therefore, to overcome this limitation, all samples with a non-Caucasian haplogroup were removed to minimize any confounding effect and reduce population stratification.

We recognise that even larger studies and studies in different ethnic groups, particularly those at high risk of diabetic retinopathy, are desirable. We only studied 7 haplogroups, while the human mitochondrial phylogenetic tree consists of hundreds of haplogroups. The hypothesis that variations in the mitochondrial genome contribute to DR risk is logical given the role that the mitochondria play in generating oxidative stress in DR.[41] Mitochondrial DNA are inherited completely from the maternal line, unlike nuclear DNA which has equal maternal and paternal contributions.[352] Risk of T1DM in the offspring varies by parental status; being two-fold lower if the mother has T1DM rather than the father.[353] Epidemiology studies show that certain ethnicities are at greater risk of DR such as people of Asian, African and Indigenous ethnic groups.[354] Complex biological and environmental factors explain this observation, and mitochondrial genetics could also play a role.

Mitochondrial haplogroup is not a specific marker for mitochondrial genetic variability. A haplogroup consists of many genetic variants that are inherited together. Therefore, if there are specific

mitochondrial variants that contribute to DR, these cannot be studied effectively. SNPs in mitochondrial genes cause diseases such as Leber hereditary optic neuropathy, and in complex diseases such as diabetes and cancer, it is increasingly recognised that small mitochondrial defects could lead to subtle bioenergetics alterations with major clinical implications.[355] Specific mitochondrial variants that have been studied for DR are nuclear genes encoding mitochondrial proteins such as *UCP2* and *Mn-SOD*.[198]

Few studies have demonstrated whether mitochondrial haplogroup directly affects mitochondrial function. Fang *et al* recently reported lower respiratory chain complex activity in haplogroup N9a compared with D4j, G4a2 and Y1, using transmitochondrial technology.[356] Mueller et al reported mitochondrial haplogroup T cell cybrids had a higher survival rate than haplogroup H cybrids under oxidative stress conditions such as when challenged with hydrogen peroxide.[357] Haplogroup K cybrids showed different gene expression levels compared with H cybrids after amyloid-beta toxicity.[358] Untreated retinal cell cybrids of H and J haplogroups also showed different gene expression and methylation status.[359] Future studies and techniques designed to explore the mitochondria genome in better detail than currently available can help us understand whether mitochondrial genetics contribute to DR risk.[360]

6.5 Conclusion

In contrast to previous studies, our much larger study found no association between the major European mitochondrial haplogroup H1, H2, UK, and DR phenotypes in either T1DM or T2DM. No significant associations were found for different severities of DR and DMO, or other subsets of mitochondrial haplogroups that were analysed by this study.

CHAPTER 7: GENETIC VARIANTS IN VEGF RECEPTOR GENES

7.1 Introduction

Genetic variations in the *VEGFR* genes could have implications for DR risk and treatment response in DMO. As described in chapter 1, there are three specific VEGF receptors; VEGFR1, VEGFR2 and VEGFR3. We performed the same targeted GWAS approach and two stage design as described in chapter 5 to explore these variants and their association with DR.

7.2 Additional methods

Four SNPs were selected from the *in-silico* analysis of the imputed GWAS data (Table 7.1A). SNPs were chosen based on the significance (p value) of their association with DR phenotypes. A literature review was carried out and previous SNPs demonstrated to be significantly associated with other ocular and non-ocular disease phenotypes and had functional changes predicted from laboratory experiments or bio-informatic tools, were also genotyped (Table 7.1 B).[361-370] Known SNPs in the VEGFR1, VEGFR2 and VEGFR3 genes were downloaded from the 1000G Genomes Project, using phase 3 EUR samples (accessed 31/08/2017). The same cohort and data set was used for analyses of microRNA related variants and VEGF receptor genes.

Table 7.1A: SNPs in VEGFR genes selected from imputed GWAS data

Gene	SNP	OR	95% CI (upper)	95% CI (lower)	P	Cases
VEGFR2	rs7667298	0.46	0.29	0.73	**8.26×10^{-4}**	T1 DM, sight threatening DR
VEGFR1	rs145121373	0.21	0.49	0.09	**3.12×10^{-4}**	T2 DM, any DR
VEGFR1	rs56138102	7.06	21.72	2.30	**6.48×10^{-4}**	T2 DM, sight threatening DR

Table 7.1B: SNPs in VEGFR genes selected from literature review

SNP	Gene	Location	Functional consequences	Study
rs664393	VEGFR1	3'-UTR	Well established polymorphism, minor allele frequency >10%, predicted to affect ESE sequence, 3'-UTR or promoter region	Scartozzi et al
rs7993418	VEGFR1	Syn: ESE		
rs74412485	VEGFR1	intron	Enhanced transcriptional level of VEGFR1	Konta et al
rs7324510	VEGFR1	intron	Enhanced transcriptional level of VEGFR1	
rs7996030	VEGFR1	intron	Reduced transcription of VEGFR1	Glubb et al
rs9943922	VEGFR1	intron	Associated with increased serum levels of VEGFR1 in neovascular aged related macular degeneration	Owen et al
rs1870377	VEGFR2	missense	Bio informatics predict nonsynonymous amino acid changes at residue 472H>Q	Wang et al
rs2305948	VEGFR2	missense	Bio informatics predict nonsynonymous amino acid changes at residue 297V>I	
rs2071559	VEGFR2	promoter	Bio informatics predict structural changes in promoter region that may decrease expression of VEGFR2	
rs7667298	VEGFR2	5'-UTR	Well established polymorphism, minor allele frequency >10%, predicted to affect ESE sequence, 3'-UTR or promoter region	Scartozzi et al
rs7667298	VEGFR3	3'-UTR	Well established polymorphism, minor allele frequency >10%, predicted to affect ESE sequence, 3'-UTR or promoter region	Scartozzi et al

SNPs shown in table 7.1A were selected from the in-silico analysis of the imputed GWAS data, based on the strength of their association with DR phenotypes.

Abbreviations: VEGFR vascular endothelial growth factor, SNP single nucleotide polymorphism, OR odds ratio, CI confidence interval, T1DM type 1 diabetes mellitus, T2DM type 2 diabetes mellitus, DR diabetic retinopathy, NPDR non-proliferative diabetic retinopathy, PDR proliferative diabetic retinopathy, CSMO clinically significant maculae oedema. Sight threatening DR defined as severe NPDR, PDR and CSMO.

7.2 Results

A total of 439 SNPs in the *VEGFR1* gene, 82 SNPs in the *VEGFR2* gene and 234 SNPs in the *VEGFR3* gene were imputed with good quality from our previous GWAS. Nominal associations between these SNPs and DR phenotypes are shown in Appendix 5 for T1DM and Appendix 6 for T2DM. No SNPs reached significance after multiple hypothesis testing (755 variants, 4 DR phenotype outcomes, $p < 1.66 \times 10^{5}$). Therefore the top SNPs across the different phenotypes ($p < 0.001$) were selected for genotyping. One variant, rs7667298 in the VEGFR2 gene, was selected for its association with sight threatening DR (OR 0.46, $p = 8.26 \times 10^{-4}$) in T1DM. Three variants in the VEGFR1 gene were chosen in the T2DM cohort: 1) rs3751395 due to its association with DMO (OR 1.25, $p = 4.1 \times 10^{-4}$) and sight threatening DR (OR 1.26, $p = 1.0 \times 10^{-4}$), 2) rs145121373 and its association with any DR (OR 0.21, $p = 3.11 \times 1^{-4}$), 3) rs56138102 and its association with sight threatening DR (OR 2.30, $p = 6.48 \times 10^{-4}$).

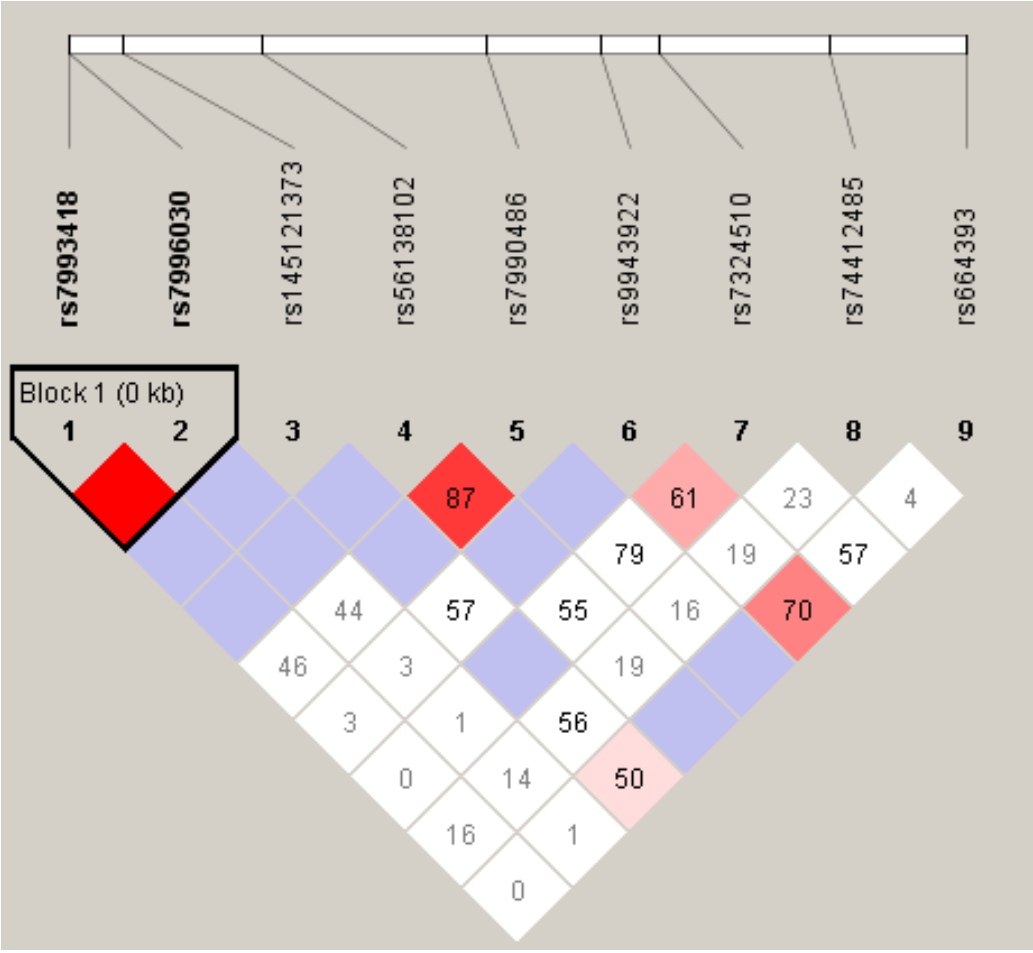
Three out of the 4 SNPs selected for genotyping from both the T1DM and T2DM cohorts were successfully genotyped in our samples. All eleven SNPs identified from the literature as having demonstrated or predicted functional consequences were successfully genotyped in our samples (Table 7.2). Nine out of the 14 SNPs were in the *VEGFR1* gene. We ran these SNPs on Haploview to assess the linkage disequilibrium (LD) between these SNPs. Figure 7.1 shows the LD block generated in Haploview using our own data. The only SNPs that appeared to be LD with each other were rs7993418 and rs7996030. The other SNPs were generally not in LD with each other, and therefore represented a good selection of tagging SNPs for the *VEGFR1* gene.

None of the SNPs from the literature were significantly associated with DR in our samples. Out of the 3 SNPs selected for genotyping from our samples, rs56138102 in the *VEGFR1* gene had the most consistent results in its association with sight threatening DR (Table 7.2). Rs56138102 in *VEGFR1* was strongly associated with sight threatening DR in T2DM with an OR of 3.97 to 4.39 across the three groups, and an adjusted p value of 4.40×10^{-3} in the GWAS group, 0.029 in the additional group

and 6.0 x 10^{-4} in the combined group. The association became more significant as the group became larger, and survived multiple hypothesis testing ($p < 3.57 \times 10^{-3}$, 14 SNPs genotyped).

The potential functional effect of rs56138102 was explored using the tool, Splicing based Analysis of Variants (SPANR). However, the analysis returned negative results as the SNP is more than 300 nucleotides away from known splice sites.[371]

Figure 7.1 Linkage disequilibrium patterns and haplotype block for the selected 9 SNPs of the VEGFR1 gene



Footnote: The magnitude of linage disequilibrium (LD) between pairs of SNPs are shown in each square. LD is measured by D' and the confidence in the value of D' is measured by the logarithm of likelihood odds ratio (LOD). The standard colour scheme of Haploview are used. Where D'=1, LD is shown as bright red (LOD>2) or blue (LOD<2). Where D'<1, LD is shown as various shades of pink (LOD>2) or white (LOD<2).

Table 7.2: Imputation, validation, replication and meta analyses of association results between significant SNPs and DR phenotypes

			Discovery - imputed		Discovery - genotyped		Additional genotyped		All samples combined	
SNP Gene	Minor Allele	DR case phenotype	MAF* cases vs controls (%)	OR 95% CI P	MAF* cases vs controls (%)	OR 95% CI P	MAF* cases vs controls (%)	OR 95% CI P	MAF* cases vs controls (%)	OR 95% CI P
rs56138102 VEGFR1	A	T2DM, Sight threatening DR	2.03 vs 0.78	7.06 2.30-21.7 **6.48×10^{-4}**	1.64 vs 0.86	4.39 1.59-12.15 **4.40×10^{-3}**	1.37 vs 0.59	4.28 1.16-15.74 **2.90×10^{-2}**	1.49 vs 0.70	3.97 1.80-8.72 **6.10×10^{-4}**
rs145121373 VEGFR1	T	T2DM, Any DR	1.19 vs 3.25	0.21 0.09-0.49 **3.12×10^{-4}**	1.41 vs 2.85	0.32 0.15-0.67 **2.70×10^{-3}**	1.22 vs 3.34	4.68 1.47-14.87 **8.95×10^{-3}**	1.70 vs 2.24	0.82 0.48-1.41 0.48
rs7667298 VEGFR2	T	T1DM, Sight threatening DR	37.58 vs 48.02	0.46 0.29-0.73 **8.26×10^{-4}**	38.78 vs 48.72	0.64 0.41-1.00 5.12×10^{-2}	44.03 vs 44.20	0.77 0.41-1.45 0.42	40.42 vs 46.83	0.71 0.50-1.01 5.88×10^{-2}

Abbreviations: VEGFR vascular endothelial growth factor, SNP single nucleotide polymorphism, DR diabetic retinopathy, MAF minor allele frequency, OR odds ratio, CI confidence interval. Sight threatening DR defined as severe NPDR, PDR and CSMO.

All analyses are adjusted for age, sex, duration of diabetes, HbA1c and hypertension. Sample sizes for each analysis are listed in Chapter 8, Table 8.3.

7.3 Discussion

In our discovery cohort, we found no imputed SNPs in the *VEGFR*1, 2 and 3 genes to be significantly associated with DR ($p < 1.66 \times 10^{-5}$ for 755 SNPs imputed). After joint analysis of the discovery and replication cohorts, rs56138102 in the *VEGFR1* gene was significantly associated with sight threatening DR in T2DM (OR 3.97, $p = 6.1 \times 10^{-4}$). This is the first study to report an association between rs56138102 and diabetic retinopathy.

Rs56138102 is an intronic variant, and its functional consequences are unknown. It could be in linkage disequilibrium with an exon variant that was not detected in our original GWAS and therefore not genotyped. A study focused on examining the whole *VEGFR1* gene to include rare alleles would be required to explore this possibility. A second hypothesis is that rs56138102 in *VEGFR1* does cause functional changes. There is growing evidence that intronic variants can affect disease pathology by controlling pre-mRNA splicing.[372] Three mechanisms have been identified: 1) mutations in the cis-acting sequence (or located within the same gene) affecting single genes 2) mutations in the trans-acting sequence affecting multiple RNA targets and 3) mutations in splicing factors that can lead to more widespread gene expression changes. According to the Human Gene Mutation Database Diseases (HGMD 2014.4), one third of all disease-causing mutations disrupt norming splicing.[373] Diseases where intronic variants have been implicated include cancer and neurodegenerative diseases such as muscular dystrophy and Parkinson's disease.[374, 375] The recent meta analyse of GWAS for DR found that the only genetic variant that surpassed genome wide level of significant was an intronic variant; rs142293996 in the nuclear VCP-like (*NVL*) gene.[191] There are currently limited resources to explore the functional effects of intronic variants.[376, 377] Two bioinformatic tools (IntSplice and SPANR) study variants within 300 nucleotides of known splice sites. Rs56138102 is unfortunately not located near these sites.[371]

*VEGFR*1 is known to have multiple splice variants. The most well-known, sFLT, is soluble and acts as a 'VEGFA trap", binding more strongly to VEGFA than its main receptor VEGFR2, and

therefore inhibits the VEGF pathways.[223] This is in opposition to the function of cellular VEGFR1 which binds to VEGFB and PIGF, contributing to angiogenesis under pathological conditions such as ischemia and inflammation.[224] Intronic variants may affect the balance of these VEGFR1 isoforms, and therefore the overall VEGF driven effect. Anti-VEGF agent aflibercept, is a recombinant fusion protein modelled on VEGFR1, and its main mechanism of action is to act as a VEGF trap. The Diabetic Retinopathy Clinical Research Network which comprises of over 100 sites in the United States, recently published results from a large randomised control trial that showed aflibercept yielded slightly superior visual outcomes than other anti-VEGF agents in eyes with DMO.[109] VEGFR1 is highly expressed in retinal epithelial cells, and could have a more important role in inflammation and neovascularisation than previously recognised. Further studies to better understand how intronic variants could contribute to complex diseases include *in silico* studies or laboratory based studies such as conventional RT-PCR analysis, sequencing of cDNA products, or direct RNA-seq analysis.

This study did not find any significant results in the T1DM cohort. Genetic risk factors may differ between the two types of diabetes. Type 1 diabetes results from autoimmune mediated destruction of insulin secreting beta cells of the pancreas, while type 2 diabetes results from insulin resistance with eventual beta cell mass decline. Insulin resistance by itself affects TNF-alpha induced NF-kb signalling pathways that contribute to DR.[378] Another reason for a lack of results in the T1DM cohort could be inadequate power as the T1DM cohort was smaller than the T2DM cohort.

There were also limitations with the T2DM cohort. Rs56138102 in *VEGFR1* has a minor allele frequency less than 1% in the cases and 1-2% in the controls of our cohort (Table 7.2). While the T2DM cohort is larger than the T1DM cohort, it still did not have adequate power to find variants with this low frequency. The SNP was selected due to its high odds ratio and significance level in the discovery cohort (OR 7.6, $p = 6.48 \times 10^{-4}$). Therefore, this result may be a false positive and requires replication in a larger population. Small sample sizes also translate to false negative results.

Four out of 439 imputed SNPs were selected for genotyping, and one SNP failed genotyping. It is possible that many potentially significant SNPs were not formally assessed in the replication cohort.

7.4 Conclusion

We conducted comprehensive analyses of SNPs in all three *VEGF* receptor genes and their association with DR. We found intronic variant rs56138102 in the *VEGFR1* gene was significantly associated with sight threatening DR in T2DM, after adjusting for clinical risk factors and replication in an additional cohort. Future studies and techniques designed to explore non-coding variants and their functional consequences are required to help us understand how rs56138102 in the *VEGFR1* gene contributes to DR risk. VEGFR1 and its genetic variations may play a more important role in the pathogenesis of sight threatening DR than previously recognised.

CHAPTER 8: CONCLUSION AND FUTURE DIRECTIONS

Diabetes and its most common complication, DR blindness, is a rising worldwide epidemic, and will remain a major global cause of blindness in the future. Current management strategies will not be adequate to reduce the significant burden it places on individuals and their communities.

The aims of this thesis were to explore two current challenges in combating DR blindness: the disproportionate burden of DR among indigenous Australians, and the complex genetic nature of DR.

Part one of this thesis focused on characterizing the burden of DR among indigenous Australian communities to explore tailored management strategies. A long-term mortality study conducted in South Australia and the Northern Territory showed that the risk of death among those with end stage DR (requiring vitrectomy surgery) was twice as high in indigenous Australians compared with non-indigenous Australians, even after age adjustment. Chronic renal disease was a significant co-morbidity. This led to a targeted epidemiology study among indigenous Australians with renal failure on dialysis; which showed 88.2% had co existing DR, yet only 66% had underwent DR screening in the last 12 months as per recommended screening guidelines. Preliminary results from a randomised controlled trial that compared the efficacy and safety of a new treatment modality, intravitreal dexamethasone, with standard treatment bevacizumab for the treatment of DMO post cataract surgery, confirmed the significant burden of DMO among remote indigenous communities, and ongoing difficulties in delivering frequent follow up and re-treatments. Early results support the preferential use and safety of dexamethasone implants.

The results of the studies presented in this thesis have direct implications for disease management. Given the higher mortality rate and significant association with renal disease, they suggest that indigenous people with diabetic retinopathy should be more closely co-managed by their physicians to screen for and treat diabetic renal complications. Additionally, awareness of this association should

be spread among indigenous communities and health care workers, and emphasised in referral pathways from optometrists, ophthalmology and general practice clinics. In Central Australia, the high proportion of diabetic renal dialysis patients who have undiagnosed DR necessitates a targeted screening strategy. Direct feedback and engagement with community leaders amongst the dialysis communities have raised awareness of this problem. Current resources in place ensure these patients are brought to the dialysis units several times per week, and feeding into this system to employ DR screening will be a cost-effective approach. Early results from our randomised controlled trial suggest a viable solution to better manage DMO in Central Australia, and this trial may change current clinical practice. Success of a new therapeutic drug in remote settings can also be extrapolated to other resource poor settings where DR and DMO are on the rise.

Future research endeavours should confirm the trends observed in this thesis. Is there also the same level of association between DR and renal disease among non-indigenous Australians, and are there different risk factors? A comparative study is currently being designed to answer this question. The randomised controlled trial comparing dexamethasone implant to bevacizumab is ongoing. A similar trial is also being conducted by our colleagues in remote indigenous communities of Western Australia, and a combined analysis of both trials will likely provide clearer results.

It is important to work closely with indigenous communities and their health workers. Research results and their potential implications should be communicated to the wider communities, so that better management strategies or funding for further meaningful research can be advocated and deployed. As part of the studies conducted in this thesis, relationships with key spokespersons have been established to facilitate this; the Central Australia Renal Advocacy Group, general practitioners for indigenous Australians (Congress), and the Baker Institute which focuses on heart and diabetes research. Ongoing relationships with these organisations will be essential to translate our research findings into clinical practice.

Part two of this thesis focused on building knowledge in the field of DR genetics in Caucasian populations, which can help us better understand disease pathways in DR. Incomplete understanding of these mechanisms is clear from clinical conundrums we face with current risk stratification and therapeutic options. Genotyping of more than 3000 well characterised diabetic individuals was done to confirm positive associations between DR and common European mitochondria haplogroups that were discovered in another study.[203, 204] No associations were replicated. Utilizing a targeted and more cost effective GWAS approach with replication and meta-analysis, associations between sight threatening DR and genetic variants in microRNA and *VEGFR1* genes were found.

The genetics work in this thesis has added important knowledge to our understanding of DR as a complex genetic disease. In the field of genetic epidemiology, it is essential to replicate results. False positives and negatives are common particularly for a disease like DR with multiple known comorbidities, clinical risk factors and different phenotypes that require separate analyses. The strongest associations between microRNA and *VEGFR1* genes were found for sight threatening disease and not for more generalised phenotype groups such as any DR. This is in keeping with severe forms of DR, such as PDR, having a higher heritability.[173] Genetic variations in microRNA genes adds to our understanding of how certain key inflammatory molecules are modulated in DR, providing us with a more upstream and possibly more effective molecular target. Genetic variations in the *VEGFR1* genes, together with our understanding of the *VEGFA* and *VEGFC* genes, may explain variable individual response to current anti-VEGF agents.

Future work can focus on expanding the findings presented in this thesis. Exploration of mitochondrial genetics in DR could be done in different ethnic groups. The Predicting Renal, Ophthalmic and Heart Events in the Aboriginal community (PROPHECY) project is a new and effective avenue to explore genetic risk factors in indigenous Australians because of collaboration with indigenous research workers, and collection of clinical and socioeconomic data to account for in genetic analyses.[379] The RADAR and GRDR genetic registries have ongoing recruitment, focusing

on increasing numbers with T1DM, PDR and CSMO. Collaboration with international colleagues can help us with replication and confirmation of significant findings. Newer techniques such as next generation sequencing and whole exome sequencing may help identify rarer variants. Patient serum and RNA samples have been collected with the potential to conduct functional studies. A sub study focusing on recruiting individuals being treated with anti-VEGF agents has been commenced to directly analyse genetic risk factors for treatment response.

It is an exciting era for genetic research as new technological advances have made the possibility of personalised medicine closer to reality. Using the cumulative effect of 'at risk' genetic variations, population screening methods for complex diseases could be developed.[380] The invention of CRISPR/Cas9 allows the possibility of gene editing to change disease risk.[381] Technical limitations, unknown side effects, controversies and ethical considerations for the use of this technology remain important issues before their widespread use.

While results from the genetic studies conducted in this thesis require further replication and confirmation of their functional consequences through laboratory experiments, they can guide the direction of future work and help develop more effective and targeted treatments for diabetic retinopathy blindness.

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