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Epidemiological and molecular risk factors for diabetic retinopathy blindness.

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EPIDEMIOLOGICAL AND MOLECULAR RISK FACTORS FOR DIABETIC RETINOPATHY BLINDNESS

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THESIS OUTCOMES

PEER REVIEWED PUBLICATIONS

Kaidonis G, Abhary S, Daniell M, Gillies M, Fogarty R, Petrovsky N, Jenkins J, Essex R, Chang JH, Pal B, Hewitt AW, Burdon KP, Craig JE. The Genetic Study of Diabetic Retinopathy: Recruitment methodology and analysis of baseline characteristics. ***Clinical and Experimental Ophthalmology***. 2014 Jul;42(5):486-93. doi: 10.1111/ceo.12239.

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Manuscripts under review:

Kaidonis G, Hassall M, Phillips R, Raymond G, Saha N, Wong GHC, Gilhotra JS, Liu E, Burdon KP, Henderson T, Newland H, Lake S, Craig JE. Rates of vitrectomy for diabetic retinopathy are five times higher amongst Indigenous Australians compared with non-Indigenous Australians.

Kaidonis G, Hassall M, Phillips R, Raymond G, Saha N, Wong GHC, Gilhotra JS, Liu E, Burdon KP, Henderson T, Newland H, Lake S, Craig JE. Visual outcomes following vitrectomy for diabetic retinopathy amongst Indigenous and non-Indigenous Australians in South Australia and the Northern Territory.

CONFERENCE PRESENTATIONS

"Genome-wide association study for diabetic retinopathy reveals association with genetic variation near the GRB2 gene in multiple populations". *The Association for Research in Vision and Ophthalmology 2014, Annual Meeting* (Orlando).

"Common sequence variation in the *VEGFC* gene is associated with diabetic retinopathy and diabetic macular edema in Caucasian patients with diabetes mellitus." *The Royal Australian and New Zealand College of Ophthalmology Annual Scientific Congress 2014* (Brisbane).

"Mir146a polymorphisms are associated with diabetic macular edema in type 2 diabetes. *"The Royal Australian and New Zealand College of Ophthalmology Annual Scientific Congress 2014* (Brisbane).

"Common sequence variation in the *VEGFC* gene is associated with diabetic retinopathy and diabetic macular edema in Caucasian patients." *The Association for Research in Vision and Ophthalmology 2015, Annual Meeting* (Denver).

"A 5 year audit of vitreoretinal surgery for diabetic retinopathy in South Australia and Northern Territory" *The Royal Australian and New Zealand College of Ophthalmology Annual Scientific Congress* 2015 (Wellington, NZ).

"Are Indigenous Australians more likely to require vitrectomy for end-stage diabetic retinopathy? : A 5 year audit of vitreoretinal surgery for diabetic retinopathy." *The Association for Research in Vision and Ophthalmology 2016, Annual Meeting* (Seattle).

PRIZES

Flinders University Best Research Higher Degree Student Publication for 2015 ($1000), for the paper titled: "Common Sequence Variation in the VEGFC Gene Is Associated with Diabetic Retinopathy and Diabetic Macular Edema".

Shortlisted for the Members in Training Outstanding Poster Award competition at *The Association for Research in Vision and Ophthalmology 2014, Annual Meeting* (Orlando), for the poster titled "Genome-wide association study for diabetic retinopathy reveals association with genetic variation near the GRB2 gene in multiple populations".

ARVO International Travel Grant (USD 1000), to present poster titled "Genome-wide association study for diabetic retinopathy reveals association with genetic variation near the GRB2 gene in multiple populations" at *The Association for Research in Vision and Ophthalmology, Annual Meeting* (Orlando).

POLICY AND PRACTICE

Design and implementation of a real-world clinical trial in a remote Indigenous Australian setting.

ABSTRACT

A recent report suggests that 3.63 million people worldwide suffer from moderate and severe vision loss due to diabetic retinopathy (DR) and its related sequelae [1]. Diabetic macular oedema (DMO), a subtype of DR, is responsible for greater visual morbidity than other vision threatening ocular diseases [2], and incurs significantly higher health care costs than other DR subtypes [3]. Despite an expansion in research and knowledge regarding risk factors that predispose to the development of DR, many questions remain unanswered for a particularly susceptible subset of patients. It is clear that patients belonging to some ethnic groups have a propensity to develop DR and this requires further investigation. Furthermore, specific risk factors for the development of DMO are less well characterised compared with other forms of DR and genetic risk factors for DMO have largely been ignored. This PhD tries to answer some of these questions by targeting two specific areas that are currently poorly understood: (1) the epidemiology of DR and DR related visual loss in Indigenous Australians; and (2) the molecular risk factors associated with the development of DMO.

A number of key findings from this PhD will contribute to reducing the burden of DR and DMO associated visual morbidity and health care costs in both Indigenous and non-Indigenous populations of Australia. The outcomes determined from the first population-based study of end-stage DR requiring vitrectomy has yielded valuable information regarding progression to vitrectomy and visual outcomes in Indigenous and non-Indigenous Australian populations. This information has guided the first Ophthalmic clinical trial in Central Australia designed as a part of this thesis and will continue to impact future initiatives aimed at improving visual outcomes in Indigenous Australians with DM. The exploration of candidate genes hypothesized to play a role in the pathogenesis of DMO in this robust genetic study has contributed to our current understanding of DMO susceptibility. The close interaction between inflammatory and angiogenic pathways in response to hypoxia is supported by our findings. Significant novel variants found within *VEGFC* and *miR-146a* validate the development of new therapeutic drugs targeting these pathways. Future evaluation of *VEGFA* variants and their interaction with environmental factors may help distinguish non-responders to current intravitreal treatments and assist clinicians employ individualized treatment strategies. The implication of our findings during an era of rapidly increasing incidence of T2DM and therefore DMO on vision related quality of life and health care costs are particularly noteworthy. The outcomes presented in this thesis are part of an ongoing research initiative that will continue to contribute to improving understanding and treatment of this global sight-threatening epidemic.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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ABBREVIATIONS

ABS	Australian Bureau Of Statistics
AGE	Advanced Glycation End Product
AKR1B1	Aldose Reductase Gene
ANDIAB	Australian National Diabetes Information Audit And Benchmarking Exercise
Ang-2	Angiopoiten-2
anti-VEGF	Antibody Against VEGFA
AusDiab	Australian Diabetes, Obesity And Lifestyle Study
BCVA	Best Corrected Visual Acuity
BMES	Blue Mountains Eye Study
BMI	Body Mass Index
BRB	Blood-Retinal Barrier
CAOHS	Central Australian Ocular Health Survey
CKD	Chronic Kidney Disease
CRT	Central Retinal Thickness
CV	Coefficient Of Variation
DBP	Diastolic Blood Pressure
DCCT	The Diabetes Control And Complications Trial
DM	Diabetes Mellitus
DMO	Diabetic Macular Oedema
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic Acid
DR	Diabetic Retinopathy
DRUID	Darwin Region Urban Indigenous Diabetes Study
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EPAS1	HIF2a Gene
ESRF	End Stage Renal Failure
ETC	Electron Transport Chain

ETDRS	Early Treatment Of Diabetic Retinopathy Study
FMC	Flinders Medical Centre
GEHS	Goldfields Eye Health Survey
GSDC	Genetic Study of Diabetic Complications
GWAS	Genome Wide Association Study
HbA1c	Glycosylated Haemoglobin (A1c)
HIF	Hypoxia Inducible Factor
HLA	Human Leukocyte Antigen
HREC	Human Research Ethics Committees
HWE	Hardy-Weinberg Equilibrium
IL	Interleukin
IOP	Intra-Ocular Pressure
IRAK-1	Interleukin-1 Receptor-Associated Kinase 1
IRMA	Intra-Retinal Microvascular Abnormalities
KRDRS	Katherine Region Diabetic Retinopathy Study
LD	Linkage Disequilibrium
LT-α	Lymphotoxin-A
LTA	Lymphotoxin-A Gene
MAF	Minor Allele Frequency
miR	Micro-RNA
mRNA	Messenger RNA
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDRS	Newcastle Diabetic Retinopathy Study
NF-κB	Nuclear Factor-κB
NHS	National Health Service
NIEHS	National Indigenous Eye Health Survey
NPDR	Non-proliferative Diabetic Retinopathy
NPL	No Perception To Light
NT	The Northern Territory
OCT	Optical Coherence Tomography
OR	Odds Ratio

PDR	Proliferative Diabetic Retinopathy
PKC	Protein Kinase-C
PRP	Pan-Retinal Photocoagulation
RADAR	Registry of Advanced Diabetic Retinopathy
RAGE	Receptor for Advanced Glycation End Product
RAH	Royal Adelaide Hospital
RCT	Randomised Controlled Trial
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SA	South Australia
SAEHP	South Australian Eye Health Program
SBP	Systolic Blood Pressure
SD	Standard Deviation
SNP	Single Nucleotide Polymorphisms
SNP SpD	SNP Spectral Decomposition
SPSS	Statistical Package For Social Sciences
STDR	Sight-Threatening Diabetic Retinopathy
STZ	Streptozotocin-Induced
T1DM	Type One Diabetes Mellitus
T2DM	Type Two Diabetes Mellitus
TNF	Tumour Necrosis Factor
TRAF6	TNF Receptor Associated Factor 6
UCL	University College London
UKPDS	The United Kingdom Prospective Diabetes Study
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VLDLR	Very Low Density Lipoprotein Receptor gene
WESDR	Wisconsin Epidemiologic Study of Diabetic Retinopathy
ZFPM2	Zinc Finger Protein Multitype 2 gene

INTRODUCTION

DIABETES MELLITUS

Diabetes Mellitus (DM) is a chronic disorder of the endocrine system, characterized by elevated blood glucose secondary to either reduced synthesis of, or an inadequate response to, insulin. The world health organization recommendations for the diagnosis of DM include the presence of at least one of the following criteria: (1) fasting plasma glucose of 7.0 mmol/L or greater; or (2) two-hour plasma glucose of 11.0 mmol/L or greater following a 75 g oral glucose load. The two most common types of DM are type 1 DM (T1DM) and type 2 DM (T2DM), and patients with these 2 types make up the target population for this PhD. Onset of T1DM is usually in children or young adults, and was previously known as juvenile-onset DM, whereas T2DM is traditionally seen in adults and therefore often called adult onset DM. However, with increasing rates of obesity in young adults and children, the incidence of T2DM in children and adolescence is rising. Furthermore, the detection of biomarkers (beta cell autoantibodies) specific to T1DM, aids in the differentiation of T1DM and T2DM, and has contributed to the understanding that T1DM can also occur in adults.

T1DM occurs when the pancreatic beta cells are unable to secrete enough insulin to maintain glucose homeostasis. Genetic risk has been shown to predict the development of autoantibodies against beta cells resulting in their autoimmune destruction. In particular, the presence of human leukocyte antigen (HLA) - DR3-DQ2 and HLA-DR4-DQ8 haplotypes, are associated with the development of beta cell autoantibodies during childhood, which increase the risk of a subsequent diagnosis of T1DM [4]. A number of environmental factors have also been associated with increased risk of T1DM in patients with the risk haplotypes, which suggests that these external factors act to trigger the development of beta autoantibodies in the context of increased genetic susceptibility. Environmental factors include viral infections, dietary factors and toxins in utero, during the postnatal period or during childhood [5]. The progressive development of two or more beta cell autoantibodies is associated with a 70% risk of progression to T1DM within 10 years [6]. Once overt T1DM has developed, lifelong treatment with exogenous insulin is required.

T2DM is the most common type of DM accounting for 90% of those with DM [7]. T2DM is caused when insulin receptors are unable to respond to circulating insulin. This occurs progressively in the context of increased adipose tissue, leading to insulin resistance. In response, insulin production is upregulated. When the body's ability to compensate is surpassed, hyperglycaemia results. Treatment with oral agents (to increase insulin sensitivity) or exogenous insulin is then required. Risk factors for the development of T2DM include central obesity, increasing age and a family history of T2DM. The estimated heritability of T2DM is 26-73%, suggesting a significant genetic component [8]. Genome wide association studies (GWAS) in multiple ethnic groups have discovered more than 80 loci associated with increased risk of T2DM [9].

DM currently affects more than 400 million people worldwide, equating to a prevalence of 8.8% [7]. In 2010, the annual cost of DM in Australia was $14.6 billion [10]. The International Diabetes Federation

predicts the number of people with DM will rise to 642 million by 2040, which will increase global health spending for DM by 19% [7]. Although this rise is predominantly due to an increase in T2DM rates, the prevalence of T1DM is also increasing worldwide, and currently represents 10-15% of DM cases [4]. Diabetes prevalence varies significantly between populations worldwide. For Indigenous populations who are no longer living a traditional lifestyle, the prevalence of DM is much greater than in the non-Indigenous population [11]. This is common to many countries, and holds true for Indigenous Australians who have an estimated DM prevalence of up to 40% [12]. This is markedly higher than the national DM prevalence in Australia which is estimated to be 5-6% [13].

DIABETIC RETINOPATHY

Hyperglycaemia is common to both T1DM and T2DM, and when sustained in a local tissue environment has toxic downstream effects resulting in microvascular damage. The predominant end organs affected include the retina, resulting in diabetic retinopathy (DR), the kidney manifesting as diabetic nephropathy (DN), and the peripheral nerves, leading to diabetic neuropathy.

DR is a progressive disease that begins in the non-proliferative phase (NPDR) characterised by microaneurysms, dot, blot or flame haemorrhages, exudates, cotton wool spots, venous beading and intraretinal microvascular abnormalities (IRMA). As the retina becomes increasingly ischaemic, new, fragile blood vessels develop. This stage of disease is known as proliferative diabetic retinopathy (PDR). Pre-retinal or vitreous haemorrhage and fibrous proliferation are complications of PDR that threaten vision. Diabetic macular oedema (DMO) is an important sight-threatening subtype of DR caused by leakage of fluid from the retinal microvasculature at the macula. DMO can be present in conjunction with any level of DR. Clinical findings include thickening of the central macula, with intraretinal fluid evident on optical coherence tomography (OCT). When oedema involves the fovea, visual acuity is compromised. PDR and DMO therefore make up the sight-threatening subtypes of DR, and most treatments of DR are directed at these two stages of disease.

The Airlie House classification system for grading DR guided the many classification systems developed in more recent times, including the Early Treatment Diabetic Retinopathy Study (ETDRS) grading criteria [14,15]. The ETDRS system is commonly used for large clinical trials but is dependent on the acquisition of fundus photographs following strict criteria. The recently developed International Clinical Diabetic Retinopathy and Diabetic Macular Oedema Severity scale is a simplified version of previous grading systems, specifically designed for use in routine clinical practice (**Table i**) [16]. Most importantly, this condensed grading system is still sensitive in identifying patients at high risk for sight-threatening complications of DR (those with intraretinal haemorrhage, IRMA or venous beading) as well as those at highest risk of progression to PDR (those patients with severe NPDR) [17]. Furthermore, this system classifies DMO as being either absent or present as any level of DMO could potentially affect vision. For these reasons, *sight-threatening diabetic retinopathy (STDR) is defined in this PhD as those patients with severe NPDR, PDR, or DMO in at least one eye.*

Table i: International Clinical Diabetic Retinopathy and Diabetic Macular Oedema Severity scale.

Grade	Findings on Ophthalmoscopy
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: More than 20 intraretinal haemorrhages in each of the four quadrants Venous beading in at least 2 quadrants IRMA in at least one quadrant AND no signs of PDR
PDR	One of the following: Neovascularisation Vitreous or preretinal haemorrhage
Macular Oedema	
Absent	No retinal thickening or hard exudates in posterior pole
Present	Retinal thickening or hard exudates in posterior pole

Abbreviations: DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; IRMA, intraretinal microvascular abnormalities. Adapted from NHMRC Guidelines for the Management of Diabetic Retinopathy 2008 [14].

DR EPIDEMIOLOGY

DR is the leading cause of blindness in working age adults [18]. The cumulative incidence of DR in patients with T2DM is approximately 66% at 10 years [19]. This is greater than the recently calculated incidence of DR in patients with T1DM at the same time-point (approximately 25% at 10 years) [20]. The incidence of DR in patients with T1DM has declined significantly since the introduction of more stringent guidelines for the management of DM resulting in better glycaemic control.

The global prevalence of DR among patients with DM is 35.4% [21]. This figure, calculated from a pooled meta-analysis, included studies conducted between 1980 and 2008 however significant variation in prevalence between the 35 individual studies was noted. The highest prevalence was among African Americans (49.6%), and lowest among Asians (19.9%) [21]. Even among studies of Caucasian patients with DM, prevalence estimates of DR vary from 32.4% in the Australian Blue Mountains Eye Study (BMES) [22] to 40% from population studies derived from the United States [23]. Multiple factors including access to DR screening and treatment, socioeconomic factors, duration of DM, glycaemic control and ethnicity related differences between the studied populations could explain these variations. The Australian National Diabetes Information Audit and Benchmarking Exercise (ANDIAB) reports that Australians with DM, with access to large DM centres for screening and treatment have a prevalence of DR of 29%, and a rate of blindness approaching 1% [24].

The prevalence of STDR, estimated at 10.2% globally, also varies greatly between sampled populations [21]. STDR prevalence is highly dependent on duration of DM, and is significantly greater in those with T1DM (32.4%) compared with those with T2DM (3.0%) [21]. This is partly a reflection of the competing mortality risk for patients with T2DM who are on average older with more comorbidities at age of DM onset, and thus have a shorter duration of disease exposure [19]. Compared with other subtypes of DR, the epidemiological data on DMO is relatively scarce. The prevalence of DMO reported in various studies ranges from 4.2% to 7.9% for patients with T1DM and up to 12.8% in patients with T2DM [19]. It must be noted that the majority of the studies reporting DMO prevalence rates used non-stereoscopic fundus photography to grade DMO which may have underestimated the true prevalence of DMO given that macular thickening is difficult to assess using this modality, compared with the current gold standard of clinical examination and OCT.

Pathogenesis of Diabetic Complications

The development of macrovascular complications secondary to DM (including cardiovascular disease and peripheral vascular disease) are predominantly the result of insulin resistance, which promotes the migration of free fatty acids from adipocytes into arterial endothelial cells [25]. This stimulates increased fatty acid oxidation resulting in the mitochondrial overproduction of reactive oxygen species (ROS), and vascular endothelial cell damage. The risk of developing macrovascular complications of DM increases 2.5 fold as HbA1c increases from 5.5% to 9.5% [26]. In contrast, the same increase in HbA1c results in a 10 fold increased risk of microvascular complications of DM, suggesting a strong pathological role of sustained hyperglycaemia in microvascular compared with macrovascular complications. The specific cells affected by intracellular hyperglycaemia are unique in that they are unable to regulate their internal glucose concentrations in the same way that the remainder of the body's cells can. These cells include mesangial cells of the kidney and endothelial cells in the vasculature of the retina and peripheral nervous system. Damage to these cells is responsible for the development of DR, DN and diabetic neuropathy respectively.

Local hyperglycaemic damage

Local intracellular hyperglycaemia results in the upregulation of 4 pathways involved in the breakdown of excess intracellular glucose and associated with the increased production of ROS [27]. The polyol pathway involves the reduction of excess glucose to sorbitol by aldose reductase, with consumption of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is required in the regeneration of the antioxidant, reduced glutathione, and this process is inhibited in the hyperglycaemic state when NADPH is unavailable. Thus the polyol pathway increases the cell's susceptibility to oxidative stress. This enhances the effects of the second pathway whereby advanced glycation end products (AGEs) are formed from bi-products of excess glucose breakdown and damage cells by modifying proteins. These include the modification of intracellular proteins that function in the regulation of gene transcription, extracellular matrix molecules and circulating proteins, which can bind and activate AGE receptors (RAGEs) producing inflammatory cytokines and growth factors. The third pathway involves the activation of protein kinase-C (PKC), which occurs due to increased production of diacyl-glycerol from the breakdown of glucose. PKC

acts by altering gene expression of a number of important factors including nitric oxide synthase, vascular endothelial growth factor (VEGF, also known as VEGF-A), transforming growth factor beta, and nuclear factor-κB (NF-κB). The final mechanism involves increased activity of the hexosamine pathway which metabolises diverted fructose-6-phophate (generated from glucose) providing another alternative to the glycolytic pathway for breakdown of excess glucose. The resultant N-acetyl glucosamine modifies transcription factors causing pathological changes in gene expression including the upregulation of angiopoietin-2 (Ang-2). The extensive work by Brownlee [27] has played a key role in our current understanding of theses pathways, and how they contribute to cellular damage. Brownlee proposed that the unifying mechanism promoting these pathways was via the generation of superoxide through the mitochondrial electron transport chain (ETC). This occurs when the maximal capacity of the ETC is surpassed (from excessive upstream glycolysis), blocking electron transfer such that electrons are instead donated to oxygen to form superoxide. Superoxide directly inhibits a critical enzyme early in the glycolytic pathway causing glycolysis intermediates to be shunted into the alternative pathways described above. The combination of ROS, inflammatory mediators and growth factors produced as a result contribute to the pathoaetiological processes involved in the development of DR described below.

Blood-retinal barrier (BRB) breakdown, pericyte loss and vasoregression

The BRB consists of retinal capillary endothelial cells with overlying pericytes (modified smooth muscle cells) and their tight adherence to the retinal pigment epithelium. The integrity of these cells and cell-cell junctions is important in maintaining vessel perfusion and in preventing leakage of molecules and fluid out of the retinal capillaries [28]. Breakdown of endothelial cell junctions occurs as a direct effect of AGE induced modification of extracellular matrix molecules in hyperglycaemic conditions [27]. Pericyte and endothelial cell apoptosis occurs in response to NF-κB which is upregulated following PKC activation [29,30]. This is thought to contribute to the formation of microaneurysms in the wall of retinal capillaries [28,31]. Pericyte apoptosis is exacerbated by migration of pericytes which is driven by Ang-2 (generated following hexosamine pathway upregulation) in the context of modest levels of VEGF [32]. Migration away from the site of capillary injury is thought to be a survival mechanism similar to that seen in traumatic brain injury. This process is known as vasoregression and results in damaged, acellular, non-perfused capillaries [31].

Intra-retinal oedema

BRB breakdown is the initial insult that promotes leakage of plasma proteins from the retinal capillaries into the retinal tissue, producing increased interstitial oncotic pressure and subsequent interstitial oedema [33]. However, there are other mechanisms that act to exacerbate this process. VEGFA functions to increase permeability of the retinal blood vessels through multiple mechanisms including through increasing levels of inflammatory cytokines and matrix metalloproteinases [34-36]. NF-κB is a transcription factor that stimulates the production of pro-inflammatory cytokines. The well-known vascular changes associated with the inflammatory cascade (increased blood flow and vascular permeability) occur at the level of the retinal

vasculature and contribute to fluid leak [28]. Endothelial nitric oxide synthase (eNOS) produced by retinal vascular endothelial cells has a direct effect on cell junction proteins causing further BRB breakdown [33]. Finally, increased hydrostatic pressure secondary to hypertension commonly coexists in patients with DM which further promotes the extravasation of fluid by disrupting the normal homeostasis of starling's forces. DMO results when these processes involve the macular.

Hypoxia and angiogenesis

Capillary nonperfusion seen during vasoregression produces local areas of hypoxia. The ischaemic retina mounts an angiogenic response in an attempt to restore oxygen to the tissue. VEGF, initially increased secondary to the effects of hyperglycaemia and further upregulated in hypoxic conditions through activation of the transcription factor, hypoxia inducible factor (HIF) [37], acts synergistically with Ang-2 to promote sprouting angiogenesis [31]. VEGF also acts as an important survival factor for endothelial cells and pericytes during vessel growth [38]. The attempt to restore oxygen to the retina through neovascularisation is referred to clinically as PDR. Increased fragility of the new vessels formed, and their ability to grow beyond the surface of the retina into the vitreous, predisposes to complications of PDR including vitreous haemorrhage.

DR TREATMENT

The first available treatment to significantly reduce the risk of vision loss in patients with STDR was laser treatment. Two large, high-quality, randomised controlled trials (RCT), the Diabetic Retinopathy Study and the ETDRS showed a 50% reduction in severe visual loss after pan-retinal photocoagulation (PRP) for patients with PDR [39], and following focal or grid laser photocoagulation for DMO [40]. Focal and grid laser treatment also increased the chance of visual improvement for those patients with DMO [40]. However PRP cannot restore vision and has the potential complications of inadvertent foveal burn, diminished visual field, increased DMO, ciliary block glaucoma, Bruch's membrane rupture, and tractional retinal detachment. These risks outweigh the benefits of performing PRP on grades of DR less than severe NPDR [39]. Migration of treatment scars towards the fovea centre is also a potential vision threatening complication of laser treatment performed for DMO.

More recently, DMO has been shown to respond to intraocular injections with anti-VEGF agents (bevacizumab, ranibizumab and aflibercept) reducing reliance on laser treatment. In fact, in patients where the very centre of the macula is involved, who have moderately reduced vision, monthly intravitreal injections with ranibizumab or bevacizumab have been shown to be superior to laser in improving vision provided a high level of compliance is achieved [41,42]. Although anti-VEGF therapy has become first line treatment for centre-involving DMO, approximately 50% of patients do not respond well to this treatment [43]. This variability may be secondary to environmental or genetic factors that affect response to anti-VEGF blockade and necessitates the development of treatments targeting other factors or pathways involved in the pathogenesis of DMO. Among patients with PDR, anti-VEGF therapy is now commonly used as an adjunct to PRP treatment. A recent RCT has shown that ranibizumab is equally effective as PRP at two years

following treatment [44]. This appears to be a reasonable alternative to laser in the short to medium term, however longer follow-up is required to determine its long-term efficacy. Intravitreal injections are considered a safe method of treatment delivery, however there are risks associated with this procedure. These include risks of bleeding, increased intraocular pressure, damage to the lens or retina, and rarely endophthalmitis [14]. There is weak evidence to suggest that some anti-VEGF agents including bevacizumab may result in systemic absorption and pose an increased risk of cardiovascular events [45]. Finally, the short half-life (and therefore short duration of action) of anti-VEGF agents developed to date means that repeated doses as frequently as monthly may be required to retain the therapeutic response [34].

Corticosteroids are anti-inflammatory agents with anti-VEGF and anti-proliferative effects. Unfortunately, increased rates of cataract and elevated intraocular pressure (IOP) are common adverse effects of intravitreal corticosteroid treatment such as triamcinolone acetonide, making this a less appealing option than anti-VEGF agents [46]. However, their efficacy has been demonstrated in a subgroup of pseudophakic patients with DMO, where triamcinolone acetonide plus laser was shown to be superior to laser alone, and equivalent to ranibizumab (alone or with laser) [46]. First line treatment with triamcinolone acetonide has also been shown to be the most cost effective option in pseudophakic patients [47]. Intravitreal triamcinolone acetonide is thus considered as an adjunct or alternative therapy to anti-VEGF agents for persistent or refractory DMO [48]. The introduction of long acting corticosteroid implants has allowed for less frequent treatments (three to six monthly) in patients who are candidates for steroid treatment [49-51].

Despite optimal early treatment of DR and DMO with laser photocoagulation and intravitreal injections with anti-VEGF and steroid agents, further progression of retinopathy often requires surgical management [52]. In the advanced stages of DR, contraction of the vitreous and the formation of contractile epiretinal fibrocellular membranes results in progressive traction on the retina and new vessels [53]. Vision is compromised when vitreous haemorrhage, tractional retinal detachment, or worsening DMO from vitreomacular traction ensue. Diabetic vitrectomy describes the surgical procedure used to remove the vitreous and eliminate traction on the retina for the complications of PDR or DMO, and as such reflects progression of DR to the most severe endpoint. Release of retinal traction during vitrectomy may also improve blood flow and reduce vessel leakage. Furthermore, the removal of vitreous and fibrous proliferation can help stabilise PDR by eliminating the scaffold that supports the growth of new vessels. The cumulative incidence of complications requiring diabetic vitrectomy at 5 years in those with DM is up to 5% [54,55]. Approximately 75% of patients have an improvement in visual acuity following vitrectomy for the complications of PDR [56]. For patients with DMO and vitreomacular traction, vitrectomy reduces retinal thickening in most eyes and improves vision in 28% and 49% of eyes [57]. Although the rate of complications following diabetic vitrectomy has decreased with improvements in surgical techniques, complications (including re-detachment of the retina requiring further surgery, development of post-operative cataract and glaucoma) occur following approximately 30% of diabetic vitrectomies [58].

RISK FACTORS FOR DR DEVELOPMENT AND PROGRESSION

Modifiable risk factors

Original landmark studies conducted between 1980 and 2000 focused on the epidemiology and treatment of DM and diabetic complications. These studies, with large patient numbers and long-term follow-up, contributed significantly to current knowledge of DM disease progression, and the importance of modifiable risk factors. Coinciding with this period of evolving treatment was a 50% reduction in the incidence of new PDR and DMO, highlighting the impact of these modifiable risk factors on DR development and progression [14]. It must be highlighted that the majority of these studies report findings in the context of DR without investigating specific subsets of the disease including DMO.

The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) [59,60], The Diabetes Control and Complications Trial (DCCT) [61] and The United Kingdom Prospective Diabetes Study (UKPDS) [26] established a causal relationship between glycosylated haemoglobin (HbA1c) levels and DR development and severity and provided a 'gold standard' target for glycaemic control (HbA1c of 7%). The DCCT found that with each 10% decrease in HbA1c, the risk of DR progression decreased by 39% [62]. Intensive glycaemic control was associated with a 58% reduction in the incidence of DMO at 4 years [63]. However, the risk of DR was not eliminated and increased with longer duration of DM despite improvements in HbA1c. The reduction of HbA1c to 6% was found to be associated with increased mortality secondary to hypoglycaemic events suggesting that levels below this are unsafe targets. Additionally, sudden reduction in HbA1c was shown to cause early worsening of DR, although this appeared to reverse at 18 months and did not result in serious visual loss in the DCCT trial [64]. The concept of metabolic memory was also established from these landmark trials. Those participants who initially had tight glucose control continued to have reduced risk of DR progression even after tight glucose control was lost compared with those who had previously had conventional therapy [62,63]. It is currently well recognised that hyperglycaemia remains the most important modifiable risk factor for DR and DMO and well controlled HbA1c early in the course of DM has long-lasting benefits.

The beneficial effects of antihypertensive treatment in slowing the development and progression of DR are now generally accepted but less well substantiated. Longitudinal studies have been inconsistent in their findings, however clinical guidelines continue to support the lowering of systolic blood pressure (SBP) to below 130 mmHg in both T1DM and T2DM patients given available data [14,65-67]. Hyperlipidaemia is a significant risk factor for the development of DR, and more specifically for hard exudates in patients with DMO [68-70]. Moreover, regression of hard exudates has been shown in patients with DMO after reduction of serum lipid concentrations [71]. Two major RCTs support the use of fenofibrate (a peroxisome proliferator activated–receptor alpha agonist) in conjunction with statins in the treatment of early DR to reduce progression [72]. Although these studies found no effect of fenofibrate therapy on the regression of DMO, some weak positive results have been reported in smaller studies [73]. Surprisingly, smoking has been associated with reduced incidence and progression of DR in some studies [74].

Early development of cataract, and higher prevalence of cataract surgery in people aged 55 to 74 with DM has been reported in large population-based studies [75]. It is well known, however, that cataract surgery poses a major increased risk of DR and DMO progression in the operated eye [76]. DR progression following cataract extraction has been correlated with glycaemic control at the time of surgery in some studies [76]. Visual outcomes following cataract surgery are often suboptimal in patients with DM, especially those with active PDR or DMO at the time of surgery [77].

Non-modifiable risk factors

Duration of DM is the strongest risk factor for the development and severity of DR. After 20 years of DM, nearly all patients with T1DM and 80% of patients with T2DM have evidence of some retinopathy regardless of other factors [59,60]. However, the DCCT trial showed that duration of DM and glycaemic control together explain only 11% of the risk of DR development, despite being the two strongest risk factors known to date [78,79]. The investigation of additional potentially contributory risk factors, including ethnicity related factors and genetic risk are explored in this thesis.

Ethnicity related risk factors

The study of DR in Indigenous populations worldwide has slowly been gaining more attention. Of particular interest are those groups very recently affected by T2DM due to changes from a traditional to a western lifestyle. It appears that Indigenous people are disproportionately affected by complications of T2DM, including DR compared with non-Indigenous people with DM. The highest rates of DR have been documented in First Nations people of Canada, with 40% of Alberta First Nations people and 43% of James Bay Cree people with DM affected [80]. This is much greater than the 27.2% DR prevalence (2.3% with PDR) in a sample population of patients with DM living in rural Alberta; 85% of whom were of European ancestry [81]. Rates of PDR in Oklahoma Indians are as high as 3.6%, with 2.5% of James Bay Cree Canadians with DM affected [80]. Exceptionally high rates of DMO of 33% have been reported in Kenya amongst those with DM [82]. Multiple factors have been found to play a role in these ethnic disparities including differences in risk factor control and access to screening and treatment [83]. A recent study found that Native American ancestry is a significant risk factor for the development of severe DR in Latino patients (who are an admixture of Native American, European and African ancestry), suggesting that there is significant genetic variability in susceptibility to DR amongst certain ethnic groups [84].

There has been an 80-fold increase in DM prevalence in Indigenous Australians in the last 30 years [85], resulting in significant morbidity secondary to diabetic complications. Quantifying the risk of DR in Indigenous Australians is difficult to study and this has led to much variation between reports [12]. There is also very limited data investigating the prevalence of DMO in this population. DR is thought to account for 9–12% of visual impairment in the Indigenous Australian population, with some reports suggesting that DR is associated with up to six times more visual loss in Indigenous than in non-Indigenous groups [86,87]. When looking at available data, the real difficulty lies in determining which factors play a significant role in increasing risk of blindness from DR in Indigenous Australians. Answering this question is of great

importance in trying to improve treatment and visual outcomes in this population, particularly given the growing proportion of Indigenous Australians affected by DM. The research presented in Part I of this thesis focuses on this poorly understood aspect of DR.

Genetic risk factors

While the risk factors described above are known to influence susceptibility to DR, the reasons that some patients with increased risk are resistant to developing STDR and some patients without traditional risk factors are particularly susceptible remains an important unanswered question. The aim of genetic research is to understand how changes in deoxyribonucleic acid (DNA) sequence relate to patient phenotypes. This method has been increasingly employed to investigate the contribution of genetic susceptibility to DR development and severity, given that there is evidence that STDR has a strong heritable component [88]. The DCCT was the first large-scale trial to investigate and determine a significant correlation between patients and their first-degree relatives in relation to the presence and severity of DR, independent of glycaemic control [89]. Significant familial aggregation of DR severity across a range of ethnic groups was subsequently demonstrated, with the degree of heritability dependent on ethnicity [88]. The heritable component of STDR has been reported to be between 25% and 50% [88,90].

The vast majority of studies so far have concentrated on determining genetic risk factors for DR and STDR. Several single nucleotide polymorphisms (SNPs) across a number of genes have been implicated. The most well studied candidate genes include *VEGFA*, aldose reductase (*AKR1B1*), eNOS and RAGE. The *AKR1B1* C/T polymorphism at rs759853 (-106) is one SNP that has been positively associated with any DR in patients with T1DM (but not with T2DM) in a meta-analysis of 17 studies, covering multiple ethnicities [91]. Replication in multiple ethnic groups implicates this gene in the pathogenesis of DR. However, findings like this highlight the fact that T1DM and T2DM are diseases of distinct pathoetiologies that may be influenced by different genetic risk factors. The efficacy of inhibiting the VEGF pathway in the treatment of DMO demonstrates the clinical relevance of research aimed at elucidating genetic risk factors, and associated molecular pathways involved in the pathogenesis of STDR. Such improved understanding is required to facilitate the development of personalized approaches, and novel therapeutic strategies to reduce the burden of DM-associated visual morbidity.

The majority of candidate gene studies have reported conflicting SNP associations with varying subtypes of DR in different ethnic groups [92]. GWAS have also been conducted to investigate the genetic contribution to DR susceptibility, but to date, no published SNPs have yet reached genome-wide significance [93-96]. Large studies with rigid case-control definitions are lacking in the literature reflecting these inconsistent results. In addition, genetic investigation of DMO as a discrete subtype of DR has largely been ignored in the literature, despite it contributing to considerable visual morbidity. The second part of this thesis focuses on the genetics of DMO with an aim to identify novel contributing mechanisms that may in future form the basis of new treatments for DMO.

CLINICAL DILEMMAS

Despite an expansion in research and knowledge regarding risk factors that predispose to the development of DR, many questions remain unanswered for a particularly susceptible subset of patients. It is clear that patients belonging to some ethnic groups have a propensity to develop DR and this requires further investigation. Furthermore, specific risk factors for the development of DMO are less well characterised compared with other forms of DR and genetic risk factors for DMO have not been adequately explored. The cases described below illustrate some of the dilemmas encountered in clinical practice that cannot be answered with current scientific knowledge.

Case 1: A 28 year old Aboriginal woman from Anthelk-Ewlpaye Town Camp in Central Australia has had T2DM for 6 years. She developed PDR requiring extensive laser treatment and neovascular glaucoma (an end-stage complication of this condition) requiring surgery. Despite reasonable glycaemic control, and short duration of DM, at the age of just 28 this patient was blind, and dialysis dependant from DM. Patients like this raise the questions: Is this outcome the norm for Indigenous Australians? Was the problem a lack of early intervention due to poor access to screening and treatment? Are there ethnicity related risk factors playing a role here?

Case 2: A 63 year old Caucasian male not known to have DM presented to his optometrist for routine review, and was found to have DR changes on eye examination. He was referred to his GP for further workup, which revealed a borderline diagnosis of T2DM with an HbA1c of 6.1%. He had no other vascular risk factors. He was referred to the ophthalmologist, where he was diagnosed with DMO and started on intravitreal anti-VEGF treatment. Within 2 months of diagnosis he developed a chronic toe ulcer requiring amputation. According to his blood glucose, this patient is hardly diabetic. So how has he managed to develop these end-stage micro and macrovascular complications of DM? Does he have a genetic predisposition to develop DMO?

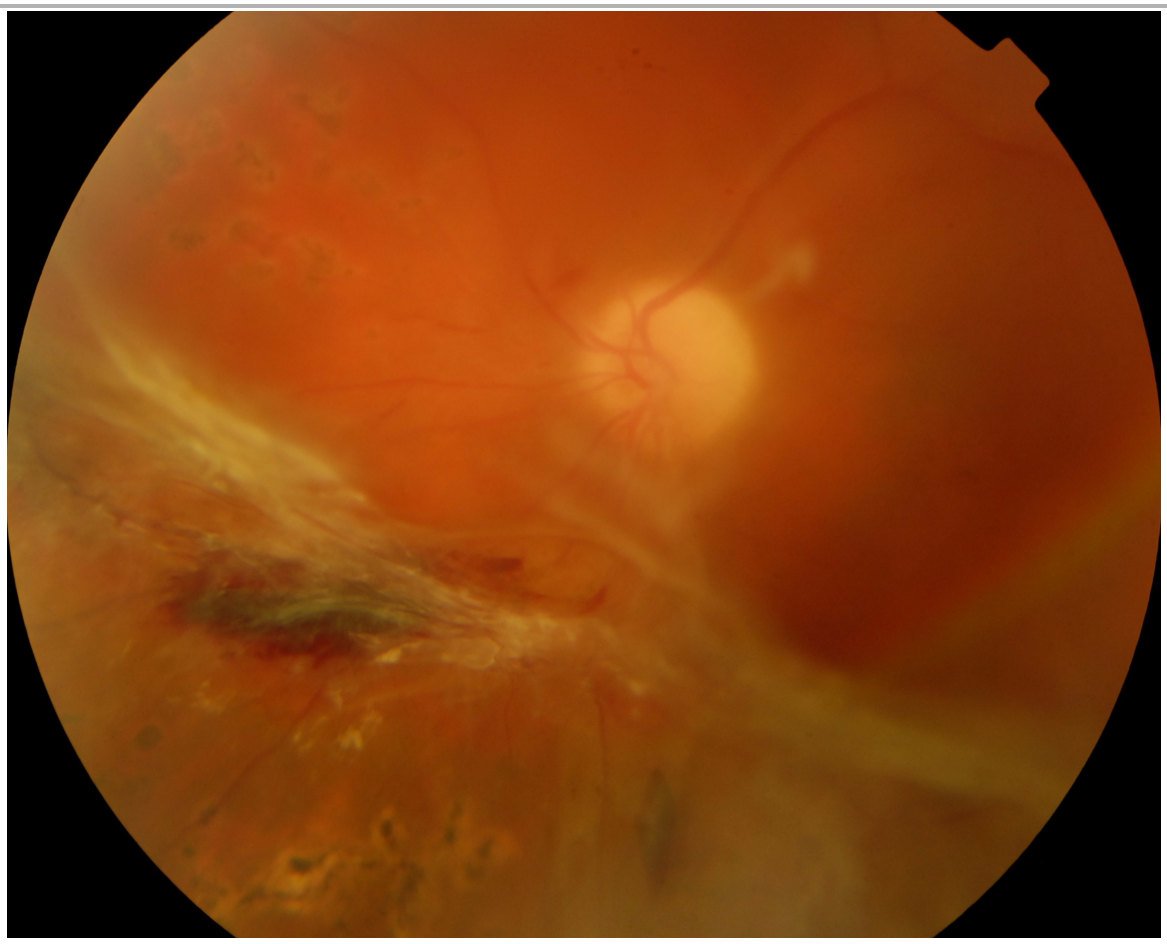
PURPOSE

The two cases described above illustrate two patients who have significant visual impairment secondary to DR, without the traditional risk factors associated with DM complications. These cases illustrate the clinical necessity for further research addressing risk factors for DR. This PhD tries to answer some of these questions by targeting two specific areas that are currently poorly understood:

1. The epidemiology of DR and DR related visual loss in Indigenous Australians; and
2. The molecular risk factors associated with the development of DMO.

Understanding risk factors and current outcomes for the development of end-stage DR in Indigenous Australia will allow for the implementation of screening and treatment facilities tailored to the needs of this population. Determining independent genetic risk factors for DMO through genomic and proteomic analysis will facilitate the development of personalized approaches, and novel therapeutic strategies. Through the

findings from this PhD we aim to contribute to reducing the burden of DR and DMO associated visual morbidity and health care costs in both Indigenous and non-Indigenous populations of Australia.



PART I

THE EPIDEMIOLOGY AND TREATMENT OF DIABETIC RETINOPATHY AND DIABETIC MACULAR OEDEMA IN INDIGENOUS AUSTRALIANS

CHAPTER 1

INTRODUCTION AND AIMS

DM is associated with high levels of morbidity across both Indigenous and non-Indigenous Australian populations [97]. The increased burden of DM on the health of Indigenous Australians is reflected in national mortality statistics, with DM associated death for Indigenous Australians at least 10 times the national average [98,99]. The greatest difference in mortality rate is within the 35- to 54-year age bracket, with Indigenous males and females 23 and 37 times more likely to die from complications of DM respectively, than non-Indigenous males and females of the same age [99]. The basis for this discrepancy is thought to be multifactorial with the effects of earlier onset of DM and increased prevalence of associated risk factors compounded by barriers to optimal care observed in the Indigenous Australian population [99]. The same factors have been proposed to contribute to increased levels of DR amongst Indigenous Australians.

Population-based epidemiological studies are inherently difficult to perform in remote areas. Numerous large population-based studies assessing DR prevalence have been performed in Australia however the majority of these have excluded very remote areas and therefore have only included small numbers of Indigenous Australians. Studies targeting Indigenous Australians with DM have been attempted however limitations in their sampling methodology are well recognised. Furthermore, the epidemiology and visual outcomes of the most severe stages of DR has not been studied previously in Indigenous Australian communities. Studies so far suggest that the prevalence of DR in Indigenous Australians is up to seven times greater than reports from non-Indigenous population-based studies [100]. The greater prevalence of DM among Indigenous Australians (37% [12] compared with less than 4% [97] for non-Indigenous Australians) would be expected to account at least in part for the observed higher complication rates, including DR. In fact, poorer control of diagnosed DM and higher rates of undiagnosed DM would be expected to lead to higher rates of DR in this population.

Given the huge prevalence of DM in the Indigenous Australian population, DR associated visual loss is a significant public health concern. There is obvious economic and social benefit in maintaining vision in working adults, justifying ongoing study to best meet the needs of this population. Understanding the outcomes of DR and its treatment is the first step in guiding future strategies for screening and management.

AIMS OF THIS WORK:

1. To compare the prevalence of DR in Indigenous and non-Indigenous Australians using data from published epidemiological studies.
2. To conduct the first truly population-based study of end-stage DR requiring surgical intervention (vitrectomy), and from this, identify the prevalence, risk factors and visual outcomes associated with diabetic vitrectomy in South Australia (SA) and The Northern Territory (NT)

3. To design and implement a treatment trial that has the potential to impact clinical practice and improve outcomes for Indigenous Australians with DMO living in remote Australia.

CHAPTER 2

ELUCIDATING THE PREVALENCE OF DIABETIC RETINOPATHY IN INDIGENOUS AUSTRALIANS.

The original work presented in this chapter has been published in the peer-reviewed literature: G Kaidonis, RA Mills, J Landers, SR Lake, KP Burdon and JE Craig. The Prevalence of Diabetic Retinopathy in Indigenous Australians. ***Clinical and Experimental Ophthalmology 2014 Dec;42(9):875-82*** [101]. *Dr Kaidonis' contributions include study conception and design, literature review, data collection, analysis and interpretation and manuscript preparation.*

AIM

This analysis compares pooled prevalence data for DR in patients known to have DM, reported in studies published within the last 30 years. The purpose of this review is to establish if current evidence suggests a difference in DR susceptibility between Indigenous and non-Indigenous Australians beyond that accounted for by differences in DM prevalence. The identification of factors predisposing some individuals to very severe outcomes will allow for targeted intervention in future.

METHODS

Literature search

A literature search was undertaken via The PubMed Database (National Centre for Biotechnology Information) using the search terms 'Australia OR Australian AND Diabetic Retinopathy AND Epidemiology'. Lit.search (Lowitja Institute, http://www.lowitja.org.au/litsearch) was used to cross-reference publications specifically relating to Indigenous Australian health using the search terms 'Diabetic Retinopathy AND Epidemiology'. All relevant studies based on review of titles and abstracts were retrieved. If multiple articles were based on the same data the publication with the most comprehensive data was included. Potentially appropriate studies were subject to the following inclusion criteria:

(i) full-text publications;
(ii) written in English;
(iii) published between 1985 and 2013;
(iv) describe the prevalence of DR in a defined population within Australia; and
(v) population-based community studies, register-based studies, or primary or secondary care clinic studies.

Studies were specifically excluded if they involved:

(i) children only;
(ii) T1DM participants only;

(iii) prevalence estimates of any DR that could not be calculated from the presented data;

(iv) self-reported DR status;

(v) DR prevalence estimates for patients with known DM or newly diagnosed DM where the data was not presented separately.

Where studies published DR prevalence data discretely for those with known DM and newly diagnosed DM, the study was included with only prevalence relating to participants with known DM incorporated in this analysis. Studies reporting combined data only were excluded. Study inclusion was not limited by method of clinical DR grading (at time of clinical examination or via retinal photographs).

Data collection

Only published data were included for this analysis. The following information was extracted from the studies where possible: study design, study period, sample size with self-reported DM, Indigenous status, target age, mean age and age range (median age is reported if mean age was not available), gender, mean duration of DM, prevalence of DR (including severity where available), and method used for DR grading.

Data analysis

Retinopathy data were compared for Indigenous and non-Indigenous studies with regards to two specific end-points: (i) any DR, including NPDR or PDR, and/or DMO; (ii) STDR defined as DMO and/or PDR. Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 20.0 (IBM SPSS Statistics for Windows Version 20.0; IBM Corp., Armonk, NY, USA). Data analysis was performed with pooled data from the included Indigenous and non-Indigenous studies. Pooled prevalence estimates for any DR were obtained for Indigenous and non-Indigenous groups using data from all studies. Prevalence rates of PDR, DMO and STDR were derived from the pooled data of all relevant studies and presented for Indigenous and non-Indigenous groups. Tests for ethnicity differences based on pooled prevalence estimates were done separately for any DR, PDR, DMO and STDR using the chi-squared test for independence. Chi-squared values with Yates' correction for continuity are presented with corresponding P-values. P values < 0.05 were considered statistically significant.

RESULTS

Characteristics of included studies

One hundred and fifteen publications were retrieved from the PubMed search, and abstracts were reviewed. Thirty-eight publications reported DR prevalence rates leading to full publication review of these. Eighteen of the 38 publications reviewed in full were excluded for the following reasons: 3 meta-analyses reported no primary Australian data; 12 studies were conducted in populations outside of Australia; 2 studies involved T1DM participants only; and 1 study involved patients with newly diagnosed DM only. The remaining 20 publications investigating DR prevalence for 10 different Australian populations satisfied criteria for inclusion for the current analysis. There were five Indigenous and five non-Indigenous populations

investigated within these 10 studies. Lit.search revealed 23 Indigenous studies, of which 11 publications reported DR prevalence estimates for seven different Indigenous Australian populations. Five of the seven studies had been identified by the PubMed search. Of the two studies not previously identified through PubMed, one study involved self-reported DR rates and was excluded. The other met inclusion and exclusion criteria and was included.

Recruitment methodology was reviewed for all 11 studies included. Study descriptions are presented in **Table 2.1**. The five studies involving non-Indigenous participants with DM included the Blue Mountains Eye Study, [22] Melbourne Visual Impairment Project, [102,103] Australian Diabetes, Obesity and Lifestyle Study (AusDiab), [104,105] Australian National Diabetes Information Audit and Benchmarking Exercise (ANDIAB), [24] and Newcastle Diabetic Retinopathy Study (NDRS) [106]. Of these five studies, three were population-based, community-derived studies, and two were primary- or secondary-care clinic-based studies. T1DM participants from AusDiab did not undergo retinopathy assessment. Because data were presented separately for T1DM and T2DM participants, T1DM cases were excluded. Forty per cent (n = 3502) of ANDIAB participants had retinal examinations for any DR, and only data for these were included in the current analysis.

Indigenous participants with DM included in the current analysis were from the following studies: National Indigenous Eye Health Survey (NIEHS) [12,107], Central Australian Ocular Health Survey (CAOHS) [100,108], Katherine Region Diabetic Retinopathy Study (KRDRS) [109,110], Darwin Region Urban Indigenous Diabetes Study (DRUID) [111], Goldfields Eye Health Survey (GEHS) [112], and the South Australian Eye Health Program (SAEHP) [113]. Included were one population-based, community-derived study; one register-based study; three secondary-care clinic, population-based studies (study sample from all the secondary care clinics in a defined geographical area); and one secondary-care non-population-based study.

Age targets varied between studies, and these data, along with mean age of participants (where available) and gender data, are presented in **Table 2.1**. Because complete data were not available in published form in a manner that could be combined, these demographic factors could not be evaluated in the pooled analysis. Mean duration of DM was available for some studies and is presented in **Table 2.1**.

Prevalence of DR

DR prevalence data from 11 Australian studies were included for the current analysis. Of the 11 studies, six reported data on Indigenous Australians and five on non-Indigenous Australians. A total of 12 666 persons with DM, of whom 2865 were Indigenous Australians and 9801 were non-Indigenous Australians contributed to the prevalence calculation for the presence of any DR. The overall prevalence of any DR was 30%. Eight of the 11 studies also reported rates of STDR, giving a total STDR prevalence of 11%. Characteristics of the participants of each study are presented in **Table 2.1**.

Table 2.1: Study design and clinical characteristics of known diabetic participants

Study title	Indigenous Studies						Non-Indigenous Studies				
	National Indigenous Eye Health Survey (NIEHS)	Central Australian Ocular Health Survey (CAOHS)	Katherine Region Diabetic Retinopathy Study (KRDRS)	Darwin Region Urban Indigenous Diabetes (DRUID)	Goldfields Eye Health Survey (GEHS)	South Australian Eye Health Program (SAEHP)	Blue Mountains Eye Study (BMES)	Melbourne Visual Impairment Project (MVIP)	Australian Diabetes, Obesity and Life- style study (Ausdiab)	Australian National Diabetes Information Audit & Benchmark-ing(ANDIAB)	Newcastle Diabetic Retinopathy study (NDRS)
Study dates	2008	2005-8	1996	2003-5	1995-2007	1999-2004	1992-4	1992-6	2003	2009	1977-88
Recruitment method	Cluster sampling of 30 sites around Australia[1]	Clinic based survey Remote Central Australia[2]	Chronic disease register of diabetics in Katherine[3]	Volunteer cohort Urban Darwin[4]	Clinic based survey remote Western Australia[3]	Clinic based survey remote South Australia[3]	Door-door census Blue Mountains[1]	Cluster sampling of 4 urban, 9 rural areas in Victoria[1]	Cluster sampling of 42 urban & rural areas in Australia[1]	Referral from adult centres & endocrine specialists in Australia[4]	Diabetic clinic and education programs in Newcastle[4]
N	394	1033	239	99	329	771	213	234	333	3502	5519
Mean duration DM (years)	9	-	-	8	-	-	6.2	9.1	-	10.9	-
Age target **Mean age (range)**	≥40 years 53*	≥20 years 50 (20-93)	All ages 49.5 (16-94)	≥15 years 53	All ages 48 (16-89)	≥15 years -	≥49 years 67.4 (49-80)	≥40 years 64.5 (42-97)	≥25 years 63	All ages 44	All ages -
Gender (% Male)	40	34	35	24	41	32	51	44	-	52	52
Prevalence DR (%)											
No DR	70.3	77.8	79.1	79	72.9	78.0	63.6	70.9	78.1	70.9	65
Any DR	29.7	22.2	20.9	21	27.1	22.0	36.4	29.1	21.9	29.1	35
Any NPDR	18.3	19.4	19.7	-	26.1	16.5	33.6	24.0	19.8	-	30
PDR	2.5	2.8	1.3	-	0.9	5.4	1.8	4.2	2.1	-	5
DMO	8.9	5.3	10	-	14.3	6.5	6.5	5.6	3.3	-	10
STDR	11.4	7.0	11.7	-	15.2	11.9	7.5	9.8	-	-	11.4
Grading method	Retinal photos	Clinical exam	Clinical exam	Clinical exam	Clinical exam	Clinical exam	Retinal photos	Retinal photos	Retinal photos	Clinical exam	Retinal photos

Study design has been coded as follows: [1]community-derived population-based study; [2]clinic-derived population-based study; [3]register-based study; clinic-derived non-population-based study.
*This figure represents median age as mean age, as data were not reported in the published data. Abbreviations: –, not available; DM, diabetes mellitus; DMO, diabetic macular oedema; DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; STDR, sight-threatening diabetic retinopathy (defined as PDR and/or DMO).

The NDRS study was conducted in 1977 with follow-up data collected in 1988, and all remaining studies were conducted after 1990. PDR and DMO prevalence rates for NDRS appear to be greater than those seen in the other four non-Indigenous studies. Tests for homogeneity confirmed a statistically significant difference between NDRS and the remaining four non-Indigenous studies for any DR (35.0% vs. 28.9%, Yates $\chi2 = 40.7$, $P < 0.001$), PDR (5.0% vs. 2.7%, Yates $\chi2 = 7.8$, $P = 0.005$) and DMO (10.0% vs. 4.9%, Yates $\chi2 = 20.6$, $P < 0.001$). A trend for a greater rate of STDR in NDRS was also seen but not confirmed to be statistically different (11.4% vs. 8.7%, Yates $\chi2 = 2.71$, $P = 0.10$). Pooled results are therefore presented both with and without inclusion of NDRS data.

Estimated prevalence rates of DR, PDR, DMO and STDR in individuals with DM are presented in **Table 2.2**. The estimated crude prevalence of any DR among Indigenous Australians with DM was 23.6% compared with 32.3% for non-Indigenous Australians with DM (Yates $\chi2 = 80.49$, $P < 0.001$). Although crude prevalence estimates for non-Indigenous participants with DM were lower when NDRS data was excluded (28.9% with any DR), the prevalence of any DR remained significantly lower for Indigenous compared with non-Indigenous persons with DM after exclusion of the NDRS data (Yates $\chi2 = 24.81$, $P < 0.001$).

With the inclusion of the NDRS study, the crude prevalence of STDR was found to be 8.6% for Indigenous persons with DM (3.2% with PDR and 7.6% with DMO) and 11.2% for non-Indigenous persons with DM (4.7% with PDR and 9.4% with DMO). These observations were confirmed to be statistically significant differences for PDR (Yates $\chi2 = 11.14$, $P = 0.001$) and DMO (Yates $\chi2 = 7.00$, $P = 0.008$) independently but not when analysed as the combined variable of STDR (Yates $\chi2 = 1.22$, $P = 0.27$). Non-Indigenous data excluding NDRS revealed lower rates of STDR, PDR and DMO, resulting in a significantly higher rate of DMO in Indigenous compared with non-Indigenous Australians (7.6% vs. 4.9%, Yates $\chi2 = 6.67$, $P = 0.01$) and no difference in prevalence of PDR (3.2% vs. 2.7%, Yates $\chi2 = 0.33$, $P = 0.56$) or STDR (10.4% vs. 8.7%, Yates $\chi2 = 0.98$, $P = 0.32$).

Table 2.2: Prevalence of DR in persons with DM by ethnicity. Chi squared P values are presented.

	Indigenous studies	**Non-Indigenous studies (all studies)**		**Non-Indigenous studies (excluding NDRS)**	
	% (N)	% (N)	P-value	% (N)	P-value
Any DR	23.6 (675)	32.3 (3170)	**<0.001**	28.9 (1238)	**<0.001**
PDR	3.2 (91)	4.7 (299)	**0.001**	2.7 (21)	0.56
DMO	7.6 (211)	9.4 (590)	**0.008**	4.9 (38)	**0.01**
STDR	10.4 (287)	11.2 (668)	0.27	8.7 (39)	0.32

Abbreviations: DR, diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; STDR, sight-threatening diabetic retinopathy (defined as PDR and/or DMO). P-values < 0.05 are highlighted in bold.

DISCUSSION

The overall prevalence of DR among Indigenous Australians with DM appeared to be lower than non-Indigenous prevalence rates and thus varied among ethnic groups. Analysis both with and without NDRS data proved not to affect this outcome. This analysis indicates that 30% of Australians with DM have DR of any level of severity, including 11% with STDR. Australian Bureau of Statistics (ABS) data from 2008 reports that 819 500 Australians (3.8%) have self reported DM [97,99]. Thus, the estimated crude prevalence of DR in the Australian population using ABS data is 1.2% or 250 000 people, with 87 000 (0.4%) of these estimated to have STDR. The prevalence of self-reported DM in Indigenous Australians has been reported to be as high as 38% in the NIEHS study sampling 30 urban and remote areas in Australia [12,100]. Within the constraints of this analysis, 24% of Indigenous Australians with DM were assessed as having DR. This suggests that there are currently up to 61 000 Indigenous Australians with any level of DR (making up approximately 9% of the Indigenous Australian population) and nearly 26 500 Indigenous Australians with STDR [12,114]. Although these extrapolations use the best available data, it must be noted that self-reported DM prevalence rates have been used, and therefore, estimated crude prevalence calculations should be interpreted with caution. This result differs from a number of studies that have evaluated ethnic disparities in the US among Caucasian, African American and Hispanic populations where prevalence of any DR was found to be similar or higher among individuals with DM across these ethnic minorities [23,86,87,115].

Ethnic differences did not persist for prevalence of STDR (after exclusion of the NDRS data), and the reasons for this are currently unclear. However, a higher rate of DMO alone was identified in the Indigenous group. Variation in prevalence of DMO among other ethnic groups has been shown previously, with data from US studies indicating a greater prevalence of DMO in Hispanics and African Americans than in non-Hispanic whites [97,116,117]. Interestingly in this analysis, inclusion of NDRS data did result in significantly lower prevalence of PDR and DMO in the Indigenous population with DM compared with non-Indigenous Australians with DM, consistent with the trends for any DR. The NDRS study was conducted during an era when evidence for strict glucose control, and risk factor management in the prevention of DM complications was only emerging. Based on observations from the DCCT [61,118] and the UKPDS [26,119], it is therefore expected that DR prevalence rates including prevalence of advanced DR reported in NDRS would be higher than those rates reported in more recent studies. Similarly, suboptimal management of DM in Indigenous Australians underlies current beliefs of increased complications of DM in this group. The latter point justifies the comparisons being performed with and without the NDRS data.

Morbidity and mortality of Indigenous Australians with DM associated with poor risk factor management (including hypertension and hyperlipidaemia) and inadequate lifestyle modifications continues to show an upward trend [98,120]. Underlying social determinants of health including access to health care and attitudes towards Western medicine and preventative health are showing gradual improvement [98]. Despite these improvements, delays in DM diagnosis, poor glycaemic control and the high morbidity and mortality attributed to DM in Indigenous populations [24,99] indicate ongoing significant issues with adherence to screening and treatment regimens. The high number of first-presentation, untreated Indigenous Australians

with DR illustrates the discrepancy between recommended and actual implementation of National Health Guidelines for the management of DR in the Indigenous population. In the GEHS, 18% of Indigenous Australians with DM were found to have any DR, and 7% found to have STDR at first eye check [112]. Furthermore, only 33% of Indigenous DR cases identified to benefit from laser photocoagulation from the SAEHP actually underwent treatment [113]. A meta-analysis of international studies conducted from 1975 to 2008 in patients not yet treated for DR showed that rates of progression to PDR and severe vision loss are substantially lower since 1985 compared with the pre-1985 era [121]. Differences are partly explained by more severe levels of DR at baseline and poorer glycaemic control prior to 1985. A more rapid progression to STDR for Indigenous Australians for the same underlying reasons may explain the equal rates of STDR between Indigenous and non-Indigenous Australians with DM, despite lower rates of any DR seen in Indigenous Australians from the current analysis.

It is difficult to gain an understanding of susceptibility to DR without comparing prevalence estimates by age groups and accounting for glycaemic control. Unfortunately these factors were not available for incorporation into the current analysis. The median age of Indigenous Australians is over 15 years lower than that for non-Indigenous Australians (21 compared with 37 years respectively) [99], as a result of high fertility rates and premature mortality among Indigenous Australian populations. It can therefore be argued that there is a subset of Indigenous Australians with DM who may have developed DR had they reached the life expectancy of their non-Indigenous Australian counterparts. This analysis is unable to account for this possibility, and DR prevalence in Indigenous Australians may therefore be underestimated when analysing the population as a whole. However, significantly earlier onset of T2DM in Indigenous Australians is also well documented [111,122,123], with Indigenous Australians thought to develop DM up to 20 years earlier than non-Indigenous Australians [124]. As duration of DM is one of the strongest predictors for the development of DR alongside glycaemic control, analysis by DM duration may be more useful than stratifying by age group and may in fact provide a better understanding of ethnic variation in DR susceptibility, particularly given the inconsistent age-group structures of these populations. Unfortunately, DM duration data were not adequately reported in the studies under review to allow for inclusion in the current analysis. Interestingly, early-onset T2DM (prior to age 45) is associated with more severe grades of DR, independent of duration of DM and glycaemic control, and is thought to suggest an inherent tissue susceptibility to hyperglycaemic damage [125]. Whether or not this finding is transferable to Indigenous Australians is worth future investigation when examining DR susceptibility in this population. In general, the younger age of diagnosis of DM in Indigenous Australians would be expected to lead to higher rates of DR in this population.

Ideally, multivariate analysis accounting for confounding risk factors (particularly DM type, glycaemic control, duration of DM, age of DM onset, hypertension, hyperlipidaemia and body mass index) is required for a more informative evaluation of ethnic variation of DR prevalence. With such data currently unavailable for multivariate analysis, results from this pooled analysis raise the possibility that genetic variation could account for a reduced initial susceptibility to any DR in Indigenous compared with non-Indigenous

Australians. In contrast, earlier age of onset of T2DM resulting in a more aggressive phenotype combined with a faster progression to STDR due to poor risk factor management in Indigenous Australian groups could explain the increased number of Indigenous Australians that ultimately progress to STDR, and in particular DMO.

A number of methodological limitations with the studies included in this analysis have been identified, including the accuracy of self-reporting and variations in sampling methods. Only data pertaining to participants with known DM were included for this pooled analysis, of which self-reported DM status was used to target patients in a number of the included studies. Significant inconsistencies have been reported when comparing self-reported rates and actual rates of health problems in Indigenous communities. The NIEHS found that 54% of those with self-reported DR had no clinical evidence of DR on examination, and of those found to have DR, 60% did not report a previous history of this [126]. The DRUID found similar results for DM diagnosis in an urban setting, with 28% of participants with a diagnosis of DM newly diagnosed in the study [111]. Variations have also been identified in non-Indigenous studies, with rates of undiagnosed DM in non-Indigenous Australians living in rural areas similar to those seen in Indigenous studies [127]. It is possible that the current analysis may underestimate the overall prevalence of DR, particularly in relation to Indigenous Australians. Future studies require more accurate diagnostic criteria in order to determine the true rate of DM and associated complications.

The challenges in conducting population-based research involving Indigenous Australians are well documented [107,128]. Indigenous Australian studies included for this analysis were limited predominantly to volunteer cohorts because of the difficulties in collecting community-derived, population-based data. A number of studies collected data in an opportunistic manner from patients attending routine eye clinics as this was seen as the most effective and culturally acceptable recruitment technique [100,110,112,113]. Thus, the potential for selection bias was high, particularly in remote areas, where eye clinics are held infrequently, and patients are increasingly likely to attend if they have a perceived visual disturbance, or are at high risk for visual loss. Conversely, an opposite bias could occur from the tendency to examine "worried well" people with DM, such as those who work around the clinics or who have been newly diagnosed with DM. This is a significant limitation of the current analysis. Furthermore, in order to provide culturally sensitive health care to Indigenous Australians, there is a tendency for both minimally invasive and streamlined practices. In addition to this, time constraints and restrictions in resources and health-care worker numbers play a role in the DR screening practices employed. This is reflected in the DR grading methods (predominantly clinical examination) used in the five out of six Indigenous studies that used opportunistic sampling methods. In contrast, four out of five non-Indigenous studies used retinal photographs, allowing for examiner blinding and reducing the risk of biased outcomes.

It must also be acknowledged that Indigenous studies conducted in specific regions produce data reflective of the health of particular communities involved (dependent on access to health care, diet, lifestyle and socioeconomic status specific to that community) and not necessarily representative of other Indigenous communities throughout Australia. Both the geographical spread of communities across remote areas of

Australia and the distinct culture of each community make it difficult to compare health outcomes both between Indigenous Australian communities and between Indigenous and non-Indigenous Australian populations. This makes it particularly difficult to establish variations in rates of DR, and may limit the validity of the calculated prevalence of DR in the Australian Indigenous population generated from this analysis. Data from a large, population-based, nationally representative sample of Indigenous Australians would make for a more valid comparison.

CONCLUSION

The observed data potentially indicate a lesser susceptibility to any DR for Indigenous compared with non-Indigenous Australians with DM. However, limitations from the available published data preclude sufficient exploration of the impact of compounding risk factors. Furthermore, methodological differences used for diagnosis and grading of DR limit the reliability of the ethnic comparisons made. Because of the high prevalence of DM in this population, Indigenous Australians still account for 23% of all Australians with STDR despite only making up 3% of the Australian population [114]. Without appropriate ophthalmic and medical intervention, one third of these are expected to reach legal blindness within 3 years [129]. Notably, the prevalence of DMO from the current analysis was found to be both significantly higher and out of proportion to the rate of DM in Indigenous Australians, and hence, further research into the pathogenesis and treatment of DMO is an urgent priority. Further study and a truly population-based design including analysis of epidemiological risk factors are required to better understand the relative risks for DR in Indigenous populations. Evaluation of uptake of screening and treatment of DR prior to the development of end-stage disease in Indigenous populations will ultimately help to determine appropriate strategies to reduce vision loss from DM in Indigenous Australians [130]. It is likely that individual susceptibility differences for the development of STDR exist, and future research needs to address the factors predisposing individuals to very severe outcomes so that appropriate interventions are directed at those individuals at highest risk before irreversible visual loss occurs.

CHAPTER 3

A POPULATION-BASED STUDY OF END-STAGE DIABETIC RETINOPATHY REQUIRING VITREORETINAL SURGERY IN SOUTH AUSTRALIA AND THE NORTHERN TERRITORY.

The original work presented in this chapter has been submitted for publication to: (1) ***The Medical Journal of Australia****: Kaidonis G, Hassall M, Phillips R, Raymond G, Saha N, Wong GHC, Gilhotra JS, Liu E, Burdon KP, Henderson T, Newland H, Lake S, Craig JE. Rates of vitrectomy for diabetic retinopathy are five times higher amongst Indigenous Australians compared with non-Indigenous Australians; and (2)* ***Retina****: Kaidonis G, Hassall M, Phillips R, Raymond G, Saha N, Wong GHC, Gilhotra JS, Liu E, Burdon KP, Henderson T, Newland H, Lake S, Craig JE. Visual outcomes following vitrectomy for diabetic retinopathy amongst Indigenous and non-Indigenous Australians in South Australia and the Northern Territory. Dr Kaidonis' contributions include study conception and design, data collection, analysis and interpretation and manuscript preparation.*

Late presentation of DR, advanced and with significant visual loss, is still a problem, particularly in remote areas of Australia [12,108]. As described in the previous chapter, despite a lesser prevalence of early DR in Indigenous compared with non-Indigenous Australian populations with DM, STDR occurs at a rate similar to non-Indigenous Australians [101]. It is well known there are considerable difficulties in analysing ethnicity as a risk factor for the development of end-stage DR using current data; particularly reflecting the challenges in conducting population-based research in Indigenous Australian Communities [107]. Local circumstances relevant to individual communities, the transient nature of residents in communities, and time constraints remain the predominant limitations to population-based sampling in remote Indigenous communities [107]. Selection bias is high in studies using volunteer cohorts, or those collecting data opportunistically from routine eye clinics. This chapter describes an alternative approach to population-based sampling in remote Australia and inturn eliminates a number of the issues that have arisen in previous studies. This has been achieved by performing a retrospective audit of surgical records in order to capture all patients undergoing diabetic vitrectomy for end-stage DR. This study successfully evaluates ethnicity related differences in patients requiring diabetic vitrectomy, which is used as a surrogate measure of DR at its most severe form.

AIM

This audit of diabetic vitrectomies encompasses all vitreoretinal operations performed for end-stage DR in SA and NT over a 5-year period from 2007 to 2011. As such, this is the first study to capture all those reaching this severe endpoint in a population-based fashion. The aims of this analysis are to (1) determine the rate of patients receiving diabetic vitrectomy in SA and NT, (2) explore the difference in prevalence of diabetic vitrectomy between Indigenous and non-Indigenous patient populations, (3) determine visual outcomes and identify factors associated with visual success in patients undergoing diabetic vitrectomy, and (4) investigate risk factors predisposing to early progression to advanced DR requiring vitrectomy in SA and

NT, including duration of DM, glycaemic control, ethnicity and the uptake of early treatment for DR. Understanding these factors is crucial in directing screening and treatment programs to aid in the management of this potentially preventable blinding disease.

METHODS

We conducted a retrospective survey of patient notes from the Royal Adelaide Hospital (RAH), Flinders Medical Centre (FMC), Royal Darwin Hospital and the private practices of all vitreoretinal surgeons in Adelaide from 1 January 2007 to 31 December 2011. Patient case files were identified by a comprehensive search of hospital and private practice surgical record databases. Search criteria included all Medicare numerical item codes for vitrectomy and related vitreoretinal procedures. As there is no permanent vitreoretinal service in the NT or regional SA, the RAH and FMC are the only public vitreoretinal services for both SA and the NT. Referral patterns and cultural preferences lead us to believe that this methodology should achieve 100% coverage of vitrectomy cases performed for the target population. Ethical approval for this study was obtained from the Southern Adelaide Clinical Human Research Ethics Committee (HREC), the Royal Adelaide Hospital HREC and the Central Australian HREC.

Files of all patients identified as having had vitreoretinal surgery during the audit period were manually examined. Any vitrectomy performed for the complications of DM was included in this study and further analysed. Diabetic vitrectomy was defined as vitrectomy performed for diabetic ocular complications, and the specific indication for surgery was categorized according to **Table 3.1**. Diabetic patients undergoing vitrectomy for non-DM related indications (eg. trauma) were not included in this analysis.

Table 3.1: Indication for vitrectomy

Inclusion criteria for 'Diabetic Vitrectomy'
1. Media opacities • Including recurrent or non-resorbing vitreous haemorrhage
2. Vitreoretinal traction (with or without haemorrhage) • Including tractional retinal detachment
3. Post-vitrectomy complications • If the primary vitrectomy was for diabetic indications
4. Other diabetic complications • Severe PDR • DMO refractory to other treatment modalities

Abbreviations: PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema.

Data were collected retrospectively and as available from case files. Details of the surgical procedure and the administration of intraoperative laser, bevacizumab or triamcinolone acetonide were recorded. Pre-operative data collected included patient demographics, ethnicity and diabetic history (type and duration of DM, and related systemic complications of DM including amputations, chronic kidney disease (CKD) and end-stage renal failure (ESRF) requiring dialysis or renal transplant). Ethnicity was determined from hospital records,

being a required field for admission to hospital, and was recorded as self-identified by the patient. Past ocular treatments for DR including PRP, macular laser and previous vitrectomy were recorded. Patients were recorded as having had timely laser treatment if they had had PRP (or macular laser for patients undergoing vitrectomy for DMO) at least 6 months prior to vitrectomy. HbA1c and blood pressure immediately prior to surgery were also collected. Best corrected visual acuity (BCVA) using a Snellen chart was collected immediately prior to surgery as well as at 6 and 12 months post-operatively. Snellen BCVA was converted to ETDRS letters for study analysis using the methods reported by Gregori et al. [131]. Patients with "count fingers" vision or worse were recorded as 0 ETDRS letters. Visual success was defined as a ≥15 ETDRS letter gain from baseline. Where required, additional information on six and twelve-month follow-up was sought from external sites involved in the patient's ongoing care in the community.

Rates of diabetic vitrectomy in Indigenous and non-Indigenous populations were determined and relative risk calculated. These calculations were based on (1) an Indigenous Australian population of 112 051 people and a non-Indigenous population of 1 827 949 people in our study population in SA and NT [132]; (2) a prevalence of measured or self reported DM of 5.1%, across Australia [13,133]; and (3) a prevalence of DM of 30% reported within Indigenous Australian communities [132,134]. Statistical analysis was carried out using SPSS version 20.0 for Mac OS X (IBM SPSS Statistics 20.0, SPSS Inc., Armonk, NY, USA). Variables for Indigenous and non-Indigenous groups were characterized using basic descriptive statistics and between group comparisons were performed using a Mann-Whitney U test (for continuous variables) or chi-square test (for dichotomous variables). A logistic regression test was used to identify factors associated with visual success, at 12 months post vitrectomy. Multivariate cox-regression survival analysis was used to explore factors that increase risk for earlier vitrectomy. For patients undergoing multiple vitrectomies, only data pertaining to the patient's first vitrectomy within the audit period was included for demographic and regression analyses. P-values < 0.05 were considered statistically significant.

RESULTS

A total of 495 vitrectomies, for 405 eyes in 335 patients, were performed for the management of DM related complications during the study period (1st January 2007 to 31st December 2011). Seventy-seven (23%) patients requiring diabetic vitrectomy were Indigenous Australians. Indigenous Australians make up 5.8% of the total study population in SA and NT [132]. The relative risk of Indigenous Australians requiring diabetic vitrectomy is 4.9 when compared with non-Indigenous Australians requiring diabetic vitrectomy in SA and NT. Accounting for the large difference in DM prevalence, the rates of diabetic vitrectomy over 5 years were 277 per 100 000 non-Indigenous patients with DM and 26 per 100 000 Indigenous patients with DM. This calculation reverses the relative risk, and Indigenous Australians with DM in SA and NT appear 10 times less likely to require diabetic vitrectomy.

In total, 66 patients had T1DM (4 Indigenous and 62 non-Indigenous patients). For those with T2DM, the mean age at DM diagnosis was nearly 7 years lower in Indigenous (37.8 years; standard deviation (SD), 10.5) compared with non-Indigenous (44.2 years; SD, 12.1) patients ($p = 0.001$) (**Table 3.2**). Females made

up 54.5% of the Indigenous Australian patient group, whereas there were more males (64.6%) in the non-Indigenous patient group (**Table 3.2**). Indigenous patients undergoing vitrectomy were more likely to have had an amputation and CKD. Glycaemic control as measured by HbA1c (mean, 8.4%; range, 5.2%-16.5%) and SBP (mean, 150 mmHg; range, 90 mmHg – 221 mmHg) were found to be poor in the whole cohort. Although the mean HbA1c in the Indigenous group was higher than that in the non-Indigenous group, the difference did not reach statistical significance (p = 0.117).

Table 3.2: Demographics and clinical characteristics of patients receiving diabetic vitrectomy by ethnicity.

Variable	n	Indigenous n (%) OR mean (SD)	n	Non-Indigenous n (%) OR mean (SD)	P-value
T1DM	**4**		**62**		
Age at DM onset (years)	4	16.50 (13.08)	52	17.63 (11.16)	0.937
Age at vitrectomy (years)	4	32.75 (12.95)	62	45.18 (17.51)	0.158
T2DM	**73**		**196**		
Age at DM onset (years)	58	37.83 (10.46)	159	44.19 (12.05)	**0.001**
Age at vitrectomy (years)	73	51.59 (9.85)	195	63.12 (10.90)	**<0.001**
All DM	**77**		**258**		
Sex (female)	77	42 (54.5)	258	91 (35.4)	**0.004**
Duration of DM (years)	62	13.90 (8.08)	212	19.93 (10.36)	**<0.001**
HbA1C (%)	51	8.85 (2.80)	172	8.20 (1.76)	0.117
Systolic blood pressure (mmHg)	76	153.45 (26.43)	192	148.00 (25.55)	0.155
Diastolic blood pressure (mmHg)	76	80.37 (11.96)	192	77.58 (12.32)	0.192
Amputation	62	15 (24.2)	220	27 (12.3)	**0.033**
CKD	71	56 (78.9)	229	116 (50.7)	**<0.001**
Dialysis	74	26 (35.1)	247	16 (6.5)	**<0.001**
Renal transplant	74	1 (1.4)	247	3 (1.2)	>0.999

Abbreviations: SD, standard deviation; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; HbA1c, haemoglobin (A1c); CKD, chronic kidney disease. n represents number of patients in each subgroup. P-values < 0.05 are highlighted in bold.

The frequency of vitrectomy per person during the 5-year audit period is illustrated in **Table 3.3.** Indigenous patients had a mean of 1.6 vitrectomies per person (SD, 0.92), which was not statistically different from the frequency of non-Indigenous diabetic vitrectomy (mean, 1.5; SD, 0.88; P = 0.062). There was no statistical difference in the number of Indigenous patients having had PRP for treatment of PDR or macular laser for treatment of DMO at least 6 months prior to vitrectomy compared with non-Indigenous patients (P = 0.774). Intra-operative adjuvant treatment with either laser, bevacizumab or triamcinolone acetonide did not vary by ethnicity (Table 3.3). **Figure 3.1** shows the number of vitrectomies by indication. The majority of vitrectomies across both ethnicities were for media opacities and vitreoretinal traction. Chi-square test revealed no significant difference in indication for vitrectomy between Indigenous and non-Indigenous Australians (P = 0.08).

Table 3.3: DR treatment at baseline and intraoperative management of patients undergoing vitrectomy by ethnicity.

Variable	n	Indigenous (n=77) n (%) OR mean (SD)	n	Non-Indigenous (n=258) n (%) OR mean (SD)	P-value
No. of vitrectomies per person between 2007 and 2011	77	1.6 (0.92)	258	1.5 (0.88)	0.062
Baseline BCVA	76	21.95 (27.31)	236	22.90 (27.74)	0.881
Prior laser treatment	69	53 (76.8)	244	181 (74.2)	0.774
Intraoperative laser	77	68 (88.3)	257	208 (80.9)	0.184
Intraoperative bevacizumab	77	5 (6.5)	257	20 (7.8)	0.897
Intraoperative triamcinolone	77	17 (22.1)	257	53 (20.6)	0.908

Abbreviations: SD, standard deviation; DR, diabetic retinopathy; BCVA, best corrected visual acuity. BCVA is measured in ETDRS letters. P-values < 0.05 are highlighted in bold.

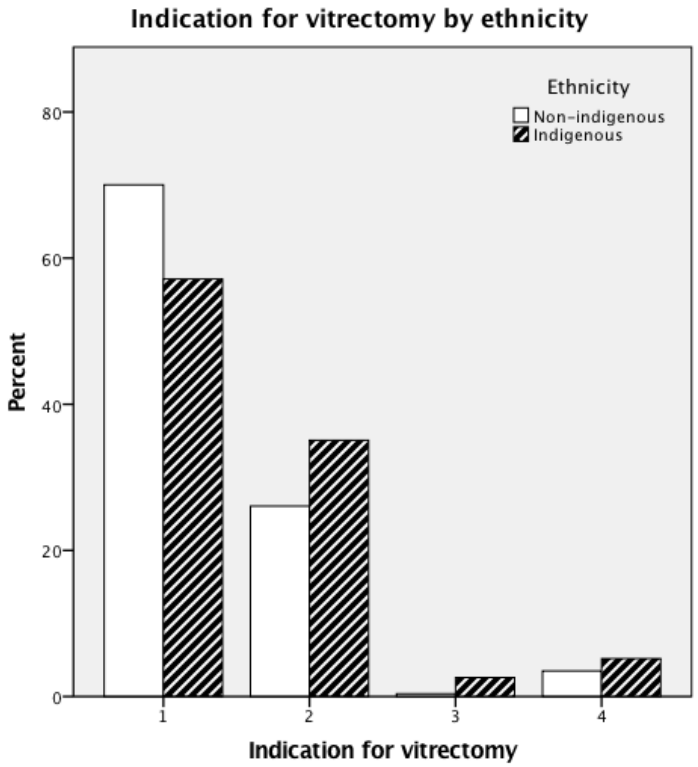


Figure 3.1: Percentage of diabetic vitrectomies performed for each indication by ethnicity (1 = Media opacities; 2 = Vitreoretinal traction; 3 = Post-vitrectomy complications; 4 = Other diabetic complications).

Visual success

Visual outcomes were assessed in all patients for whom baseline BCVA and post-operative BCVA (at 6 and 12 months) was available. Visual outcomes at 6 months were calculated for 185 patients (287 eyes when more than one diabetic vitrectomy per person was included). Visual outcomes at 12 months were calculated for 162 patients, which totalled to 255 eyes when all diabetic vitrectomies were included.

Table 3.4 shows visual outcomes at 6 and 12 months post vitrectomy by indication for vitrectomy. Baseline BCVA was not significantly different between the 4 groups (P = 0.086). At 12 months post-operatively, eyes undergoing vitrectomy for media opacity were the most likely to have stable or improved vision (93.2% of eyes lost less than 15 ETDRS letters from baseline), compared with those with vitreoretinal traction (85.3%), post vitrectomy complications (82.4%) and those requiring vitrectomy for other diabetic indications (56.3%). Statistically significant differences in visual outcomes between these indications were found at 12 months.

Table 3.4: Visual outcomes following vitrectomy at 6 and 12 months by indication for vitrectomy (all eyes).

	Media opacity	**Vitreoretinal traction**	**Post vitrectomy complications**	**Other**	**P-value**
Baseline BCVA Mean ETDRS letters (SD)	20.3 (27.4)	23.4 (27.6)	12.0 (20.5)	29.8 (27.1)	0.086
≥ 15 letter gain					
6 months, n (%)	116 (64.1)	39 (50.0)	5 (33.3)	2 (15.4)	**<0.001**
12 months, n (%)	91 (61.9)	29 (38.7)	4 (23.5)	4 (25.0)	**<0.001**
± 15 letters					
6 months, n (%)	56 (30.9)	35 (44.9)	9 (60.0)	9 (69.2)	**0.003**
12 months, n (%)	46 (31.3)	35 (46.7)	10 (58.8)	5 (31.3)	**0.034**
≥ 15 letter loss					
6 months, n (%)	9 (5.0)	4 (5.1)	1 (6.7)	2 (15.4)	0.646
12 months, n (%)	10 (6.8)	11 (14.7)	3 (17.6)	7 (43.8)	**<0.001**

Abbreviations: BCVA, best-corrected visual acuity; ETDRS, Early Treatment Diabetic Retinopathy Study; SD, standard deviation. P-values < 0.05 are highlighted in bold.

Table 3.5: Visual outcomes following vitrectomy by ethnicity (first eye).

	Indigenous		**Non-Indigenous**		
	n	**Mean (SD)**	**n**	**Mean (SD)**	**P-value**
Baseline VA	76	21.6 (27.3)	236	22.9 (27.7)	0.881
Final VA at 6 months	31	52.6 (25.7)	165	49.5 (24.9)	0.347
Mean change VA	20	24.8 (25.1)	156	27.2 (31.3)	0.856
Visual success at 6 months	20	19 (63.3)	156	93 (59.6)	0.859
Final VA at 12 months	29	46.2 (32.3)	145	48.1 (29.4)	0.998
Mean change VA	28	19.9 (37.4)	135	25.5 (36.2)	0.589
Visual success at 12 months	28	17 (60.7)	135	73 (54.1)	0.664

Abbreviations: SD, standard deviation; BCVA, best-corrected visual acuity. n represents number of patients in each subgroup. BCVA is measured in ETDRS letters. Visual success represents a gain of at least 15 ETDRS letters. P-values < 0.05 are highlighted in bold.

There was no statistically significant difference in BCVA between Indigenous and non-Indigenous patients at baseline (P = 0.881), or at 6 (P = 0.347) and 12 months (P = 0.998) postoperatively (**Table 3.5**). At 12 months post vitrectomy, Indigenous patients gained, on average, 20 (SD 37.4) letters and non-Indigenous patients gained 26 (SD 36.2) letters (P = 0.589). Visual success at 12 months (≥15 ETDRS letter gain) was

achieved in 60.7% of Indigenous patients and 54.1% of non-Indigenous patients for whom BCVA data was available at this time-point (**Table 3.5**). A total of 22 operated eyes (8.1% of those for which 12 month BCVA data was available) had no perception of light (NPL) at 12 months post-operatively. Thirteen of these operations were performed for 7 (4.8%) non-Indigenous patients and 9 operations were performed for 8 (27.6%) Indigenous patients. Twenty-nine patients were legally blind (Snellen BCVA of 6/60 (35 ETDRS letters) or worse in the better seeing eye) at 12 months following their first vitrectomy. This included 24 (17.0%) non-Indigenous patients and 5 (17.9%) Indigenous patients (P > 0.999). There was no significant difference between the number of Indigenous (23%) and non-Indigenous patients (21.5%) who were legally blind at baseline prior to their first vitrectomy (P = 0.910).

Table 3.6: Logistic regression analysis for "visual success" (≥ 15 letter gain at 12 months)

Variable	OR (95% CI)	P-value
Sex, Female	1.25 (0.36-4.37)	0.728
Ethnicity, Indigenous	4.75 (0.79-28.70)	0.090
DM type, T1DM	0.70 (0.16-3.09)	0.640
Duration of DM	0.99 (0.93-1.05)	0.785
Amputation	1.57 (0.12-19.80)	0.729
CKD	1.01 (0.26-3.87)	0.988
Dialysis	0.49 (0.06-4.40)	0.527
HbA1c	1.15 (0.79-1.68)	0.471
Systolic blood pressure	0.99 (0.96-1.01)	0.385
Diastolic blood pressure	0.98 (0.91-1.05)	0.508
Prior laser	4.90 (1.17-20.54)	**0.030**
Pre-operative BCVA	0.97 (0.94-0.99)	**0.010**
Indication for vitrectomy (VH)	1.84 (0.03-103.96)	0.767
Intraoperative laser	2.67 (0.60-11.80)	0.199
Intraoperative bevacizumab	1.88 (0.33-10.85)	0.480
Intraoperative triamcinolone	5.22 (0.55-49.17)	0.149

Abbreviations: OR (95%CI), odds ratio with 95% confidence interval; DM, diabetes mellitus; T1DM, type 1 DM; CKD, chronic kidney disease; HbA1c, haemoglobin (A1c); BCVA, best-corrected visual acuity; VH, vitreous haemorrhage. BCVA is measured in ETDRS letters. P-values < 0.05 are highlighted in bold.

Logistic regression was performed to ascertain the effects of clinical risk factors on the likelihood of visual success in patients undergoing diabetic vitrectomy (**Table 3.6**). Variables entered into the regression included sex, ethnicity, DM type, duration of DM, amputation, CKD, dialysis, pre-operative HbA1c, pre-operative SBP and diastolic blood pressure (DBP), timely laser treatment in the eye undergoing vitrectomy, indication for vitrectomy and the use of intraoperative adjuvant treatment (laser, bevacizumab or triamcinolone acetonide). The overall logistic regression model was not statistically significant, $\chi^2(17)=24.114$, $P = 0.116$ (Cox and Snell $R^2=0.275$, Nagelkerke $R^2=0.370$). Patients with lower pre-

operative BCVA were less likely to achieve visual success (P = 0.010), with a 3% reduction in likelihood of visual success for each 1 ETDRS letter worsening of BCVA at baseline. Patients who were treated with laser photocoagulation at least 6 months prior to vitrectomy were nearly 5 times more likely to have gained 15 or more letters at 12 months post vitrectomy than those who had not undergone timely laser treatment (P = 0.030). These were the only statistically significant factor found to predict visual success in our model.

Risk factors for earlier vitrectomy

Multivariate cox regression survival analysis was performed to investigate factors that may predispose to earlier vitrectomy. Patients with complete data for the following covariates: sex, ethnicity, DM type, age of DM diagnosis, HbA1c at time of vitrectomy, amputation, CKD, dialysis and timely laser in the eye undergoing vitrectomy were included, and two time variables were assessed. Results from both analyses are presented in **Table 3.7**.

Table 3.7: Cox regression survival analysis evaluating risks for earlier vitrectomy

	Time of DM onset to vitrectomy		**Time of PDR onset to vitrectomy**	
Variable	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
Sex, Female	0.90 (0.65-1.23)	0.494	0.99 (0.71-1.37)	0.929
Ethnicity, Indigenous	2.74 (1.69-4.43)	**<0.001**	1.67 (1.05-2.65)	**0.029**
DM type, T1DM	2.13 (1.25-3.64)	**0.006**	1.21 (0.66-2.20)	0.543
Age DM diagnosed	1.06 (1.04-1.08)	**<0.001**	1.01 (0.99-1.03)	0.228
Amputation	0.76 (0.45-1.26)	0.281	0.73 (0.42-1.28)	0.273
CKD	0.85 (0.61-1.18)	0.333	1.04 (0.74-1.46)	0.827
Dialysis	1.63 (0.98-2.73)	0.062	0.90 (0.53-1.53)	0.704
HbA1c	1.02 (0.93-1.12)	0.692	1.04 (0.94-1.14)	0.453
Lack of prior laser	0.94 (0.65-1.34)	0.718	1.72 (1.20-2.49)	**0.004**

Abbreviations: DM, diabetes mellitus; PDR, proliferative diabetic retinopathy; OR (95%CI), odds ratio with 95% confidence interval; T1DM, type 1 DM; CKD, chronic kidney disease; HbA1c, haemoglobin (A1c). P-values < 0.05 are highlighted in bold.

The first time variable assessed was from diagnosis of DM to time of first vitrectomy, for which complete data were available for 173 patients. The overall model was statistically significant ($\chi^2(9)=61.57$; P < 0.001). Age at DM onset (OR, 1.06; CI, 1.04-1.08; P < 0.001), T1DM (OR, 2.13; CI, 1.25-3.64; P = 0.006) and Indigenous Australian ethnicity (OR, 2.74; CI, 1.69-4.43; P < 0.001) were independently associated with earlier vitrectomy. **Figure 3.2** shows survival function patterns for Indigenous and non-Indigenous patients. Indigenous patients had 2.7 times the risk of requiring vitrectomy compared with non-Indigenous patients when all other factors remained equal. This risk increased to 3.1 times (OR, 3.13; CI, 1.73-5.71; P < 0.001) when evaluating only patients with T2DM in the same model (results not presented). The second time variable assessed was from diagnosis of PDR to time of first vitrectomy, for which complete data were available for 158 patients (**Table 3.7**). The overall model was statistically significant ($\chi^2(9)$ =16.71; P =

0.048). Lack of timely laser treatment prior to vitrectomy (OR, 1.72; CI, 1.20-2.49; P = 0.004) and Indigenous Australian ethnicity (OR, 1.67; CI, 1.05-2.65; P = 0.029) were the predominant factors found to be associated with earlier vitrectomy.

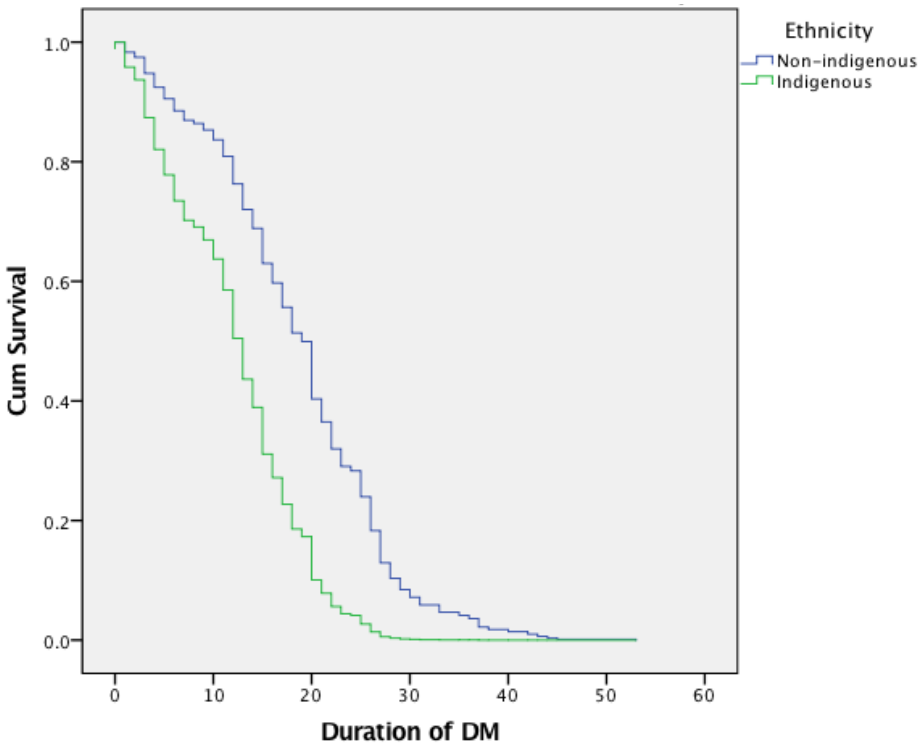


Figure 3.2: Survival analysis from onset of DM to vitrectomy for patients with all types of DM.
Abbreviations: DM, diabetes mellitus.

DISCUSSION

Of the 335 patients receiving diabetic vitrectomy during the audit period, 77 were Indigenous Australian patients. Indigenous Australians make up 5.8% of the total population in SA and NT [132], but account for 23% of patients requiring diabetic vitrectomy (relative risk 4.9), reflecting a large burden of disease secondary to end-stage DR in the Indigenous Australian population. However, when we account for the six times greater prevalence of DM in Indigenous Australian communities, the relative risk reverses, and Indigenous Australians with DM in SA and NT appear 10 times less likely to require diabetic vitrectomy. This is the first study to report such findings. It is unclear whether this reflects a lower susceptibility to retinal complications of DM amongst Indigenous Australians, reduced life expectancy or differing access to care or indications for surgery, or a combination of these factors. Access to public tertiary services is readily available throughout the study population through outreach services.

Patients with DM reaching such severe ocular endpoints often suffer equally advanced microvascular and macrovascular diabetic complications of other end organs. As such, 5 year survival rates for patients undergoing diabetic vitrectomy have been shown to range from 75% to 85%; up to 10% lower than survival rates in the standard DM population [135]. It appears from this population-based study that Indigenous patients requiring diabetic vitrectomy have significantly more DM related comorbidities than non-Indigenous

patients requiring diabetic vitrectomy. The proportion of patients having undergone amputation was approximately double in the Indigenous versus non-Indigenous group. Rates of amputation have been reported previously in Indigenous populations in Far North Queensland. Between 1998 and 2008, 52% of major amputations secondary to DM were performed for Indigenous patients at a major tertiary hospital, servicing a population where Indigenous Australians account for 9.6% of the total population [136]. Furthermore, the current population-based study found that 79% of Indigenous Australians requiring diabetic vitrectomy had CKD at the time of surgery and 35% were on dialysis. This is more than 5 times greater than the 6.5% of non-Indigenous Australians on dialysis at time of vitrectomy. It is unclear from these data whether Indigenous patients on dialysis are subsequently more likely to access care for other diabetic complications than those who are not dialysis dependent, or that Indigenous patients are more susceptible to developing DN than DR. It is noteworthy that poor DM control reflected by the HbA1c levels was present in both Indigenous and non-Indigenous groups. It is possible that other environmental risk factors (smoking or infection) could differ between the groups, or the data may reflect genetic differences in complication susceptibility. Interestingly, it seems that a higher incidence of CKD compared with other DM complications is not unique to Indigenous Australians with DM, but is common to ethnic minorities and First Nations people world-wide [137,138]. In addition, the cumulative incidence of ESRF increases with decreasing age of DM onset among First Nations people with DM in Canada [138], and may also hold true for the Indigenous Australian population studied here. Despite the significantly higher rate of CKD and dialysis in the Indigenous Australian group, there was no significant difference in the number of patients receiving renal transplant between Indigenous and non-Indigenous Australians undergoing diabetic vitrectomy. Low rates of kidney transplantation amongst Indigenous Australians have been reported previously and are thought to reflect lower rates of live kidney donation as well as higher mortality during dialysis treatment [139].

The mean age of Indigenous Australians with T2DM undergoing diabetic vitrectomy was 51.6 years, nearly 12 years younger than the mean age of non-Indigenous Australians (63 years). This could in part be explained by the earlier age of onset of T2DM in the Indigenous Australian group. A study of young people (aged less than 25) in the Top End of the NT found that only 17% of the 70 cases of T1DM represented Indigenous Australians where as 84% of the young people with T2DM were Indigenous Australians [140]. However, mean duration of T2DM at vitrectomy was also significantly lower in the Indigenous Australian group. There are two plausible explanations for this difference. Firstly, onset of T2DM prior to age 45 has been postulated to result in a more aggressive phenotype and appears to be highly associated with the premature development of STDR [125]. This theory supports the notion that the earlier age of DM onset is in fact driving this increased susceptibility to end-stage DR requiring vitrectomy at such an early age. Secondly, the shorter duration of DM may be purely a reflection of the shorter life expectancy of Indigenous Australians. The median age at death in 2013 for Indigenous males and females was 48.8 years and 55.3 years respectively in SA and 52.5 years and 58.0 years respectively in the NT [141]. Non-Indigenous males and females had a median age at death of 80 years and 85.5 years respectively in SA and 69.9 years and 71.4 years respectively in NT [141]. Furthermore, when stratifying by cause of death, DM accounts for 7.9% of Indigenous deaths compared with only 2.6% of non-Indigenous deaths [142]. Therefore, patients with DM

are likely to be among those with the highest discrepancy in life expectancy between Indigenous and non-Indigenous Australians. Considering a mean age of 38 years at Indigenous DM onset found in this study, and the median age at death of Indigenous males and females presented above, Indigenous patients surviving with duration of DM greater than 12 years (males) and 20 years (females) may be relatively uncommon. This also raises the possibility that the rate of Indigenous vitrectomy would actually be much greater if Indigenous Australians with DM had a similar life expectancy to non-Indigenous Australians.

The gender discrepancy in those requiring diabetic vitrectomy showed opposing trends in the Indigenous versus non-Indigenous group. Thirty-five percent of non-Indigenous patients were females compared with fifty-five percent of Indigenous patients. A number of factors are likely to contribute to the increased number of Indigenous females undergoing vitrectomy. Firstly, multiple studies investigating Indigenous DM prevalence report a greater prevalence of T2DM in Indigenous Australian females compared with males [143]. This may be partly because the rate of gestational DM is higher in Indigenous Australian women compared to the general Australian population (despite a younger age of giving birth), resulting in a greater number of women subsequently developing DM [144,145]. Indigenous Australian women with gestational DM also have a higher cumulative incidence of T2DM after pregnancy than non-Indigenous women, with some studies reporting a four fold greater risk [144,146]. Additionally, the higher median age at death of Indigenous females means that females are living with DM longer than their male counterparts, and are therefore more likely to develop end-stage DR requiring vitrectomy.

The overall number of patients (55.2%) achieving visual success at 12 months in our study, was similar to that reported at 6 months in a South African cohort [147], but lower than the 60-75% reported at 12 months post-operatively in various studies conducted in the UK [56,58,148]. Our study found that 39.7% of patients had a BCVA of ≥6/12 (70 ETDRS letters) 12 months following the first operated eye, which was similar to that reported in the DRIVE-UK study (38%) [58]. Our finding was greater than that reported by Yorston et al. (11%) [56] and that reported by Rice et al. (33%) [147] in their South African cohort. Reports of long term visual outcomes following diabetic vitrectomy indicate that 42% to 75% of patients maintain stable vision 5-10 years after surgery [149]. This figure is promising given the number of good outcomes reported from the current study.

This study is the first to compare visual prognosis following diabetic vitrectomy between Indigenous and non-Indigenous Australians. We did not find a significant difference in number of letters gained, or in the proportion of patients achieving visual success between these ethnicities. Other studies around the world investigating the effect of ethnicity on visual success have also failed to find a difference [58]. Visual success was equal across ethnicities in our study despite the significantly higher rates of comorbidities in the Indigenous group. This supports the findings by Yorston et al. [56] who concluded that age, duration of DM, the presence of systemic risk factors and other diabetic complications were not associated with visual prognosis following diabetic vitrectomy. However, other studies in the literature have reported an association between duration of DM, SBP, ischaemic heart disease and older age at surgery and visual success in their cohorts [58,147]. The only factors associated with visual success in a multivariate regression model in our

cohort was timely laser treatment prior to vitrectomy and better baseline BCVA in the operated eye, the latter of which has also been reported in the literature previously [56].

Our study revealed that 17% of patients were legally blind (≤6/60 vision in the better seeing eye) at 12 months post-operatively and we found no difference in the number of Indigenous and non-Indigenous patients reaching this endpoint. The DRIVE-UK study reported 6.4% of patients reaching legal blindness [58] where as Yorston et al. [56] and Rice et al. [147] reported approximately 25% of patients reaching this endpoint. A total of 8% of operated eyes in our study had NPL vision at 12 months post-operatively. This was greater than other studies conducted in South Africa and the UK who reported 3-4% of eyes with resultant NPL vision [58,147]. We found that the proportion of patients who were NPL in the operated eye at 12 months post-operatively was significantly greater in the Indigenous (28% of patients) compared with the non-Indigenous group (5% of patients) in our study. This may reflect the complexity of surgery in the Indigenous group, which is dependent on the degree of macular traction or detachment, and has been shown to be an important prognostic indicator [56]. The extent of vitreoretinal traction was not assessed in the current study and could not be evaluated as a compounding factor for visual failure. It is also probable that Indigenous patients travelling from remote areas had a greater delay from the time of listing for vitrectomy to time of surgery (although this was also not quantified in our study) and this may have affected visual outcomes. There may have also been significant delay between the onset of visual deterioration and time of presentation for ophthalmic review in those living in remote communities. Rice et al. [147] found an increase in morbidity while patients waited for surgery in their South African cohort and this may have also played a role in our study, leading to an increased number of Indigenous patients with NPL vision in the operated eye.

Looking more closely at patients with visual failure (loss of 15 or more letters from baseline) revealed significant differences in Indigenous and non-Indigenous outcomes. Of the 174 patients for which we had 12-month follow-up data for from the time of their first vitrectomy, the number of blind eyes (counting fingers or worse) decreased from 80 to 38. This means that for every 4 patients undergoing diabetic vitrectomy, blindness will be reversed for 1 patient. The number needed to treat specifically for non-Indigenous patients in our study was 3. These figures are comparable to the South African cohort examined by Rice et al. [147] who reported a 1 in 5 rate of reversing blindness with surgery. Unfortunately, when we examine the Indigenous cohort alone, the results are not as promising. We found 8 Indigenous patients with a blind eye prior to their first vitrectomy and 9 patients with a blind eye following surgery (3 of whom had a blind eye both pre-operatively and post-operatively). This means that more operated eyes are blind following surgery than prior to surgery. The number needed to harm was 29. As mentioned previously, this finding may be related to the complexity of surgery in the cases performed for Indigenous Australians, but this requires further investigation. These analyses suggest that although the number of good outcomes as determined by the rate of visual success are equal between Indigenous and non-Indigenous Australians, the number of poor outcomes are greater in Indigenous patients receiving diabetic vitrectomy.

Indigenous Australians were also found to be more likely to need vitrectomy earlier in the course of DM compared with non-Indigenous Australians once other systemic risk factors were accounted for. This was

also true for patients with T1DM compared with T2DM. Once PDR had been diagnosed, the only two factors found to influence time to diabetic vitrectomy in this study was whether or not patients had PRP laser at least 6 months prior to vitrectomy and Indigenous Australian ethnicity. The completeness of PRP was not evaluated and could have influenced this outcome. Ostri et al. [55] found that full PRP did not significantly influence the need for vitrectomy in T1DM patients with PDR, however they did not assess whether it influences the time to vitrectomy. The validity of these survival analyses also relies on the accuracy of the time variable data collected. Delayed presentation of disease commonly seen in the Indigenous Australian population is likely to have influenced both the documented date of DM onset as well as the documented date of PDR onset.

The major strength of this study is the ability to capture all diabetic vitrectomies performed in SA and NT between 2007 and 2011. The methodology used here addresses many of the challenges normally encountered when conducting population-based studies in Indigenous communities, allowing for an accurate representation of Indigenous and non-Indigenous patients with end-stage DR requiring vitrectomy. This is the first population-based study of diabetic vitrectomies in Australia. However, there are some limitations. Firstly, data was collected retrospectively from patient files. Data relating to patients' DM history, ocular history and co-morbidities were not always complete. In particular, patients transferred from remote areas for surgery were more likely to have missing data in these fields, which may have skewed the results. For example, BCVA follow up data were available from case notes for about 60% of non-Indigenous patients and 38% of Indigenous patients at 12 months post-operatively. This was predominantly due to the fact that the surgeon performing the vitrectomy or location at which the vitrectomy was performed was not always where the patient attended follow-up. This is particularly applicable to the patients travelling from remote areas who attended follow-up in their communities. Furthermore, given that late diagnosis of DM is a significant issue in remote communities, DM duration as calculated from patient case files may have been greater than that recorded.

There were also some factors that have been shown to predict visual outcomes following diabetic vitrectomy in previous studies that we did not take into account in the current analysis. Yorston et al. [56] found that macular involving tractional detachment correlated with visual prognosis. As stated previously, we did not assess anatomical outcomes or extent of macular traction in our study. We also did not record the completeness of pre-operative PRP treatment, or the cataract grading at post-operative visits, which could have been an additional factor affecting the success of vitrectomy and the BCVA at follow-up. Finally, we could not include patients for whom vitrectomy was indicated, but not undertaken due to reasons including patient refusal of surgery, other comorbidities, or patients lost to follow-up. Under diagnosis of the clinical need for vitrectomy in those patients who did not engage in screening and ophthalmic care could further contribute to the comparatively small number of Indigenous Australians reaching the endpoint of end-stage DR requiring vitrectomy calculated from this data, although all areas of the study population are serviced by regular outreach services.

CONCLUSION

This is the first population-based study to capture all patients with end-stage DR undergoing diabetic vitrectomy in SA and NT. Indigenous Australians have a five-fold increased risk of requiring diabetic vitrectomy, reflecting a large burden of disease secondary to end-stage DR in the Indigenous Australian population. Indigenous patients are just as likely to have improved vision following diabetic vitrectomy as non-Indigenous Australians. Indigenous patients with blind eyes (counting fingers or worse) prior to vitrectomy are significantly less likely to improve from surgery but may still have some benefit in stabilising their ocular status over no surgery. This study gives some insight into the characteristics of Indigenous patients undergoing diabetic vitrectomy and highlights the need for focused screening and early intervention in order to reduce the number of young Indigenous patients with T2DM suffering poor visual outcomes following diabetic vitrectomy. The greater prevalence of DM, and earlier age of disease onset in the Indigenous Australian population are thought to contribute to the increased risk of diabetic vitrectomy. The prevalence of CKD and diabetic neuropathy in Indigenous patients with end-stage DR requiring vitrectomy is striking and requires increased awareness and attention. The development of a common care pathway in conjunction with endocrine and renal physicians is recommended for young Indigenous patients with T2DM presenting for DR treatment.

CHAPTER 4

A REAL WORLD CLINICAL TRIAL FOR THE MANAGEMENT OF CATARACT SURGERY-INDUCED DMO IN INDIGENOUS AUSTRALIAN PATIENTS LIVING IN REMOTE COMMUNITIES

Multiple factors can contribute to visual loss for patients with DM. The complications and treatment of PDR and DMO, as well as the premature development of cataracts are well known causes [150]. Furthermore, the development or progression of DMO after cataract surgery is frequently observed, particularly in patients with pre-existing DR or DMO [151,152]. A large retrospective study of 81 984 eyes undergoing cataract surgery in the UK quantified the risk of post-operative DMO to be 4 times greater in patients with DM compared with those without DM [153]. The risk of post-operative DMO was also found to be directly related to the severity of DR [153].

For patients with DM requiring cataract surgery, studies have shown short term benefit of adjuvant therapy with either intravitreal bevacizumab or triamcinolone acetonide administered intraoperatively, in reducing the development of post-operative DMO [154]. This has been shown for both patients with pre-existing DMO, as well as in patients with DR but without DMO [154]. However, ongoing intravitreal injections with these agents is often required post-operatively to keep DMO from re-emerging. In particular, anti-VEGF agents commonly require monthly injections or evaluations at least for the first 6-12 months of treatment. Triamcinolone acetonide is used off-label when injected intravitreally, and has a significant risk of adverse effects that limit its use. These include sterile endophthalmitis characterised by intraocular inflammation (occurs in up to 12% of patients [155]), visual impairment from the drug itself (which is white and opaque) settling on the macula, increased IOP, and the development of cataract in phakic patients (a side effect that is common to all steroid preparations, but not anti-VEGF agents) [156]. Direct application of these treatments is impractical in some populations where compliance with follow-up is a significant issue, and access to ophthalmology services is variable. In Central Australia communication barriers, extreme travel distances and competing priorities result in non-attendance, making visual outcomes in real world clinical situations inferior to those seen in strictly run clinical trials. In addition, potential adverse outcomes are particularly important to consider when administering treatment to patients that may be poorly compliant with follow-up. It is therefore imperative that systems are tailored to the specific needs of this population.

The biodegradable 0.7mg dexamethasone intravitreal implant (Ozurdex, Allergan, Irvine, CA, USA) is a slow release preparation of dexamethasone (a highly potent steroid with short half-life) with intravitreal concentration peaking within 3 months and sustained for up to 6 months post injection [157]. This translates clinically to less frequent injections than conventional treatment with monthly intravitreal bevacizumab, and without the inflammatory adverse effects of triamcinolone acetonide [49,50,158]. While initially FDA approved for macular oedema secondary to retinal vein occlusion and non-infectious posterior uveitis, increasing short-term evidence of its efficacy in the treatment of DMO has resulted in the approval of

dexamethasone intravitreal implant for this indication [158-161]. The BEVORDEX study showed that in a metropolitan setting, with regular and frequent follow up, significantly less frequent administration of dexamethasone intravitreal implant (mean 2.8 injections per year) was required to achieve similar visual and anatomic outcomes as bevacizumab treatment (9.1 injections per year) for patients with DMO [158]. The MEAD study showed that dexamethasone intravitreal implant treatment was able to improve vision following cataract surgery and prevent worsening of DMO that often occurs with cataract surgery [160]. A retrospective report of dexamethasone intravitreal implant used at the time of cataract surgery in patients with DR but no DMO showed that a single dexamethasone intravitreal implant was able to prevent the development of DMO post-operatively in the short-term (at 1 week, 1 month and 3 months post-operatively) [162].

With the advent of a well-tolerated, slow release steroid preparation such as the dexamethasone intravitreal implant, it would be valuable to determine if it is possible to gain adequate control of DMO following cataract surgery with less frequent reviews and treatment than with the use of the currently used anti-VEGF bevacizumab. As a part of this PhD, I have designed, gained ethics approval and implemented a non-inferiority clinical trial for Indigenous patients in Central Australia requiring cataract surgery who have coexistent DMO or DR. At the time of cataract surgery, patients are randomized to receive either adjuvant intravitreal bevacizumab or dexamethasone intravitreal implant. Six and twelve month outcomes will be investigated. The purpose of this study is to compare efficacy of two treatments, proven to be effective for the treatment of DMO in patients already having cataract at a level where surgery is required, in a real world setting, in remote Central Australia.

INTRA-OPERATIVE ADMINISTRATION OF DEXAMETHASONE INTRAVITREAL IMPLANT (OZURDEX) VERSUS INTRAVITREAL BEVACIZUMAB (AVASTIN) DURING CATARACT SURGERY FOR THE MANAGEMENT AND PREVENTION OF DMO IN CENTRAL AUSTRALIA: A REAL WORLD CLINICAL TRIAL.

AIMS

1. To determine if intraoperative administration of dexamethasone intravitreal implant (measured by BCVA and central retinal thickness (CRT) at 6 and 12 months) is not inferior to intraoperative administration of bevacizumab in improving vision and reducing retinal thickness in patients with DMO undergoing cataract surgery.
2. To determine if intraoperative administration of dexamethasone intravitreal implant (measured by BCVA and CRT at 6 and 12 months) is not inferior to intraoperative administration of bevacizumab in preventing post-operative DMO in patients with DR, but no active DMO following cataract surgery.
3. To determine whether intraoperative dexamethasone intravitreal implant is a safe and practical alternative to intraoperative bevacizumab for treatment and prevention of DMO in a Central Australian

population where there are significant barriers to delivering frequent follow up and treatment to patients with DM.

HYPOTHESES

1. Dexamethasone intravitreal implant can be delivered safely to remote Australian populations through existing clinical care pathways.
2. Dexamethasone intravitreal implant is at least as effective in improving vision and CRT compared to intravitreal bevacizumab when administered during cataract surgery in remote Australian populations with DMO.
3. Dexamethasone intravitreal implant is at least as effective in maintaining vision and CRT compared to intravitreal bevacizumab when administered during cataract surgery in remote Australian populations with DR without DMO.
4. In patients with DR or DMO, those who are administered intravitreal injection with dexamethasone intravitreal implant during cataract surgery require less frequent postoperative intervention (laser or intravitreal injections) than those receiving intravitreal bevacizumab during cataract surgery.

SAMPLE POPULATION AND INCLUSION CRITERIA

This is a prospective, non-inferiority trial of 40 diabetic patients with DR and/or DMO, randomized to receive either cataract surgery combined with intravitreal bevacizumab (1.25mg) or cataract surgery combined with dexamethasone intravitreal implant (0.7mg).

Inclusion criteria:

- Adult patients treated by the Central Australian & Barkly Integrated Eye Health Service, who fit either of the following two treatment groups:
  A. Patients with active DMO (defined as macular involving DR, with retinal thickening as assessed on clinical examination), or
  B. Patients with DR involving the macular (maculopathy), without active DMO
- Participants with visually significant lens opacity (more than grade 3 for any type of cataract associated with symptoms attributable to cataract) and scheduled to undergo cataract surgery at the time of enrolment into the study.
- Participants with reduced vision (BCVA impaired to at least the level of 75 ETDRS letters) in the eye included for the study.

Exclusion criteria:

- Patients who have undergone intervention for DR within the preceding 3 months;
- Patients with a history of open-angle glaucoma or steroid induced IOP elevation that required IOP-lowering treatment, or, IOP ≥25; and/or
- Patients with concurrent ocular pathology other than cataract or DMO causing visual loss.

Where both eyes were potentially suitable for inclusion, the worse eye at presentation (as determined by BCVA) will be included for the study. Patients with only one functioning eye are not specifically excluded if the remaining criteria are satisfied, as both anti-VEGF and dexamethasone intravitreal implants have been proven effective for DMO.

Sample size

Previous studies have shown that intravitreal bevacizumab given to patients with DR at the time of cataract surgery results in a mean improvement of 25 ETDRS letters at 3 months post operatively, which is significantly greater than the average increase of 17 ETDRS letters in those receiving no adjuvant treatment at the time of cataract surgery (control group) [163]. The proposed study aims to recruit 40 participants (20 participants in each group). This sample size allows for 80% power to detect a mean BCVA in the dexamethasone intravitreal implant group that is non-inferior to the mean BCVA in the bevacizumab treated group (assuming a non-inferiority limit of 15 EDTRS letters, and a standard deviation of 15 ETDRS letters) (online power calculator: https://www.sealedenvelope.com/power/continuous-noninferior) [164].

STUDY PROTOCOL

Participants meeting inclusion criteria are randomised to treatment groups at the time of initial assessment via numbered concealed envelopes. Envelopes containing the treatment allocation have been randomly numbered by a statistician not involved as an investigator in the study. Participants receive intravitreal injection of either 1.25mg bevacizumab (20 participants) or 0.7mg dexamethasone implant (20 participants) at the time of cataract surgery according to their group allocation. The treating ophthalmologist is not blinded to the treatment allocation due to the limitations in staff in remote clinic locations and the fact that the treating surgeon is required to administer the drug, and the dexamethasone implant is visible after administration. **Figure 4.1** illustrates the study protocol.

Patients will be followed via outpatient clinic review and further monitoring and treatment administered on an ‘as required’ basis. BCVA, CRT and IOP will be measured at each clinic visit, by clinic staff blinded to the treatment allocation. Administration of intravitreal injections and/or laser treatment post-operatively will be at the discretion of the treating ophthalmologist, based on the presence of DMO on clinical examination affecting BCVA. Retreatment with intravitreal agents will be on an as required basis and dependent on the group allocation: minimum time between post-operative bevacizumab is 1 month; minimum time between post-operative dexamethasone intravitreal implant is 4 months (based on the BEVORDEX study retreatment criteria [158]). Macular laser and PRP have been shown to be effective treatments and will be performed if indicated in normal clinical practice. IOP will be measured at each follow-up visit and treated as clinically indicated.

The aim is for monthly follow-up, but at least 6 monthly clinical assessments for data collection will be performed. Infrequent clinic attendance (due to cultural business, travel difficulties and other causes) has previously been high and will likely limit plans for a more rigorous treatment regime and follow up.

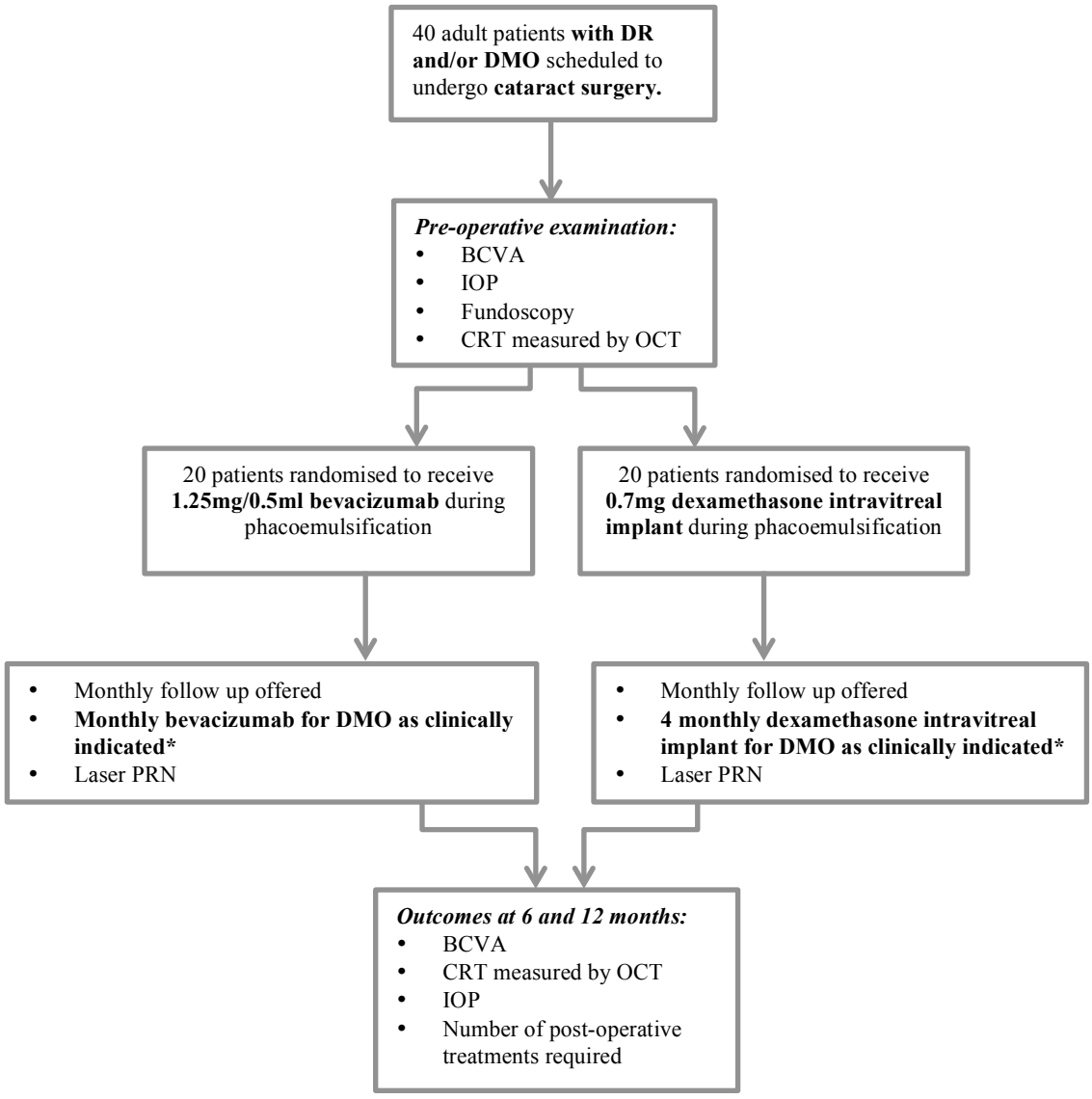


Figure 4.1: Study protocol

*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.

OUTCOME MEASURES

The primary outcome measure for success of treatment is the mean change in BCVA. BCVA will be measured for all study participants, at each clinic visit. Secondary outcome measures include: (1) change in CRT as measured by OCT (will be performed bi-annually for patients attending clinic at Alice Springs Hospital only, as OCT is unavailable in community clinics. All patients will undergo a baseline OCT pre-operatively (when the level of cataract allows); (2) number of intravitreal injections required post-operatively; (3) number of laser treatments required; (4) adverse events. Outcomes will be reported at 6 months, and 12 months.

IMPLEMENTATION

The design of this clinical trial was initiated in 2014 when data supporting the efficacy of dexamethasone intravitreal implant for DMO was emerging. Prior to this, I had participated in outreach ophthalmology

clinics servicing the Anangu Pitjantjatjara Yankunytjatjara lands in remote SA. Ophthalmologists Prof. Richard Mills and Dr. Stewart Lake from Flinders University are amongst the few Ophthalmologists who donate their time to provide twice yearly ophthalmology clinics in this region and other regions in remote SA. Dr. Tim Henderson is the only permanent ophthalmologist in Alice Springs and services remote areas in Central Australia. All patients from remote SA and Central Australia who require cataract surgery are referred to the Ophthalmology Departments at either Alice Springs Hospital or FMC.

The idea of performing a real world clinical trial dedicated to the treatment and prevention of cataract surgery-induced DMO in those living in remote Central Australia was proposed, and I drafted a research plan. A working group was established including Dr. Stewart Lake, Dr. Tim Henderson, Prof. Jamie Craig, Prof. Richard Mills and myself. This allowed the design of a study that is directly applicable and beneficial to the population given the services available for treatment and follow-up. In addition, cultural integrity and mutual respect for patients and their beliefs was considered an important factor in the success of this study and incorporated into the research plan. Enrolment packs (including consent forms, patient information sheets, study protocol and data collection forms) that I designed were approved by the working group.

Two ethics committees were involved in approving this study. I initially applied for ethics approval through the Central Australian HREC so that any concerns directly related to Indigenous Australians could be reviewed and addressed. This included the development of specialised consent forms and patient information sheets. Secondary ethics was gained from the Southern Adelaide Clinical HREC given that the investigators were associated with Flinders University. I registered the study as a clinical trial with the Australian New Zealand Clinical Trials Registry and the World Health Organisation International Clinical Trials Registry Platform (Universal Trial Number: U1111-1166-2630). The proprietors of dexamethasone intravitreal implant (Ozurdex, Allergan, Irvine, CA, USA) were approached regarding funding for this study. The study proposal was approved and Allergan (Irvine, CA, USA) signed an agreement to supply the experimental treatment (dexamethasone intravitreal implant, Ozurdex) free of charge for this study. Bevacizumab is FDA approved for DMO and readily available for use in Central Australia. A timeline of events relating to study implementation is presented in **Figure 4.2.**

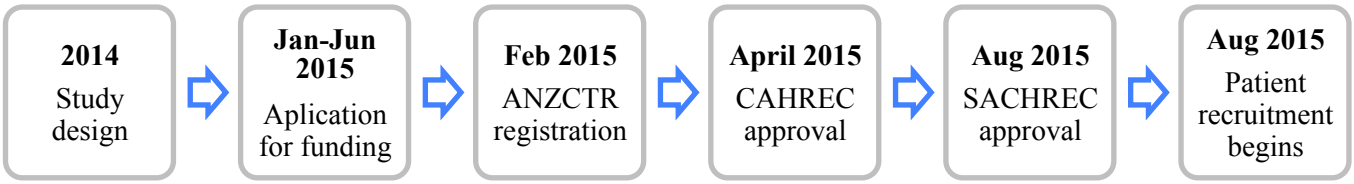


Figure 4.2: Timeline of events

Abbreviations: ANZCTR, Australian New Zealand Clinical Trials Registry; CAHREC, Central Australian Human Research Ethics Committee; SACHREC, Southern Adelaide Clinical Human Research Ethics Committee.

The study was finalised and approved to begin recruitment in August 2015. Recruitment is currently ongoing. Participants have been recruited during both their regular ophthalmology clinic visits in Alice Springs and during "Cataract week". "Cataract week" is an intensive surgical treatment week offering

cataract surgery to patients from Central Australia who have previously been identified, consented and listed for surgery. Ophthalmology fellows funded by the Fred Hollows Foundation assist in order to perform a greater number of operations than would otherwise be possible with a single Ophthalmologist on site. Patients attending Alice Springs Hospital for "Cataract week" are screened for eligibility for the current study. Those who are eligible for enrolment are approached and offered participation in the study. Recruitment to date has been challenging secondary to difficulties in scheduling patients for surgery (poor patient attendance on the day scheduled for surgery) and loss to follow up. Comorbid conditions including ESRF requiring dialysis, makes scheduling inherently more difficult.

Expansion of this study to other remote areas of Australia, including remote Western Australia may be considered if recruitment continues to be a significant issue in future. In addition, patients from remote SA who are referred to FMC in Adelaide (rather than Alice Springs Hospital) for cataract surgery could also be included if ethics approval is expanded to include this site. These strategies are currently being considered by the working group.

Preliminary data

Fifteen participants have been recruited to date with 6 participants generating data at or beyond 6 months post-operatively. Vision at or beyond 6 months was greater than 75 ETDRS letters for all 6 patients. Those randomized to dexamethasone intravitreal implant have had fewer re-treatments (mean of 1.3 retreatments) compared with those randomized to bevacizumab (mean of 2 retreatments) at 6 months post-operatively. No rise in IOP over 10mmHg from baseline has been observed in either group. This preliminary data suggests that dexamethasone intravitreal implants have so far been safe to use in this population. A trend towards a reduced number of post-operative treatments is also emerging in favor of dexamethasone intravitreal implant, however no conclusions regarding treatment effects can be made with so few participants included in this preliminary analysis.

DISCUSSION

As described in chapter 1, due to extreme rates of T2DM, Indigenous Australians account for 16% of all Australians with vision threatening DR despite only making up 2.5% of the Australian population [101]. DMO accounts for a significant percentage of the vision loss associated with DR in this population. "The Population-based study of end-stage diabetic retinopathy requiring vitreoretinal surgery in SA and the NT" described in Chapter 3 indicates that surgery performed for end-stage DR in SA and NT is occurring frequently for Indigenous Australians. One of the significant factors driving this high rate of vitrectomy is the difficulty in providing timely and frequent treatment in the early stages of the disease in remote community settings. This prospective, randomised, non-inferiority trial comparing dexamethasone intravitreal implants with intravitreal bevacizumab at the time of cataract surgery was designed with an understanding of the geographical and cultural obstacles often experienced in Central Australia, in order to optimise management of DMO following cataract surgery in patients with DM living in remote Australia.

Safety

The main safety concerns associated with use of intravitreal steroids, including dexamethasone, is the progression of cataracts and the development of ocular hypertension (measured as an increased IOP), which can lead to open-angle glaucoma. Given that this study involves administration of dexamethasone intravitreal implant during cataract surgery, the risk of cataract progression is eliminated. Long-term results from dexamethasone intravitreal implant trials indicate that changes in IOP secondary to dexamethasone are transient and predictable. Boyer et al. showed that the mean IOP peaked at a similar level and returned to baseline levels by 6 months after each dexamethasone intravitreal implant injection [160]. Furthermore, the incidence of increased IOP did not increase after subsequent treatments over 3 years. These results indicate that there was no cumulative effect of dexamethasone intravitreal implant on IOP [160].

Studies comparing the safety of intravitreal steroid agents have associated the dexamethasone intravitreal implant with a lesser incidence of IOP increase compared with other agents including triamcinolone acetonide [165]. Differences in the pharmacologic and pharmacokinetic profiles are thought to account for these differences. In particular, dexamethasone and triamcinolone acetonide have been shown to activate different patterns of gene expression in human trabecular meshwork cell lines [166]. Risk factors associated with ocular hypertension following intravitreal steroids include phakic individuals, pre-existing glaucoma, baseline IOP>15mmHg, higher steroid dose, larger volume of injection, uveitis and previous steroid response [165].

Interestingly, the normative range of IOP is low in the Central Australian Indigenous population, with mean IOP 12.8mmHg, compared with 17.1mmHg for Caucasian populations, 14.5mmHg for Asian populations, and 18.7mmHg for Black African populations [167]. Furthermore, the prevalence of glaucoma varies between racial groups. Studies have found a prevalence of glaucoma among Indigenous Australians living in Central Australia of 0.4-0.5% [168,169]. This is one-third of the prevalence found among non-Indigenous Australians [170]. The lower incidence of glaucoma in Indigenous Australians seems to occur despite high rates of risk factors normally associated with glaucoma, including pseudoexfoliation syndrome. Among non-Indigenous Australians, 14.2% of those with pseudoexfoliation syndrome may also have glaucoma compared with 1.7% of those without [170]. However, Landers et al. found no participants with glaucoma or with raised IOP among Indigenous Australians of Central Australia with pseudoexfoliation syndrome in the CAOHS [167]. This appears to be a unique feature of the Indigenous Australian population.

Genetic or environmental factors specific to Indigenous Australians may be protective against the development of glaucoma and in particular increased IOP in this population. This may be due to anatomical and physiological differences in the trabecular outflow tract between Indigenous and non-Indigenous Australians. Furthermore, the risk of IOP increase with dexamethasone intravitreal implant is less than that with commonly used intravitreal steroids such as triamcinolone acetonide, IOP changes are transient and there is no cumulative effect with recurrent treatments [160]. For these reasons we believe that dexamethasone intravitreal implant is likely to be a safe treatment to use in Indigenous Australians of Central

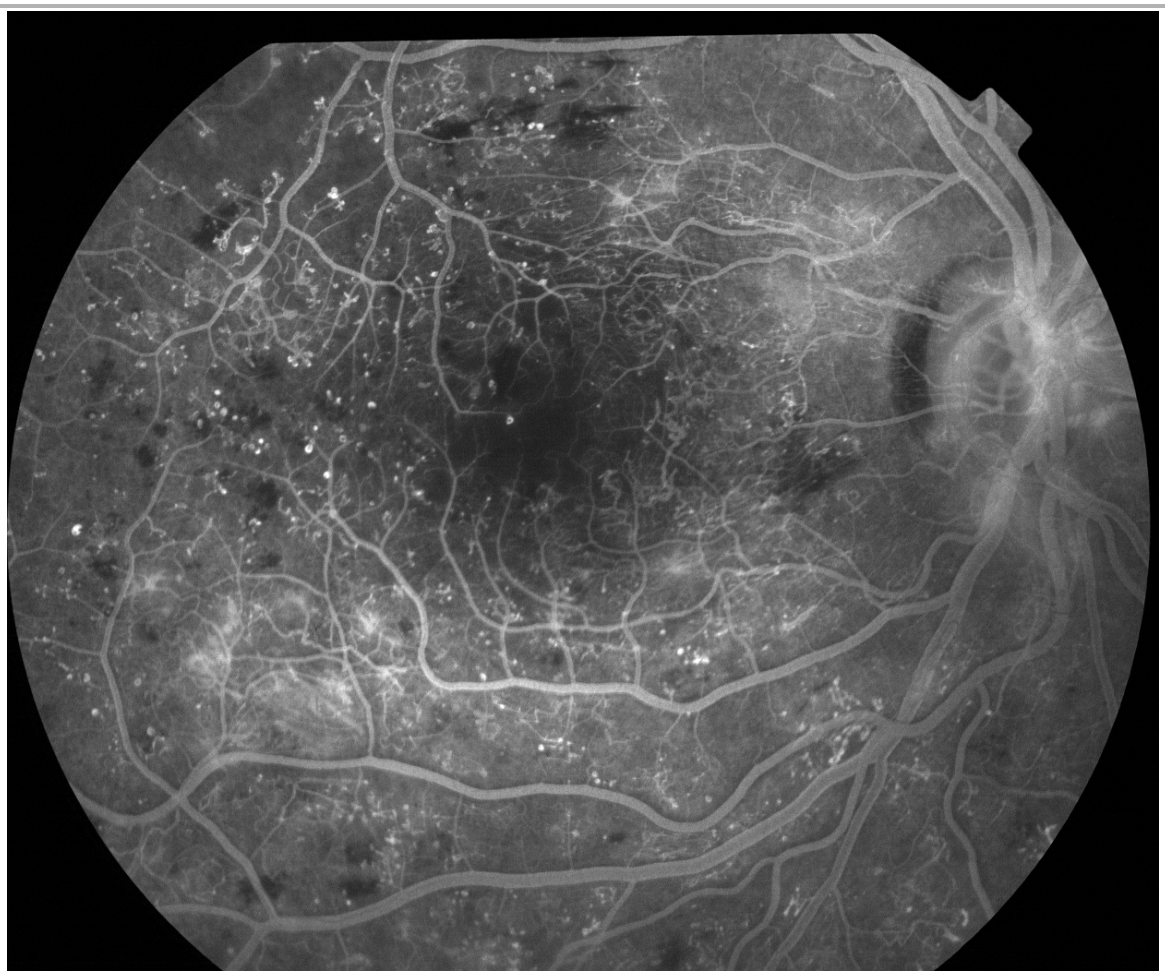
Australia, and follow-up regimes are adequate to detect and treat potential adverse events associated with dexamethasone intravitreal implant including raised IOP. Furthermore, the risk of treatment is outweighed by the greater risks of not receiving treatment and any treatment regime that allows at least equal results with less frequent review and re-treatment is a valuable improvement in care both for patients and in relation to cost.

Significance

This clinical trial has immediate relevance to clinical practice in Central Australia, and may challenge or reinforce current practice. Long-term benefits anticipated include appropriate distribution of resources and allocation of funding, which will have an impact on the screening and treatment offered in Indigenous communities in remote SA and NT and throughout Australia. Ultimately, a better treatment regime may support local resourcing to bring care nearer to home. Furthermore, we expect the results from this study to be generalizable to other resource poor settings with increasing levels of DM, where infrequent attendance and difficult management of DR is also a significant issue.

CONCLUSIONS

Any intervention that can prevent progression of DMO following cataract surgery, with one third of the treatments than currently used bevacizumab [158] may help to reduce the discrepancy in health outcomes between Indigenous and non-Indigenous Australians. If a practical regimen is found to work at least as well as the existing combination of laser and bevacizumab injections, then it will have significant implications for managing DMO in remote Australia. Preliminary data so far has not revealed any safety concerns related to either treatment, and supports the completion of this study. The results from this real world clinical trial will help us work towards reducing numbers of Indigenous Australian patients living in remote Central Australia losing vision from DM.



PART II

MOLECULAR RISK FACTORS FOR DIABETIC MACULAR OEDEMA

CHAPTER 5

INTRODUCTION AND AIMS

THE GENETIC STUDY OF DIABETIC COMPLICATIONS (GSDC): PREVIOUS FINDINGS

The GSDC established in 2006, is a repository of DNA samples from a well characterised group of patients with DM. Participants recruited for this study are a current representation of Australian patients with T1DM and T2DM from an era in which the importance of glycaemic control and systemic vascular risk factors have been well understood. The target population includes individuals with DM affected by potentially sight-threatening complications of DM, as well as individuals who have DM without DR. The eventual outcome of this study is to investigate the influence of genetics on the development and severity of DR, specifically using GWAS methodology to identify candidate SNPs. An understanding of the molecular pathogenesis of DR has significant implications for screening strategies and the development of novel therapies.

Genome wide genotyping has been performed for the first 1000 cases (with STDR) and controls (with no DR or minimal NPDR) recruited into this study. This phase 1 GWAS identified a SNP (rs9896052) on chromosome 17 associated with STDR in the discovery cohort as well as in 3 independent replication cohorts [171]. This is the first published GWAS to show consistent association at this locus across both T1DM and T2DM, and in different ethnic groups. *GRB2* is a gene in the region of this SNP, and it's involvement in insulin, VEGF and EPO signalling makes it a promising candidate for DR [172,173]. Further functional work performed by our group showed expression of GRB2 in the human retina, and demonstrated that GRB2 expression is upregulated in the retina of mice following retinal stress resulting in proliferative retinopathy [171]. These genotyped samples have also contributed significantly to a meta-analysis of GWAS studies investigating association with "Any DR", conducted by an international consortium (analysis in progress).

A candidate gene study approach has been successfully applied to investigate specific genes thought to play a role in the pathogenesis of DR. *VEGFA* SNPs have been significantly associated with STDR in patients with T2DM and with DMO in a combined group of both T1DM and T2DM patients [174]. This was the first study to show that genetic variation within the *VEGFA* gene is associated with DMO. Abhary et al. [175] also found that DR was significantly associated with SNPs in the *EPO* gene.

The success of this study so far has been partly attributable to the fact that patients have been predominately recruited through ophthalmology clinics, allowing for accurate characterization of their DR phenotype, and covering the whole spectrum of STDR sub-phenotypes. We hypothesise that different genes are involved in the pathogenesis of each component of the DR phenotype and that these phenotypes in T1DM and T2DM may have slightly different pathoaetiologies. The main limitation of this sample is that it lacks statistical power to produce significant results on analysis of sub-types of DM (in particular T1DM) and sub-phenotypes of STDR (including DMO and PDR) individually, for both GWAS and for candidate gene analysis.

Developing a new recruitment strategy for severe cases of DR

Suboptimal power remains problematic in the genetic literature relating to DR, and as a result, genome-wide significance has not been reached in any published study, nor has there been any consistency in genes identified between studies [90,93-96]. Studies so far have typically included samples from the entire range of DR severity, with case-control definitions comparing no DR with any DR, or any DR with severe DR. Given that the DR variants under investigation are likely to have small to moderate effect sizes, large sample sizes are required in order to reach statistical significance when using population sampling.

There is good recent evidence to suggest that focusing on recruitment and analysis of phenotypic extremes results in systematically larger effect sizes than the overall population effect size [176]. This strategy has specifically been used in the discovery of rare variants, and is postulated to reduce the required sample size by as much as four-fold compared with sampling from the general population [176]. The case-control definitions adopted for the current study differ from GWAS and candidate gene studies reported in the literature by involving analysis of phenotypic extremes (comparing those with STDR with those with no DR or minimal NPDR).

In 2013, and as part of this PhD, the Registry of Advanced Diabetic Retinopathy (RADAR; projectradar.com.au) was developed, to extend our existing study, GSDC. RADAR is based on the successful Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG), which recruits patients with advanced glaucoma from all over Australia, and New Zealand using the postal system for sample collection. The aim of this recruitment strategy was to develop a novel and efficient Australasian disease registry approach for recruitment of highly selected advanced DR cases. This strategy focuses on those patients who have undergone intervention (including laser, intravitreal injections, or surgery) for the treatment of STDR. Through this approach, participants are able to have DNA and ribonucleic acid (RNA) collected remotely, and without placing a large burden on the ophthalmologist or the clinic. RADAR was developed to increase power of the current study, particularly in the analysis of STDR sub-phenotypes.

Choice of candidate genes: Angiogenic and inflammatory hypotheses

The success of anti-VEGF agents in the treatment of PDR and DMO supports the contribution of VEGF-A in the pathogenesis of DR [44,177]. However, given that anti-VEGF treatment is not associated with total regression of retinal neovascularisation in patients with PDR, or total resolution of DMO, it has been proposed that other inflammatory molecules involved in BRB breakdown are also implicated [178]. In fact, patients with DMO who are resistant to treatment with anti-VEGF agents often show a favourable response to intravitreal steroid injections [179].

Sohn et al. [180] report a study in which they successfully treated patients with bilateral DMO with intravitreal anti-VEGF in one eye and intravitreal steroid in the other. They showed that after treatment with intravitreal steroids, aqueous levels of inflammatory cytokines and growth factors (including IL-6, interferon gamma-induced protein 10 (or IP-10), monocyte chemoattractant protein-1, platelet derived growth factor-

AA and VEGF-A) that are increased in those with DMO compared to controls, reduced significantly. Treatment with intravitreal anti-VEGF resulted in a greater reduction in VEGF-A than steroid treatment, but had minimal effect on aqueous levels of other inflammatory cytokines. This finding correlates clinical treatment response with local biochemical changes that support a combined pathway linking angiogenesis and inflammation in the pathogenesis of DMO. Fogli et al. [181] illustrate the interaction between these mechanisms and propose that VEGF-A may in fact be the common link between pro-angiogenic and pro-inflammatory pathways (**Figure 5.1**). The candidate genes explored as part of this PhD focus on mediators involved in these 2 key pathways.

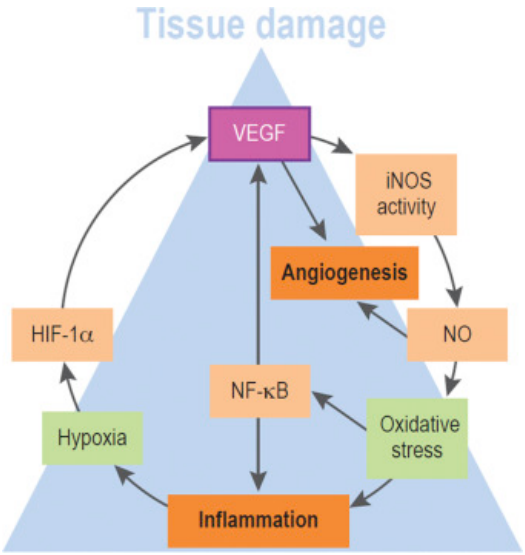


Figure 5.1: Combined mechanisms of angiogenesis and inflammation. The figure represents our viewpoint of how angiogenesis and inflammation may be part of the same network of events ultimately leading to diabetic tissue damage.

Abbreviations: VEGF, vascular endothelial growth factor; HIF-1a, hypoxia inducible factor-1a; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; NO, nitric oxide. Figure reproduced from Fogli et al. [181].

The complex integration between genetic susceptibility and environmental risk factors in the development of diabetic microvascular complications requires further investigation with large sample sizes and clear a priori case and control definitions in order to obtain meaningful results. Specific analysis of DMO is warranted given the lack of current understanding of the genetic risk factors involved, and the associated visual morbidity of this type of DR. We expect results of the current study to be sufficiently powered for detection of susceptibility loci for STDR and its sub-phenotypes PDR and DMO in T1DM and T2DM groups individually. The potential outcome of this study is the identification of candidate genes involved in the development and progression of PDR and DMO, to assist in the development of novel management strategies targeting this blinding disease of increasing prevalence.

AIMS OF THIS WORK:

To further investigate genetic risk factors for the development of STDR and in particular DMO by:

1. Examining SNPs of pro-inflammatory mediators upregulated in the retina secondary to hyperglycaemia induced oxidative stress.
2. Exploring genetic variation within the *VEGFA* genes, and in genes encoding factors upstream and downstream of VEGF-A in the hypoxia and angiogenesis pathway.
3. Determining whether implicated SNPs in the *VEGFA* gene have a functional effect in changing levels of serum protein expression.

CHAPTER 6

METHODOLOGY AND BASELINE CHARACTERISTICS

The original work presented in this chapter has been published in the peer-reviewed literature: G Kaidonis, S Abhary, M Daniell, MC Gillies, R Fogarty, N Petrovsky, A Jenkins, RW Essex, JH Chang, B Pal, AW Hewitt, KP Burdon, JE Craig. Genetic study of diabetic retinopathy: recruitment methodology and analysis of baseline characteristics. ***Clinical and Experimental Ophthalmology. 2014 Jul;42(5):486-93****.* [182] *Dr Kaidonis' contributions include patient recruitment, database and data collection design, data analysis and interpretation and manuscript preparation. This manuscript describes the baseline characteristics with patients recruited as a part of GSDC only. The equivalent analysis of baseline characteristics is presented in this chapter, updated to reflect the whole cohort of participants from GSDC and RADAR included for candidate gene study analysis in this thesis.*

RECRUITMENT METHODOLOGY

THE GENETIC STUDY OF DIABETIC COMPLICATIONS

The following centres were involved in patient recruitment for this study, with study approval by their respective HREC: Flinders Medical Centre (Southern Adelaide Clinical HREC), The Repatriation General Hospital (Southern Adelaide Clinical HREC), The Royal Adelaide Hospital (Royal Adelaide Hospital HREC), The Queen Elizabeth Hospital (The Queen Elizabeth Hospital HREC), The Royal Melbourne Hospital (Royal Melbourne Hospital HREC), Royal Victorian Eye and Ear Hospital (Royal Victorian Eye and Ear Hospital HREC), St Vincent's Hospital, Melbourne (St Vincent's Hospital HREC), Sydney Eye Hospital (South Eastern Sydney Illawarra HREC), and Canberra Hospital (Australian Capitol Territory Health HREC) in Australia, and The National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital National Health Service (NHS) Foundation Trust and University College London (UCL) Institute of Ophthalmology, London, UK (The NHS Health Research Authority in London).

In 2007 recruitment of participants began from three tertiary hospitals in metropolitan Adelaide, South Australia: FMC, RAH and the Queen Elizabeth Hospital. The project was expanded to the Royal Melbourne Hospital (Melbourne, Victoria) in 2009, the Sydney Eye Hospital (Sydney, New South Wales) in 2010, and the Repatriation General Hospital (Adelaide, South Australia) and The Canberra Hospital (Canberra, Australian Capital Territory) in 2011. Moorfields Eye Hospital (National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, London, United Kingdom) has become the first international recruitment centre for this study.

Ophthalmology, endocrinology and renal clinics of these hospitals were used to identify and recruit participants with DM meeting eligibility criteria. A combination of sequential recruitment, and opportunistic recruitment with a bias towards patients with more severe complications of DM occurred. Written, informed

consent was obtained from all participants following explanation of the nature and possible consequences of the study. The project conformed to the tenets of the Declaration of Helsinki.

Eligibility criteria

Eligibility criteria for this study required that participants were at least 18 years of age and had either T1DM of any duration, or T2DM of at least 5 years duration. All participants were taking oral hypoglycaemic medication, or were on insulin therapy, or both. Individuals with diet-controlled DM were excluded.

Case-control definitions

Clinical ophthalmic examination was performed for each participant. Participants were categorized according to their worst-ever DR grading using a modified ETDRS criteria grading system with the following stages: no DR, minimal NPDR, mild NPDR, moderate NPDR, severe NPDR, PDR and/or DMO [15,16]. **Figure 6.1** illustrates the two case-control definitions used in this thesis (STDR versus controls; and Any DR versus No DR). "Any DR" was defined as the presence of at least mild NPDR, PDR, or DMO in at least one eye. "STDR" described those with severe NPDR, PDR, or DMO in at least one eye. "Controls" were defined as those whose retinopathy grading has never been worse than minimal NPDR, with no history of DMO in either eye. A subset of the controls with no history of DR or DMO were labelled "no DR."

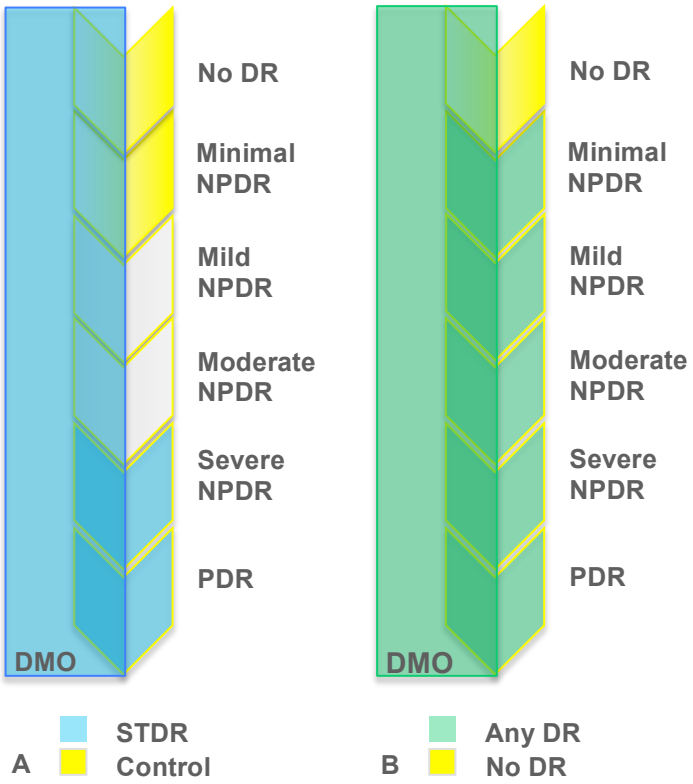


Figure 6.1: Case-control definitions. A. STDR versus Control. B. Any DR versus No DR.

Abbreviations: DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; STDR, sight-threatening diabetic retinopathy.

Clinical data collection

Full ophthalmic examination was performed according to existing site-specific clinical practice. Visual acuity was assessed with either Snellen or logMAR charts, and recorded in the database as Snellen equivalent. Examination of the anterior segment included specific assessment of any lens opacity, and also the presence or absence of rubeosis. Intraocular pressure was measured with either a Goldmann tonometer, or an iCare tonometer (iCare Finland Oy, Revenio Group Corporation, Helsinki, Finland). Dilated retinal examination was performed with slit lamp biomicroscopy and retinopathy status was clinically graded.

Participants with active disease were documented with colour photography, fundus fluorescein angiography, and OCT of the macula as clinically indicated at the discretion of the treating clinician, but the clinical grading as determined at the time of slit lamp biomicroscopy was used for classification. Ophthalmologists were contacted to obtain ophthalmic examination data of recruited participants who attended private ophthalmology clinics.

A detailed questionnaire was administered to collect relevant information regarding social, demographic and medical history. Information collected included sex, age, ethnicity and lifestyle factors (cigarette smoking and alcohol history). Medical history data included DM type and duration, family history of DM, coexisting risk factors (systemic hypertension, hypercholesterolemia), history of vascular disease (ischaemic heart disease, cerebrovascular accident or transient ischaemic attack, peripheral vascular disease), and history of diabetic complications (peripheral neuropathy, DN and details of renal transplant or dialysis). Ophthalmic history was completed by the treating ophthalmologist and included details of past cataract surgery, age-related macular degeneration, glaucoma, retinal detachment, vitreous haemorrhage, rubeosis/iris neovascularisation and past treatment for DR (focal laser, macular grid laser, PRP, intravitreal injections and vitrectomy). The year of development of STDR was also documented.

Height, weight and blood pressure measurements were taken at the time of recruitment. Individuals were classified as having hypertension if they were on pharmacologic treatment for hypertension, or they had a systolic or diastolic blood pressure reading greater than or equal to 140 mmHg or 90 mm Hg, respectively. Body mass index (BMI) was calculated and recorded.

Results of the participants' previous blood tests were accessed from their medical record where available. The most recent serum lipid profile results (total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides) were recorded. Hypercholesterolemia was defined as total cholesterol greater than or equal to 5.5 mM, or current use of lipid-lowering medication. Renal function tests (serum creatinine, urine albumin and albumin–creatinine ratio) were also obtained. The most recent renal function results available were used for analyses except for those who were on dialysis or who had received a renal transplant, in which case results immediately prior to the start of dialysis or transplantation were used. Nephropathy was defined as those who were on dialysis, or who had received a renal transplant for DN, or those with a 24-hour urine albumin of at least 30 mg/d or eGFR < 60 mls/min. The mean of three HbA1c levels was used for each participant. The three most recent values immediately prior to recruitment were

used, except in cases of STDR where three HbA1c values in the year of onset of STDR development were taken.

Sample collection

A venous blood sample was obtained from each participant by a trained venipuncturist. Two 9 ml Vacuette EDTA tubes of venous blood were collected and stored at 4°C. DNA was extracted from whole blood using QiaAmp Blood Maxi Kits (Qiagen, Doncaster, Victoria, Australia) and stored at −20°C for future analysis. An 8 ml Vacuette serum tube was collected from a subset of approximately half the participants when the proximity of the recruitment site facilitated the timely processing of the sample. Blood was centrifuged at 2700 g for 10 minutes within 2 hours of collection. Serum was aliquoted into 1 ml microfuge tubes and stored at −80°C for future analysis. Three millilitres of blood was collected from each participant using Tempus (Life Technologies) blood RNA tubes. Tubes were kept at room temperature for 4 days following collection and then stored at -80°C for future RNA extraction and analysis.

Data storage

All data recorded onto the data collection form were entered into a password-protected Microsoft Office Access 2003 version 5.1 for Windows XP professional (Microsoft Corporation, Redmond, WA, USA) electronic database.

THE REGISTRY OF ADVANCED DIABETIC RETINOPATHY

This study was approved by the Southern Adelaide HREC. Adult patients with DM currently undergoing or who have previously received treatment (including surgery, laser or intravitreal injections) for DR were eligible for RADAR.

In 2013, an iPhone program ("App") was developed specifically for the referral of patients by ophthalmologists. The content and layout of the app were established as a part of this PhD and used to guide app developers who built the app. The RADAR App is a unique referral method that enables the referring clinician to provide RADAR staff with information related to the patient's DR grading, past ocular treatment and visual morbidity as well the patient's verbal consent to be contacted by registry staff. This is an efficient point of care referral system that can be completed by the referring clinician within 2 minutes and provides registry staff with instantaneous patient data, required for the next stage of recruitment.

Clinical data and sample collection

Eligible participants referred by the RADAR App were contacted by the RADAR coordinator, who explained the study and guided the patient through the recruitment process. A pack was sent to the participant containing written information, a consent form, a patient questionnaire and the exact blood tubes required for sample collection. The patient questionnaire collects the same data as that of GSDC (described above) to allow cases from RADAR to be compared with controls from GSDC. In addition, information

regarding the patient's visual morbidity (eg. the impact of DR associated blindness on their ability to drive) was also collected as part of the questionnaire.

The participant's general practitioner or local pathology service provider collected a venous blood sample (2 9ml Vacuette EDTA tubes). The pack (including blood tubes, completed questionnaire and signed consent form) was mailed back to the RADAR coordinator using the provided plastic canister.

Data storage

All data received from the App referral or recorded onto the patient questionnaire were entered into a password-protected Microsoft Office Access 2003 version 5.1 for Windows XP professional (Microsoft Corporation, Redmond, WA, USA) electronic database. The RADAR database was electronically linked to the GSDC database so that participants from each study could be analysed as one common cohort of cases and controls.

CANDIDATE GENES: SNP SELECTION AND GENOTYPING

Candidate genes were selected based on factors involved in angiogenic and inflammatory pathways thought to play a role in the pathogenesis of DR. Further justification of the specific genes chosen is included in sections reporting candidate gene study results.

Tag SNPs

Utah residents with ancestry from northern and western Europe (CEU) samples genotyped as part of the International HapMap Project [183] were used as the basis for linkage disequilibrium patterns. Tag SNPs across the candidate gene of interest, including the promoter region, were selected using the tagger program in Haploview 4.2 [184]. SNPs with a minor allele frequency (MAF) >5% in HapMap were considered.

Genotyping

Tag SNPs were genotyped using iPLEX Gold chemistry on an autoflex mass spectrometer (Sequenom, San Diego, CA) at Geneworks (Adelaide, Australia). Assay designs, optimization and testing were out-sourced to this facility.

MEASUREMENT OF SERUM PROTEIN CONCENTRATION

Serum samples from 180 participants with T1DM (80 with STDR and 100 controls) were tested for VEGF-A levels by quantitative sandwich enzyme-linked immunosorbent assay (ELISA), using R&D DuoSet^{TM} ELISA kit for human VEGF-A according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). All patients included had been genotyped for *VEGFA* tag SNPs as part of the candidate gene study. Each patient sample was tested in duplicate.

Serum concentration of VEGF-A (presented in pg/ml) was calculated using MyAssays (http://www.myassays.com), an online analysis software. A Four Parameter Logistic fit was used to calculate

serum concentrations from the mean of each sample in accordance with the R&D kit instructions. Samples outside the range of the standards or the fit were excluded. The coefficient of variation (CV), SD and standard error were calculated for each replicated sample using MyAssays. Samples with a CV >20% were excluded. Mann-Whitney U test using SPSS version 20.0 for Mac OS X (IBM SPSS Statistics 20.0, SPSS Inc., Armonk, NY, USA) was performed to compare serum VEGF-A levels between cases and controls for patients with T1DM. PLINK (version 1.06) was used to test for association of *VEGFA* SNPs and haplotypes with serum levels of VEGF-A. Multivariate analysis, adjusting for age, sex, duration of DM, HbA1c, hypertension, DN and presence of STDR, PDR or DMO were performed with linear regression analysis.

STATISTICAL ANALYSIS

Basic descriptive statistical analysis was carried out using SPSS version 20.0 for Mac OS X (IBM SPSS Statistics 20.0, SPSS Inc., Armonk, NY, USA). Case and control groups were compared for differences in baseline demographics and clinical variables using a Mann–Whitney *U*-test and a chi-squared test, for continuous and dichotomous variables respectively. $P < 0.05$ was considered statistically significant.

Power calculations were made with the online program Genetic Power Calculator [185]. SNP association studies were designed to have at least 80% power to detect a minimal odds ratio (OR) of 1.3, based on an allelic model, and assuming a disease prevalence of STDR of 7% amongst individuals with DM. Where alternative models are investigated, power calculations have been presented in the corresponding sections relating to these analyses.

SNPs were tested for Hardy-Weinberg equilibrium (HWE) in our cohort using the chi-square test. PLINK (version 1.06) was used to test for association of SNPs with cases versus controls. Univariate association based on an allelic model was performed with the chi-square test. Where alternative models are presented, the chi-square test was performed for dominant and recessive models and the Cochran-Armitage test for the additive model. Multivariate analysis, adjusting for age, sex, DM type, duration of DM, HbA1c, hypertension, DN and smoking were performed with binary logistic regression analysis. Multiple testing of individual SNPs was adjusted for using Nyholt's SNP spectral decomposition (SNP SpD) method [186], modified by Li and Ji [187] for multiple SNPs in a single gene. Where SNPs in more than one gene were investigated, the sum of the independent tests from each gene were used. Linkage Disequilibrium between markers was calculated using Haploview 4.0. Haplotype associations were undertaken in PLINK and adjusted for the number of haplotypes in the block using the Bonferroni test.

BASELINE CHARACTERISTICS

Cases and controls

Caucasian patients with T1DM or T2DM (n=2899) were included for genotyping. 980 participants had 'No DR' (205 T1DM and 775 T2DM). Of the 1919 participants with 'Any DR', 1123 had NPDR (206 T1DM

and 917 T2DM), 734 had PDR (252 T1DM and 482 T2DM) and 909 had DMO (140 T1DM and 769 T2DM). 499 of those with DMO also had co-existing PDR or severe NPDR.

1153 participants were classified as controls (258 with T1DM and 895 with T2DM), and 1315 participants satisfied criteria for STDR (330 with T1DM and 985 with T2DM). The remaining 480 participants had mild or moderate NPDR and were not included in case control analyses. Of those with STDR, 731 participants had PDR (with 251 T1DM and 480 with T2DM) and 1026 had DMO (170 T1DM and 856 with T2DM). 639 of the cases with STDR were recruited since the initiation of RADAR.

The number of participants in each subgroup as described above reflects those included in univariate analyses throughout this thesis. Multivariate analyses were performed with only those participants who had complete data for the covariates included. The number of participants in each subgroup included in multivariate analyses is shown in **Table 6.1**.

Table 6.1: Number of participants in each subgroup included in multivariate analyses.

	All DM	T1DM	T2DM
Any DR	1197	345	852
STDR	790	231	559
PDR	433	184	249
DMO	528	90	438
Controls	951	240	711
No DR	798	187	611

Abbreviations: DM, diabetes mellitus; T1DM, type 1 DM; T2DM, type 2 DM; DR, diabetic retinopathy; STDR, sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema. STDR defined as severe non-proliferative DR (NPDR) or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. No DR defined as no NPDR, PDR or DMO. Patients with DMO can have co-existing PDR or NPDR.

Clinical variables

Duration of DM ranged from 0 to 70 years with a mean duration of 17.7 years (SD 10.9 years). The prevalence of comorbidities was high, with 65% of participants having hypertension, 61% of participants having hyperlipidaemia and a mean body mass index of 30.6 (SD 6.6). Suboptimal diabetic control as determined by HbA1C was also common, with the sample having a mean HbA1C of 8.0% (SD 1.7%). Duration of DM, total cholesterol, SBP, HbA1C and body mass index were all unimodally distributed.

Other complications of DM (DN, neuropathy, ischaemic heart disease or stroke) were noted in 50% of the sample. Of 905 patients with DN, 38 of these patients were on dialysis and 13 had received a renal transplant. Twenty-two per cent of patients had neuropathy, 18.5% had ischaemic heart disease and 7% had a history of stroke. Clinical characteristics of cases and controls by type of DM are presented in **Table 6.2**.

Amongst participants with T1DM, those with STDR were older, had longer duration of DM, and had significantly greater risk factors (including increased HbA1c, increased BMI, hyperlipidaemia, hypertension and smoking history) than controls. They were also more likely to have other diabetic microvascular

complications including DN. In the T2DM group, those with STDR were more likely to be male, and of younger age than controls. Longer duration of DM and worse glycaemic control (as measured by HbA1c) was seen in the STDR group, who were also more likely to be on insulin treatment for their DM. There was a significantly higher rate of DN in those with STDR. Those with STDR were more likely to have a current or previous smoking history, but there was no difference between groups with respect to other cardiovascular risk factors, including BMI, hypertension and hyperlipidaemia.

Table 6.2: Demographic and clinical characteristics of participants with STDR and controls. T1DM and T2DM groups are presented separately.

	T1DM			T2DM		
Clinical characteristics	**STDR n=330**	**Controls n=258**	**P value**	**STDR n=985**	**Controls n=895**	**P value**
Female, n (%)	155 (46.8)	124 (47.7)	0.900	410 (41.5)	433 (48.1)	**0.005**
Age, years, mean (SD)	48.96 (15.3)	37.2 (15.5)	**<0.001**	64.8 (11.0)	67.1 (12.0)	**<0.001**
Duration of DM, years, mean (SD)	30.3 (12.7)	15.4 (10.5)	**<0.001**	18.6 (9.4)	13.0 (8.0)	**<0.001**
Insulin treatment, n (%)	-	-	-	553 (61.0)	333 (39.9)	**<0.001**
HbA1c, % (mmol/mol), mean (SD)	8.7 (72) (1.7)	8.1 (65) (1.7)	**<0.001**	8.5 (69) (1.8)	7.5 (59) (1.4)	**<0.001**
BMI, mean (SD)	27.7 (6.2)	26.7 (4.5)	**0.032**	32.0 (6.5)	31.7 (6.6)	0.316
Hyperlipidemia, n (%)	190 (57.9)	77 (29.7)	**<0.001**	660 (68.5)	628 (69.9)	0.571
Hypertension, n (%)	197 (61.0)	77 (30.6)	**<0.001**	752 (80.4)	663 (78.9)	0.468
Smoking history, n (%)	158 (52.1)	99 (39.6)	**0.004**	439 (51.3)	451 (57.2)	**0.019**
Ischaemic heart disease, n (%)	34 (18.6)	11 (4.5)	**<0.001**	184 (31.0)	234 (29.7)	0.646
Stroke, n (%)	12 (6.6)	6 (2.4)	0.061	63 (10.6)	97 (12.3)	0.368
Neuropathy, n (%)	54 (29.7)	20 (8.1)	**<0.001**	248 (42.0)	211 (26.8)	**<0.001**
Nephropathy, n (%)	116 (36.0)	29 (11.4)	**<0.001**	353 (37.4)	210 (23.7)	**<0.001**
Dialysis, n (%)	3 (1.1)	2 (0.8)	0.999	14 (1.7)	6 (0.7)	0.103
Transplant, n (%)	1 (0.4)	1 (0.4)	0.999	0 (0.0)	7 (0.8)	**0.030**

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; STDR, sight-threatening diabetic retinopathy; SD standard deviation; BMI, body mass index; HbA1c, haemoglobin (A1c). Nephropathy defined as those with 24-hour urine albumin ≥30mg/d, or eGFR <60 mls/min, or a history of dialysis or transplant for DN. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. Patients with DMO can have co-existing PDR or NPDR. n represents number of participants in each group. P-values < 0.05 are highlighted in bold.

The correlation of clinical variables with STDR was examined (**Table 6.3**). PDR and DMO were also assessed in separate models. Statistically significant correlations with one or more subgroups of STDR were found for duration of DM, DM type, hypertension, hyperlipidaemia, DN, neuropathy and HbA1c. Correlations were strongest for HbA1c and duration of DM. Hyperlipidaemia was significantly correlated with DMO (P = 0.047) but not PDR (P = 0.191). DM type also differed in its correlation with PDR and DMO, with PDR correlating with T1DM (P < 0.001) and DMO correlating with T2DM (P < 0.001).

Table 6.3: Correlation of clinical variables with retinopathy grading. Pearson correlations (r) are presented with corresponding p-values.

	STDR		PDR		DMO	
Variable	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**
Duration of DM	0.37	**<0.001**	0.44	**<0.001**	0.30	**<0.001**
T2DM	-0.03	0.123	-0.13	**<0.001**	0.08	**<0.001**
Hypertension	0.09	**<0.001**	0.06	**0.007**	0.06	**0.005**
Hyperlipidaemia	0.05	**0.009**	0.03	0.191	0.04	**0.047**
Nephropathy	0.18	**<0.001**	0.23	**<0.001**	0.16	**<0.001**
Neuropathy	0.18	**<0.001**	0.21	**<0.001**	0.14	**<0.001**
HbA1c	0.26	**<0.001**	0.29	**<0.001**	0.24	**<0.001**
BMI	0.02	0.323	0.02	0.409	0.04	0.114

Abbreviations: STDR, sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; DM, diabetes mellitus; T2DM, type 2 diabetes; HbA1c, haemoglobin (A1c); BMI, body mass index. STDR defined as severe NPDR or PDR, and/or DMO. P-values < 0.05 are highlighted in bold.

DISCUSSION

This large, cross-sectional study of Caucasian patients with DM has been designed to explore genetic risk factors affecting those patients most likely to go blind from DR. The stringent criteria used to define cases (those with STDR) have identified patients most likely to carry high-risk alleles. In addition, controls (those with no DR or minimal NPDR) have a mean duration of DM of 15 years for those with T1DM and 13 years for those with T2DM suggesting a reduced propensity to developing DR, and are theoretically more likely to carry protective alleles. The resultant increase in power reflects the inflated difference in risk allele frequency between case and control groups.

Limitations of the current study design must also be acknowledged. Firstly, the cross sectional nature of this study does not allow for long-term follow-up or longitudinal analysis. Thus, patients recruited with no DR and a 6 year history of DM are treated equally whether they go on to develop PDR at 7 years or at 30 years following DM diagnosis. It has been proposed by some authors that DR controls should have a long duration of DM in order to construct a control group with relative resistance to developing DR [188]. Comparing this subgroup with patients who develop STDR early in the course of DM (ideally with relatively good glycaemic control) would be an added method of examining genetic susceptibility to STDR. With longitudinal follow-up, recruited patients could be categorised on an ongoing basis as they reach these milestones. Secondly, progression of DR and response to treatment could be analysed as additional outcomes.

Missing data is another limitation encountered through the data collection methodology employed in this study. In particular, data relating to lab results (including HbA1c, serum lipids and renal function tests) were collected via searching patient records or contacting the patient's primary care doctor. Patients were not referred for lab tests at the time of recruitments as part of this study as this is a costly exercise. Patients for whom this data was not available, were considered to contribute to random missing data and were unlikely to bias study results. However, missing data does significantly affect the power of multivariate analysis, as only patients with complete data sets across all covariates were included. Smaller sample sizes in multivariate

compared with univariate analyses may contribute to a higher rate of false negative results following adjustment for clinical covariates.

The clinical characteristics of the cohort described here generally reflect the common risk factors associated with DM and its complications. Interestingly, PDR was correlated with T1DM, whereas DMO was correlated with T2DM. This finding is consistent with data from long-term prospective population-based studies indicating that those with T2DM may be more susceptible to DMO than those with T1DM, and that the difference in the incidence of DMO between these groups becomes more pronounced as duration of DM increases [189,190]. Descriptive statistics of the present study sample are consistent with this finding, with 1.7 times the rate of DMO in T2DM compared with T1DM participants. The weak correlation between hyperlipidaemia and DMO (but not PDR) could reflect the increased risk of DMO in those with hyperlipidaemia as documented in the literature [68-70]. Alternatively, this could reflect the increased number of T2DM patients (with metabolic syndrome and hyperlipidaemia) with DMO in our cohort. The implication of these associations during an era of rapidly increasing incidence of T2DM, on vision-related quality of life and health-care costs is particularly noteworthy. DMO is the major vision-threatening complication of DR and thus determining independent genetic risk factors for DMO is imperative in achieving improved treatment strategies and outcomes.

CHAPTER 7

INFLAMMATORY PATHWAYS: GENETIC RISK FOR STDR AND DMO

Multiple mechanisms have been postulated to contribute to the pathogenesis of microvascular complications of DM, many of which involve hyperglycaemia-induced elevation of oxidative stress [27]. Oxidative stress is a key factor involved in the formation of AGEs, and in the activation of NF-κB, resulting in a chronic inflammatory process and tissue damage [27,181].

The interaction of AGEs with RAGEs (their receptors) on the surface of macrophages and endothelial cells results in an increase in the synthesis and secretion of pro-inflammatory cytokines including tumour necrosis factor (TNF, previously known as TNF-α) [191]. TNF has been implicated in the pathogenesis of DR, through its contribution to BRB breakdown and neovascularisation [191]. Another pro-inflammatory cytokine lymphotoxin-α (LT-α, previously known as TNF-β) binds the same TNF receptors, with similar downstream effects [192].

TNF promoter polymorphisms have been extensively studied across a range of diseases with rs1800629 (TNF -308) and rs361525 (TNF -238) being the most widely investigated SNPs. Individuals homozygous for the less common A allele at rs1800629 have been shown to have higher circulating TNF levels than those homozygous for the G allele, and have worse outcomes in response to infectious diseases [193]. The SNP at rs361525 has also been implicated in a number of disease states with the A allele conferring protection against severe forms of autoimmune diseases including rheumatoid arthritis [194]. Both the *TNF* and *LTA* genes are located on chromosome 6 in close proximity to each other, in the HLA class III region [195]. A number of SNPs in this region have been studied in relation to insulin resistance, DM, and diabetic complications. Variations within the *TNF* gene have specifically been associated with DN [196] and cardiovascular disease [197]. *LTA* gene variants have been associated with PDR in an Indian cohort of patients with T2DM [198].

NF-κB, a nuclear transcription factor, regulates multiple gene pathways involved in inflammation, immune response and apoptosis, and has been implicated in the development of diabetic complications [27,29]. NF-κB functions as an important regulator of endothelial cell apoptosis by activating pro-apoptotic factors in response to cellular stressors such as hyperglycaemia [29,199]. Studies have consistently shown that NF-κB activation correlates with the apoptosis of retinal pericytes in the presence of sustained hyperglycaemia, and precedes histopathological changes characteristic of DR such as pericyte ghosts [29,30].

MicroRNAs (miRs) are short, noncoding RNA molecules, which regulate mRNA stability and translation by binding to their target messenger RNAs (mRNAs) [200]. Following transcription, pre-miRs assume a hairpin structure which is then cleaved to form two mature miR duplex strands, each given a suffix of either -5p or -3p [200,201]. By targeting negative regulators of angiogenic signalling pathways, miRs play an important

role in modulating endothelial cell proliferation and vascular development in response to both physiological and pathological events [202].

NF-κB responsive miRs, including miR-146a, miR1-55, miR-132 and miR-21 are upregulated in retinal endothelial cells of Streptozotocin (STZ) induced diabetic rats [203]. This finding by Kovacs et al. [203] is suggestive of a miR signature of NF-κB activation, that may modulate the inflammatory response in diabetic retinal endothelial cells (**Figure 7.1**).

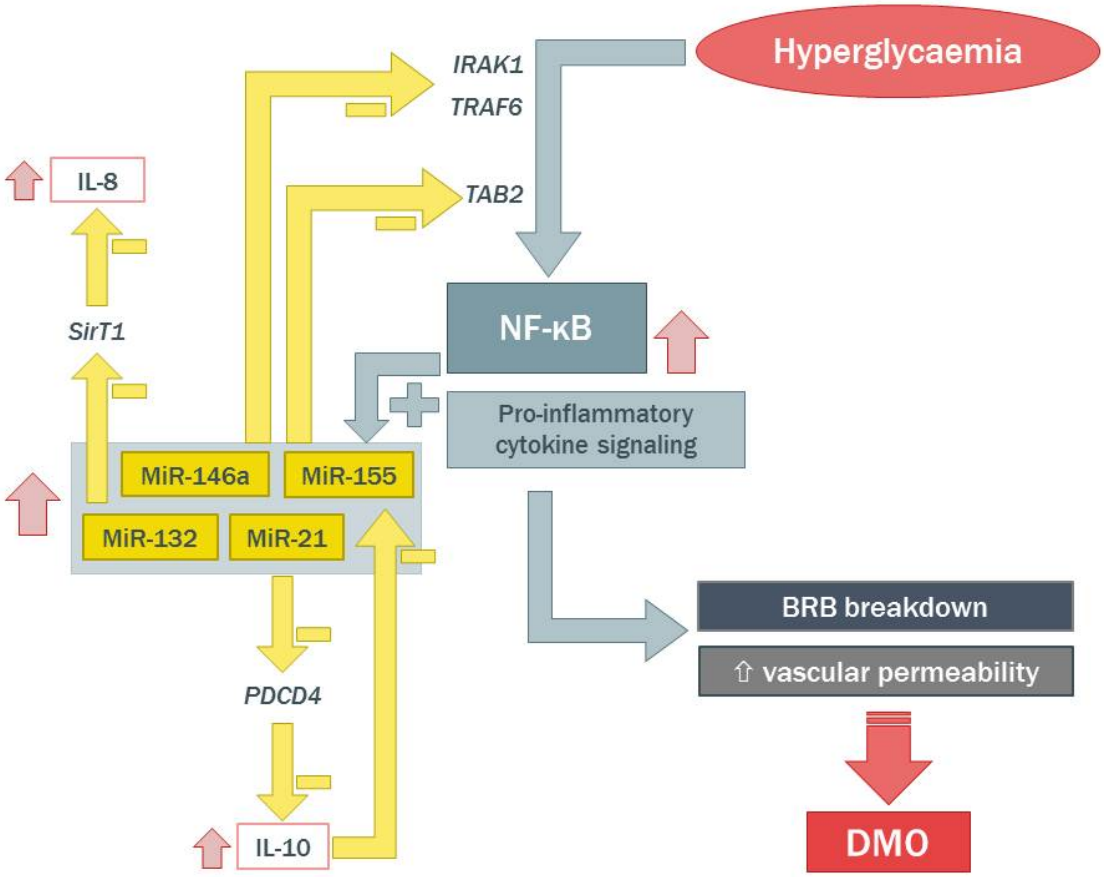


Figure 7.1: The role of NF-κB responsive miRs in inflammation. Hyperglycaemia results in the activation of a number of biochemical and molecular pathways. The associated increase in NF-κB affects pro-inflammatory cytokine signalling which contributes to blood retinal barrier (BRB) breakdown in the pathogenesis of diabetic macular oedema (DMO). NF-κB responsive microRNAs (miRs) including miR-146a, miR-155, miR-132 and miR-21 are known to be upregulated in the diabetic retina. These miRs negatively regulate inflammation via a number of pathways. MiR-146a inhibits further NF-κB activation through its target genes, interleukin-1-receptor-associated kinase-1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) [204]. MiR-155 modulates NF-κB levels through its target TAK1-binding protein 2 (TAB2) mRNA [205]. Induction of miR-132 decreases SirT1 expression, which in turn increases interleukin-8 (IL-8 production) [206]. MiR-21 targets the mRNA encoding programmed cell death 4 (PDCD4), resulting in increased interleukin-10 (IL-10) production [205].

Of these NF-κB responsive miRs, miR-146a has been well studied, both from a functional and genetic point of view. It plays an important role in the innate immune response and is associated with autoimmune diseases [207] and inflammation [204]. MiR-146a is located on chromosome 5q33 and, in common with most miRs, has multiple gene targets [208]. Post-transcriptional gene silencing by miR-146a inhibits the NF-κB inflammatory cascade via a number of known pathways [209,210]. Mir-146a also targets fibronectin, an extracellular matrix (ECM) protein deposited in end organs (including retina, kidney, heart) affected by DM [211]. Rs2910164 is a common SNP located within the seed sequence of miR-146a-3p and is predicted to lead to perturbation of pairing of the hairpin strands [212]. The presence of the minor allele (C) reduces the processing efficacy of pre-miR-146a into the mature miR-146a form, resulting in an overall reduction in mature miR-146a [212]. This SNP is also the only common variant located within the *MIR146A* gene and has been studied previously in relation to other diseases. There are several other very rare variants mapping to this gene; however there is currently no evidence of their functionality. MiR-155, miR-21 and miR-132 are known to function in the inflammatory pathway, via increasing NF-κB [205], interleukin-10 (IL-10) [205] and interleukin-8 (IL-8) [206] respectively. Polymorphisms within these miR genes are rare and have not been investigated in relation to their miR function or any disease states.

In this chapter we explore the genetic association of genes thought to contribute to retinal inflammation in DM, including SNPs known to regulate *TNF* and *LTA* genes, and SNPs within or near NF-κB responsive miRs.

7.1 TUMOUR NECROSIS FACTOR

The original work presented in this section has been published in the peer-reviewed literature: G Kaidonis, JE Craig, MC Gillies, S Abhary, RW Essex, JH Chang, B Pal, M Pefkianaki, M Daniell, S Lake, N Petrovski and, KP Burdon. Promoter polymorphism at the tumour necrosis factor / lymphotoxin-alpha locus is associated with type of diabetes but not with susceptibility to sight–threatening diabetic retinopathy. ***Diabetes and Vascular Disease Research. 2016, Vol. 13(2), pp.164-167*** [213]. *Dr Kaidonis' contributions include study design, patient recruitment, sample preparation, data analysis and interpretation and manuscript preparation.*

Aim

We conducted this study to further investigate the regulatory region of *TNF* and *LTA* genes on chromosome 6, by investigating 2 promoter polymorphisms (rs1800629 and rs361525) known to have a functional role in *TNF* and *LTA* expression, and their relationship to DR in a large and well-characterised cohort of Caucasian patients with T1DM and T2DM.

Results

Genotyped SNPs (rs1800629 and rs361525) were in HWE in our cohort (**Table 7.1.1**). Adjustment for the 2 SNPs tested resulted in a P-value of less than 0.025 required for significance. Genotype counts of controls were compared with patients with STDR, and PDR and DMO individually. Genotype frequencies for STDR and controls are given in **Table 7.1.1**.

Table 7.1.1: Genotype frequencies, shown as n (%) for STDR and controls by DM type.

SNP	Genotype	T1DM		T2DM		HWE P-value
		Controls	STDR	Controls	STDR	
rs1800629	AA/GA/GG	13/87/156 (5.1/34.0/60.9)	13/128/188 (4.0/38.9/57.1)	28/238/623 (3.1/26.8/70.1)	34/253/696 (3.5/25.7/70.8)	0.356
rs361525	AA/GA/GG	3/31/222 (1.2/12.1/88.7)	2/40/288 (0.6/12.1/87.3)	4/99/788 (0.4/11.1/88.4)	4/112/868 (0.4/11.4/88.2)	0.095

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; Chr, Chromosome; SNP, single nucleotide polymorphism; STDR, sight-threatening diabetic retinopathy. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. SNPs with HWE $P < 0.05$ are shown in bold. *SNPs that remained statistically significant for HWE testing after correction for multiple SNPs Bonferroni correction ($P < 0.025$).

The allele frequencies of SNPs 1800629 and rs361525 did not differ between patients with STDR and controls, in patients with T1DM or T2DM (**Table 7.1.2**). No association was found between either of the two SNPs and PDR or DMO alone, in either type of DM. In a multivariate logistic regression analysis controlling for age, sex, duration of DM, HbA1c, hypertension and DN, no association was found between STDR, PDR or DMO cases and controls in T1DM or T2DM groups (**Table 7.1.2**).

Table 7.1.2: Allelic association of rs1800629 (-308G>A) and rs361525 (-238G>A) with STDR, PDR and DMO for T1DM and T2DM. Results for both univariate and multivariate analyses are presented. Uncorrected P-values are shown.

	T1DM				T2DM			
	Univariate		Multivariate[#]		Univariate		Multivariate[#]	
SNP	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
rs1800629: A								
STDR	1.08 (0.82-1.42)	0.590	0.95 (0.64-1.42)	0.812	0.99 (0.83-1.17)	0.864	0.95 (0.76-1.19)	0.672
PDR	1.18 (0.88-1.58)	0.257	1.02 (0.66-1.57)	0.944	0.94 (0.76-1.17)	0.579	0.91 (0.69-1.21)	0.536
DMO	0.99 (0.70-1.41)	0.967	1.39 (0.84-2.30)	0.199	0.94 (0.78-1.14)	0.540	0.95 (0.75-1.21)	0.675
rs361525: A								
STDR	0.92 (0.58-1.44)	0.708	0.67 (0.33-1.35)	0.264	1.02 (0.78-1.33)	0.905	1.00 (0.69-1.43)	0.976
PDR	0.82 (0.50-1.35)	0.433	0.60 (0.27-1.32)	0.200	0.86 (0.61-1.21)	0.380	0.67 (0.40-1.13)	0.137
DMO	1.15 (0.67-1.98)	0.616	0.83 (0.36-1.89)	0.653	0.97 (0.73-1.30)	0.834	0.90 (0.61-1.33)	0.602

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; SNP, single nucleotide polymorphism; OR (95%CI), odds ratio with 95% confidence interval; STDR, sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. [#]Adjusted for age, sex, duration DM, HbA1c, hypertension, DN, smoking. P-values < 0.05 are highlighted in bold. *Significant association following Bonferroni correction for multiple testing (P < 0.025).

Discussion

This study found no association between SNPs at rs1800629 and rs361525 and STDR, PDR or DMO in patients with either T1DM or T2DM, in a multivariate logistic regression analysis.

The rs1800629 promoter SNP has previously been investigated in relation to DR, in a large study including 742 T1DM and 2957 T2DM Caucasian patients by Lindholm et al. [214]. Cases with STDR were compared with controls with no DR or NPDR and no significant association between this promoter polymorphism and STDR risk was found. This finding is comparable to the results from our study, which evaluated differences between phenotypic extremes by comparing controls with no DR or minimal NPDR to cases with STDR. Furthermore, rs1800629 has been studied in smaller Chinese and Japanese cohorts with T2DM. Wang et al. investigated 3 SNPs in the *TNF/LTA* promoter region including rs1800629 and found no relationship between any of these SNPs and DR (any DR, NPDR or PDR) in a Chinese population of patients with T2DM [215]. A study of 251 Japanese patients with T2DM also found no association between the rs1800629 promoter variant and DR [216]. Our results are consistent with findings reported in these two ethnic groups. However, rs1800629 has been investigated in a cohort of Brazilian Caucasian patients, with conflicting findings to these previous studies [217]. Sesti et al. recently reported a positive association between the A allele of rs1800629 and an increased risk of PDR [217]. To date no association has been found between rs361525 and DR [217], consistent with findings from the current study.

Functional studies have suggested a role for TNF as a biomarker for DR. Circulating levels of TNF are increased in the serum of patients with T1DM and PDR compared to patients with DM but without DR [218]. TNF concentration in tears has been shown to correlate with DR severity and DN in patients with DM [219]. mRNA expression of *TNF* as well as the level of soluble TNF receptors, are elevated in the vitreous of patients with PDR [220]. Furthermore, inhibition of TNF with angiopoietin-1 has shown promising outcomes in preventing early DR in a diabetic rat model [221].

There is increasing evidence to suggest that a complex interaction exists between *TNF, LTA* and other genes in the HLA class III region, and may explain the discrepancy between genetic and functional work to date. Lindholm et al. investigated 3 SNPs in the MHC complex on chromosome 6, including *TNF* 308G/A, *LTA* T60NC/A and *RAGE* 374T/A variants [214]. These authors found that the *TNF, LTA* and *RAGE* SNPs were associated with the HLA-DQB1 risk genotype for DR, but the allele frequencies for each individual SNP did not differ between patients with and without STDR in T1DM or T2DM groups [214]. Alleles within the MHC region are in strong linkage disequilibrium making a direct association between SNPs in this region and TNF phenotypes less likely. It has been postulated that alternative pathways may be responsible for TNF expression including regulation by linked genes, interaction between the 3'UTR outside the TNF promoter and the -308 element, and epigenetic control via TNF promoter methylation [193,222]. Further investigation of the relationship between these genes and factors modulating their expression is required to gain a better understanding of the role of these pro-inflammatory cytokines in the pathogenesis of DR.

Conclusion

In conclusion, we found no association between either of the 2 polymorphisms in the promoter region of *TNF* and *LTA* and STDR (including individual analysis of sub-phenotypes DMO or PDR), in patients with either T1DM or T2DM. It is clear that increased levels of TNF, both locally and systemically, are associated with DR risk, however further investigation of the complex interplay between *TNF,* and *LTA* genes with regards to the HLA haplotypes, and epigenetic modifications is required to identify the specific effect of these variants on microvascular complications of DM, including DR, and determine the underlying biological drivers of these elevated levels.

7.2 NF-KB RESPONSIVE MICRORNAS

MICRORNA-146A

The original work presented in this section has been published in the peer-reviewed literature: G Kaidonis, MC Gillies, S Abhary, E Liu, RW Essex, JH Chang, B Pal, S Sivaprasad, M Pefkianaki, M Daniell, S Lake, N Petrovsky, AW Hewitt, A Jenkins, EL Lamoureux, JM Gleadle, JE Craig, KP Burdon. A single-nucleotide polymorphism in the MicroRNA-146a gene is associated with diabetic nephropathy and sight-threatening diabetic retinopathy in Caucasian patients. ***Acta Diabetologica. 2016, Vol. 53(4), pp.643-650*** [223]. *Dr Kaidonis' contributions include study design, patient recruitment, sample preparation, data analysis and interpretation and manuscript preparation.*

Aim

Given the function of miR-146a as an inhibitor of NF-κB-activated inflammation and fibronectin gene transcription, changes in miR-146a levels secondary to the rs2910164 SNP could plausibly have functional implications in the pathogenesis of diabetic microvascular complications. This study aimed to investigate whether rs2910164 is associated with STDR, in two large and well-characterised cohorts of Caucasian patients T1DM and T2DM.

Results

Genotype frequencies were in HWE in our cohort (P = 0.668). Genotype frequencies for controls and cases with STDR are shown in **Table 7.2.1**.

Table 7.2.1: Genotype frequencies, shown as n (%) for Controls and cases with STDR, for each type of DM.

Phenotype	Genotype	Controls	STDR
T1DM	CC/GC/GG	13/89/156 (5.0/34.5/60.5)	21/119/190 (6.4/36.0/57.6)
T2DM	CC/GC/GG	55/327/513 (6.1/36.5/57.3)	70/393/522 (7.1/39.9/53.0)

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM type 2 diabetes mellitus; STDR, sight-threatening diabetic retinopathy. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR.

Rs2910164 was tested for association with STDR. Where there was *a priori* evidence of an association with STDR, testing for association for STDR sub-phenotypes (PDR and DMO) versus controls was performed. Adjustment for the 2 hypotheses tested (PDR and DMO), resulted in a P-value of less than 0.025 required for significance. For the subgroup of patients with T2DM, this study was 80% powered to detect a minimal OR of 1.3 (for dominant and additive models), with the significance level set at 0.025 (to account for multiple comparisons) [224]. The recessive model requires a larger OR to be detectable in this study. For patients with T1DM, and a significance level of 0.025, this study was powered (80%) to detect an OR of 1.3 for an additive model, for STDR analyses only [224].

Table 7.2.2: Association of *miR-146a* SNP rs2910164 (C allele) with STDR and its subtypes PDR and DMO, for T1DM and T2DM groups individually. Univariate and multivariate analyses, for additive, dominant and recessive models are presented. Uncorrected P-values are shown.

	STDR		PDR		DMO	
T1DM						
Univariate	**CATT or X^2**	**P value**	**CATT or X^2**	**P value**	**CATT or X^2**	**P value**
Additive	Z=0.75	0.689	Z=2.07	0.355	Z=0.71	0.701
Dominant	X^2=0.50	0.480	X^2=2.05	0.152	X^2=0.58	0.446
Recessive	X^2=0.47	0.495	X^2=0.07	0.786	X^2=0.01	0.907
Multivariate	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
Additive[#]	1.23 (0.84-1.79)	0.293	1.24 (0.81-1.89)	0.324	0.87 (0.54-1.42)	0.583
Dominant[#]	1.15 (0.72-1.85)	0.562	1.25 (0.74-2.09)	0.409	0.84 (0.46-1.52)	0.558
Recessive[#]	1.99 (0.78-5.04)	0.148	1.54 (0.53-4.46)	0.430	0.89 (0.25-3.16)	0.853
T2DM						
Univariate	**CATT or X^2**	**P value**	**CATT or X^2**	**P value**	**CATT or X^2**	**P value**
Additive	Z=3.63	0.163	Z=4.49	0.106	Z=6.27	**0.043**
Dominant	X^2=3.54	0.059	X^2=4.34	**0.037**	X^2=5.47	**0.019***
Recessive	X^2=0.70	0.403	X^2=0.05	0.819	X^2=2.44	0.118
Multivariate	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
Additive[#]	1.17 (0.97-1.43)	0.107	1.22 (0.94-1.57)	0.137	1.25 (1.03-1.53)	**0.025***
Dominant[#]	1.21 (0.95-1.54)	0.127	1.37 (1.00-1.87)	0.051	1.29 (1.01-1.65)	**0.044**
Recessive[#]	1.27 (0.78-2.07)	0.338	0.90 (0.45-1.78)	0.758	1.46 (0.90-2.37)	0.122

Abbreviations: STDR, sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; CATT, Cochran-Armitage Trend Test; OR (95%CI), odds ratio with 95% confidence interval. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. [#]Adjusted for age, sex, duration DM, HbA1c, hypertension, DN, smoking. P-values < 0.05 are highlighted in bold. *Significant association following Bonferroni correction for multiple testing (P < 0.025).

In the T2DM group, univariate analysis with a dominant model showed a trend towards an association with STDR (P = 0.059) (**Table 7.2.2**). When the composite phenotype of STDR was then analysed by its sub-phenotypes PDR and DMO, a stronger association was found between those with genotypes containing the minor allele (dominant model) and PDR (P = 0.037), as well as DMO (P = 0.019). In an additive model analysis, the C allele was also found to be associated with DMO (P = 0.043). The significant associations with DMO were maintained after adjustment for age, sex, duration of DM, HbA1c, hypertension and DN (additive model: OR, 1.25; CI, 1.03-1.53; P = 0.025; dominant model: OR, 1.29; CI, 1.01-1.65; P = 0.044), and in the additive model, the association with DMO survived adjustment for multiple testing. After multivariate analysis, the association with PDR was of only borderline significance (P = 0.051). No significant association was found between rs2910164 and STDR in the smaller T1DM subgroup alone.

Discussion

This is the first study to investigate the common miR-146a SNP at rs2910164 and its relationship with DR. The frequency of the C allele was found to be greater in patients with STDR than in diabetic controls in those with T2DM. Subtype analyses showing similar effect sizes in both PDR and DMO phenotypic subgroups suggest that this SNP may increase susceptibility to retinal damage via a pathway involved in both angiogenesis and blood retinal barrier breakdown. The functionally important C allele specifically confers significant risk of developing DMO in patients with T2DM. PDR and DMO subgroup analyses in the T1DM group may have been underpowered to detect an association.

The literature relating to rs2910164 presents contrasting results regarding the risk allele across varying diseases and ethnicities. The CC genotype has been associated with increased risk of a number of diseases including coronary artery disease in Indian males [225]. On the other hand, the C allele and CC genotype have been found to be protective against the development of cardiovascular diabetic autonomic neuropathy in a Caucasian population [226]. The opposite (protective) effect of the C allele found by Ciccaci et al. compared with the current study may be due to the study size (only 11 cases with cardiovascular diabetic autonomic neuropathy compared with 100 controls). Alternatively, there may in fact be different genetic risks of various diabetic complications.

It is well accepted that altered expression of miR-146a in the hyperglycaemic (and pro-inflammatory) environment negatively regulates NF-κB expression via a negative feedback loop [203,211]. Jazdzewski et al. correlated miR-146a genotypes with mature miR-146a levels. They showed a 1.8 fold decrease in mature miR-146a from the C allele compared to the G allele at rs2910164 [212]. They also found that the presence of the C allele results in less inhibition of Interleukin-1 receptor-associated kinase 1 (IRAK-1) and TNF Receptor Associated Factor 6 (TRAF6) than the G allele, confirming a direct functional effect of rs2910164 on miR-146a target genes [212]. These findings imply that the C allele has impaired ability to dampen NF-κB-mediated inflammation, which could be of relevance to the pathological role of the C allele in the development of DMO suggested by the current study.

Feng et al. [211] investigated the role of miR-146a on fibronectin expression, a second target of miR-146a. They found that reduced miR-146a expression directly increases synthesis of fibronectin, an ECM glycoprotein found in microvascular endothelial cells. Increased fibronectin synthesis was seen in a number of end organs including retina, kidney and heart in a STZ rat model [211]. Enhanced production of ECM proteins, including fibronectin, is characteristic of DM microvascular pathology. The deposition of excess fibronectin in human retina has been shown to bind endothelial cell transmembrane receptors, integrins (also located on chromosome 5q), which play a significant role in vascular permeability and ocular neovascularisation [227]. Increasing the availability of miR-146a with intravitreal injection of a miR-146a mimic was able to block DM-induced fibronectin upregulation in a diabetic rat model [211]. Further investigation of this, and other miR-146a targets is warranted to gain a better understanding of how miR-146a could be manipulated for therapeutic use.

There have been a number of studies investigating the effects of hyperglycaemia on miR-146a function and expression. Human kidney tissue from patients with DN (stage IV-V) showed a 4.87 fold upregulation of miR-146a compared with non-diabetic kidney tissue, with highest expression in glomerular endothelial cells (ECs), mesangial areas and tubular sections of the diabetic kidney [228]. The increase in miR-146a expression in kidney tissue has been reported to correlate with progression of DN in STZ rats [228]. Similar increases in miR146a expression have been found in retinal endothelial cells of rats 2-3 months after STZ-induced DM [203]. It is possible that miR-146a is upregulated in end organ tissues in an attempt to protect against the increase in hyperglycaemia-induced inflammation that occurs during the progression of microvascular complications of DM. It would be interesting to explore the rate of progression of end organ damage in those with and without the miR-146a risk genotype reported in this study.

The predominant limitation of this study was the lack of power for PDR and DMO sub-analyses in the smaller T1DM group. This may explain the negative findings in the T1DM analysis for these phenotypes. The small to moderate effect size assessed in this study (OR=1.3) was based on the fact that DM complications are likely to be a result of complex gene-environment interactions, and the impact of a single SNP is therefore likely to be small. It may be that an even smaller effect size needs to be considered for the impact of this SNP and that the T2DM cohort was underpowered to detect an effect of this size. Thus, it is possible that the DMO association in T2DM patients found in the current study (of borderline significance and small OR of 1.25) is in fact a false positive result. Further studies with a larger cohort size are required to more accurately explore these specific phenotypes with relation to miR-146a SNPs. Given that T1DM and T2DM are distinct diseases with differing aetiologies, association of SNPs specific to one type of DM is also a plausible explanation. The development of DR is influenced by environmental factors that may be more likely to occur in the context of a specific type of DM.

Conclusion

This study reports for the first time that rs2910164 is associated with STDR and its subgroup DMO in Caucasian patients with T2DM. There is prior evidence for a direct link between the CC genotype, decreased levels of mature miR-146a and downstream failure of regulation of NF-κB-mediated inflammation. Predictive studies measuring mir-146a levels or identifying those with the rs2910164 risk genotype have potential to contribute to the identification of DM patients at high risk of complications. Furthermore, manipulation of miR-146a has therapeutic potential for the treatment or prevention of STDR.

OTHER NF-KB RESPONSIVE MICRORNAS

Given the association of rs2910164 with DMO in patients with T2DM, we hypothesised that miR polymorphisms of other NF-κB responsive miRs (miR-155, miR-132 and miR-21) may also be associated with STDR and DMO. A search to identify SNPs within the pre-miR sequences of these miRs using the dbSNP database from The National Centre for Biotechnology Information (NCBI) was performed. However, only rare variants were identified (MAF<0.05) for which this study would not have power to detect an association. MiR synthesis and expression can be altered via a number of mechanisms other than through pre-miR SNPs. These include SNPs outside of the pre-miR coding region, and SNPs in the promoter region of a single miR or a specific miR cluster [229]. We therefore explored SNPs within 500bp of the miRs of interest, and with MAF > 0.05 in Caucasian populations.

Aim

This study aimed to investigate 3 common SNPs, rs1547354 (353bp downstream of miR-155), rs2957924 (165bp downstream of miR-132) and rs1292037 (210bp downstream of miR-21) near NF-κB responsive miRs, and their association with STDR and DMO.

Results

All 3 tag SNPs were successfully genotyped. Multiple SNP testing correction for 3 independent tests was performed, with a P-value of less than 0.017 required for significance. Genotype frequencies in patients with STDR and controls are shown in **Table 7.2.3** for both T1DM and T2DM participants. Genotype frequencies for STDR subtypes PDR and DMO are also included. None of the 3 SNPs tested were in HWE in our cohort (rs1547354 HWE P = 0.002; rs2957924 HWE P = 1.2×10^{-6}; rs1292037 HWE P = 0.001) and thus results from this analysis should be interpreted with caution. No participants had the AA genotype at rs1547354 in the T2DM group and only 2 expressed this genotype in the T1DM group, so this SNP was excluded from further analyses.

Table 7.2.3: Genotype frequencies, shown as n (%) for controls and cases with STDR, for each type of DM.

		T1DM		T2DM		
SNP	**Genotype**	**Controls**	**STDR**	**Controls**	**STDR**	**HWE P-value**
rs1547345 (miR-155)	AA/GA/GG	1/0/257 (0.4/0.0/99.6)	1/1/323 (0.3/0.3/99.4)	0/1/891 (0.0/0.1/99.9)	0/10/967 (0.0/1.0/99.0)	**0.002***
rs2957924 (miR-132)	TT/GT/GG	2/64/186 (0.8/25.4/73.8)	2 /73/247 (0.6/22.7/76.7)	2/205/655 (0.2/23.8/76.0)	5/235/723 (0.5/24.4/75.1)	**<0.001***
rs1292037 (miR-21)	GG/AG/AA	13/84/160 (5.1/32.7/62.3)	12/107/211 (3.6/32.4/63.9)	42/282/572 (4.7/31.5/63.8)	71/306/607 (7.2/31.1/61.7)	**0.001***

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; Chr, Chromosome; SNP, single nucleotide polymorphism; STDR, sight-threatening diabetic retinopathy. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. SNPs with HWE P < 0.05 are shown in bold. *SNPs that remained statistically significant for HWE testing after correction for multiple SNPs Bonferroni correction (P < 0.017).

Both SNPs were tested for association with STDR and its subtypes PDR and DMO in T1DM and T2DM patients. Univariate and multivariate analyses are presented in **Table 7.2.4**. No significant associations were identified in univariate analyses. The G allele of rs2957924 was weakly associated with DMO in a multivariate analysis in the T1DM group only (OR, 0.48; CI, 0.23-0.99; P = 0.046), however this association did not survive multiple testing correction. No other significant associations were identified after adjustment for clinical covariates.

Table 7.2.4: Association of *miR-132* SNP rs2957924 (T allele) and *miR-21* SNP rs1292037 (G allele) with STDR and its subtypes PDR and DMO, for T1DM and T2DM groups individually. Univariate and multivariate analyses are presented. Uncorrected P-values are shown.

	STDR		PDR		DMO	
T1DM	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
rs2957924						
Univariate	0.87 (0.61-1.24)	0.437	0.86 (0.59-1.25)	0.433	0.80 (0.52-1.22)	0.301
Multivariate[#]	0.61 (0.35-1.06)	0.077	0.71 (0.39-1.28)	0.254	0.48 (0.23-0.99)	**0.046**
rs1292037						
Univariate	0.91 (0.68-1.21)	0.514	0.93 (0.68-1.25)	0.614	0.82 (0.58-1.16)	0.259
Multivariate[#]	0.83 (0.55-1.26)	0.390	0.94 (0.60-1.49)	0.805	0.70 (0.42-1.16)	0.167
T2DM						
rs2957924						
Univariate	1.06 (0.87-1.29)	0.585	0.93 (0.72-1.19)	0.542	1.04 (0.85-1.28)	0.709
Multivariate[#]	0.98 (0.73-1.31)	0.871	0.89 (0.60-1.33)	0.571	1.01 (0.74-1.37)	0.943
rs1292037						
Univariate	1.15 (0.98-1.34)	0.082	1.11 (0.92-1.34)	0.287	1.16 (0.99-1.36)	0.071
Multivariate[#]	1.02 (0.82-1.26)	0.870	1.04 (0.78-1.38)	0.783	1.01 (0.80-1.27)	0.936

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; STDR, sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; OR (95%CI), odds ratio with 95% confidence interval. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. [#]Adjusted for age, sex, duration DM, HbA1c, hypertension, DN, smoking. P-values < 0.05 are highlighted in bold. *Significant association following Bonferroni correction for multiple testing (P < 0.017).

Discussion

The investigation of miR-155, miR-132 and miR-21 was stimulated by our positive findings in miR-146a. Together, this cluster forms a signature of NF-κB responsive miRs. We found no significant association between rs2957924 (downstream of miR-132) or rs1292037 (downstream of miR-21) and STDR (or its subtypes PDR and DMO) in patients with T1DM or T2DM. Very few patients in this study expressed the minor allele at rs1547354 (downstream if miR-155) and this SNP could not be explored fully in a case-control analysis. None of the three SNPs tested were distributed in HWE in our pooled group of cases and controls. Given that all patients recruited had DM, it is possible that HWE may not be reflected for SNPs that

have differing frequencies in populations with DM. The occurrence of technical errors during genotyping must also be considered as an alternative explanation.

Despite the negative findings of this study, there is mounting evidence that theses miRs play a role in the development and progression of DR. MiR-21 is present at higher levels in the vitreous of patients with PDR compared with non-diabetic controls [230], and is also increased in the serum of patients with PDR compared with patients with NPDR [231]. There is also a consecutive increase of miR-155 in the serum of patients with DM as DR progresses from no DR, to NPDR, and finally to PDR [232]. Retinal endothelial cells of STZ induced diabetic rats have shown an upregulation of NF-κB responsive miRs (miR-146a, miR-155, miR-132, and miR-21), as well as VEGF responsive miRs [203]. Interestingly, miR-21 and miR-155 appear to be induced by both VEGF and NF-κB, which has been hypothesized to be due to the close relationship and interaction between these 2 factors [203]. Furthermore, inflammatory cytokines have been shown to directly increase miR-155 expression in the human retina [233].

A number of factors could have contributed to the lack of association found in this investigation. Firstly, the position of the chosen SNPs (although nearby their target miRs) may have no impact on the function or expression of these miRs. There are no reports in the literature of an association of the SNPs tested here with any disease state, an in fact they have been minimally investigated in general. The location of functional SNPs are more likely to be within the pri-miR transcript, or within the 5'-seed region affecting miR binding to its target gene [234]. For example, Thompson et al. [235] have mapped an NF-κB-responsive element approximately 178 bp upstream of the miR-155 transcription start site, which may be more likely to include SNPs with functional roles on miR-155 binding. Thirdly, miR target site polymorphisms can impact miR binding and function [234] and investigation of miR target sites in the NF-κB mRNA could be interesting. Lastly, the pathological role of these miRs may be due to modulation by other molecular or environmental changes related to hypoxia or hyperglycaemia, and may not be influenced by sequence variation at all.

Conclusion

We found no significant associations of 3 common SNPs near NF-κB responsive miRs, namely miR-155, miR-132 and miR-21, and STDR or DMO. Further exploration of these miRs is required to gain a better understanding on how they may impact on the development of DR.

CHAPTER 8

HYPOXIA AND ANGIOGENESIS: GENETIC RISK FOR STDR AND DMO

The VEGF family consists of VEGF-A, -B, -C and -D and placental growth factor, all of which are able to bind to different combinations of the three tyrosine kinase VEGF receptors (VEGFR-1, -2 and -3) [236] (**Figure 8.1**). VEGF-A, a ligand for VEGFR-2, is the best studied of these growth factors in relation to DR, and like other factors in the VEGF protein family, is upregulated in response to the hypoxic conditions of the diabetic retina. In non-proliferative stages of DR local VEGF-A production increases in areas of capillary non-perfusion, ultimately causing leakage of these retinal capillaries [35]. Studies of diabetic human vitreous have also shown that the balance between pro-angiogenic isoforms (VEGF-A_{xxx}) and anti-angiogenic isoforms (VEGF-A_{xxxb}), stemming from alternative exon splicing, changes to favour a pro-angiogenic environment [237]. This contributes to the later stages of DR where VEGF-A is known to promote proliferation of endothelial cells and has been implicated in the pathological growth of new vessels [238].

The molecular structure of the *VEGFA* gene allows for the effects of its key regulator, oxygen tension. Hypoxia increases *VEGFA* transcription through transcription factors that interact with the *VEGFA* core promoter as well as enhancers and repressors outside the promoter [239]. Of particular importance is the hypoxia response element (HRE), located 1000bp upstream from the transcription initiation site and the site where HIF-1 binds and contributes to *VEGFA* regulation [240,241]. The majority of positive findings associating genetic risk with the development of DR have so far been concentrated on SNPs within these *VEGFA* promoter and regulatory regions [242].

The highly polymorphic nature of *VEGFA* (resulting in multiple isoforms) is another important factor potentially allowing for functional changes of VEGF-A from gene polymorphisms [243]. A number of SNPs at the *VEGFA* locus have also been associated with the development of DR, including STDR and its subtype PDR [244]. To date, only one publication from the GSDC has shown a significant association between a *VEGFA* SNP and DMO [174]. Significance was reached in a combined T1DM and T2DM cohort, with lack of power in the smaller T1DM cohort (24 cases with DMO) being the predominant limitation for testing in the T1DM group alone. Furthermore, no association of *VEGFA* SNPs with STDR (n=76) in the T1DM group survived correction for multiple SNP testing in this study. With a significantly larger sample of T1DM patients (331 cases with STDR and 260 controls) in this thesis, we aimed to further investigate *VEGFA* SNPS in T1DM patients for association with STDR, PDR and DMO.

The therapeutic use of anti-VEGF-A antibodies has been shown to favourably influence the course of DR [43]. However, inhibition of VEGF-A does not appear to completely prevent the angiogenic process [245]. Investigation of additional factors in the VEGF-A pathway and their genetic regulation is important to provide further insights into pathogenesis and opportunities to improve therapy. VEGF-C functions in lymphangiogenesis through its activation of VEGFR-3, but it also shares a common downstream pathway

with VEGF-A by binding VEGFR-2; through which it contributes to the angiogenic process [236] (**Figure 8.1**). VEGF-C expression is markedly increased in retinal vessels of patients with DM, particularly in those with PDR [246]. To date there has been no investigation of genetic variation in the *VEGFC* gene in relation to DM microvascular complications.

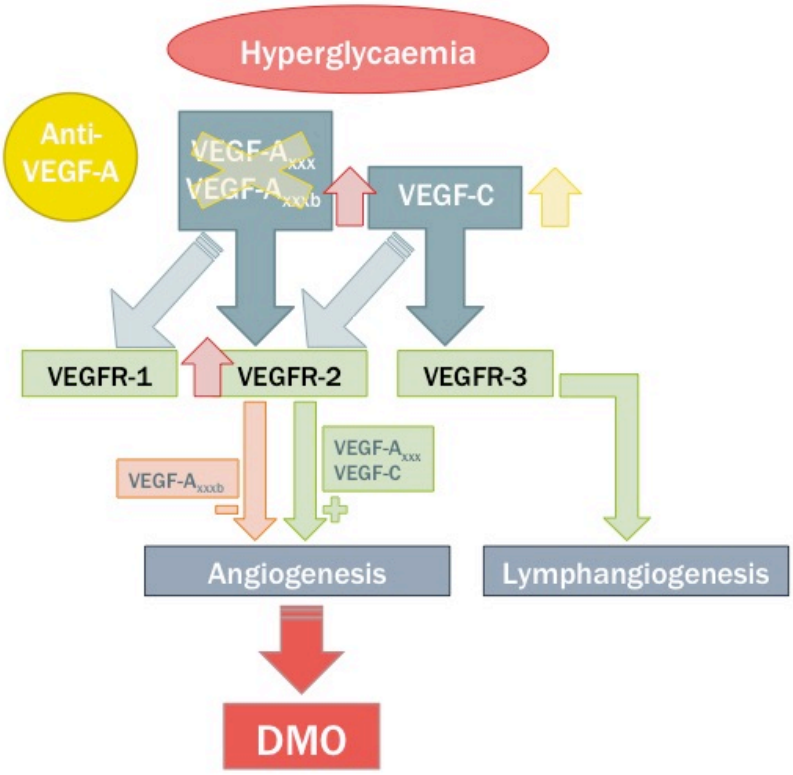


Figure 8.1: Common VEGF-A and VEGF-C pathway in the pathogenesis of DMO. Vascular endothelial growth factor (VEGF) –A (isoforms xxx and xxxb) and VEGF-C together bind a combination of 3 VEGF receptors (VEGFR-1, -2 and -3). Through the downstream effects of VEGFR-2 activation, VEGF-A_{xxx} and VEGF-C contribute to retinal angiogenesis. VEGF-A_{xxxb} is an anti-angiogenic isoform of VEGF-A that binds to, but is unable to fully activate VEGFR-2. In the diabetic human retina, *VEGFC, VEGFA* and *VEGFR-2* expression increase (pale red arrows). Inhibition of VEGF-A_{xxx} and VEGF-A_{xxxb} with anti-VEGF agents leads to enhanced VEGF-C secretion (yellow arrow) compensating for the reduced VEGF-A_{xxx} and VEGF-A_{xxxb} levels, and allowing for sustained activation of the VEGFR-2 pathway despite VEGF-A blockade.

HIFs are transcription factors that regulate many genes, including those promoting angiogenesis, anaerobic metabolism and resistance to apoptosis, in response to tissue hypoxia [37]. HIFs are heterodimers consisting of 2 subunits: an oxygen regulated HIF-α subunit, and a common HIF-1β subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT) [247]. In the presence of oxygen, HIF-α ubiquitination mediates its proteasomal degradation. However in the hypoxic state, HIF-α accumulates, dimerises with HIF-β, binds to DNA, and transcriptionally regulates hypoxia-responsive genes (**Figure 8.2**). Wright et al. [248] demonstrated that retinal HIF-2α levels are increased in the early stages of DM in the DM rat, before the onset of hypoxia. It has been proposed that in the hyperglycaemic state, HIFs have increased protection against proteasomal degradation, enhancing the normal cellular response to hypoxia [249].

VEGFA is one of the best characterized target genes of the HIFs, however they are also known to upregulate many other proangiogenic factors including platelet derived growth factor BB (PDGF-BB) in hypoxic retinal pigment epithelial cells, and VEGF-C in cancer cells [250]. HIF-1α and HIF-2α are the predominant mediators of the hypoxic response involving VEGF, each having specific temporal and functional roles [37]. HIF-1α is responsible for the initial upregulation of *VEGFA* in response to hypoxia (<24hrs) however in states of chronic hypoxia, hypoxia associated factor and heat shock protein 70 cause HIF-1α degradation and promote HIF-2α transactivation [37]. HIF-1α and HIF-2α are found at increased levels in the retina of rats with STZ induced DM [251], and in mice subject to hypoxia, where HIF levels correlate with retinal increase in VEGF-A expression [252]. The role of *HIF1A* has been studied in relation to other retinal diseases including age-related macular degeneration but genetic variation in neither *HIF1A* or *EPAS1* (encodes HIF-2a) genes have been studied in the context of DR.

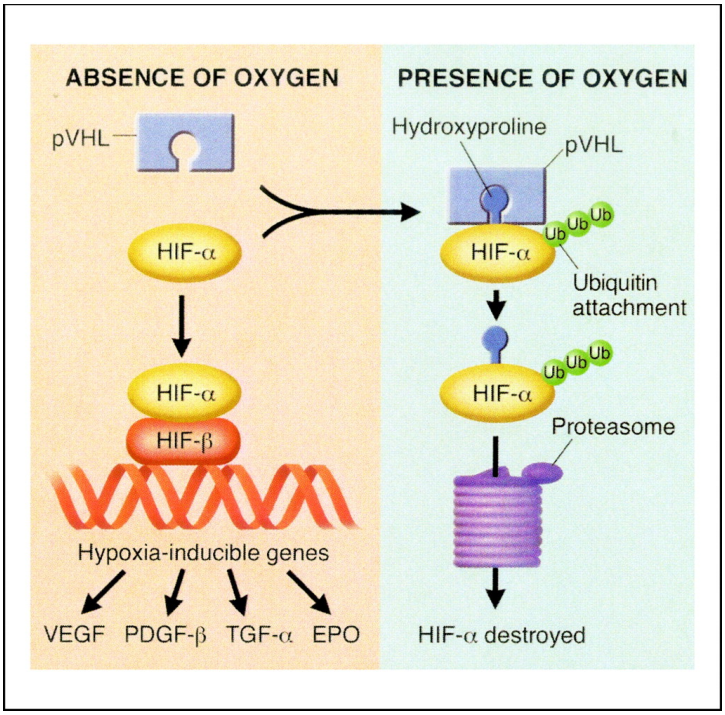


Figure 8.2: Post-translational modifications of HIF-α in hypoxic and normoxic conditions. In the absence of oxygen, HIF-α accumulates, dimerises with HIF-β, binds to DNA, and transcriptionally regulates hypoxia-responsive genes. In the presence of oxygen and iron, specific proline residues in HIF-α are hydroxylated, increasing the binding of the Von Hippel-Lindau (VHL) tumour suppressor protein. This targets HIF-α for ubiquitination and mediates its proteosmal degradation. (Figure from http://flipper.diff.org/app/pathways/1169)

This chapter explores genetic variation in the *VEGFA* gene in T1DM in an attempt to extend our previous work involving *VEGFA*. It also investigates genes in the hypoxia-induced angiogenesis pathway, both upstream of VEGFA (*HIF1A* and *EPAS1*) as well as *VEGFC* which binds the common VEGFR-2 receptor and thus influences the pathway downstream of VEGFA.

8.1 VASCULAR ENDOTHELIAL GROWTH FACTOR A

Aim

The GSDC, and other groups, have successfully investigated the effect of genetic variation within the *VEGFA* gene on STDR and DMO in T2DM patients and in combined cohorts of T1DM and T2DM patients. However, the GSDC cohort of T1DM participants was previously underpowered to detect an association between *VEGFA* SNPs and DMO. The current investigation aimed to specifically study *VEGFA* in a significantly larger cohort of patients with T1DM. Furthermore, the functional effect of the top ranked SNPs were measured by correlating genetic findings with serum VEGF-A concentrations in this T1DM patient group.

Results

Twenty-six tag SNPs (rs866236, rs833057, rs1547651, rs833058, rs699946, rs833060, rs699947, rs833061, rs3024987, rs833068, rs833070, rs3024994, rs2146323, rs3025000, rs3025007, rs3025020, rs3025030, rs3025035, rs10434, rs998584, rs6899540, rs6905288, rs9394964, rs879825, rs12204488, rs1885659) capturing 34 alleles reaching an r^2 threshold of 0.8 (mean $r^2 = 0.976$) were genotyped in participants with T1DM. Nyholt's SNP SpD method [186], modified by Li and Ji [187] estimated 18 independent tests for the 26 SNPs. P-value less than 0.003 was required for significance.

Association of VEGFA SNPs with STDR, PDR and DMO in T1DM patients:

Genotype frequencies in patients with STDR and controls are shown in **Table 8.1.1** for T1DM patients. A number of SNPs had HWE p-values less than 0.05, however only two of these SNPs (rs833057 and rs2146323) survived SNP SpD correction. Subsequent analyses presented for these two SNPs should be interpreted with caution. The remaining 24 SNPs were in HWE.

Two *VEGFA* SNPs (rs3025020 and rs1885659) were found to be associated with STDR in patients with T1DM, after adjustment for clinical covariates (**Table 8.1.2**). Of these, only rs3025020 remained statistically significant after correction for multiple SNP testing (OR, 2.08; CI, 1.39-3.10; P = 3x10-4). The minor allele (T) at rs3025020 was also significantly associated with both PDR (OR, 2.25; CI, 1.43-3.54; $P = 4x10^{-4}$) and DMO (OR, 2.24; CI, 1.38-3.64; P = 0.001) in multivariate analysis and after SNP SpD correction. A further 2 SNPs (rs10434 and rs2146323) were nominally associated with DMO in multivariate analysis but did not survive correction for multiple SNP testing.

Table 8.1.1: Genotype frequencies, shown as n (%) for STDR and controls with T1DM.

	SNP	Genotype	STDR n (%)	Controls n (%)	HWE P-value
1	rs866236	TT/TC/CC	19/110/200 (5.8/33.4/60.8)	18/101/140 (6.9/39.0/54.1)	**0.004**
2	rs833057	GG/GT/TT	12/45/179 (5.1/19.1/75.8)	4/49/108 (2.5/30.4/67.1)	**<0.001***
3	rs1547651	TT/TA/AA	9/95/226 (2.7/28.8/68.5)	13/66/177 (5.1/25.8/69.1)	**0.003**
4	rs833058	TT/TC/CC	41/149/137 (12.5/45.6/41.9)	42/107/110 (16.2/41.3/42.5)	0.341
5	rs699946	GG/GA/AA	12/96/212 (3.8/30.0/66.3)	9/77/166 (3.6/30.6/65.9)	0.131
6	rs833060	TT/TG/GG	25/102/122 (10.0/41.0/49.0)	22/107/95 (9.8/47.8/42.4)	0.267
7	rs699947	CC/CA/AA	68/152/92 (21.8/48.7/29.5)	66/122/66 (26.0/48.0/26.0)	0.154
8	rs833061	TT/TC/CC	72/157/94 (22.3/48.6/29.1)	66/124/67 (25.7/48.2/26.1)	0.406
9	rs3024987	TT/TC/CC	6/63/261 (1.8/19.1/79.1)	4/62/190 (1.6/24.2/74.2)	**0.038**
10	rs833068	AA/AG/GG	26/123/158 (8.5/40.1/51.5)	21/112/113 (8.5/45.5/45.9)	**0.013**
11	rs833070	GG/GA/AA	74/164/90 (22.6/50.0/27.4)	68/124/67 (26.3/47.9/25.9)	0.565
12	rs3024994	TT/TC/CC	1/26/302 (0.3/7.9/91.8)	0/16/243 (0.0/6.2/93.8)	0.831
13	rs2146323	AA/AC/CC	128/76/124 (39.0/23.2/37.8)	96/56/104 (37.5/21.9/40.6)	**<0.001***
14	rs3025000	TT/TC/CC	27/124/165 (8.5/39.2/52.2)	16/110/120 (6.5/44.7/48.8)	0.130
15	rs3025007	TT/TC/CC	72/154/93 (22.6/48.3/29.2)	46/135/66 (18.6/54.7/26.7)	**0.024**
16	rs3025020	TT/TC/CC	31/147/146 (9.6/45.4/45.1)	13/112/132 (5.1/43.6/51.4)	0.698
17	rs3025030	CC/CG/GG	7/77/238 (2.2/23.9/73.9)	8/52/194 (3.1/20.5/76.4)	0.300
18	rs3025035	TT/TC/CC	2/32/283 (0.6/10.1/89.3)	1/30/220 (0.4/12.0/87.6)	0.225
19	rs10434	AA/AG/GG	41/115/126 (14.5/40.8/44.7)	32/130/75 (13.5/54.9/31.6)	0.869
20	rs998584	GG/GT/TT	84/168/78 (25.5/50.9/23.6)	63/120/76 (24.3/46.3/29.3)	0.408
21	rs6899540	CC/CA/AA	8/90/224 (2.5/28.0/69.6)	6/54/196 (2.3/21.1/76.6)	0.372
22	rs6905288	GG/GA/AA	60/158/112 (18.2/47.9/33.9)	45/121/93 (17.4/46.7/35.9)	0.765
23	rs9394964	GG/GA/AA	2/46/273 (0.6/14.3/85.0)	0/38/218 (0.0/14.8/85.2)	0.555
24	rs879825	GG/GA/AA	3/48/278 (0.9/14.6/84.5)	0/39/216 (0.0/15.3/84.7)	0.237
25	rs12204488	TT/TC/CC	24/111/187 (7.5/34.5/58.1)	13/76/166 (5.1/29.8/65.1)	0.232
26	rs1885659	AA/AG/GG	18/120/190 (5.5/36.6/57.9)	18/94/144 (7.0/36.7/56.3)	0.319

Abbreviations: SNP, single nucleotide polymorphism; STDR, sight-threatening diabetic retinopathy; HWE, Hardy-Weinberg equilibrium. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal non-proliferative diabetic retinopathy. SNPs with HWE $P < 0.05$ are shown in bold. *SNPs that remained statistically significant for HWE testing after correction for multiple SNPs using SNP SpD correction ($P < 0.003$).

Table 8.1.2: Allelic association of *VEGFA* Tag SNPs with STDR, PDR and DMO, for patients with T1DM. Results from multivariate analysis are presented. Uncorrected P-values are shown.

		STDR		PDR		DMO	
SNP	**Minor allele**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI**	**P value**
rs866236	T	0.97 (0.66-1.44)	0.881	0.78 (0.50-1.21)	0.263	1.09 (0.67-1.75)	0.732
rs833057	G	0.87 (0.50-1.53)	0.635	0.77 (0.42-1.39)	0.384	0.91 (0.44-1.89)	0.796
rs1547651	T	1.13 (0.74-1.75)	0.572	0.91 (0.55-1.51)	0.719	1.34 (0.81-2.23)	0.260
rs833058	T	0.82 (0.58-1.15)	0.249	0.89 (0.61-1.29)	0.536	0.81 (0.53-1.24)	0.333
rs699946	G	0.84 (0.54-1.30)	0.423	0.97 (0.60-1.56)	0.891	0.92 (0.54-1.59)	0.767
rs833060	T	0.87 (0.57-1.32)	0.506	0.94 (0.58-1.53)	0.815	0.79 (0.47-1.32)	0.365
rs699947	C	0.80 (0.58-1.12)	0.195	0.84 (0.59-1.22)	0.364	0.81 (0.52-1.24)	0.328
rs833061	T	0.83 (0.59-1.15)	0.255	0.88 (0.61-1.26)	0.483	0.85 (0.56-1.30)	0.460
rs3024987	T	0.71 (0.42-1.23)	0.222	0.68 (0.37-1.23)	0.200	0.77 (0.41-1.44)	0.409
rs833068	A	0.86 (0.59-1.25)	0.423	0.88 (0.58-1.34)	0.552	0.91 (0.57-1.45)	0.687
rs833070	G	0.74 (0.53-1.04)	0.084	0.78 (0.54-1.13)	0.189	0.76 (0.49-1.16)	0.201
rs3024994	T	1.14 (0.47-2.75)	0.778	1.18 (0.46-3.04)	0.737	1.00 (0.33-3.09)	0.994
rs2146323	A	1.16 (0.89-1.52)	0.268	1.05 (0.78-1.41)	0.756	1.44 (1.03-2.01)	**0.032**
rs3025000	T	0.76 (0.52-1.12)	0.170	0.83 (0.54-1.27)	0.385	0.81 (0.50-1.30)	0.382
rs3025007	T	0.99 (0.71-1.40)	0.977	1.09 (0.74-1.61)	0.647	0.97 (0.63-1.49)	0.890
rs3025020	T	2.08 (1.39-3.10)	**0.0003 ***	2.25 (1.43-3.54)	**0.0004 ***	2.24 (1.38-3.64)	**0.001***
rs3025030	C	0.89 (0.55-1.46)	0.656	1.00 (0.59-1.71)	0.994	1.05 (0.58-1.88)	0.877
rs3025035	T	0.80 (0.41-1.56)	0.505	0.58 (0.26-1.29)	0.181	1.25 (0.58-2.68)	0.573
rs10434	A	0.69 (0.47-1.02)	0.061	0.74 (0.49-1.13)	0.162	0.61 (0.37-1.00)	**0.048**
rs998584	G	0.89 (0.64-1.23)	0.484	0.89 (0.62-1.28)	0.531	0.91 (0.61-1.36)	0.651
rs6899540	C	1.22 (0.77-1.92)	0.402	1.15 (0.69-1.92)	0.587	1.27 (0.72-2.23)	0.412
rs6905288	G	0.82 (0.59-1.15)	0.249	0.84 (0.58-1.22)	0.357	0.84 (0.55-1.27)	0.398
rs9394964	G	1.02 (0.54-1.90)	0.960	1.13 (0.58-2.20)	0.718	0.78 (0.32-1.90)	0.586
rs879825	G	0.89 (0.49-1.64)	0.718	1.05 (0.55-2.00)	0.880	0.68 (0.28-1.62)	0.382
rs12204488	T	1.27 (0.88-1.83)	0.209	1.36 (0.90-2.05)	0.141	1.18 (0.74-1.88)	0.486
rs1885659	A	0.62 (0.41-0.94)	**0.023**	0.61 (0.38-0.96)	**0.034**	0.67 (0.40-1.11)	0.116

Abbreviations: SNP, single nucleotide polymorphism; STDR, sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; SNP, single nucleotide polymorphism; OR (95%CI), odds ratio with 95% confidence interval. STDR defined as severe non-proliferative diabetic retinopathy (NPDR) or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. [#]Adjusted for age, sex, duration DM, HbA1c, hypertension, DN, smoking. P-values < 0.05 are highlighted in bold. *Significant association following correction for multiple SNPs using SNP SpD correction (P < 0.003).

Association of VEGFA haplotypes with STDR in T1DM patients:

The linkage disequilibrium pattern between SNPs is presented in **Figure 8.1.1.** Three main blocks of linkage disequilibrium were observed; block 1 comprising SNPs 3 to 12, block 2 comprising SNPs 16 to 19 and block 3 comprising SNPs 20 to 26. Haplotypes from each block were analysed for association with STDR, in participants with T1DM (**Table 8.1.3**). Following adjustment for clinical covariates, there were two haplotypes found to be associated with STDR; haplotype 6 of block 2 (TGCG) (OR, 2.09; CI, 1.38-3.18; P = $6x10^{-4}$), and haplotype 3 of block 3 (GAGAACA) (OR, 0.59; CI, 0.39-0.89; P = 0.013). The block 2 TGCG haplotype was the only one that survived Bonferroni correction for the haplotypes in each block.

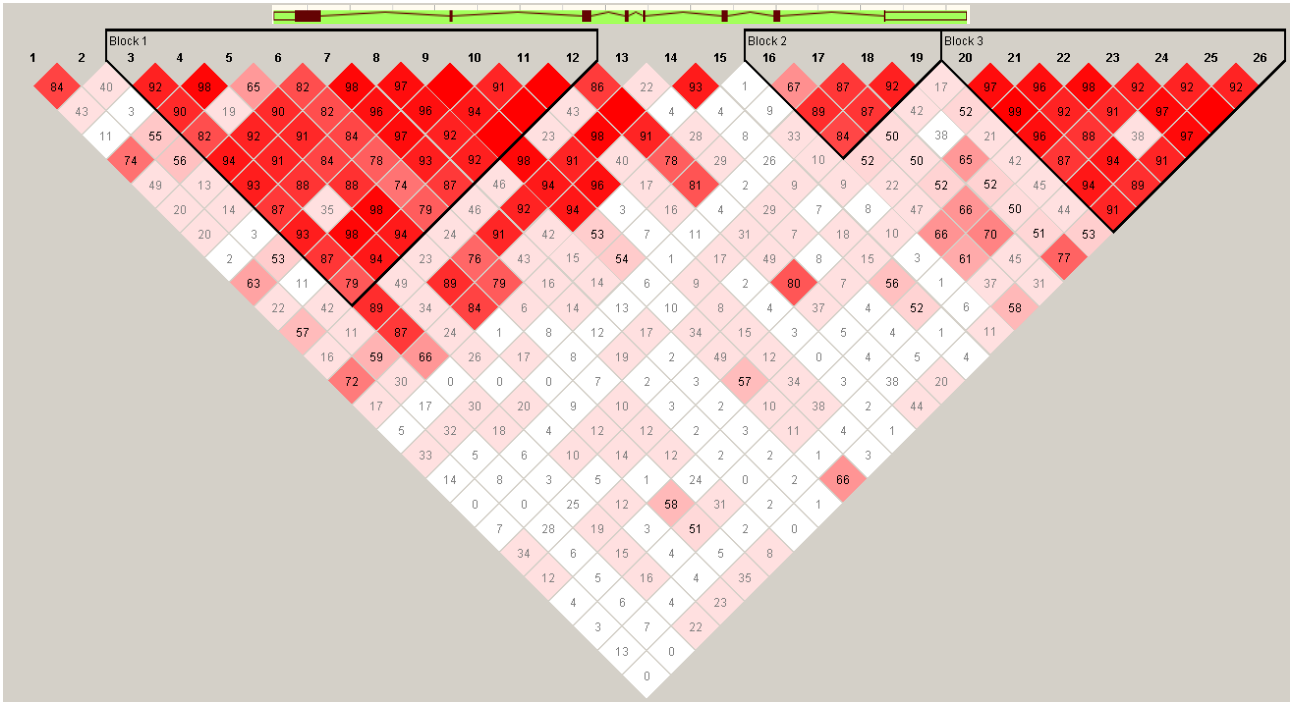


Figure 8.1.1: Linkage disequilibrium between genotyped tag SNPs in and adjacent to the *VEGFA* gene. SNPs are numbered as per Table 10.1. The D' value for each pair of SNPs is given, multiplied by 100. A blank cell indicates D'=1.0. *VEGFA* gene schematic included above linkage disequilibrium plot.

Table 8.1.3: Haplotype associations adjusted for clinical covariates (including age, sex, duration DM, HbA1c, hypertension, DN and smoking) with STDR for participants with T1DM. SNPs are numbered as in Table 8.1.1. Uncorrected P-values are shown.

Haplotype	Alleles										Haplotype freq.	Control freq.	STDR freq.	OR (95%CI)	P-value
Block 1	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**					0.642
1	A	T	G	T	C	T	C	A	G	C	0.128	0.148	0.146	1.07 (0.65-1.76)	0.805
2	A	C	A	G	A	C	C	G	A	C	0.310	0.311	0.357	1.29 (0.90-1.86)	0.169
3	A	T	G	G	C	T	C	A	G	C	0.035	0.033	0.037	0.58 (0.20-1.69)	0.316
4	A	C	A	T	A	C	C	G	A	C	0.029	0.032	0.033	0.50 (0.13-1.96)	0.321
5	A	C	A	T	C	T	C	A	G	C	0.109	0.133	0.112	0.80 (0.49-1.30)	0.370
6	T	C	A	G	A	C	C	G	A	C	0.147	0.172	0.167	1.23 (0.78-1.94)	0.380
7	A	T	A	G	C	T	C	G	G	T	0.030	0.027	0.034	1.01 (0.35-2.88)	0.988
8	A	T	A	G	C	T	T	G	G	C	0.106	0.129	0.108	0.75 (0.42-1.34)	0.328
9	A	T	A	G	C	T	C	G	G	C	0.011	0.015	0.008	1.51 (0.28-8.24)	0.636
Block 2	**16**	**17**	**18**	**19**											**0.036**
1	T	G	C	A							0.014	0.011	0.020	2.17 (0.30-16.00)	0.447
2	C	G	C	A							0.373	0.408	0.355	0.71 (0.49-1.03)	0.074
3	C	G	T	G							0.057	0.064	0.052	0.76 (0.38-1.51)	0.428
4	T	C	C	G							0.013	0.010	0.012	1.10 (0.12-10.20)	0.934
5	C	C	C	G							0.122	0.116	0.125	0.92 (0.53-1.58)	0.755
6	T	G	C	G							0.273	0.251	0.288	2.09 (1.38-3.18)	**0.0006***
7	C	G	C	G							0.140	0.141	0.148	0.60 (0.32-1.12)	0.110
Block 3	**20**	**21**	**22**	**23**	**24**	**25**	**26**								0.158
1	T	A	A	A	A	C	G				0.484	0.533	0.493	1.10 (0.80-1.53)	0.553
2	G	A	A	G	G	T	G				0.068	0.066	0.076	1.10 (0.58-2.10)	0.778
3	G	A	G	A	A	C	A				0.232	0.259	0.233	0.59 (0.39-0.89)	**0.013**
4	G	A	G	A	A	C	G				0.027	0.020	0.039	1.37 (0.54-3.48)	0.504
5	G	C	G	A	A	T	G				0.136	0.122	0.159	1.14 (0.72-1.82)	0.571

Abbreviations: STDR, Sight-threatening diabetic retinopathy; OR (95%CI), odds ratio with 95% confidence interval. STDR defined as severe non-proliferative diabetic retinopathy (NPDR) or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. P-values < 0.05 are highlighted in bold. *Haplotypes that remained statistically significant after Bonferroni correction for multiple haplotype testing (Block 1: P < 0.006; Block 2: P < 0.007; Block 3: P < 0.01).

Serum VEGF-A concentration in patients with T1DM:

Serum was available for a total of 180 patients with T1DM that had been genotyped for this analysis. Eighty of these had STDR and 100 were controls. Of the 80 T1DM cases with STDR, 56 had PDR and 44 had DMO. Serum concentration of VEGF-A was measured by quantitative ELISA as described previously in the methods (Chapter 6). Only those samples with a CV of less than 20% were included in this analysis. Serum concentration of VEGF-A in cases and controls were compared using Mann-Whitney U test.

Patients with T1DM and STDR (mean, 280.64 pg/ml; SD, 213.50 pg/ml) were found to have significantly higher serum VEGF-A concentrations than controls (mean, 252.07 pg/ml; SD, 371.97 pg/ml; P = 0.004) (**Table 8.1.4**). A higher VEGF-A serum concentration was also seen in cases with PDR (mean, 280.98 pg/ml; SD 178.23 pg/ml) compared with controls (P = 0.006). The subgroup of patients with DMO, were found to have lower VEGF-A serum concentrations than controls (mean 235.55 pg/ml; SD, 116.7 pg/ml; P = 0.031). **Figure 8.1.2** shows the spread of VEGF-A serum concentrations in cases and controls. This illustrates the large variation in serum concentration, in both case and control groups.

Table 8.1.4: VEGF-A serum concentration in T1DM cases and controls.

	Case Serum VEGF-A Concentration (pg/ml)		Control Serum VEGF-A Concentration (pg/ml)		
	n	**Mean (SD)**	**n**	**Mean (SD)**	**P-value**
STDR	69	280.64 (213.50)			**0.004**
PDR	47	280.98 (178.23)	79	252.07 (371.97)	**0.006**
DMO	41	235.55 (116.7)			**0.031**

Abbreviations: pg/ml, picograms per millilitre; STDR, Sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema. STDR defined as severe non-proliferative diabetic retinopathy (NPDR) or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. P-values < 0.05 are highlighted in bold.

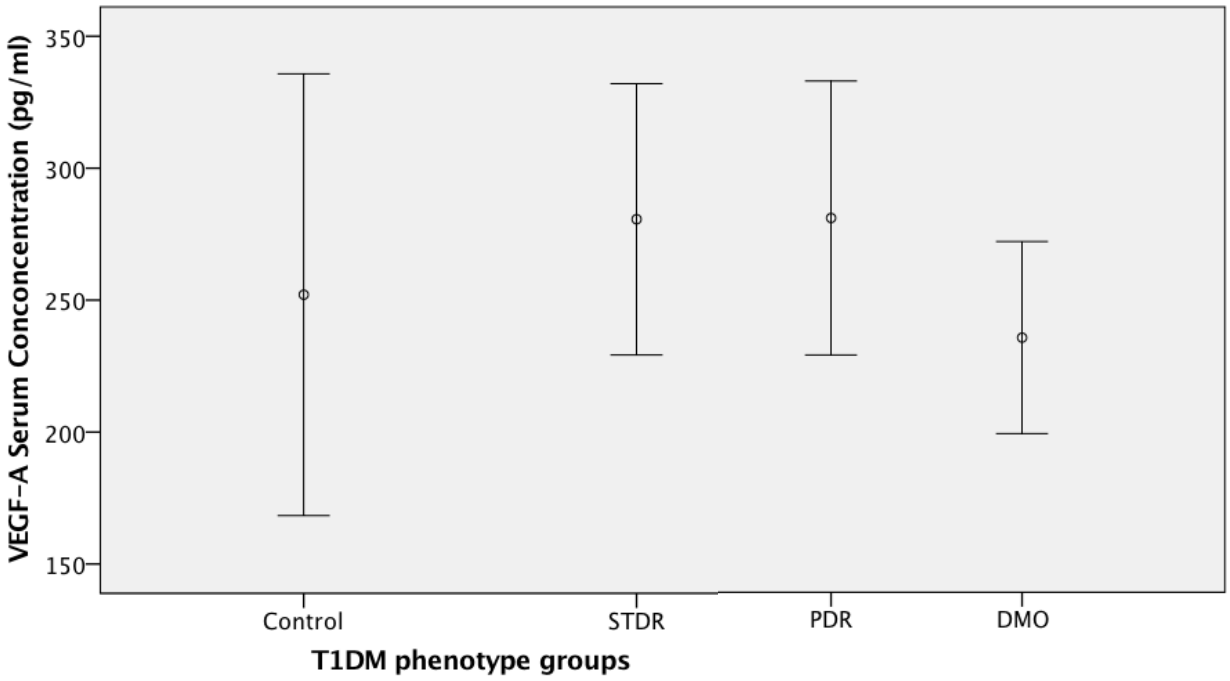


Figure 8.1.2: Graph showing the range of VEGF-A serum concentrations within 2 standard errors (T bars) of the mean (central circles) in patients with T1DM. Cases with STDR as well as subgroups with PDR and DMO are shown next to the control group.

A subset of the patients with PDR were known to have concomitant DMO. Given the opposing trends of serum VEGF-A levels in those with PDR compared with DMO, the PDR group was also tested excluding those patients who also had a history of DMO. This analysis found that the mean VEGF-A concentration in the PDR only group was 315.59 pg/ml (SD 214.11 pg/ml), which was significantly greater than the control group (P = 0.019). Levels of VEGF-A in the PDR only group compared with the control group is illustrated in **Figure 8.1.3**.

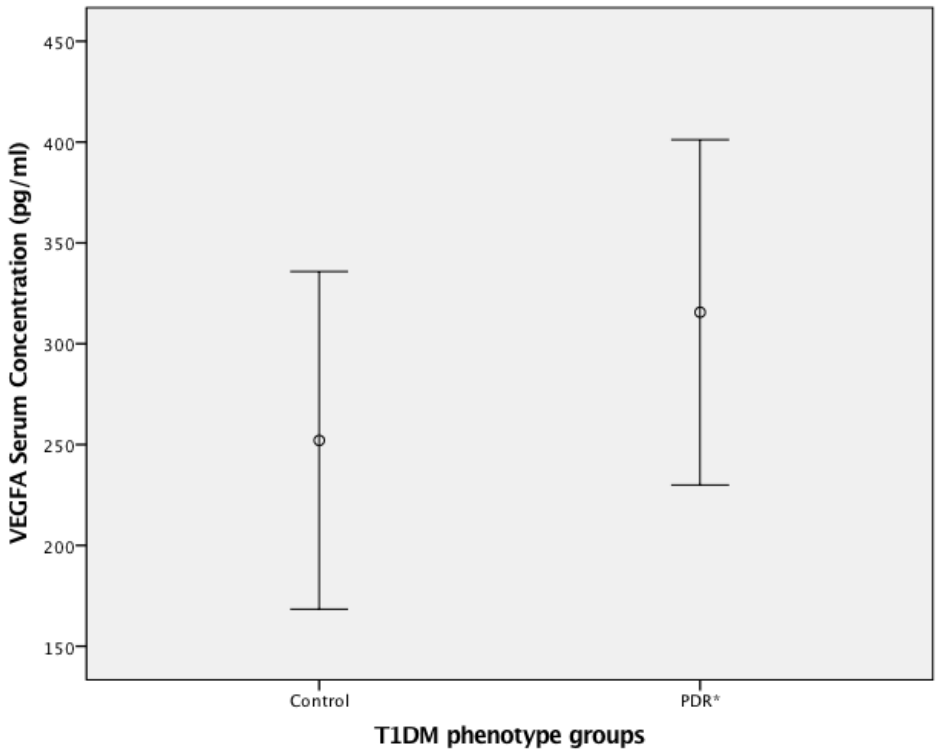


Figure 8.1.3: Graph showing the range of VEGF-A serum concentrations within 2 standard errors (T bars) of the mean (central circles) in patients with T1DM. Cases with PDR (with no history of DMO) are shown next to the control group.

The top SNP (rs3025020) found to be associated with STDR, PDR and DMO in this T1DM cohort was further investigated to determine its effect on VEGF-A serum concentration in these patients (**Table 8.1.5**). In a multivariate analysis adjusting for basic clinical covariates (including age, sex, duration of DM, HbA1c, hypertension, DN and smoking) as well as DMO status, the T allele at rs3025020 was significantly associated with increased serum VEGF-A (P = 0.036). When adjusting for basic clinical covariates as well as STDR or PDR status, no significant association was found. Haplotype analysis of block 2 (which contains rs3025020) showed similar results for the haplotype TGCG (haplotype 6 of block 2). This haplotype was associated with VEGF-A serum concentration in a multivariate analysis after adjustment for basic clinical covariates as well as DMO status (P = 0.011) but not after adjustment for PDR (P = 0.077) or STDR (P = 0.276) status.

Table 8.1.5: Multivariate SNP association and haplotype association for the top ranked SNP and haplotype with serum VEGF-A concentration in the T1DM cohort. All analyses are adjusted for basic clinical covariates (age, sex, duration DM, HbA1c, hypertension, DN and smoking) as well as the presence of either STDR, PDR or DMO as indicated.

	Adjusted for STDR			Adjusted for PDR			Adjusted for DMO		
SNP	**Beta**	**Stat.**	**P value**	**Beta**	**Stat.**	**P value**	**Beta**	**Stat.**	**P value**
rs3025020	0.07[#]	0.79	0.428	0.14[#]	1.45	0.150	0.21[#]	2.12	**0.036**
16-19 (TGCG)	57.12	1.21	0.276	89.07	3.20	0.077	132.10	6.82	**0.011**

Abbreviations: STDR, Sight-threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; SNP, single nucleotide polymorphism; Stat., Wald test statistic. STDR defined as severe non-proliferative diabetic retinopathy (NPDR) or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. [#]Standardised Beta regression coefficient. P-values < 0.05 are highlighted in bold.

Discussion

This analysis found the T allele at rs3025020 to predict a two-fold increase in risk in the development of PDR and DMO in patients with T1DM, after adjusting for clinical covariates. This finding was supported by both the independent SNP analysis as well as the haplotype analysis in which the T allele at rs3025020 contributed to the risk haplotype. These results remained positive following stringent correction for multiple SNPs, in an attempt to reduce false positive results. This is the first study to report a significant SNP association in patients with T1DM and DMO after multiple SNP correction.

The previous report from GSDC involving a significantly smaller sample size, identified rs833086 to be associated with PDR and DMO in patients with T1DM prior to correction for multiple SNPs [174]. This result was unable to be replicated in the current, larger study and may have represented a false positive result from the previous analysis. Another SNP, rs10434 was previously found to be associated with DMO after correction for multiple SNPs in the combined T1DM and T2DM cohort [174]. This SNP was nominally associated with DMO in the current cohort of patients with T1DM, but did not resist multiple testing correction. The relationship between rs10434 and DMO in patients with T1DM is unclear from these results. Rs3025021 was significantly associated with increased STDR risk in the T2DM GSDC cohort, and did survive multiple SNP testing [174]. This SNP was unable to be typed in the current experiment. However, rs3025021 and rs3025020 are known to be in strong linkage disequilibrium (LD), with a D' value of 0.99. This potentially implicates a third SNP, also in high LD with these tag SNPs, in the development of STDR in both T1DM and T2DM. Al Kateb et al. [253] found that rs3025021 (P = 0.002) and rs3025020 (P = 0.005) were associated with time to severe retinopathy in a multivariate analysis in a large cohort of patients with T1DM from the DCCT study. Rs3025021 however, was the only SNP to survive Bonferroni correction in that study [253]. Further studies are required to investigate the relationship between these SNPs and determine their role in the pathogenesis of STDR and DMO in patients with T1DM.

Rs3025020 is located at intron 6 of the *VEGFA* transcript. The functional effects of this SNP on VEGF-A structure and function are currently unknown. However, it can be postulated that *VEGFA* introns may play a role in alternative splicing or contain transcription factor binding sites in the regulation of *VEGFA*

expression. Alternative splicing of exons 6, 7 and 8 generate various VEGF isoforms, of which $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$ were amongst the first to be discovered [254]. An alternative exon 8 (originally named exon 9) was later discovered by Bates et al., who noted that when $VEGF_{165}$ contained the alternative exon 8 ($VEGF_{165b}$) it was able to inhibit vascular proliferation mediated by $VEGF_{165}$ [255]. VEGF is now considered to have 2 families of isoforms, pro-angiogenic isoforms denoted $VEGF_{xxxa}$, and anti-angiogenic isoforms, $VEGF_{xxxb}$. Intraocular administration of $VEGF_{165b}$ in a mouse model of oxygen induced retinopathy is able to reduce pre-retinal neovascularisation indicating that anti-angiogenic splice variants have therapeutic potential for proliferative eye diseases including DR [256]. Splicing factors regulate alternative splicing of exon 8 by binding exonic splice enhancer sites proximal and distal to the splice site [257]. However, the exonic splice enhancer sites surrounding the exon 8 splice site are highly conserved and no nearby SNPs have been implicated in the development of PDR or neovascular age-related macular degeneration to date [258].

Multiple studies have investigated the effect of VEGFA polymorphisms on serum VEGF-A levels. Al-Habboubi et al. [259] investigated VEGF-A serum levels in relation to *VEGFA* genotypes in a healthy cohort of Bahraini individuals. The MAF of *VEGFA* SNPs in this population were comparable to those established for Caucasian populations. The TT genotype of rs3025020 was associated with increased serum VEGF-A in this study. Similar findings were also reported in 3 remote villages in Italy [260]. Conversely, Al-Khateeb et al. found that increased minor allele (T) and genotype frequencies of rs3025020 correlated with reduced serum VEGF concentrations [261]. This finding was examined in the context of recurrent spontaneous miscarriage where rs3025020 was associated with increased disease risk. This suggests that rs3025020 may have differing effects on circulating VEGF-A in certain disease states or in response to local stimuli (eg. hypoxia) compared with healthy controls. This may help to explain the significantly lower levels of VEGF-A found in patients with DMO compared with controls in the current study.

The use of anti-VEGF treatment may also explain the lower serum VEGF-A level found in our DMO group. Ma et al. [262] investigated vitreous and plasma VEGF-A levels in patients with PDR undergoing vitrectomy. They found that those patients who had not been administered intravitreal bevacizumab pre-operatively had the highest VEGF-A levels in both vitreous and plasma compared with patients who had received bevacizumab pre-operatively. Those given bevacizumab within 7 days of vitrectomy (mean 4 days) had lower VEGF-A levels in both vitreous and plasma than those given bevacizumab greater than 7 days (mean 35 days) prior to surgery. The number of patients with DMO in our cohort who had received intravitreal anti-VEGF treatment prior to venipuncture was not recorded and therefore could not be adjusted for in our analysis. Our finding needs to be interpreted in light of this potential influence. Results from our SNP analysis support this theory, as rs3025020 and the TGCG haplotype were significantly associated with higher serum VEGF-A levels in the model accounting for DMO status only.

A GWAS of circulating VEGF-A levels in 3527 Caucasian individuals found that genetic variants associated with increased serum VEGF-A reaching genome-wide significance were located downstream from the *VEGFA* gene (on chromosome 6) or within introns of *ZFPM2* (zinc finger protein multitype 2 gene on

chromosome 8) and *VLDLR* (very low density lipoprotein receptor on chromosome 9) [263]. This region on chromosome 6, downstream from *VEGFA* could contain functional SNPs that alter *VEGFA* expression. The *ZFPM2* gene encodes a member of the GATA transcription factor family, and the *VLDLR* gene is thought to contribute to the network of genes activated in response to hypoxia [263]. These variants were able to explain half of the phenotypic variation in circulating VEGF-A levels, and are therefore worth exploring in patients with STDR and DMO in future. This GWAS was also able to replicate 2 previously reported associations within the VEGFA promoter (rs699947 and rs833061) at $P < 5x10^{-7}$, however these did not reach genome wide significance [263]. Neither of these promoter variants were found to be associated with STDR or its subtypes in our cohort of patients with T1DM.

Despite significant results found here, the translation of serum VEGF-A levels from this study towards clinical utility are somewhat limited. Although the mean VEGF-A concentration differed between cases and controls, there was wide variation in the serum concentration measured in both case and control groups. The variation in the control group was particularly noteworthy. This is also common to other studies reporting serum VEGF-A levels and may reflect a large variation in VEGF-A in the normal population [263]. This means that there was no obvious level of VEGF-A in the serum, above which could be thought of as being associated with either STDR, or the rs3025020 variant. It must also be noted that a significant proportion of VEGF-A levels measured in case and control samples in the current study were towards the lower end of the detection capabilities of the ELISA kit. This floor effect may have affected the accuracy of the serum concentrations found to be in this lower range. Another important limitation of this study involved the measurement of total serum VEGF-A levels without comparing the relative levels of pro- and anti-angiogenic VEGF-A isoforms. The R&D DuoSet^{TM} ELISA kit for human VEGF-A used measures rhVEGF$_{165a}$, VEGF$_{165b}$, and VEGF$_{121}$. Although total VEGF-A levels were found to vary between case and control groups, and influenced by genetic variation, the angiogenic potential of VEGF-A between groups and genotypes could not be determined. Determining the levels of different isoforms requires the use of isoform specific quantification methods and appropriate controls as described by Bates et al. [264], and should be considered for future studies.

Conclusion

In conclusion, this study adds to the current literature surrounding *VEGFA* polymorphisms and DR by reporting the first SNP (rs3025020) associated with DMO in patients with T1DM. This SNP was also found to be associated with increased serum VEGF-A levels after accounting for DMO status, allowing for a functional explanation for this risk genotype. However, the angiogenic potential of VEGF-A is highly dependent on the presence of pro-angiogenic isoforms, which is independent of the total VEGF-A level. This was not investigated in the current study, and significantly limits the impact of the conclusions drawn here.

8.2 VASCULAR ENDOTHELIAL GROWTH FACTOR C

The original work presented in this section has been published in the peer-reviewed literature: G Kaidonis, KP Burdon, MC Gillies, S Abhary, RW Essex, JH Chang, B Pal, M Pefkianaki, M Daniell, S Lake, N Petrovsky, AW Hewitt, A Jenkins, EL Lamoureux, JM Gleadle and JE Craig. Common sequence variation in the VEGFC gene is associated with diabetic retinopathy and diabetic macular oedema in Caucasian patients. ***Ophthalmology 2015, Vol. 122(9), pp.1828-36****.* [265]. *Dr Kaidonis' contributions include study design, patient recruitment, sample preparation, data analysis and interpretation and manuscript preparation.*

Aim

Through binding VEGFR-2, VEGF-C is able to activate the same downstream angiogenic pathways as VEGF-A, making it an attractive DR susceptibility gene candidate. This study aimed to determine whether tag SNPs in the *VEGFC* gene are associated with DR in this large, well characterized, Caucasian cohort of T1DM and T2DM participants described previously.

Results

Thirteen tag SNPs (rs17697305, rs2046462, rs17697419, rs7664413, rs6828869, rs17697515, rs1485766, rs11947611, rs9654285, rs3775195, rs3775194, rs1564922, rs2333526) capturing 104 alleles reaching an r^2 threshold of 0.8 (mean r^2 = 0.973) were genotyped in individuals with T1DM and T2DM. One SNP, rs1564922, did not type successfully and was excluded from all subsequent analyses. All 12 successfully typed SNPs were in HWE (**Table 8.2.1**). Nyholt's SNP SpD method [186], modified by Li and Ji [187] estimated nine independent tests for the 12 genotyped SNPs. P-value less than 0.006 was required for significance.

Association of VEGFC SNPs with 'Any DR':

Genotype frequencies in patients with and without DR are shown in **Table 8.2.1** for both T1DM and T2DM participants. 'Any DR' was nominally associated with 5 *VEGFC* SNPs when populations with T1DM or T2DM were combined. Three of these (rs17697419, rs17697515 and rs2333526) survived multiple testing correction for 9 independent tests (P < 0.006, **Table 8.2.3**). These top three SNPs also showed significant associations with 'Any DR' following adjustment for clinical covariates (DM type, age, sex, duration of DM, HbA1c, hypertension, DN and smoking): rs17697419: OR, 0.67; CI, 0.52-0.85; P = 0.001; rs17697515: OR, 0.62; CI, 0.47-0.81; P = 0.001; and rs2333526: OR, 0.69; CI, 0.54-0.09; P = 0.005.

Analyses stratified by DM type showed similar trends (**Table 8.2.2**). After adjustment for clinical covariates, the top three SNPs identified from the combined analysis (rs17697419, rs17697515 and rs2333526) reached significance in the T2DM cohort, which included 852 participants with 'Any DR' (rs17697419: P = 0.003; OR, 0.65; CI, 0.49-0.86; rs17697515: P = 0.003; OR, 0.62; CI, 0.45-0.85; and rs2333526: P = 0.003; OR, 0.65; CI, 0.49-0.86). Rs17697419 and rs17697515 showed similar odds ratios in the T1DM cohort after

adjustment for clinical covariates ('Any DR' n=345), however statistical significance was not reached. Rs6828869 was found to be nominally associated with 'Any DR' in the TIDM group following multivariate analysis (OR, 0.69; CI, 0.49-0.97; P = 0.035), but did not survive SNP SpD correction for multiple SNP testing.

Table 8.2.1: Genotype frequencies, shown as n (%) for 'No DR' and 'Any DR' by DM type.

			T1DM		T2DM		
	SNP	**Genotype**	**No DR n (%)**	**Any DR n (%)**	**No DR n (%)**	**Any DR n (%)**	**HWE P-value**
1	rs2046462	CC/CT/TT	21/79/104 (10.3/38.7/51.0)	34/186/240 (7.4/40.4/52.2)	73/338/356 (9.5/44.1/46.4)	136/561/739 (9.5/39.1/51.5)	0.181
2	rs17697305	CC/CT/TT	1/12/134 (0.7/8.2/91.2)	1/31/340 (0.3/8.3/91.4)	1/44/592 (0.2/6.9/92.9)	1/78/1242 (0.1/5.9/94.0)	0.381
3	rs17697419	AA/AG/GG	3/43/149 (1.5/22.1/76.4)	2/88/365 (0.4/19.3/80.2)	11/144/593 (1.5/19.3/79.3)	13/205/1182 (0.9/14.6/84.4)	0.521
4	rs7664413	TT/TC/CC	6/57/141 (2.9/27.9/69.1)	14/131/321 (3.0/28.1/68.9)	31/231/508 (4.0/30.0/66.0)	63/438/943 (4.4/30.3/65.3)	0.269
5	rs6828869	CC/CG/GG	41/110/52 (20.2/54.2/25.6)	82/222/161 (17.6/47.7/34.6)	157/381/232 (20.4/49.5/30.1)	293/662/481 (20.4/46.1/33.5)	0.134
6	rs17697515	TT/TC/CC	2/38/163 (1.0/18.7/80.3)	1/64/397 (0.2/13.9/85.9)	3/123/643 (0.4/16.0/83.6)	5/171/1260 (0.3/11.9/87.7)	0.275
7	rs1485766	CC/CA/AA	42/104/57 (20.7/51.2/28.1)	113/213/134 (24.6/46.3/29.1)	171/393/202 (22.3/51.3/26.4)	370/701/353 (26.0/49.2/24.8)	0.971
8	rs11947611	AA/AG/GG	38/71/41 (25.3/47.3/27.3)	101/170/102 (27.1/45.6/27.3)	156/320/170 (24.1/49.5/26.3)	295/673/365 (22.1/50.5/27.4)	0.670
9	rs9654285	TT/TA/AA	2/44/155 (1.0/21.9/77.1)	7/94/363 (1.5/20.3/78.2)	10/174/578 (1.3/22.8/75.9)	13/277/1146 (0.9/19.3/79.8)	0.591
10	rs3775195	AA/AC/CC	16/86/98 (8.0/43.0/49.0)	36/172/255 (7.8/37.1/55.1)	62/293/410 (8.1/38.3/53.6)	115/538/784 (8.0/37.4/54.6)	**0.036**
11	rs3775194	GG/GC/CC	36/97/70 (17.7/47.8/34.5)	73/216/176 (15.7/46.5/37.8)	129/368/272 (16.8/47.9/35.4)	228/648/564 (15.8/45.0/39.2)	**0.035**
12	rs2333526	TT/TC/CC	1/34/166 (0.5/16.9/82.6)	1/77/386 (0.2/16.6/83.2)	10/141/611 (1.3/18.5/80.2)	9/206/1222 (0.6/14.3/85.0)	1.000

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg Equilibrium. Any DR defined as any non-proliferative diabetic retinopathy or proliferative diabetic retinopathy, and/or diabetic macular oedema. Controls (No DR) defined as no diabetic retinopathy or diabetic macular oedema. SNPs with HWE P < 0.05 are shown in bold. *SNPs that remained statistically significant for HWE testing after correction for multiple SNPs using SNP SpD correction (P < 0.006).

Table 8.2.2: Allelic association of *VEGF-C* Tag SNPs with 'Any DR' (versus 'no DR') for all cases combined, as well as T1DM and T2DM groups individually. Results for both univariate and multivariate analyses are presented. Uncorrected P-values are shown.

		All DM				T1DM				T2DM			
		Univariate		Multivariate[#]		Univariate		Multivariate[#]		Univariate		Multivariate[#]	
SNP	**Minor allele**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
rs2046462	C	0.88 (0.79-0.99)	**0.040**	0.86 (0.74-1.01)	0.067	0.91 (0.70-1.17)	0.444	0.92 (0.63-1.32)	0.641	0.89 (0.77-1.01)	0.078	0.87 (0.73-1.03)	0.103
rs17697305	C	0.87 (0.63-1.19)	0.381	1.09 (0.73-1.65)	0.666	0.93 (0.49-1.76)	0.820	0.79 (0.31-2.04)	0.630	0.83 (0.58-1.21)	0.333	1.10 (0.69-1.76)	0.684
rs17697419	A	0.75 (0.63-0.90)	**0.002***	0.67 (0.52-0.85)	**0.001***	0.78 (0.54-1.13)	0.192	0.75 (0.44-1.27)	0.285	0.72 (0.58-0.89)	**0.002***	0.65 (0.49-0.86)	**0.003***
rs7664413	T	1.00 (0.87-1.15)	0.958	1.01 (0.84-1.22)	0.891	1.01 (0.74-1.38)	0.947	0.95 (0.60-1.50)	0.819	1.03 (0.88-1.21)	0.687	1.03 (0.84-1.26)	0.766
rs6828869	C	0.90 (0.81-1.00)	0.060	0.89 (0.77-1.03)	0.122	0.79 (0.63-1.00)	**0.050**	0.69 (0.49-0.97)	**0.035**	0.93 (0.82-1.06)	0.285	0.96 (0.82-1.12)	0.579
rs17697515	T	0.72 (0.59-0.88)	**0.002***	0.62 (0.47-0.81)	**0.001***	0.67 (0.44-1.00)	**0.049**	0.66 (0.36-1.18)	0.160	0.74 (0.58-0.93)	**0.010**	0.62 (0.45-0.85)	**0.003***
rs1485766	C	1.10 (0.99-1.23)	0.075	1.20 (1.04-1.39)	**0.013**	1.06 (0.84-1.34)	0.635	1.10 (0.79-1.53)	0.561	1.11 (0.98-1.26)	0.098	1.19 (1.01-1.40)	**0.038**
rs11947611	A	0.96 (0.85-1.08)	0.470	0.92 (0.79-1.08)	0.290	1.04 (0.79-1.35)	0.800	1.01 (0.68-1.48)	0.973	0.94 (0.82-1.07)	0.363	0.93 (0.77-1.10)	0.382
rs9654285	T	0.84 (0.71-1.00)	**0.045**	0.80 (0.63-1.00)	0.052	0.97 (0.68-1.40)	0.875	0.87 (0.53-1.47)	0.641	0.81 (0.67-0.98)	**0.030**	0.77 (0.60-1.01)	0.055
rs3775195	A	0.95 (0.84-1.07)	0.377	0.97 (0.83-1.14)	0.694	0.86 (0.66-1.11)	0.237	0.80 (0.55-1.16)	0.233	0.97 (0.85-1.12)	0.704	1.00 (0.83-1.19)	0.970
rs3775194	G	0.91 (0.81-1.01)	0.077	0.93 (0.81-1.08)	0.341	0.89 (0.71-1.13)	0.354	0.93 (0.67-1.30)	0.683	0.91 (0.80-1.03)	0.124	0.92 (0.78-1.08)	0.323
rs2333526	T	0.75 (0.63-0.91)	**0.003***	0.69 (0.54-0.90)	**0.005***	0.95 (0.63-1.43)	0.792	0.98 (0.53-1.80)	0.938	0.72 (0.58-0.89)	**0.002***	0.65 (0.49-0.86)	**0.003***

Abbreviations: DM, diabetes mellitus; T1DM, type 1 DM; T2DM, type 2 DM; SNP, single nucleotide polymorphism; OR (95%CI), odds ratio with 95% confidence interval. [#]Adjusted for age, sex, duration DM, HbA1c, hypertension, DN, smoking. P-values < 0.05 are highlighted in bold. *Significant association following correction for multiple SNPs using SNP SpD correction (P < 0.006).

The linkage disequilibrium pattern between SNPs is presented in **Figure 8.2.1**. Two main blocks of linkage disequilibrium were observed; block 1 comprising the first six SNPs, and block 2 comprising the remaining six SNPs. Haplotypes from each block were analysed for association with 'Any DR', with T1DM and T2DM participants combined (**Table 8.2.3**). After adjustment for clinical covariates, there were two haplotypes found to be associated with 'Any DR': haplotype 1 of block 1 (CTACCT) (OR, 0.59; CI, 0.44-0.79; P = $3x10^{-4}$), and haplotype 1 of block 2 (CATCGT) (OR, 0.65; CI, 0.49-0.86; P = 0.002). These two associated haplotypes survived Bonferroni correction for the haplotypes in each block. A third haplotype, haplotype 8 of block 2, was also associated with 'Any DR', but did not survive Bonferroni correction.

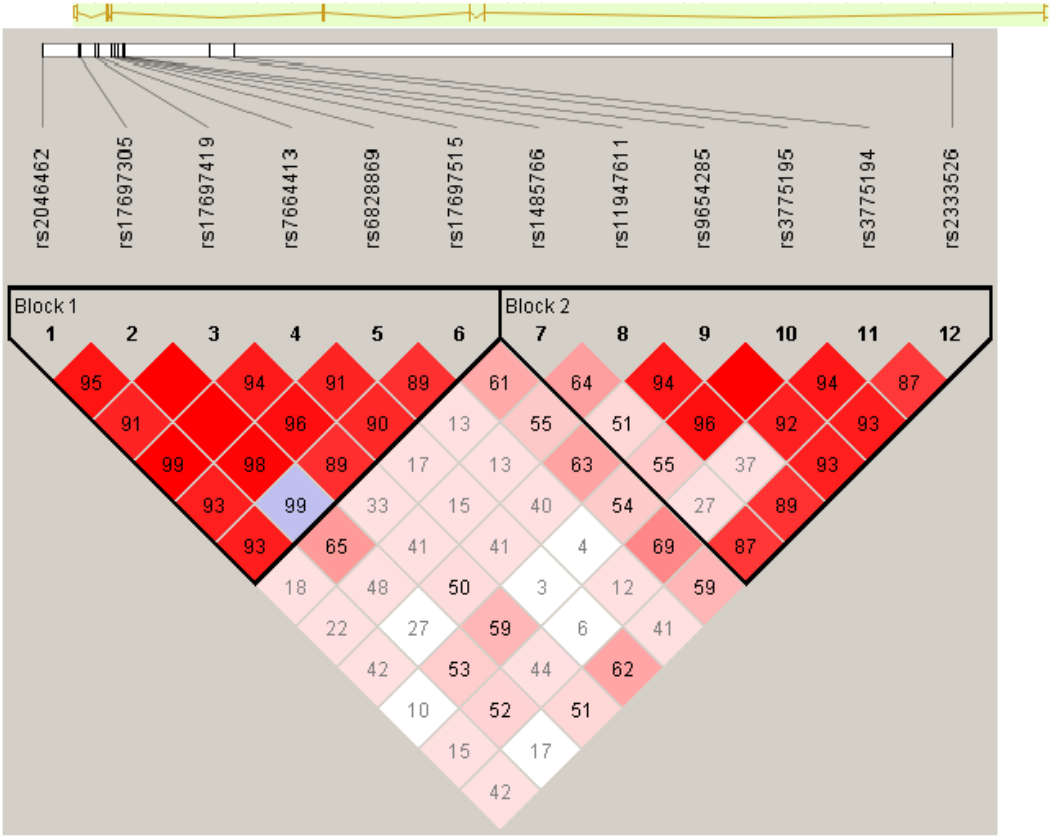


Figure 8.2.1: Linkage disequilibrium between genotyped tag SNPs in and adjacent to the *VEGF-C* gene. The D' value for each pair of SNPs is given, multiplied by 100. A blank cell indicates D'=1.0. *VEGF-C* gene schematic included above linkage disequilibrium plot.

Association of VEGFC SNPs with PDR and DME:

The top three SNPs associated with 'Any DR' were investigated for association with PDR and DMO subtypes (**Table 8.2.4**). Rs17697515 was nominally associated with PDR (n=433) and DMO (n=425) in the 'all DM' analysis following adjustment for covariates and correction for multiple SNP testing (P = 0.007 and 0.009 respectively). In the T2DM cohort alone, which included 361 participants with DMO, this SNP was found to be significantly associated with DMO following adjustment and correction (OR, 0.53; CI, 0.35-0.82; P = 0.004). There was no association found for rs1769515 with PDR or DMO in the TIDM group. Rs17697419 and rs2333526 were not found to be associated with PDR or DMO in the multivariate analysis in any group.

Table 8.2.3: Haplotype associations adjusted for clinical covariates (including DM type, age, sex, duration DM, hypertension, DN, HbA1c and smoking) with any type of DR for T1DM and T2DM participants combined. SNPs are numbered as in Table 8.2.1. Uncorrected P-values are shown.

Haplotype	Alleles						Haplotype frequency	No DR frequency	Any DR frequency	OR (95%CI)	P-value
Block 1	**1**	**2**	**3**	**4**	**5**	**6**					
1	C	T	A	C	C	T	0.065	0.085	0.061	0.59 (0.44-0.79)	**0.0003***
2	C	T	G	T	C	C	0.181	0.183	0.182	0.98 (0.81-1.18)	0.835
3	C	T	A	C	C	C	0.025	0.028	0.024	0.86 (0.54-1.35)	0.513
4	T	C	G	C	C	C	0.035	0.040	0.042	0.99 (0.66-1.48)	0.969
5	C	T	G	C	C	C	0.011	0.009	0.008	1.37 (0.61-3.08)	0.446
6	T	T	G	C	C	C	0.116	0.114	0.118	1.04 (0.83-1.32)	0.722
7	T	T	G	C	G	C	0.544	0.543	0.566	1.10 (0.95-1.27)	0.188
Block 2	**7**	**8**	**9**	**10**	**11**	**12**					
1	C	A	T	C	G	T	0.074	0.092	0.072	0.65 (0.49-0.86)	**0.002***
2	A	G	A	A	G	C	0.207	0.221	0.229	1.01 (0.85-1.21)	0.876
3	C	G	A	A	G	C	0.049	0.059	0.048	0.80 (0.56-1.14)	0.209
4	A	A	T	C	G	C	0.021	0.021	0.023	1.15 (0.66-2.01)	0.609
5	A	G	A	C	G	C	0.010	0.007	0.010	2.36 (0.94-5.95)	0.056
6	A	A	A	C	C	C	0.052	0.052	0.054	0.95 (0.65-1.39)	0.795
7	C	A	A	C	C	C	0.303	0.326	0.316	0.94 (0.80-1.10)	0.421
8	A	G	A	C	C	C	0.187	0.177	0.197	1.24 (1.02-1.51)	**0.031**
9	C	G	A	C	C	C	0.049	0.046	0.052	1.12 (0.74-1.69)	0.604

Abbreviations: DR, diabetic retinopathy; OR (95%CI), odds ratio with 95% confidence interval. Any DR defined as any non-proliferative diabetic retinopathy or proliferative diabetic retinopathy, and/or diabetic macular oedema (DMO). Controls (No DR) defined as no DR or DMO. P-values < 0.05 are highlighted in bold. *Haplotypes that remained statistically significant after Bonferroni correction for multiple haplotype testing (Block 1: P < 0.007; Block 2: P < 0.006).

Table 8.2.4: Allelic association of the top 3 *VEGF-C* Tag SNPs with PDR and DMO, for all diabetics combined, as well as T1DM and T2DM groups individually. Results from multivariate analysis are presented. Uncorrected P-values are shown.

	All DM		T1DM		T2DM	
SNP	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI**	**P value**
rs17697419: A						
PDR	0.80 (0.57-1.13)	0.206	1.24 (0.70-2.19)	0.462	0.66 (0.43-1.03)	0.065
DMO	0.81 (0.60-1.11)	0.184	1.07 (0.50-2.29)	0.857	0.76 (0.53-1.07)	0.112
rs17697515: T						
PDR	0.76 (0.45-0.97)	**0.007**	1.04 (0.54-2.00)	0.899	0.52 (0.31-0.88)	**0.015**
DMO	0.61 (0.42-0.89)	**0.009**	1.18 (0.51-2.69)	0.701	0.53 (0.35-0.82)	**0.004***
rs2333526: T						
PDR	0.88 (0.62-1.24)	0.473	1.38 (0.71-2.68)	0.342	0.79 (0.52-1.20)	0.273
DMO	0.75 (0.55-1.03)	0.079	1.21 (0.54-2.75)	0.642	0.70 (0.49-1.00)	0.052

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; PDR, proliferative diabetic retinopathy; DMO, diabetic macular oedema; DM, diabetes mellitus; OR (95%CI), odds ratio with 95% confidence interval. Controls defined as no DR or minimal NPDR. Adjusted for age, sex, duration DM, HbA1c, hypertension, DN, smoking. P-values < 0.05 are highlighted in bold. *Significant association following correction for multiple SNPs using SNP SpD correction (P < 0.006).

Discussion

Despite extensive research demonstrating a role for the *VEGFA* gene in neovascularisation pathogenesis, and more specifically DR, there is a paucity of data on the potential influence of other VEGF family genes including *VEGFC*. To our knowledge, this is the first study to specifically investigate genetic variation in the *VEGFC* gene in patients with DR.

After adjustment for DM type, age, sex, DM duration, HbA1c, hypertension, DN and smoking history, the A allele of rs17697419, T allele of rs17697515, and T allele of rs2333526 were shown in the current study to be protective against the development of DR. The association was strongest when both T1DM and T2DM participants were combined in the same analysis. Analysis by DM type showed similar trends, with all three SNPs again reaching significance in the T2DM cohort after correction for multiple SNP testing. The lack of association of rs17697419 and 17697515 seen in the T1DM cohort is likely secondary to reduced power due to the smaller size of the T1DM sample, as suggested by the similar odds ratios at these SNPs observed in both T1DM and T2DM subsets. Haplotype analysis confirmed a protective role of these top three ranked SNPs. The minor alleles of rs17697419 and rs17697515 were present in haplotype 1 of block 1, and together conferred significant protection against DR. The minor allele of rs2333526, was represented in a second protective haplotype in block 2.

These results are further supported by the strong association of rs17697515 with DMO in the T2DM cohort, which also suggests an important protective role for the T allele of this *VEGFC* SNP in the subset of patients with DMO. To our knowledge, there have been only two previous studies to report a positive SNP association for DMO, both of which involved SNPs in the *VEGFA* gene. Awata *et al*. found that a *VEGFA* C-634G (rs2010963) polymorphism was associated with DMO (n=63) in a cohort of Japanese subjects with DM [266], and Abhary *et al.* found an association between the G allele of rs10434 and DMO in Caucasian subjects with DM [174].

VEGF-C is thought to contribute to retinal angiogenesis and increased vascular permeability through downstream effects on VEGFR-2 activation. However, the interaction between VEGFR-2, and its two ligands VEGF-A and VEGF-C is complex and not well elucidated. In the human diabetic retina, VEGFR-2 expression has been shown to be greater than in non-diabetic retinas [238,267], and is concentrated in microvascular endothelial cells in two areas: the tips of angiogenic vessels in the peripheral retina, and the macular region [238,267,268]. Along with the VEGFR-2 receptor, there is a pathological increase in VEGF-C and VEGF-A mRNA seen in the microvascular endothelial cells of individuals with DM [267].

AGEs, produced in conditions of sustained hyperglycaemia, bind to RAGE, which induce transcription of pro-inflammatory cytokines including TNF. Upregulation of RAGE has also been directly implicated in the increase in VEGF-A but not VEGF-C secretion [269]. The mechanism by which VEGF-C is increased also depends on increased levels of TNF [267]. Zhao *et al*. showed that TNF found in the vitreous of patients with PDR is adequate to stimulate *VEGF-C* and *VEGFR-2* expression in microvascular endothelial cells, but does not alter expression of *VEGFR-3* [267]. In response to VEGF-A and VEGF-C binding VEGFR-2 on

endothelial cells, nitric oxide production is increased, and this results in increased blood vessel permeability, and proliferation of endothelial cells [238,268]. Furthermore, VEGF-C through binding VEGFR-2 but not VEGFR-3 has been shown to prevent TNF and hyperglycaemia induced apoptosis of microvascular endothelial cells, and thus potentiate the angiogenic action of VEGF-A [267].

It is well known that a proportion of patients with DMO treated with anti-VEGF agents are resistant to treatment [43]. It can be hypothesized that upregulation of other VEGFR-2 ligands such as VEGF-C, or changes in the expression of VEGFR-2 contribute to the sustained activation of the VEGFR-2 molecular pathway in such resistant patients, despite inactivation of VEGF-A. Indeed, inhibition of VEGF-A with bevacizumab has been shown to lead to enhanced VEGF-C secretion, which thereby likely compensates for the reduced VEGF-A levels [269]. Although anti-VEGF agents have revolutionized the management of neovascular retinal diseases, including age-related macular degeneration and fovea-involving macular oedema, there is increasing evidence that treatments targeting additional pathways may be required for better treatment effect. Multikinase inhibitors such as sorafenib and pazopanib, are promising new agents that target a number of tyrosine kinase receptors in the VEGFR family, and have been shown to significantly reduce the expression of VEGFR-1 and VEGFR-2 in human retinal pigment epithelial cells [270]. Phase IIa therapeutic trials using topical pazopanib eye drops for the treatment of neovascular age-related macular degeneration have shown favourable results [271]. The efficacy of pazopanib for the treatment of DR has so far been limited to a STZ rat model in which topical pazopanib was shown to reduce DR-associated BRB breakdown [272]. Fenofibrate, a peroxisome proliferator–activated receptor alpha agonist, has multiple actions including the inhibition of endothelial VEGFR-2 expression [273]. Although traditionally used as systemic therapy for patients with DM and dyslipidaemia, recent experimentation in a STZ rat model supports the use of topical fenofibrate eye drops as a potential therapeutic agent in DR to prevent retinal inflammation, neovascularisation and oedema [274]. Exploration of VEGFR-2 genotypes could also be of interest based on these therapeutic trials and results from the current study. Genetic variation in the *VEGFC* gene likely alters VEGF-C function or expression, which could help to explain differing responses to VEGF-A treatment. Further investigation of the functional effect of the *VEGFC* risk haplotype reported here for the first time, is necessary to gain a better understanding of the role of VEGF-C in DR, and thereby assist development of better treatment strategies, which in future could be tailored according to patient genotype. The manipulation of VEGF-C, either directly or at the ligand-receptor level, presents a worthwhile research direction, and is enhanced by the results of this large study suggesting that genetic variation at this locus has a significant influence on DR development.

Conclusion

This study is the first to evaluate SNPs across the *VEGFC* gene in diabetic patients with and without DR. We found that genetic variation within the *VEGFC* gene is significantly associated with any type of DR, as well as with DMO in patients with T2DM. This adds to other evidence that VEGF-C and its interaction with VEFG-A and VEGFR-2 play a major functional role in the pathogenesis of DR.

8.3 HYPOXIA INDUCIBLE FACTORS

Aim

HIF-1α and HIF-2α are both known to play roles in the regulation of *VEGFA* expression [275]. This study aimed to investigate SNPs in the *HIF1A* (located on chromosome 14) and *EPAS1* (codes for HIF2a and is located on chromosome 2) genes and their association with STDR, and in particular DMO in a Caucasian cohort of T1DM and T2DM participants.

Results

Thirty-one *EPAS1* tag SNPs (rs6708838, rs6739083, rs1867786, rs2346417, rs6720535, rs4953340, rs9973653, rs6753302, rs2034327, rs7582701, rs4953342, rs4953344, rs11694197, rs12712973, rs9679290, rs4953347, rs6726454, rs12614710, rs4953353, rs1868084, rs10199201, rs7589621, rs6712143, rs13412887, rs3768727, rs2346176, rs1992846, rs7568285, rs7557402, rs17035085 and rs11689694) capturing 117 alleles reaching an r^2 threshold of 0.7 (mean $r^2 = 0.883$) were genotyped. SNP SpD method [186], modified by Li and Ji [187] estimated 20 independent tests for the 31 *EPAS1* SNPs tested. Seven *HIF1A* tag SNPS (rs2301106, rs4899056, rs1957757, rs12434438, rs11158358, rs2301113 and rs1319462) capturing 18 alleles reaching an r^2 threshold of 0.8 (mean $r^2 = 0.969$) were genotyped. SNP SpD method [186], modified by Li and Ji [187] estimated 4 independent tests for the 7 *HIF1A* SNPs. A total of 24 independent tests across the 2 genes were included in this analysis resulting in a P-value of less than 0.002 required for significance.

Genotype frequencies in patients with STDR and controls are shown in **Table 8.3.1** for both T1DM and T2DM participants. A number of SNPs had HWE p-values less than 0.05, however none survived Bonferroni correction, and thus all genotyped SNPs were in HWE. A single *HIF1A* tag SNP, rs2301113, was nominally associated with STDR in the T1DM group (OR, 1.73; CI, 1.18-2.54; P = 0.005) (**Table 8.3.2**). This association did not survive multiple testing correction. Rs2301113 was tested for association with STDR subtypes PDR (OR, 1.68; CI, 1.10-2.54; P = 0.015) and DMO (OR, 1.72; CI, 1.09-2.71; P = 0.02) in the T1DM group, but again failed to survive multiple SNP correction in a multivariate analysis. None of the other 37 *EPAS1* and *HIF1A* SNPs tested showed evidence of association with STDR in either the T1DM or T2DM groups.

Table 8.3.1: Genotype frequencies, shown as n (%) for STDR and controls by DM type.

SNP	Genotype	T1DM		T2DM		HWE
		STDR n (%)	Controls n (%)	STDR n (%)	Controls n (%)	P-value
EPAS1						
rs6708838	AA/AG/GG	0/41/286 (0/12.5/87.5)	0/29/227 (0/11.3/88.7)	5/106/864 (0.5/10.9/88.6)	5/134/742 (0.6/15.2/84.2)	>0.999
rs6739083	CC/CT/TT	50/149/126 (15.4/45.8/38.8)	32/121/101 (12.6/47.6/39.8)	132/438/408 (13.5/44.8/41.7)	127/414/344 (14.4/46.8/38.9)	0.187
rs1867786	CC/CT/TT	55/166/106 (16.8/50.8/32.4)	38/140/81 (14.7/54.1/31.3)	203/461/319 (20.7/46.9/32.5)	175/452/269 (19.5/50.4/30.0)	0.827
rs2346417	AA/AT/TT	72/157/100 (21.9/47.7/30.4)	62/117/77 (24.2/45.7/30.1)	208/500/275 (21.2/50.9/28.0)	195/442/255 (21.9/49.6/28.6)	0.691
rs6720535	GG/GA/AA	4/61/263 (1.2/18.6/80.2)	3/54/198 (1.2/21.2/77.6)	14/199/768 (1.4/20.3/78.3)	7/163/719 (0.8/18.3/80.9)	>0.999
rs4953340	CC/CG/GG	44/161/122 (13.5/49.2/37.3)	39/112/104 (15.3/43.9/40.8)	142/470/367 (14.5/48.0/37.5)	123/391/375 (13.8/44.0/42.2)	0.396
rs9973653	TT/TG/GG	28/146/152 (8.6/44.8/46.6)	24/100/130 (9.4/39.4/51.2)	82/401/481 (8.5/41.6/49.9)	83/360/444 (9.4/40.6/50.1)	>0.999
rs6753302	CC/CA/AA	3/42/284 (0.9/12.8/86.3)	1/26/229 (0.4/10.2/89.5)	4/102/875 (0.4/10.4/89.2)	6/106/777 (0.7/11.9/87.4)	**0.029**
rs2034327	GG/GC/CC	91/144/93 (27.7/43.9/28.4)	63/123/72 (24.4/47.7/27.9)	239/509/234 (24.3/51.8/23.8)	216/433/244 (24.2/48.5/27.3)	0.692
rs7582701	CC/CG/GG	2/33/293 (0.6/10.1/89.3)	1/42/215 (0.4/16.3/83.3)	9/124/844 (0.9/12.7/86.4)	5/112/776 (0.6/12.5/86.9)	**0.027**
rs4953342	GG/GA/AA	27/127/173 (8.3/38.8/52.9)	23/107/125 (9.0/42.0/49.0)	72/390/517 (7.4/39.8/52.8)	72/355/464 (8.1/39.8/52.1)	0.720
rs4953344	CC/CT/TT	8/93/228 (2.4/28.3/69.3)	4/58/194 (1.6/22.7/75.8)	31/270/682 (3.2/27.5/69.4)	25/239/628 (2.8/26.8/70.4)	0.321
rs11694197	TT/TC/CC	0/22/302 (0/6.8/93.2)	0/18/237 (0/7.1/92.9)	0/67/910 (0/6.9/93.1)	0/63/826 (0/7.1/92.9)	**0.049**
rs12712973	AA/AC/CC	80/161/87 (24.4/49.1/26.5)	55/128/74 (21.4/49.8/28.8)	196/529/245 (20.2/54.5/25.3)	212/417/260 (23.8/46.9/29.2)	0.447
rs9679290	CC/CG/GG	70/157/102 (21.3/47.7/31.0)	43/135/78 (16.8/52.7/30.5)	185/511/282 (18.9/52.2/28.8)	196/418/272 (22.1/47.2/30.7)	0.402
rs4953347	AA/AG/GG	80/163/82 (24.6/50.2/25.2)	57/128/70 (22.4/50.2/27.5)	208/530/246 (21.1/53.9/25.0)	220/423/246 (24.7/47.6/27.7)	0.329
rs6726454	AA/AG/GG	77/163/79 (24.1/51.1/24.8)	54/127/67 (21.8/51.2/27.0)	211/499/239 (22.2/52.6/25.2)	212/412/240 (24.5/47.7/27.8)	0.441
rs12614710	TT/TG/GG	79/159/91 (24.0/48.3/27.7)	52/127/78 (20.2/49.4/30.4)	182/519/279 (18.6/53.0/28.5)	193/414/277 (21.8/46.8/31.3)	0.610
rs4953353	TT/TG/GG	49/130/142 (15.3/40.5/44.2)	34/108/99 (14.1/44.8/41.1)	130/456/368 (13.6/47.8/38.6)	120/368/353 (14.3/43.8/42.0)	0.289
rs1868084	GG/GC/CC	9/91/229 (2.7/27.7/69.6)	11/78/167 (4.3/30.5/65.2)	20/284/679 (2.0/28.9/69.1)	29/253/606 (3.3/31.5/64.5)	0.851
rs10199201	CC/CT/TT	12/91/220 (3.7/28.2/68.1)	8/78/169 (3.1/30.6/66.3)	39/318/616 (4.0/32.7/63.3)	35/278/569 (4.0/31.5/64.5)	0.217
rs7589621	AA/AG/GG	26/119/180 (8.0/36.6/55.4)	28/103/128 (10.8/39.8/49.4)	84/377/513 (8.6/38.7/52.6)	79/334/479 (8.9/37.4/53.7)	**0.006**
rs6712143	GG/GA/AA	30/119/180 (9.1/36.2/54.7)	28/101/127 (10.9/39.5/49.6)	87/389/508 (8.8/39.5/51.6)	81/343/469 (9.1/38.4/52.5)	**0.014**
rs13412887	GG/GC/CC	14/103/208 (4.3/31.7/64.0)	13/77/166 (5.1/30.1/64.8)	43/280/650 (4.4/28.8/66.8)	44/266/573 (5.0/30.1/64.9)	**0.008**
rs3768727	GG/GA/AA	2/79/249 (0.6/23.9/75.5)	4/54/198 (1.6/21.1/77.3)	28/257/697 (2.9/26.2/71.0)	14/221/657 (1.6/24.8/73.7)	0.942
rs2346176	CC/CT/TT	48/153/125 (14.7/46.9/38.3)	48/131/80 (18.5/50.6/30.9)	182/481/314 (18.6/49.2/32.1)	160/420/313 (17.9/47.0/35.1)	0.318
rs1992846	TT/TC/CC	19/116/190 (5.8/35.7/58.5)	15/94/137 (6.1/38.2/55.7)	90/364/518 (9.3/37.4/53.3)	61/319/479 (7.1/37.1/55.8)	**0.013**

rs7568285	AA/AT/TT	78/154/95 (23.9/47.1/29.1)	67/122/66 (26.3/47.8/25.9)	245/484/253 (24.9/49.3/25.8)	216/431/240 (24.4/48.6/27.1)	0.181
rs7557402	GG/GC/CC	85/163/81 (25.8/49.5/24.6)	57/126/73 (22.3/49.2/28.5)	228/492/263 (23.2/50.1/26.8)	207/445/238 (23.3/50.0/26.7)	0.914
rs17035085	GG/GA/AA	0/22/306 (0/6.7/93.3)	2/25/232 (0.8/9.7/89.6)	6/78/898 (0.6/7.9/91.4)	1/72/815 (0.1/8.1/91.8)	0.076
rs11689694	TT/TC/CC	78/164/86 (23.8/50.0/26.2)	60/126/70 (23.4/49.2/27.3)	254/496/237 (25.7/50.3/24.0)	222/440/234 (24.8/49.1/26.1)	0.615
HIF1A						
rs2301106	CC/CT/TT	6/59/215 (2.1/21.1/76.8)	3/46/134 (1.6/25.1/73.2)	13/226/671 (1.4/24.8/73.7)	12/159/586 (1.6/21.0/77.4)	0.613
rs4899056	TT/TC/CC	4/66/257 (1.2/20.2/78.6)	2/50/206 (0.8/19.4/79.8)	21/181/776 (2.1/18.5/79.3)	8/201/683 (0.9/22.5/76.6)	0.296
rs1957757	TT/TC/CC	4/60/265 (1.2/18.2/80.5)	1/41/217 (0.4/15.8/83.8)	15/160/808 (1.5/16.3/82.2)	6/167/721 (0.7/18.7/80.6)	0.155
rs12434438	GG/GA/AA	21/101/198 (6.6/31.6/61.9)	10/90/156 (3.9/35.2/60.9)	57/344/580 (5.8/35.1/59.1)	49/300/539 (5.5/33.8/60.7)	**0.029**
rs11158358	GG/GC/CC	17/91/220 (5.2/27.7/67.1)	6/76/174 (2.3/29.7/68.0)	32/298/651 (3.3/30.4/66.4)	29/253/609 (3.3/28.4/68.4)	0.581
rs2301113	CC/CA/AA	38/110/180 (11.6/33.5/54.9)	12/83/158 (4.7/32.8/62.5)	57/345/568 (5.9/35.6/58.6)	62/329/502 (6.9/36.8/56.2)	0.054
rs1319462	GG/GA/AA	20/87/221 (6.1/26.5/67.4)	9/63/184 (3.5/24.6/71.9)	28/292/658 (2.9/29.9/67.3)	23/240/629 (2.6/26.9/70.5)	0.146

Abbreviations: T1DM, type 1 diabetes mellitus T2DM, type 2 diabetes mellitus; STDR, sight-threatening diabetic retinopathy. STDR defined as severe non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR), and/or diabetic macular oedema (DMO). Controls defined as no DR or minimal NPDR. SNPs with HWE $P < 0.05$ are shown in bold. *SNPs that remained statistically significant for HWE testing after correction for multiple SNPs using Bonferroni correction ($P < 0.001$).

Table 8.3.2: Allelic association (additive model) of *EPAS1* and *HIF1A* Tag SNPs with STDR, for T1DM and T2DM groups individually. Results from multivariate analysis are presented. Uncorrected P-values are shown.

SNP	Minor allele	T1DM		T2DM	
		OR (95%CI)	P-value	OR (95%CI)	P-value
EPAS1					
rs6708838	A	0.81 (0.37-1.76)	0.591	0.93 (0.77-1.11)	0.408
rs6739083	C	1.23 (0.86-1.76)	0.249	1.01 (0.85-1.21)	0.877
rs1867786	C	1.06 (0.75-1.52)	0.734	0.99 (0.83-1.19)	0.938
rs2346417	A	1.03 (0.74-1.43)	0.845	1.21 (0.91-1.61)	0.183
rs6720535	G	1.29 (0.77-2.16)	0.331	1.13 (0.94-1.36)	0.191
rs4953340	C	1.10 (0.78-1.54)	0.594	0.91 (0.74-1.11)	0.340
rs9973653	T	1.04 (0.71-1.50)	0.853	0.82 (0.57-1.18)	0.281
rs6753302	C	1.40 (0.67-2.93)	0.375	1.04 (0.87-1.24)	0.691
rs2034327	G	0.90 (0.66-1.24)	0.532	1.01 (0.72-1.41)	0.966
rs7582701	C	0.76 (0.41-1.41)	0.389	0.92 (0.75-1.13)	0.446
rs4953342	G	0.80 (0.54-1.17)	0.244	1.05 (0.83-1.32)	0.684
rs4953344	C	1.46 (0.91-2.35)	0.120	1.22 (0.76-1.95)	0.422
rs11694197	T	0.68 (0.28-1.69)	0.407	1.04 (0.87-1.24)	0.690
rs12712973	A	1.05 (0.75-1.47)	0.769	0.97 (0.81-1.16)	0.729
rs9679290	C	1.06 (0.76-1.49)	0.725	1.00 (0.84-1.20)	0.983
rs4953347	A	1.06 (0.75-1.48)	0.747	1.07 (0.89-1.28)	0.470
rs6726454	A	0.97 (0.69-1.38)	0.879	1.01 (0.84-1.20)	0.925
rs12614710	T	1.09 (0.78-1.53)	0.600	1.09 (0.91-1.32)	0.353
rs4953353	T	1.05 (0.74-1.48)	0.783	0.97 (0.76-1.25)	0.830
rs1868084	G	0.68 (0.43-1.05)	0.081	1.14 (0.92-1.42)	0.231
rs10199201	C	0.95 (0.63-1.43)	0.789	0.98 (0.80-1.19)	0.808
rs7589621	A	0.88 (0.62-1.25)	0.466	0.96 (0.79-1.16)	0.644
rs6712143	G	0.94 (0.66-1.33)	0.716	0.92 (0.73-1.14)	0.435
rs13412887	G	0.89 (0.60-1.33)	0.575	1.20 (0.94-1.54)	0.144
rs3768727	G	1.02 (0.62-1.67)	0.950	1.08 (0.90-1.29)	0.425
rs2346176	C	0.80 (0.57-1.12)	0.193	1.12 (0.91-1.36)	0.283
rs1992846	T	1.04 (0.70-1.55)	0.830	1.09 (0.91-1.30)	0.349
rs7568285	A	0.95 (0.68-1.31)	0.744	0.97 (0.81-1.16)	0.731
rs7557402	G	1.17 (0.84-1.63)	0.353	1.07 (0.70-1.61)	0.766
rs17035085	G	0.76 (0.36-1.60)	0.467	1.09 (0.91-1.30)	0.355
rs11689694	T	0.97 (0.70-1.35)	0.869	1.36 (1.03-1.80)	0.029
HIF1A					
rs2301106	C	0.84 (0.46-1.53)	0.566	0.84 (0.63-1.12)	0.237
rs4899056	T	1.03 (0.61-1.74)	0.921	0.76 (0.56-1.05)	0.094
rs1957757	T	1.26 (0.72-2.22)	0.416	1.03 (0.84-1.28)	0.753
rs12434438	G	1.37 (0.93-2.01)	0.108	1.03 (0.82-1.30)	0.811
rs11158358	G	1.30 (0.85-2.00)	0.221	0.86 (0.70-1.06)	0.162
rs2301113	C	1.73 (1.18-2.54)	**0.005**	1.16 (0.91-1.47)	0.221
rs1319462	G	1.61 (1.07-2.43)	0.021	0.93 (0.77-1.11)	0.408

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; STDR, sight-threatening diabetic retinopathy; OR (95%CI), odds ratio with 95% confidence interval. STDR defined as severe NPDR or PDR, and/or DMO. Controls defined as no DR or minimal NPDR. #Adjusted for age, sex, duration DM, HbA1c, hypertension, DN, smoking. P-values < 0.05 are highlighted in bold. *Significant association following correction for multiple SNPs using SNP SpD correction (P < 0.002).

Discussion

This study is the first to investigate *HIF1A* and *EPAS1* polymorphisms and their association with DR. We found no significant association following multiple SNP correction between 38 *HIF1A* and *EPAS1* SNPs and STDR in T1DM or T2DM patients. A single tag SNP, rs2301113, in the *HIF1A* gene was nominally associated with STDR and its subtypes PDR and DMO in the T1DM group, but did not survive correction due to the large number of SNPs tested.

Rs2301113 is an intronic SNP with no known functionality. Though widely investigated in the cancer literature, it so far only shows promise as a prognostic indicator following treatment for thymic malignancies [276] and non-small cell lung cancer [277]. Two SNPs in exon 12 of *HIF1A* gene, at rs11549465 and rs11549465 have been the focus of most published studies relating to *HIF1A* to date. Both these SNPs result in amino acid substitutions in the N-terminal transactivation domain of HIF-1α, altering the affinity of Von Hippel-Lindau (VHL) for this degradation domain and enhancing the hypoxic stabilisation of HIF-1α [278]. Both variants cause significantly higher transcription activity under normoxic and hypoxic conditions [278]. These SNPs have been associated with risk for a number of diseases including cancer [279], diabetic neuropathy [280] and T1DM and T2DM [281,282]. In the current study, rs11549464 was indirectly captured via the tag SNP at rs2301106, which showed no evidence of an association with STDR. rs11549467 was excluded as it had a MAF of 0.009, below our cut off for SNPs that this study was powered to detect.

Xin et al. [283] have shown that HIF-1α blockade can reduce oedema in a mouse model of oxygen-induced retinopathy. Furthermore they showed that HIF-1α promotes increased vascular permeability independent of its effect on *VEGFA* upregulation. These findings support a significant role for HIF-1α in the pathogenesis and treatment of DMO, despite the fact that no genetic associations were found in the current study. This also raises the question that genetic variation in *HIF1A* and *EPAS1* may be relevant to treatment response in patients with DMO treated with anti-VEGF agents rather than in the development of DMO itself. Both rs11549465 and rs11549465 have been investigated for association with age-related macular degeneration [284] and anti-VEGF treatment response in patients with age-related macular degeneration [285], but no statistically significant association has been found to date. *EPAS1* SNPs have also been investigated in patients with age-related macular degeneration receiving anti-VEGF treatment with conflicting results. The IVAN study [286] found a nominal association between rs9679290 and treatment responders, although this positive association was not able to be replicated in the CATT trial [287]. Further investigation of *HIF1A* and *EPAS1* polymorphisms and their role in anti-VEGF treatment response in DMO is warranted.

The complex pathway involving HIFs in the retina is currently only partly understood. Mechanisms other than genetic variation by which HIF-1α and HIF-2α could impact on downstream gene targets also need further evaluation. The stabilisation of HIFs is a key event that occurs prior to translocation to the nucleus and allows HIFs to subsequently act as transcription factors of angiogenic cytokines. Any factor or condition that alters HIF stability, such as hyperglycaemia, will increase transcription of factors such as VEGF-A and angiopoietin-like 4 that play a role in increased vascular permeability and retinal oedema [283]. Secondly,

binding of HIF to the hypoxia response element of its target gene is another environment dependent factor that can be modulated. For example, insulin treated diabetic rats exhibit greater binding of HIF-1α to the hypoxia response element of *VEGFA* compared with diabetic rats not treated with insulin [288]. This finding supports the well known clinical observation that patients started on intensive insulin therapy subsequently experience acute worsening of DR and DMO. Investigation of *HIF1A* and *EPAS1* SNPs in insulin-dependent diabetics compared with non-insulin dependent diabetics was not evaluated in the current study, but should be considered in future studies. Finally, miRs are known to play a role in the regulation of HIF by targeting HIFs as well as HIF hydroxylases (HIF regulating factors) [289] and justify another worthwhile research direction.

Conclusion

We found no genetic association between *HIF1A* and *EPAS1* and risk for STDR in this large cohort of T1DM and T2DM patients. The investigation of molecular mechanisms other than genetic variation may give more insight into the role of HIFs in the pathogenesis of DMO.

CONCLUSIONS

A recent report suggests that 3.63 million people worldwide suffer from moderate and severe vision loss due to DR and its related sequelae [1]. DMO alone is responsible for greater visual morbidity than other vision threatening ocular diseases [2], and incurs significantly higher health care costs than other DR subtypes [3]. With the rising prevalence of T2DM in both first-world and third-world populations in Australia and worldwide, STDR has become a huge public health concern.

This PhD informs about factors that affect visual outcomes secondary to DR and DMO in Indigenous Australian communities. This work is the first to propose that Indigenous Australian patients may have a unique predisposition to STDR and DMO in the context of lower rates of less severe grades of DR. This observation, in the setting of earlier DM onset in Indigenous Australians is notable because it alludes to a potential molecular mechanism underlying ethnicity based disparities in DR. Given this finding, we sought to assess the prevalence of diabetic vitrectomy for end-stage DR in SA and the NT in a population-based fashion. The ability to capture all patients reaching this endpoint through the methodology applied is unique, and gives our study greater validity than other population-based studies assessing vision and DR in Indigenous Australians.

This project has contributed to the literature by determining the rates and success of vitrectomy for end-stage DR in Indigenous and non-Indigenous Australians and by determining factors contributing to visual success in these populations. It is encouraging to see that Indigenous Australians undergoing diabetic vitrectomy have similar rates of visual success post-operatively compared with non-Indigenous Australians. However, of those whose vision does not improve, the discrepancy in the number of Indigenous patients with blind eyes following diabetic vitrectomy is significant. Understanding and recognising that there may be ethnicity-related differences in disease severity or response to treatment is the first step in helping to enhance existing services, through clinical decision making, outreach services and access. Identification of risk factors for poor visual outcomes will assist health care workers and treating ophthalmologists directly involved in the care of Indigenous Australians, identify patients who require close monitoring and earlier intervention such as laser treatment. In doing so, it will initiate an evidence base for changing current practices for managing Indigenous Australians with diabetic eye disease and permit rational use of limited resources, with an aim to reduce the likelihood of late presentation of advanced disease. Controlling the severity of DR complications by timely and adequate treatment ultimately improves visual outcomes and reduces the likelihood of interstate transfer and the potential for cultural dislocation.

There remain many unanswered questions relating to the susceptibility of STDR and other microvascular complications of DM in Indigenous Australians. It is clear from our population-based study that rates of CKD are disproportionally high in Indigenous patients with end-stage DR. Similarly, amongst amputees in Far North Queensland, Indigenous patients were 5 times more likely to have CKD than non-Indigenous patients [136]. These studies contribute to the mounting evidence that Indigenous Australians are highly

susceptible to developing CKD in the context of DM compared with other complications of DM including retinopathy and neuropathy. Many factors are likely to contribute to this observation including multiple insults to the kidney from non-diabetic causes early in life. These include low birth weight resulting in lower renal volume, high frequency of bacterial infections and post-infectious glomerulonephritis [290]. A genetic predisposition may further help to explain the high incidence of CKD and lower incidence of DR seen in this population compared with the non-Indigenous Australian population and other Caucasian populations studied in the literature. Epigenetic factors are also of particular interest given the relatively acute onset of T2DM in this population. T2DM (and DR) were virtually non-existent at the time of the first nation-wide eye health screening performed in the 1970s [169]. This holds true for many ethnic minorities and first-nation people worldwide, who interestingly suffer equally high rates of CKD compared with DR [138]. Although there is further scope to examine some of these factors using an epidemiological approach, this methodology will not allow for the exploration of genetic risk.

The controversies of performing genetic research in Indigenous Australian populations continue to limit the use of this research methodology. Historical events involving Indigenous genomic studies around the world have contributed to racial stereotyping and genetic discrimination in the context of longstanding oppression of Indigenous people [291]. Fears of inappropriate use of human samples, as well as cultural beliefs have added to the opposition of genetic research in Indigenous communities. These factors have made ethical approval of genetic studies in Indigenous Australia difficult. Unfortunately this means that Indigenous Australians have been excluded from the majority of genetic research to date, including the benefits of these studies in the understanding of disease states. More recent efforts in collaboration with Indigenous communities have allowed for genetic studies to be successfully undertaken and have focused on diseases of high priority to Indigenous communities. The first GWAS in an Australian Indigenous population was recently published and provides some insights into the development of T2DM in Indigenous Western Australian communities [292]. The high burden and severity of DM complications is the most immediate concern from a community perspective in South Australian communities. The Aboriginal Diabetes Study is an NHMRC funded research project that aims to understand the determinants of disparity in DM and its complications in Aboriginal people in South Australia. This study commenced recruitment in 2016 and is the first to employ a genetic and epigenetic approach to investigate risk for diabetic complications including DR in Indigenous Australians. This project has the ability to link genetic findings and environmental risks with phenotypes specific to Indigenous Australians living with DM in SA.

Primary and secondary prevention of DR are currently the most important strategies in maintaining good vision. The design and implementation of the clinical trial described in this PhD was in response to the knowledge gained from our prior work. This is the first randomised controlled trial in the field of ophthalmology designed specifically for patients living in remote Central Australia. The nature of DM, DR, and access to care are all unique factors in this population and for this reason we believe that the results from this study are likely to be specifically relevant to the communities involved, and lead to rapid translation into clinical practice. All research related to this study involving Indigenous health uses the existing services to

deliver the most appropriate care to patients. All investigators represent a strong collaboration of service providers for individuals requiring DR screening and treatment in SA and NT. Increasing awareness of DMO and its treatment in Indigenous Australian communities might also aid in improving attendance and referrals. The equality of all patients with diabetic eye disease is acknowledged and consequently the right to access treatment for avoidable blindness should be available to all. This clinical trial is the first step to providing relevant, evidence based care tailored to this population.

The principle molecular findings from this PhD include the novel association of SNPs within *miR-146a*, *VEGFA* and *VEGFC* with the development of DMO. These findings further support the interaction between inflammatory and angiogenic pathways involved in the pathogenesis of DMO. Decreased resistance to hyperglycaemic damage secondary to ageing and in the context of background inflammation are likely to enhance susceptibility to DMO when combined with high local levels of VEGF in the diabetic retina.

Leucocyte-mediated endothelial cell injury and death is an inflammatory process that contributes to the increase in vascular permeability in DR and DMO [293]. Systemic, low-grade chronic inflammation has been linked to both T1DM and T2DM, and has specifically been associated with the development of microvascular diabetic complications in the context of insulin resistance [294-296]. Hietala et al. report that patients with higher age at T1DM onset (greater than 15 years of age) have significantly higher cumulative incidence and risk of developing DMO, despite accounting for other risk factors including HT, than those with younger age of T1DM onset [297]. This study also found that the highest CRP concentrations were found in older patients with DMO and PDR compared with those with T1DM without DR, suggesting an inflammatory mediated process that is exacerbated by increasing age [297]. This finding is further supported by the consensus that DMO is found at increased rates in patients with T2DM compared with those with T1DM [189]. We found a functional SNP within the *miR-146a* gene to be associated with DMO in patients with T2DM. Interestingly, this SNP was also associated with DN in our cohort [223]. This is supported by the strong relationship between insulin resistance (which is found earlier in the course of T2DM) and susceptibility to CKD and DR [296,298]. In the context of the current literature, our findings support the theory that both DMO and DN occur in a pro-inflammatory environment associated with DM.

VEGFA is a well-studied gene in the DR literature. The significant effects of anti-VEGF agents in the treatment of DMO and more recently PDR, continue to make this molecule of great interest to clinicians and researchers [44]. This PhD reports a direct and functional association between the *VEGFA* polymorphism at rs3025020 and DMO in patients with TIDM. This is the first time that T1DM patients with DMO have been studied in large enough numbers to find a significant association with a SNP within the *VEGFA* gene after correction for multiple SNP testing. Furthermore, this polymorphism was shown to be associated with increased serum VEGF-A levels in our T1DM cohort. Further examination of this SNP in patients being treated with anti-VEGF agents would provide insight into whether this result is of clinical benefit in predicting treatment response in these patients.

The future of DMO therapeutics lies in the ability to develop new drugs that function synergistically with currently used anti-VEGFA agents, to target those patients who have a suboptimal response to current treatments. This PhD was the first study to investigate *VEGFC* in patients with DR. Our findings showed that genetic variation within the *VEGFC* gene is associated with DMO risk in patients with T2DM. This novel finding presents a potential therapeutic target that could reduce the downstream effects of VEGF-A via an indirect pathway. There are currently 2 drugs that target VEGF-C undergoing therapeutic trials. VGX-100 is a VEGF-C antibody that has so far only been utilised in the colorectal cancer population [299]. VGX-300, a soluble form of VEGFR-3, binds and inhibits VEGF-C and -D. VGX-300 mediated blockade of VEGF-C/-D significantly inhibits choroidal neovascularization and vascular leakage comparably to aflibercept (an anti-VEGFA agent) in the laser-induced mouse model of wet macular degeneration [300]. This finding implicates VEGF-C in the pathogenesis of retinal oedema and suggests that VEGF-C antagonists have therapeutic potential either as a single agent or in conjunction with anti-VEGFA agents. Although these have not yet been trialled in patients with DM, the results from this PhD indicate VEGF-C blockade as a promising future treatment of DMO.

It is clear that with the very recent expansion of treatment options for DMO, management dilemmas are emerging for clinicians. As novel treatments are developed, it will become increasing important to identify individual patient factors that make a specific treatment option more desirable and to determine prognosis. Furthermore, the total mean cost per patient for currently approved intravitreal anti-VEGF treatments for 1 year range from $4000 to $26000 depending on the specific agent used [301]. The risks associated with intravitreal injections (including a serious risk of blinding infection) as well as the potential morbidity associated with adverse events of the drugs used also need to be considered. Ongoing research aimed at determining the role of genetic risk factors for the development and progression of DR, as well as response to treatment are important in the quest for administering personalized medicine to achieve the safest and most effective treatment option for each patient.

Many studies in the literature have focused on elucidating genetic risk factors for the development of DR, with relatively few positive results able to be reproduced [302]. Multiple factors are likely to contribute to this including differences in ethnicity, varying case-control definitions and comparisons across different types of DM. High false positive rates often related to low statistical power and other biases have been shown to frequently contaminate genetic association studies [303]. Low statistical power occurs in the context of low sample size and small effect size and increases the likelihood that a nominally significant result is actually a false positive finding [304]. The methodology used in this PhD was specifically designed to increase power by including a large, well-characterized sample of Caucasian patients with T1DM and T2DM, concentrated at the extremes of the DR phenotype. In particular, our cohort of patients with DMO is significantly larger than that reported in previous studies [174]. RADAR, a world leading registry and DNA repository of STDR cases was initiated as a part of this PhD. This efficient point of care referral system is available as a Smartphone App. for clinicians around Australia and has significantly increased recruitment of those cases with STDR for the current study. Finally, our approach has taken into account factors known to

be associated with increased risk of STDR and employed stringent techniques for correction of multiple SNP testing in order to reduce the risk of type 1 errors.

The negative findings presented in this thesis could be related to a number of explanations. Firstly, it could be that these are true negative findings in genes that have alternative mechanisms of upregulation in the pathogenesis of DMO. Alternatively, the interactive effects of the environment may have profound effects on the development and progression of DMO and may influence genetic pathways in a way that cannot be accounted for using a candidate gene approach. For example, it has recently been reported that epigenetic changes such as persistent DNA methylation at key genomic loci are responsible for the well-known phenomenon of 'metabolic memory' [305]. Secondly, this study may have failed to detect some of the common variants with small effect sizes despite the sample size and extreme phenotype sampling employed here. Alternatively, the candidate gene methodology used in this thesis may not be sensitive enough to detect rare variants associated with this complex disease. The recent advances in sequencing technologies and the resulting next-generation sequencing data have allowed for better detection of rare variants using this methodology [306]. This approach has not yet been employed in the field of DR genetics and could be a useful alternative to GWAS or candidate gene analysis in the detection of rare variants.

In conclusion, this PhD has successfully targeted two specific aspects of DR susceptibility that have so far been poorly studied in the literature, despite being associated with disproportionally high levels of visual morbidity. The implication of our findings during an era of rapidly increasing incidence of T2DM and therefore DMO on vision related quality of life and health care costs are particularly noteworthy. This research extends previous reports in several respects. The outcomes determined from the first population-based study of end-stage DR requiring vitrectomy has yielded valuable information regarding progression to vitrectomy and visual outcomes in Indigenous and non-Indigenous Australian populations. This information has guided the first Ophthalmic clinical trial in Central Australia designed as a part of this thesis and will continue to impact future initiatives aimed at improving visual outcomes in Indigenous Australians with DM. The exploration of candidate genes hypothesized to play a role in the pathogenesis of DMO in this robust genetic study has contributed to our current understanding of DMO susceptibility. The close interaction between inflammatory and angiogenic pathways in response to hypoxia is supported by our findings. Significant novel variants found within *VEGFC* and *miR-146a* validate the development of new therapeutic drugs targeting these pathways. Future evaluation of *VEGFA* variants and their interaction with environmental factors may help distinguish non-responders to current intravitreal treatments and assist clinicians employ individualized treatment strategies. The outcomes presented in this thesis are part of an ongoing research initiative that will continue to contribute to improving understanding and treatment of this global sight-threatening epidemic.

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