The Genetic Study of Diabetic Retinopathy

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Summary

Diabetic retinopathy (DR) is a microvascular complication of diabetes mellitus (DM). It is the fifth most common causes of blindness in the world, accounting for approximately 4.8% of global blindness and is also the leading cause of blindness in working age adults. The pathogenesis of DR is complex and multifactorial, but at a biochemical level is related to altered glucose metabolism. Established risk factors in the development of DR include prolonged hyperglycemia, increased duration of DM, uncontrolled hypertension and hyperlipidemia. It has become evident through familial aggregation studies that susceptibility to DR also has a heritable component, independent of other established risk factors. The aim of this thesis was to further explore genetic risk factors in the development of DR in type 1 DM and type 2 DM. A meta-analysis of all of the published candidate gene studies for DR has been undertaken and a total of 34 variants in 20 genes have been analysed, with 5 genes found to be significantly associated with DR. Six candidate gene studies have been undertaken, including replication studies of two of the genes associated with DR in the meta-analysis. In particular, variation in the *vascular endothelial growth factor* and *erythropoietin* gene were found to be significantly associated with DR, especially sight-threatening DR. A serum protein study investigating the nitric oxide pathway was undertaken and found asymmetric and symmetric dimethylarginines and L-arginine to be significantly associated with sight-threatening DR. Finally, a genome-wide association study was undertaken and identified several novel susceptibility genes for sight-threatening DR. This study advances understanding of DR pathogenesis, and may assist in refinement of genetic screening programs to identify individuals at particularly high risk of DR. Data from this study may also assist in the identification of novel therapeutic targets for DR.

Signed 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.

Acknowledgements

This thesis is dedicated to all Persian lives lost or politically detained in the fight for human rights and democracy for their country. Although pervading with intelligence and talents, due to circumstances faced by the country they have not been as privileged as I have to live out their dreams. As descendents of world renowned academics including Avicenna, Zakariya Razi, historical greats including Cyrus the Great, Ferdowsi, Rumi and Hafez , I hope this work inspires others to live out their dreams in a free Iran.

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Abbreviations

ACE	Angiotensin converting enzyme
ADMA	Asymmetric dimethylarginine
AKR1B1	Aldose reductase
BMI	Body mass index
CA1	Carbonic anhydrase 1
CI	Confidence interval
CSME	Clinically significant macular edema
DDAH	Dimethylarginine dimethylaminohydrolase
DM	Diabetes mellitus
DR	Diabetic retinopathy
EPO	Erythropoietin
GWAS	Genome-wide association study
HbA1c	Hemoglobin A1c
HWE	Hardy-Weinberg Equilibrium
NO	Nitric oxide
eNOS	Endothelial nitric oxide synthase
NOS	Nitric oxide synthase
NPDR	Non-proliferative diabetic retinopathy
PKA	Protein kinase A
PKC	Protein kinase C
OR	Odds ratio
PDR	Proliferative diabetic retinopathy
SDMA	Symmetric dimethylarginine
SE	Standard error

SNP	Single nucleotide polymorphism
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A

CHAPTER 1

Introduction

Definition of Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterised by chronic hyperglycemia. The criteria for the diagnosis of diabetes mellitus defined by the World Health Organisation is blood glucose levels of greater than or equal to 7.0 mmol/l of fasting venous plasma, or greater or equal to 11.1 mmol/l for venous plasma 2 hours post 75 grams of oral glucose load[1]. DM results from defects in insulin secretion, insulin action or both[2], and although there are several forms of DM, this thesis examines diabetic ocular complications in the two most common types of diabetes – type 1 DM (T1DM) and type 2 DM (T2DM).

Type 1 Diabetes Mellitus

T1DM accounts for approximately 10% of diabetes cases[3]. It most often has an onset in childhood, commonly between the ages of 10-14 years and usually before the age of 30.

T1DM has been well established as a complex heterogenous disease. The precise etiology of T1DM is uncertain and a multifactorial model of genetic and environmental risk factors has been proposed. Concordance rates of T1DM in twin studies range from 23-53% in monozygotic twins to 5-11% in dizygotic twins[4, 5]. Approximately 45% of the genetic susceptibility to T1DM is due to inheritance of genes located within the major histocompatibility complex (MHC) HLA (human

leukocyte antigen) class II region on chromosome 6p21, with HLA-DR3 and DR4 playing a major role[6-8]. Recent genome-wide association studies have also indicated the involvement of at least ten loci in the pathogenesis of T1DM, including the *IFIH1* and *KIAA0350* gene regions and several chromosomal regions, particularly involving chromosome 12[9-12].

In addition to genetic influences, environmental factors such as viral exposure and diet appear to be involved. Congenital rubella is the only proven causal viral factor in T1DM[13, 14]. Other environmental factors including enterovirus[15] and rotavirus[16] infections and early infant diet containing cow's milk[17] have been investigated as environmental triggers associated with T1DM disease pathogenesis in some studies. However to date, none have been convincingly implicated in the pathogenesis of T1DM.

Genetic and environmental factors lead to the autoimmune destruction of pancreatic islet cells, which are responsible for the production of insulin in response to rising blood glucose levels[18]. This leads to a lack of production of endogenous insulin, such that individuals with T1DM require life long injections of insulin.

Type 2 Diabetes Mellitus

T2DM accounts for up to 90% of cases of DM globally, resulting from a combination of obesity, physical inactivity and genetic factors[19]. Positive family history leads to a 2.4 fold increased risk for T2DM development[20]. Twin studies have shown higher concordance rates for T2DM, ranging from 34-58% in monozygotic, to 16-37% in dizygotic twins[4, 21-23]. Recent genome-wide association studies have

demonstrated at least ten identified loci that are associated with the development of T2DM. These include disease susceptibility loci present in or surrounding genes *TCF7L2*, *SLC30A8* and *CDKAL1* [24-28].

Obesity and physical inactivity have been shown to result in changes in the levels of circulating hormones, cytokines and non-esterified free fatty acids (that originate in adipocytes). These factors modulate insulin action, leading to insulin resistance, defective insulin action and hyperglycemia[29]. Management of hyperglycemia in T2DM may include a combination of diet and exercise, oral hypoglycemic agents and insulin[30].

Incidence of Diabetes Mellitus

DM has reached epidemic proportions worldwide. Global diabetes prevalence data indicates 171 million people were diagnosed with diabetes in 2000, with prevalence expected to double by 2030 to 366 million[31]. The proportional increase in DM is greater in developing countries[32]. The prevalence of both type T1DM and T2DM diabetes is predicted to rise substantially over the next few decades[30, 31, 33]. Globally, India is estimated to have the highest number of people with diabetes in the world, followed by China and the USA. These countries are predicted to remain the top three ranking countries in DM prevalence in 2025-2030[31, 32]. The increase in the incidence and prevalence of DM may reflect an increase in rates of diagnosis, however it is likely to be due also to increasing incidence of risk factors such as sedentary lifestyle and obesity[34].

Approximately 100,000 Australians are diagnosed with DM each year[35]. The latest report from the Australian Bureau of Statistics (ABS) regarding DM reported 700,000 people or 3.5% of the Australian population to have DM in 2004-2005, of which 0.5% have been reported to have T1DM and 3.0% T2DM[36]. The AusDiab study reports a significantly higher prevalence of T2DM at 7.2%[35]. Not only has there been a substantial rise from 404,000 people (2.4% of the population) in 1995, but the prevalence of diabetes in Australia has already exceeded predictions made in 1995 to be 3.3% in 2025[32]. The ABS report has indicated 13% of people with DM have T1DM and 83% T2DM, with most of the increase in prevalence of diabetes since 1995 shown to be due to an increase in T2DM.

Diabetic Retinopathy Definition

Diabetic retinopathy (DR) is a sight-threatening microvascular complication of DM and a major cause of morbidity. DR is defined by the presence of retinal microvascular lesions. Early retinal signs in non-proliferative diabetic retinopathy (NPDR) include the following:

- Microaneurysms - these occur as a result of physical weakening of the capillary walls due to pericyte loss. Microaneurysms are at risk of rupture and may bleed (leading to dot haemorrhages), become occluded (leading to retinal nerve fibre layer infarction) or leak (leading to retinal edema).
- Hard exudates – these are caused by the breakdown of the blood-retinal barrier, allowing leakage of serum proteins, lipids and protein from the blood vessels.

- Cotton wool spots / infarcted nerve fibre layer - these are areas of retinal nerve fibre layer death due to a local lack of oxygen as a result of damaged retinal blood vessels.
- Intraretinal microvascular abnormalities – these are formations of arteriovenous shunts as a result of capillary occlusion.
- Venous beading – this occurs as a result of venous dilatation and engorgement and is a sign of retinal ischemia and a powerful predictor of conversion to proliferative DR (PDR)[37, 38].

It is important to note that none of these changes are specific for DR, as they may occur in other disease processes such as hypertension, hyperviscosity, inflammation or radiation.

The growth of *abnormal new blood vessels* in the retina secondary to retinal ischemia frequently leads to pre-retinal and vitreous haemorrhage and is the principal hallmark of PDR. Capillary leakage in the macular or perimacular region results in retinal thickening or diabetic macular edema, defined as thickening located within two disc diameters of the centre of the macula. When retinal thickening is present within 500µm of the fovea or when hard exudates are present within 500µm of the fovea with adjacent thickening, it is termed clinically significant macular edema (CSME)[39]. Visual impairment in DR occurs secondary to pre-retinal or vitreous hemorrhage, retinal detachment and diabetic maculopathy (either macular edema or macular ischemia)[40].

Prevalence of Diabetic Retinopathy

Global population-based data indicates that DR is the fifth most common cause of blindness in the world and accounts for approximately 4.8% of global blindness[41-43]. DR is also a leading cause of blindness in industrialised countries[44] and the most common cause in working age adults[45]. The incidence of DR is likely to increase with increasing frequency of DM[46, 47]. Combined analysis of data from 8 population studies (including 2 Australian studies) of participants aged over 40 years and of varying ethnicities has revealed the overall prevalence of any level of DR among persons with DM to be as high as 40.3%, with 8.2% being sight-threatening[46]. Examination of the findings from three population-based studies and over 11,000 participants revealed the overall prevalence of DR in Australian individuals with DM to range from 9.6-15.8%[48]. The prevalence of DR is higher in patients with T1DM, with sight-threatening DR reported to be up to 2.5 times more frequent than in those with T2DM[49-53]. Conversely, the incidence of macular edema has been reported to be up to two times higher amongst those with T2DM[54, 55].

Diabetic Retinopathy Grading

Several grading systems for DR have been devised. The Early Treatment Diabetic Retinopathy Study (ETDRS) staging system[56] (based on the Modified Airlie House Classification) is still regarded as the gold standard for grading in clinical trials and epidemiologic studies[40], and a simplified ETDRS (Wisconsin Level) classification is recommended for clinical classification by the National Health and Medical Research Council (NHMRC) (Table 1.1).

Table 1.1- Clinical classification of diabetic retinopathy (Wisconsin level)

Retinopathy stage	**Definition**
Minimal NPDR	Ma only
Mild NPDR	Ma and one or more of: retinal haem, HEx, CWS, but not meeting Moderate NPDR definition
Moderate NPDR	H/Ma ≥ std photo 2A in at least one quadrant and one or more of: CWS, VB, IRMA, but not meeting Severe NPDR definition
Severe NPDR *preproliferative*	Any of : H/Ma >std photo 2A in all four quadrants, IRMA >std photo 8A in one or more quadrants, VB in two or more quadrants
PDR	Any of: NVE or NVD <std photo 10A, vitreous/ preretinal haem and NVE <½ disc area (DA) without NVD
High-risk PDR	Any of: NVD>¼ to ⅓ disc area, or with vitreous/ preretinal haem, or NVE>½ DA with vitreous/ preretinal haem
Advanced PDR	High-risk PDR with tractional detachment involving macula or vitreous haem obscuring ability to grade NVD and NVE
Macular Oedema	Retinal thickening within 2 disc diameters of macular centre
Clinically Significant Macular Oedema (CSME)	Retinal thickening within 500μm of macular centre or hard exudates within 500μm of macular centre with adjacent thickening

Definitions: Ma (microaneurysm), H (dot haemorrhage) Hex (hard exudates) NVD (new vessels on the disc), NVE (new vessels elsewhere), CWS (cotton wool spot), VB (venous beading), IRMA (intra-retinal microvascular abnormality).
(Modified and reproduced table from the NHMRC Guidelines for the Management of Diabetic Retinopathy 1997[57])

Pathogenesis of Diabetic Retinopathy

The pathogenesis of DR is complex and multifactorial, with its biochemical component being primarily due to altered glucose metabolism[58]. Microvascular

damage occurs as an end result of hyperglycemic damage on the retinal vascular endothelium. Several pathways have been implicated in the pathogenesis of DR, including the polyol pathway, oxidative stress, advanced glycation end products, renin angiotensin system and the cytokine network leading to damage of pericytes, thickening of retinal vascular endothelial basement membranes and increased vascular permeability and proliferation of endothelial cells[59]. These pathways are discussed in detail in the first chapter.

Clinical Risk Factors for Diabetic Retinopathy

There are several established risk factors in the development of DR. Large longitudinal prospective studies have confirmed that prolonged hyperglycemia is the most important single determinant of risk[60-62]. The duration of DM has also been strongly associated with the development and severity of DR[53, 63-65]. Uncontrolled hypertension has been established as a risk factor for the progression of DR[66, 67], and hyperlipidemia for the development of DR[68, 69], in particular for CSME[70]. Other risk factors including nephropathy[71, 72], and smoking[67], have also been reported to play a role in the development of DR.

Management of Diabetic Retinopathy

The mainstay of treatment of the non-blinding forms of DR remains optimal control of its associated risk factors. The NHMRC guidelines recommend optimal glycemic control of patients with DR, with a target of HbA1c less than 7.0% and systolic blood pressure of less than 130 mmHg[40]. There is evidence from randomised controlled trials (RCTs) for the use of lipid lowering agents as an adjunct to laser treatment for macular edema[73-75]. For severe NPDR and PDR, large RCTs[76, 77] have shown pan-

retinal photocoagulation to reduce the risk of moderate and severe visual loss by 50%. Similarly focal or grid laser treatment for macular edema reduces the risk of moderate visual loss by at least 50%[39, 78, 79]. A meta-analysis of all relevant RCTs has provided level 1 evidence (systematic review) of vision preserving benefits of laser treatment in both PDR and macular edema[80].

Reports of significant improvement in diabetic macular edema with intravitreal corticosteroids[81, 82] and PDR and macular edema with anti-VEGF treatments[83, 84] have more recently been reported in RCTs. However, larger clinical trials are required for confirmation of beneficial effects, particularly as intravitreal corticosteroids can accelerate cataract formation and glaucoma in susceptible individuals[85]. It is important to note that available treatments largely focus on preventing further loss of vision and there are no treatments currently available to consistently restore lost vision. There are various treatments for specific complications of advanced PDR (such as vitreous haemorrhage and retinal detachments) but their details will not be a focus of this thesis. Visual outcomes in advanced PDR are typically poor with permanent reduction in visual acuity, visual field or both.

The Role of Genetics in Diabetic Retinopathy

Evidence for a role of genetics in DR

It has become evident through familial aggregation studies that susceptibility to DR has a heritable component, independent of established risk factors including glycemic control and duration of diabetes. The FIND-eye study has been the largest familial aggregation study to report a significant familial connection for DR severity

in 767 families[86]. The majority of the 2368 diabetic subjects enrolled were of Mexican American decent with T2DM and showed a sib-sib correlation of 0.1359 (±0.02) for DR with DR heritability estimated at approximately 27%.

In other studies specifically investigating T2DM participants, first degree relatives of Indian participants with DR have been shown to be at significantly higher risk of developing any DR (OR 3.37 [95% CI 1.56-7.29], p=0.002)[87]. These findings have been replicated in Mexican Americans, particularly for severe NPDR or PDR (OR 1.72 [95% CI 1.03-2.88])[88].

Similarly, DR heritability has been investigated and found in participants with T1DM. The Diabetes Control and Complications Trial is the largest trial to investigate this, and revealed a familial tendency for severe DR in 304 American and British T1DM participants with 241 affected relatives[89]. This study showed an increased risk for severe DR (severe NPDR, CSME, or laser treatment) among relatives of DR positive subjects when compared to DR negative T1DM subjects (OR 3.1 [95% CI 1.2-7.8], p<0.05). Finnish participants with T1DM have also shown familial clustering of DR, with increased risk of PDR in siblings of probands (OR 2.76 [95% CI 1.25-6.11], p=0.01)[90].

Genetic association studies

Most studies investigating genetic associations with DR susceptibility have been candidate gene studies. In fact over 160 candidate gene studies have been carried out, mostly investigating genes in well established candidate pathways for DR, including the polyol, nitric oxide, advanced glycation end products and the renin-angiotensin-

aldosterone pathway. Although some convincing evidence exists for specific genetic associations, due to significant variation in study design, often including sub-optimal study power, different DR grading scales, the failure to undertake multivariate analyses for known clinical risk factors, and failure to correct for multiple hypothesis testing, it is difficult to draw solid conclusions from many of the candidate gene studies in the literature.

A genome-wide linkage study, whereby 516 microsatellite markers were scanned in 322 Pima Indian sib-ships with T2DM, has shown evidence of linkage to 1p36 for DR[88]. Another genome-wide linkage study was undertaken in 282 affected Mexican-American sibling pairs with T2DM[91]. In this study 360 markers were investigated and suggestive linkage to chromosomes 3, 5, 6, 12, 19 and 20 was found. The biggest limitation of these linkage studies was the inability to identify associated genes due to low resolution. Associated chromosomal regions require extensive candidate gene follow up in order to identify disease associated genes. No genome wide SNP association studies have as yet been published for DR. Thus no specific genetic risk factor to date has been conclusively and reproducibly implicated in the development of DR.

Aims

The aim of this thesis was to recruit a large cohort of well characterised Australian individuals with T1DM or T2DM in order to identify genetic risk factors in the development of DR, with a particular focus on sight-threatening DR. A meta-analysis of all relevant candidate gene studies for DR was undertaken to establish the most significantly associated genes with DR based on existing literature. Utilising the

findings of the meta-analysis, candidate gene studies were undertaken to explore associations in participants with T1DM and T2DM specifically recruited as part of this PhD project. In addition, other novel candidate gene studies were carried out for genes believed to play biologically plausible roles in the development of sight-threatening DR. Serum biomarkers involved in established pathways for DR were analysed. Finally a genome-wide association study was undertaken to identify novel DR susceptibility loci.

CHAPTER 2

A systematic meta-analysis of genetic association studies for diabetic retinopathy

The original work presented in this chapter has been published in the peer-reviewed literature: S Abhary, AW Hewitt, KP Burdon, JE Craig A systematic meta-analysis of genetic association studies for diabetic retinopathy. Diabetes 2009 Sep;58(9):2137-47[92]. Dr Abhary's contributions include conception and design of the study, review of the literature, data collection, analysis and interpretation of data and writing of the manuscript.

Introduction

Attempts to further understand the pathogenesis of DR have been made in over 160 candidate gene studies for DR in T1DM and T2DM of different ethnic cohorts. The majority of genetic studies have investigated genes in well established pathways for DR. Individual studies have provided some degree of evidence for the association of various genes with DR. However, many studies have been undertaken in small cohorts with sub-optimal power and have had considerable fundamental variations in study design, including the use of non-standardised DR grading scales and the failure to control for relevant clinical covariates in multivariate analyses. Therefore no genetic risk factors thus far have been irrefutably implicated in the development of DR in T1DM or T2DM.

The aim of this systematic meta-analysis was to analyse all published studies that met specified inclusion criteria and investigated the association between genetic polymorphisms and the development of DR. We sought to determine which of the previously investigated genetic variants are significantly associated with the development of DR in T1DM or T2DM under meta-analysis and to examine the strength of these associations. The findings were then used to inform the selection of a number of genes for further analysis in the well powered cohort being recruited for this thesis.

Methods

Literature Search and Data collection:

A systematic literature search was performed to identify all studies published between January 1990 and August 2008, which investigated the association of genetic variants with the development of any form of DR. The Pubmed database (National Center for Biotechnology Information; NCBI), ISI Web of Knowledge (v4.5) and the Cochrane Library were explored using the following keyword strings; "genetic" AND "diabetic retinopathy" and "gene" AND "diabetic retinopathy". All retrieved publications written in English were further reviewed. Studies that contained sufficient case (subjects with diabetes and DR) and control (subjects with diabetes but without the complication of DR) genotype information, such as allele or genotype frequency, were included. The reference list of each relevant publication was also examined to identify additional studies appropriate for inclusion in the meta-analysis. Polymorphisms were included in the meta-analysis if a minimum of two studies had assessed association with DR development.

Data Analysis:

Data were entered into a database developed specifically for the meta-analysis. Publication bias was assessed in Stata (version 10.1, Stata Corporation, USA) and all other statistical analyses were performed using RevMan software (version 4.2, Oxford, UK).

Publicly available genomics resources Ensemble (http://www.ensembl.org/index.html), and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) were used to locate rs# identifiers for the genetic variants to ensure consistency of naming. When an rs# identifier could not be located the most commonly used name for that specific variant or single nucleotide polymorphism (SNP) was selected.

Analyses were performed for all cases with any form of DR compared to all diabetic subjects without DR (controls). Sub-analysis of the DR subtypes non-proliferative DR (NPDR), and proliferative DR (PDR) was subsequently undertaken where possible, including the comparison of NPDR with PDR. Major and minor allele frequencies were calculated from the available genetic data of all reported variants in the included studies and odd ratios (ORs) and 95% confidence intervals (CIs) were calculated. The allelic association of microsatellite markers was also investigated, whereby the risk conferred by each allele was compared against all other alleles as well as against specific alleles. The Der Simonian and Laird random-effects model was used[93, 94]. This model utilises weights which incorporate both within-study and between-study variance. Heterogeneity between studies was calculated as the inverse variance estimate. To minimize genetic heterogeneity, sub-analysis was performed

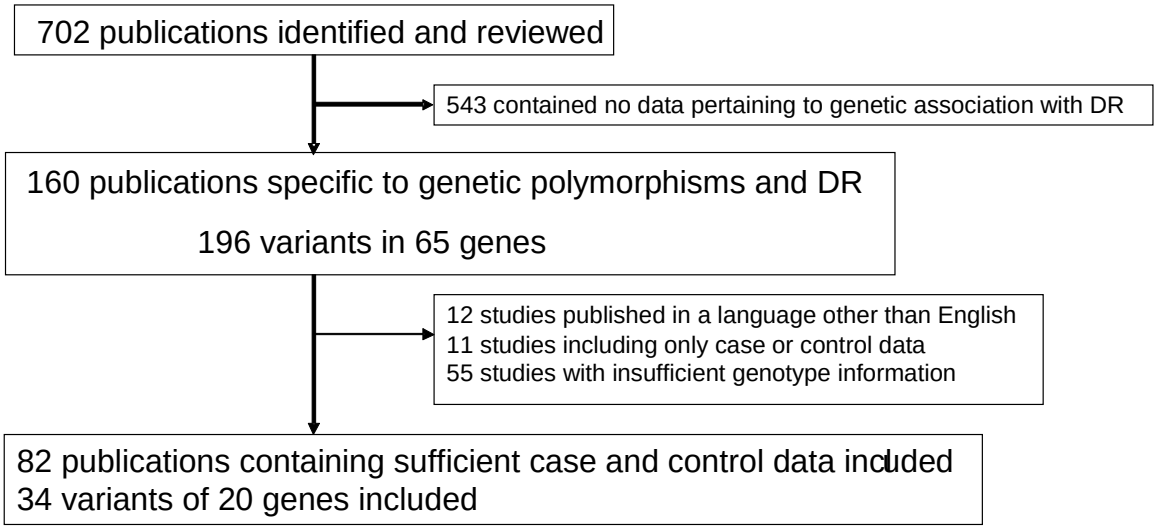
for all cohorts of Caucasian origin and for datasets in which inter-study heterogeneity remained, outlying studies were then removed in a step-wise fashion until homogeneity was achieved[95].

For the purpose of this meta-analysis, 'Caucasian ancestry' was defined as being of European descent. Studies including subjects of Caucasian and non-Caucasian ancestry have had their cohorts divided and analysed individually. Funnel plots were constructed in Revman and Egger's test[96] was applied in Stata to investigate publication bias. A p-value of <0.05 was considered statistically significant in all analyses, except for publication bias (Egger's test), where a p value of <0.1 was considered as statistically significant. No adjustments were made for multiple testing.

Results

Seven hundred and two publications were identified. One hundred and sixty of these were specific to genetic polymorphisms and the development of DR (Figure 2.1). Twelve of these studies were in a language other than English and were excluded, and a final total of 82 studies were suitable for inclusion as a result of having presented sufficient case and control genotype information. One hundred and ninety six polymorphisms were identified with 34 of these having adequate genotype data for inclusion in the meta-analysis. In total, genetic data from 87,187 individuals were included in this meta-analysis.

Figure 2.1 – Flow chart of study selection process and included studies.



Visual inspection of the funnel plots revealed a symmetrical inverted V shape and Egger's test did not detect significant publication bias ($p>0.1$) for all polymorphisms examined by more than 5 studies (Appendix 1).

Five polymorphisms were investigated by more than five studies and are discussed in detail (Table 2.1) and details of their study design are provided in an appendix (Appendix 2).

Forty eight studies were included in the analyses for the five polymorphisms, 20 (42%) of which included subjects of Caucasian ancestry (Table 2.2).

Table 2.1 – Meta-analysis of genetic variants and risk for DR in T1DM, T2DM and both types of diabetes regardless of participant's ethnicity.

All variants have been examined by a minimum of 5 studies. Sub-analyses have been performed for NPDR and PDR if data were available.

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
ACE	INS/DEL at intron 16	287 base pair deletion	No DR vs Any DR	Type 1	651 (55)	458 (52)	32.76	1.06 (0.88−1.27)	0.09	6	3.67	0.5402	97-102
				Type 2	986 (44)	931 (43)	67.24	1.01 (0.89−1.15)	0.06	7	2.67	0.866	99, 103-108
				Total	1637 (47)	1389 (45)	100.00	1.03 (0.93−1.14)	0.05	13	6.51	0.6249	
			No DR vs NPDR	Type 2	276 (35)	438 (36)	100.00	0.91 (0.75−1.12)	0.10	3	1.51	0.3786	105, 106, 108
			No DR vs PDR	Type 1	387 (60)	220 (55)	48.63	1.19 (0.77−1.86)	0.23	4	8.01	0.4355	97, 98, 100, 101
				Type 2	221 (41)	438 (36)	51.37	1.12 (0.89−1.42)	0.12	3	0.21	0.3237	105, 106, 108
				Total	608 (51)	658 (40)	100.00	1.17 (0.94−1.44)	0.11	7	8.55	0.1515	
			NPDR vs PDR	Type 1	120 (64)	72 (45)	17.02	2.21 (1.44−3.39)	0.22	1	<0.01	0.0003	100, 109
				Type 2	353 (45)	481 (41)	82.98	1.12 (0.92−1.35)	0.1	6	4.75	0.2512	105, 106, 108, 110-112
				Total	473 (49)	553 (41)	100	1.25 (0.95−1.65)	0.14	7	12.77	0.1064	
NOS3	rs3138808	393 base pair insertion	No DR vs Any DR	Type 1	21 (11)	42 (22)	9.91	0.43 (0.24−0.76)	0.29	1	<0.01	0.0036	113
				Type 2	498 (21)	705 (26)	90.09	0.93 (0.74−1.17)	0.12	7	13.63	0.5408	114-120
				Total	519 (21)	747 (26)	100.00	0.87 (0.68−1.11)	0.13	8	19.38	0.2578	
			No DR vs NPDR	Type 1	21 (11)	42 (22)	32.47	0.43 (0.24−0.76)	0.29	1	<0.01	0.0036	113
				Type 2	95 (20)	116 (24)	67.53	0.53 (0.12−2.46)	0.78	2	22.10	0.421	114, 120
				Total	116 (17)	158 (24)	100.00	0.5 (0.19−1.31)	0.49	3	22.99	0.157	
			No DR vs PDR	Type 2	43 (17)	116 (24)	100.00	0.42 (0.05−3.69)	1.11	2	28.95	0.4341	114, 120
			NPDR vs PDR	Type 2	43 (17)	95 (20)	100.00	0.79 (0.42−1.5)	0.33	2	2.61	0.4723	114, 120

OR = odds ratios, CI = confidence interval and SE = standard error

Table 2.1 continued

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
VEGF	rs2010963	G	No DR vs Any DR	Type 2	1204 (55)	1144 (59)	100.00	0.86 (0.7−1.05)	0.10	7	14.65	0.1407	[119, 121-126]
			No DR vs NPDR	Type 2	242 (53)	328 (65)	100.00	0.62 (0.48−0.81)	0.14	3	1.93	0.0005	[121, 125, 126]
			No DR vs PDR	Type 2	212 (56)	643 (59)	100.00	0.8 (0.6−1.06)	0.14	4	0.92	0.1166	[121, 123, 125, 126]
			NPDR vs PDR	Type 2	211 (56)	242 (53)	100.00	1.32 (0.99−1.76)	0.15	3	0.81	0.0559	[121, 125, 126]
AKR1B1	rs759853	T	No DR vs Any DR	Type 1	114 (29)	149 (45)	33.33	0.49 (0.36−0.68)	0.16	3	0.02	<0.0001	[127-129]
				Type 2	508 (34)	460 (26)	66.67	1.16 (0.98−1.36)	0.08	5	3.96	0.0885	[130-133]
				Total	622 (33)	609 (29)	100.00	0.9 (0.66−1.22)	0.16	8	25.19	0.4857	
			No DR vs NPDR	Type 1	8 (27)	28 (43)	14.13	0.49 (0.19−1.27)	0.48	1	<0.01	0.143	[129]
				Type 2	175 (43)	147 (39)	85.87	1.17 (0.88−1.55)	0.15	2	0.09	0.2945	[130]
				Total	183 (42)	175 (39)	100.00	1.02 (0.7−1.5)	0.20	3	3.00	0.9099	
			No DR vs PDR	Type 1	7 (25)	28 (43)	12.07	0.45 (0.17−1.21)	0.50	1	<0.01	0.1144	[129]
				Type 2	123 (35)	147 (39)	87.93	0.84 (0.6−1.19)	0.18	2	1.19	0.3285	[130]
				Total	130 (34)	175 (39)	100.00	0.79 (0.55−1.13)	0.18	3	2.51	0.1949	
			NPDR vs PDR	Type 1	7 (25)	8 (27)	6.65	0.92 (0.28−2.98)	0.60	1	<0.01	0.8848	[129]
				Type 2	123 (35)	175 (43)	93.35	0.76 (0.47−1.2)	0.24	2	1.95	0.2364	[130]
				Total	130 (34)	183 (42)	100.00	0.73 (0.54−0.99)	0.16	3	2.12	0.0428	
AKR1B1	(CA)n dinucleotide repeat	z	No DR vs Any DR	Type 1	344 (36)	313 (32)	39.83	1.09 (0.89–1.35)	0.11	6	5.24	0.3945	[127, 129, 134-137]
				Type 2	436 (29)	612 (25)	60.17	1.04 (0.70–1.57)	0.21	9	46.91	0.8330	[133, 138-145]
				Total	780 (32)	925 (27)	100.00	1.05 (0.81–1.35)	0.13	15	52.88	0.7264	

OR = odds ratios, CI = confidence interval and SE = standard error

Table 2.1 continued

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
AKR1B1	(CA)n dinucleotide repeat	z	No DR vs NPDR	Type 1	25 (41)	20 (31)	28.59	1.55 (0.75–3.23)	0.37	1	<0.01	0.2366	[129]
				Type 2	97 (27)	81 (37)	71.41	0.65 (0.45–0.94)	0.19	2	0.03	0.0215	[139, 145]
				Total	122 (29)	101 (36)	100.00	0.83 (0.49–1.42)	0.27	3	4.38	0.4937	
		z	No DR vs PDR	Type 1	132 (43)	147 (37)	37.17	1.29 (0.95–1.75)	0.15	2	0.30	0.0979	[129, 136]
				Type 2	179 (31)	171 (38)	62.83	0.77 (0.59–1.00)	0.13	4	1.63	0.0482	[138-140, 145]
				Total	311 (35)	318 (37)	100.00	0.94 (0.72–1.24)	0.14	6	8.40	0.6738	
		z	NPDR vs PDR	Type 1	25 (41)	20 (31)	28.59	1.55 (0.75–3.23)	0.37	1	<0.01	0.2366	[129]
				Type 2	97 (27)	81 (37)	71.41	0.65 (0.45–0.94)	0.19	2	0.03	0.0215	[139, 145]
				Total	122 (29)	101 (36)	100.00	0.83 (0.49–1.42)	0.27	3	4.38	0.4937	
		z-2	No DR vs Any DR	Type 1	362 (37)	261 (27)	41.98	1.95 (1.04–3.66)	0.32	6	40.66	0.0367	[129, 136]
				Type 2	385 (26)	171 (7)	58.02	2.64 (1.39–5.01)	0.33	9	69.93	0.0029	[138-140, 145]
				Total	747 (30)	432 (13)	100.00	2.33 (1.49–3.64)	0.23	15	120.27	0.0002	
		z-2	No DR vs NPDR	Type 1	22 (36)	23 (35)	22.71	1.03 (0.50–2.13)	0.37	1	<0.01	0.9400	[129, 137]
				Type 2	137 (38)	55 (25)	77.29	1.87 (1.29–2.71)	0.19	2	0.20	0.0010	[138-140, 145]
				Total	159 (38)	78 (28)	100.00	1.64 (1.14–2.35)	0.18	3	2.26	0.0075	
		z-2	No DR vs PDR	Type 1	41 (24)	35 (23)	24.74	1.18 (0.69–2.01)	0.27	2	0.31	0.5363	[129, 137]
				Type 2	173 (30)	85 (19)	75.26	1.64 (1.21–2.22)	0.16	4	2.50	0.0016	[138-140, 145]
				Total	214 (29)	120 (20)	100.00	1.51 (1.16–1.97)	0.14	6	3.89	0.0023	
		z-2	NPDR vs PDR	Type 1	19 (18)	12 (14)	18.52	1.39 (0.64–3.04)	0.40	1	<0.01	0.4076	[137]
				Type 2	137 (38)	55 (25)	81.48	1.87 (1.29–2.71)	0.19	2	0.20	0.0010	[139, 145]
				Total	156 (34)	67 (22)	100.00	1.77 (1.26–2.48)	0.17	3	0.64	0.0009	

OR = odds ratios, CI = confidence interval and SE = standard error

Table 2.1 continued

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
AKR1B1	(CA)n dinucleotide repeat	z+2	No DR vs Any DR	Type 1	153 (16)	224 (23)	40.68	0.58 (0.36–0.93)	0.24	6	18.16	0.0243	127, 129, 134-137
				Type 2	331 (22)	521 (21)	59.32	0.97 (0.57–1.65)	0.27	9	64.22	0.9038	133, 138-145
				Total	484 (20)	745 (22)	100.00	0.79 (0.53–1.17)	0.20	15	104.32	0.2388	
		z+2	No DR vs NPDR	Type 1	7 (12)	13 (20)	22.07	0.52 (0.19–1.40)	0.51	1	<0.01	0.1955	137
				Type 2	34 (10)	29 (14)	77.93	0.71 (0.42–1.20)	0.27	2	0.07	0.2001	139, 145
				Total	41 (10)	42 (15)	100.00	0.66 (0.41–1.05)	0.24	3	0.36	0.0820	
		z+2	No DR vs PDR	Type 1	48 (16)	70 (18)	38.34	0.84 (0.50–1.40)	0.26	2	1.26	0.4971	129, 136
				Type 2	82 (14)	98 (22)	61.66	0.62 (0.39–0.99)	0.24	4	4.71	0.0464	138-140, 145
				Total	130 (15)	168 (20)	100.00	0.71 (0.51–0.98)	0.17	6	6.92	0.0383	
		z+2	NPDR vs PDR	Type 1	7 (12)	13 (20)	22.07	0.52 (0.19–1.40)	0.51	1	<0.01	0.1955	137
				Type 2	34 (10)	29 (14)	77.93	0.71 (0.42–1.20)	0.27	2	0.07	0.2001	139, 145
				Total	41 (10)	42 (15)	100.00	0.66 (0.41–1.05)	0.24	3	0.36	0.0820	

OR = odds ratios, CI = confidence interval and SE = standard error

Table 2.2 – Meta-analysis of genetic variants and risk for diabetic retinopathy in patients with T1DM, T2DM and both types of diabetes, for studies of Caucasian populations. Sub-analyses were been performed for NPDR and PDR if data were available.

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
ACE	INS/DEL at intron 16	287 base pair deletion	No DR vs Any DR	Type 1	651 (55)	458 (52)	50.73	1.06 (0.88−1.27)	0.09	6	3.67	0.5402	97-102
				Type 2	504 (53)	501 (54)	49.26	0.99 (0.83−1.19)	0.09	4	1.43	0.9182	99, 103, 104, 106
				Total	1155 (54)	959 (53)	100	1.02 (0.9−1.16)	0.07	10	5.35	0.7157	
			No DR vs PDR	Type 1	387 (60)	220 (55)	76.84	1.19 (0.77−1.86)	0.23	4	8.01	0.4355	97, 98, 100, 101
				Type 2	75 (53)	78 (49)	23.15	1.14 (0.73−1.79)	0.23	1	<0.01	0.5617	106
				Total	462 (59)	298 (53)	100	1.19 (0.86−1.66)	0.17	5	8.11	0.2995	
			NPDR vs PDR	Type 1	120 (64)	72 (45)	33.91	2.21 (1.44−3.39)	0.22	1	<0.01	0.0003	100
				Type 2	156 (54)	224 (50)	66.08	1.23 (0.9−1.67)	0.16	2	0.83	0.1905	106, 111
				Total	276 (58)	296 (49)	100	1.52 (1−2.32)	0.22	3	5.55	0.0509	
NOS3	rs3138808	393 base pair insertion	No DR vs Any DR	Type 1	21 (11)	42 (22)	47.02	0.43 (0.24−0.76)	0.29	1	<0.01	0.0036	113
				Type 2	123 (22)	58 (21)	52.97	1.09 (0.77−1.55)	0.18	1	<0.01	0.6248	118
				Total	144 (19)	100 (21)	100	0.71 (0.28−1.75)	0.46	2	7.49	0.4512	
VEGF	rs2010963	G	No DR vs Any DR	Type 2	387 (55)	247 (62)	100	0.83 (0.65−1.07)	0.13	2	0.43	0.1601	124, 125

OR = odds ratios, CI = confidence interval and SE = standard error

Table 2.2 continued

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
AKR1B1	rs759853	T	No DR vs Any DR	Type 1	99 (29)	121 (46)	45.13	0.5 (0.35−0.71)	0.18	2	0.01	0.0001	[127, 128]
				Type 2	324 (42)	202 (41)	54.86	1.05 (0.83−1.33)	0.12	2	0.91	0.6756	[130]
				Total	423 (38)	323 (43)	100	0.76 (0.5−1.16)	0.22	4	12.83	0.2061	
AKR1B1	(CA)n dinucleotide repeat	z	No DR vs Any DR	Type 1	277 (42)	247 (39)	75.24	1.11 (0.88–1.39)	0.117	3	0.88	0.3785	[127, 134, 136]
				Type 2	79 (32)	67 (42)	24.75	0.66 (0.44–1.00)	0.21	1	<0.01	0.05	[139]
				Total	356 (39)	314 (40)	100	0.97 (0.73–1.27)	0.14	4	5.47	0.8076	
		z	No DR vs PDR	Type 1	107 (43)	127 (38)	51.90	1.24 (0.89–1.74)	0.17	1	<0.01	0.2008	[136]
				Type 2	79 (32)	67 (42)	48.09	0.66 (0.44–1.00)	0.21	1	<0.01	0.05	[139]
				Total	186 (38)	194 (39)	100	0.92 (0.5–1.70)	0.314	2	5.42	0.7872	
		z-2	No DR vs Any DR	Type 1	226 (34)	167 (27)	74.10	1.83 (0.85–3.95)	0.393	3	15.42	0.1238	[127, 134, 136]
				Type 2	97 (40)	43 (27)	25.89	1.78 (1.15–2.74)	0.22	1	<0.01	0.0091	[139]
				Total	323 (36)	210 (27)	100	1.80 (1.06–3.06)	0.271	4	15.89	0.0301	
		z-2	No DR vs PDR	Type 1	87 (35)	121 (36)	52.35	0.96 (0.68–1.35)	0.175	1	<0.01	0.8023	[136]
				Type 2	97 (40)	43 (27)	47.64	1.78 (1.15–2.74)	0.22	1	<0.01	0.0091	[139]
				Total	184 (37)	164 (33)	100	1.29 (0.7–2.36)	0.309	2	4.85	0.4166	
		z+2	No DR vs Any DR	Type 1	103 (16)	159 (25)	76.34	0.51 (0.25–1.06)	0.372	3	12.85	0.0722	[127, 134, 136]
				Type 2	28 (12)	24 (15)	23.65	0.73 (0.41–1.31)	0.299	1	<0.01	0.2958	[139]
				Total	131 (15)	183 (23)	100	0.56 (0.32–0.97)	0.282	4	13.71	0.039	
		z+2	No DR vs PDR	Type 1	41 (17)	57 (17)	63.94	0.97 (0.62–1.50)	0.224	1	<0.01	0.8814	[136]
				Type 2	28 (12)	24 (15)	36.05	0.73 (0.41–1.31)	0.299	1	<0.01	0.2958	[139]
				Total	69 (14)	81 (17)	100	0.87 (0.62–1.24)	0.179	2	0.56	0.455	

OR = odds ratios, CI = confidence interval and SE = standard error

Inter-study heterogeneity was eliminated in the sub-analyses of studies with participants of Caucasian ancestry without the requirement of removal of outlying studies, indicating that ethnicity was a major source of heterogeneity.

Data on the remaining 27 SNPs analysed in the current meta-analysis, which have been examined by a minimum of 2 and maximum of 5 cohorts, are presented in Table 2.3. The rs2910964 SNP of the α2β1 integrin *(ITGA2)* gene (OR 1.65 [95% CI: 1.26-2.15], $p=2x10^{-4}$), and rs13306430 of the intercellular cell adhesion molecule 1 *(ICAM1)* gene (OR 0.56 [95% CI: 0.39-0.81], $p=1.70x10^{-3}$) were significantly associated with DR, both being examined in T2DM only and by 2 studies in each case.

Table 2.3 – Meta-analysis of 27 SNPs and risk for DR in T1DM, T2DM and both types of diabetes. All SNPs have been examined by a minimum of 2 and maximum of 5 studies. Sub-analyses have been performed for NPDR and PDR if data were available.

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
AGTR1	rs5186	C	No DR vs Any DR	Type 1	98 (29)	200 (33)	73.99	0.82 (0.61-1.10)	0.15	2	1.87	0.1852	[102, 108]
				Type 2	22 (4)	46 (5)	34.26	0.73 (0.43-1.22)	0.26	1	<0.01	0.2251	[108]
				Total	142 (14)	246 (15)	100.00	0.8 (0.62-1.03)	0.13	3	2.05	0.0788	
ITGA2	rs2910964	A	No DR vs Any DR	Type 2	258 (46)	137 (34)	100.00	1.65 (1.26-2.15)	0.14	2	0.05	0.0002	[146, 147]
ADRB3	rs4994	C	No DR vs Any DR	Type 1	62 (7)	12 (10)	48.01	0.73 (0.38-1.39)	0.33	1	<0.01	0.3320	[148, 149]
				Type 2	49 (27)	34 (15)	51.99	2.16 (1.32-3.51)	0.25	1	<0.01	0.0020	[148, 149]
				Total	111 (11)	46 (13)	100.00	1.28 (0.44-3.72)	0.54	2	6.95	0.6516	
			No DR vs NPDR	Type 1	27 (7)	12 (10)	47.68	0.71 (0.35-1.44)	0.36	1	<0.01	0.3420	[148, 149]
				Type 2	21 (22)	34 (15)	52.32	1.71 (0.94-3.14)	0.31	1	<0.01	0.0811	[148, 149]
				Total	48 (10)	46 (13)	100.00	1.12 (0.47-2.67)	0.44	2	3.44	0.7900	
			No DR vs PDR	Type 1	35 (7)	12 (10)	48.89	0.74 (0.37-1.47)	0.35	1	<0.01	0.3892	[148, 149]
				Type 2	28 (31)	34 (15)	51.11	2.68 (1.51-4.75)	0.29	1	<0.01	0.0008	[148, 149]
				Total	63 (11)	46 (13)	100.00	1.43 (0.41-5.03)	0.64	2	7.95	0.5797	
			NPDR vs PDR	Type 1	35 (7)	27 (7)	61.48	1.04 (0.62-1.76)	0.27	1	<0.01	0.8706	[148, 149]
				Type 2	28 (31)	21 (22)	38.52	1.56 (0.81-3.01)	0.34	1	<0.01	0.1829	[148, 149]
				Total	63 (11)	48 (10)	100.00	1.22 (0.81-1.83)	0.21	2	0.89	0.3399	

OR = odds ratios, CI = confidence interval and SE = standard error

Table 2.3 Continued

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
AGT	rs4762	C	No DR vs Any DR	Type 1	111 (55)	189 (63)	37.28	0.73 (0.51-1.05)	0.18	1	<0.01	0.0875	[102, 108]
				Type 2	90 (14)	161 (17)	62.72	0.84 (0.63-1.11)	0.14	1	<0.01	0.2102	[102, 108]
				Total	201 (24)	350 (27)	100.00	0.79 (0.64-0.99)	0.11	2	0.35	0.0418	
APOE	ε2/ε3/ε4	ε2	No DR vs Any DR	Type 1	21 (14)	19 (10)	19.55	1.46 (0.75-2.83)	0.34	1	<0.01	0.2620	[150, 151]
				Type 2	223 (10)	51 (11)	80.45	1.05 (0.76-1.45)	0.17	2	0.05	0.7681	[150, 151]
				Total	244 (11)	70 (11)	100.00	1.12 (0.84-1.5)	0.15	3	0.81	0.4470	
FGF2	rs41456044	A	No DR vs PDR	Type 2	31 (5)	47 (5)	100.00	1.19 (0.74-1.91)	0.24	2	0.27	0.4672	[152, 153]
FGF2	rs308395	G	No DR vs PDR	Type 2	90 (14)	131 (13)	100.00	1.08 (0.81-1.45)	0.15	2	0.29	0.5882	[152, 153]
NOS3	rs1799983	G	No DR vs Any DR	Type 2	393 (30)	478 (24)	100.00	1.11 (0.94-1.31)	0.08	4	1.69	0.2279	[114-117]
NOS3	rs41322052	C	No DR vs Any DR	Type 1	105 (42)	106 (42)	25.37	1.01 (0.71-1.44)	0.18	1	<0.01	0.9525	[154]
				Type 2	313 (27)	334 (24)	74.63	1.04 (0.75-1.45)	0.17	3	4.33	0.7956	[114, 116]
				Total	418 (29)	440 (27)	100.00	1.06 (0.85-1.33)	0.11	4	4.77	0.5962	
			No DR vs PDR	Type 1	105 (42)	106 (42)	77.27	1.01 (0.71-1.44)	0.18	1	<0.01	0.9525	[154]
				Type 2	16 (12)	29 (14)	22.73	0.83 (0.43-1.59)	0.33	1	<0.01	0.5686	[114, 116]
				Total	121 (31)	135 (29)	100.00	0.97 (0.71-1.32)	0.16	2	0.28	0.8263	
SLC2A1	rs841853	XbaI-	No DR vs Any DR	Type 1	227 (35)	142 (64)	66.08	0.41 (0.13-1.23)	0.57	2	8.27	0.1118	[155, 156]
HLA	DR1-8	DR1	No DR vs Any DR	Type 1	45 (14)	40 (15)	100.00	0.99 (0.62-1.56)	0.2359	2	0.2	0.9506	[157, 158]
HLA	DR1-8	DR7	No DR vs Any DR	Type 1	39 (12)	38 (14)	100.00	0.59 (0.10-3.60)	0.9247	2	1.86	0.5658	[157, 158]
ICAM1	rs13306430	G	No DR vs Any DR	Type 2	150 (36)	89 (50)	100.00	0.56 (0.39-0.81)	0.18	2	0.01	0.0017	[159, 160]
MTHFR	rs1801133	T	No DR vs Any DR	Type 2	318 (44)	405 (37)	100.00	1.39 (0.99-1.94)	0.17	4	8.34	0.0563	[132, 161-163]
NPY	rs16139	C	No DR vs Any DR	Type 2	26 (9)	16 (5)	100.00	2.62 (0.9-7.61)	0.54	2	1.38	0.0759	[164, 165]
PAI-1	rs1799768	5G	No DR vs Any DR	Type 2	355 (44)	252 (45)	100.00	1.06 (0.85-1.32)	0.11	3	<0.01	0.6017	[106, 132, 166]
PON1	rs662	G	No DR vs Any DR	Type 2	310 (54)	175 (48)	100.00	0.99 (0.44-2.27)	0.42	2	8.48	0.9896	[166, 167]

OR = odds ratios, CI = confidence interval and SE = standard error

Table 2.3 continued

Gene	Variant	Risk Allele	Comparison	Type of Diabetes	Total cases with risk allele (%)	Total controls with risk allele (%)	Weight	OR (95% CI)	SE	Number of included studies	Chi2	P value	References
PON2	rs7493	G	No DR vs Any DR	Type 1	192 (57)	191 (49)	54.02	1.37 (1.03-1.84)	0.15	1	<0.01	0.0322	[167, 168]
				Type 2	144 (72)	144 (78)	45.98	0.72 (0.46-1.15)	0.23	1	<0.01	0.1685	[167, 168]
				Total	336 (62)	335 (58)	100.00	1.02 (0.55-1.91)	0.32	2	5.33	0.9422	
PPARG	rs1801282	G	No DR vs Any DR	Type 1	33 (13)	108 (14)	29.35	0.85 (0.56-1.29)	0.21	1	<0.01	0.4413	[169-172]
				Type 2	124 (14)	273 (16)	70.65	0.83 (0.62-1.11)	0.15	4	3.33	0.2135	[169-172]
				Total	157 (14)	381 (16)	100.00	0.83 (0.67-1.05)	0.12	5	3.34	0.1173	
AGER	rs1800624	A	No DR vs Any DR	Type 2	361 (33)	112 (18)	100.00	1.89 (0.53-6.75)	0.65	3	46.09	0.3271	[173-175]
			No DR vs NPDR	Type 2	217 (37)	60 (12)	100.00	2.94 (0.46-18.76)	0.95	2	26.55	0.2540	[174, 175]
			No DR vs PDR	Type 2	67 (26)	93 (20)	100.00	0.93 (0.62-1.38)	0.20	2	0.88	0.7055	
AGER	rs1800625	C	No DR vs Any DR	Type 2	125 (13)	94 (15)	100.00	0.94 (0.7-1.27)	0.15	3	0.32	0.7035	[173-175]
			No DR vs PDR	Type 2	35 (14)	76 (15)	100.00	0.99 (0.64-1.55)	0.23	2	0.99	0.9803	
VDR	rs10735810	C	No DR vs Any DR	Type 1	207 (40)	86 (34)	57.59	1.27 (0.93-1.74)	0.16	1	<0.01	0.1282	[176]
				Type 2	85 (50)	169 (47)	42.41	1.15 (0.8-1.66)	0.19	1	<0.01	0.4415	[177]
				Total	292 (42)	255 (42)	100.00	1.22 (0.96-1.55)	0.12	2	0.17	0.0978	
VEGF	rs25648	T	No DR vs Any DR	Type 2	86 (16)	56 (14)	100.00	1.48 (0.42-5.19)	0.64	2	9.06	0.5363	[119, 121]
VEGF	rs1570360	A	No DR vs Any DR	Type 2	78 (12)	81 (13)	100.00	0.93 (0.67-1.3)	0.17	2	0.22	0.6740	[121, 122]
VEGF	rs3095039	T	No DR vs Any DR	Type 2	86 (16)	52 (13)	100.00	1.1 (0.47-2.62)	0.44	2	4.47	0.8230	[121, 126]
			No DR vs NPDR	Type 2	45 (14)	52 (13)	100.00	0.97 (0.33-2.85)	0.55	2	5.03	0.9560	[121, 126]
			No DR vs PDR	Type 2	41 (18)	52 (13)	100.00	1.44 (0.88-2.36)	0.25	2	1.13	0.1491	[121, 126]
			NPDR vs PDR	Type 2	41 (18)	45 (14)	100.00	1.22 (0.76-1.96)	0.24	2	1.01	0.4146	[121, 126]
VEGF	rs35569394	-2549DEL	No DR vs Any DR	Type 1	73 (58)	68 (52)	46.01	1.25 (0.77-2.04)	0.25	1	<0.01	0.3724	[178]
				Type 2	252 (65)	81 (45)	53.99	2.28 (1.59-3.26)	0.18	1	<0.01	1.0x10-5	[179]
				Total	325 (63)	149 (48)	100.00	1.73 (0.96-3.11)	0.30	2	3.77	0.0677	
VEGF	rs699947	A	No DR vs Any DR	Type 2	204 (29)	280 (29)	100.00	1.01 (0.56-1.83)	0.30	2	7.61	0.9741	[122, 123]

OR = odds ratios, CI = confidence interval and SE = standard error

Angiotensin Converting Enzyme *(ACE)*

The gene encoding the angiotensin I converting enzyme (*ACE*) is located on chromosome 17q23[180]. Included in the meta-analysis were six studies that examined the insertion/deletion (INS/DEL) polymorphism in intron 16 of the *ACE* gene in patients with T1DM and seven studies in patients with T2DM. The 287 base pair deletion was treated as the risk variant and there was no statistically significant association with this polymorphism and the development of any form of DR (Table 2.1).

Ten studies of subjects with Caucasian origin were sub-analysed for all DR comparisons. The 287 base pair deletion was not found to be significantly associated with DR or its subtypes in type 1 or T2DM (Table 2.2).

Aldose Reductase (*AKR1B1* - also known as *ALR*)

The aldo-keto reductase family 1 member B1 (*AKR1B1*) gene (also known as *ALR*) is located on chromosome 7q35[180]. Associations of two *AKR1B1* SNPs with DR have been reported in the literature; the promoter SNP rs759853, and the (CA)n microsatellite polymorphism located 5' of the *AKR1B1* gene. Six studies have examined the association between the *AKR1B1* (CA)n microsatellite with DR in type 1 and nine studies in T2DM (Table 2.1). The three most commonly investigated *AKR1B1* alleles (z, z+2 and z-2) in the literature were included for analysis.

There was a significant association with the z-2 allele and the development of any DR (OR 2.33 [95% CI 1.49–3.64], $p=2x10^{-4}$). Sub-analyses revealed a significant association between the z-2 allele and DR in patients with T2DM (OR 2.64 [95% CI

1.39–5.01], p=2.9x10^{-3}), with weaker but statistically significant association also being found for patients with T1DM (OR 1.95 [95% CI 1.04–3.66], p=0.04). A significant association was also found in the NPDR and PDR subgroups.

No statistically significant association was found between the z allele and the development of any DR (OR 1.05 [95% CI 0.81–1.35], p=0.73). However, in the sub-analysis for type of DR, the z allele was significantly protective against NPDR development in T2DM (OR 0.65 [95% CI 0.45–0.94], p=0.02). A significant difference between NPDR and PDR development for the presence of z allele was found also in T2DM (OR 0.65 [95% CI 0.45–0.94], p=0.02).

Similarly, the z+2 allele was found to be significantly protective against the development of DR (OR 0.58 [95% CI 0.36–0.93], p=0.02).

Only four studies examining the *AKR1B1* (CA)n microsatellite have included subjects of Caucasian ancestry (Table 2.2), with the majority of studies including subjects of only Asian ancestry. In the analyses of studies of Caucasian origin, only the z-2 (OR1.80 [95% CI 1.06–3.06], p=0.03) and z+2 (OR: 0.56 [95% CI 0.32–0.97], p=0.04) polymorphisms remained significantly associated with DR, with the z-2 allele conferring risk and z+2 conferring protection against DR.

Three studies examined the association of a second *AKR1B1* polymorphism (promoter SNP rs759853) with DR in T1DM and 5 studies in T2DM (Table 2.1). The T allele was the minor allele and considered to be the risk variant. Interestingly however, analyses revealed protection against DR with the T allele in T1DM (OR

0.49 [95% CI 0.36-0.68], $p<1.00x10^{-4}$). There was no statistically significant association between DR and rs759853 in T2DM. However, a borderline association with PDR was found when compared to those with NPDR (OR 0.73 [95% CI 0.54−0.99], p=0.04).

Four studies investigating the *AKR1B1* rs759853 included subjects of Caucasian ancestry. A significant protection of the T allele against DR in T1DM (OR 0.5 [95% CI 0.35−0.71], $p=1.00x10^{-4}$) remained. Insufficient studies were available for sub-analysis of DR subtypes.

Vascular Endothelial Growth Factor *(VEGF)*

The *VEGF* gene is located on chromosome 6p12[180]. Six *VEGF* polymorphisms were included in this meta-analysis, with the rs2010963 polymorphism being the most frequently studied. No studies examining this polymorphism in T1DM and DR were located. Seven studies examining this polymorphism in T2DM and DR were included in the analyses (Table 2.1). The G allele has been considered as the risk variant. A significant association between patients with no DR and those with NPDR (OR 0.62 [95% CI: 0.48−0.81], $p=5.0x10^{-4}$) was identified, yet no significant differences were found between NPDR and PDR development. Meta-analysis revealed no significant association between the *VEGF* polymorphisms: rs25648, rs1570360, rs3095039, rs35569394, or rs699947 and any type of DR (Table 2.3).

Only two studies of participants were available for inclusion in the sub-analysis for Caucasian ancestry (Table 2.2). No statistically significant association of the *VEGF*

rs2010963 polymorphism with DR was found (OR 0.83 [95% CI 0.65−1.07], p=0.16) and insufficient studies were available for DR subtype analyses.

Endothelial nitric oxide synthase *(NOS3)*

The *NOS3* gene is located on chromosome 7q35-36[180]. Three *NOS3* SNPs (rs1799983, rs41322052 and rs3138808) met the inclusion criteria for meta-analysis. The rs3138808 variant has been the most commonly examined *NOS3* DR polymorphism and the 393 base pair insertion has been classified as the risk variant. One study examining this polymorphism and DR in T1DM and seven studies in T2DM were included in the analysis (Table 2.1). There was no statistically significant association between rs3138808 and any form of DR. Additionally, no significant association between any form of DR and the SNP rs1799983 (OR 1.11 [95% CI 0.94-1.31], p=0.23) or rs41322052 (OR 1.06 [95% CI 0.85-1.33], p=0.60) was identified (Table 2.3).

Only two studies were available to be included in the sub-analysis for subjects of Caucasian ancestry (Table 2.2). No statistically significant association was found with the *NOS3* rs3138808 polymorphism and DR development (OR 0.71 [95% CI 0.28−1.75], p=0.45) and insufficient studies were available for DR subtype analysis.

Discussion

This meta-analysis comprehensively assessed the risk of DR in relation to every published candidate gene meeting inclusion criteria. Genetic data from over 87,000 individuals in 82 studies examining 20 genes and 34 SNPs were analysed. Pathways

involved in the pathogenesis of DR relevant to the genes with the most studied polymorphisms in this meta-analysis are described in detail below.

Renin-Angiotensin System

The angiotensin I-converting enzyme (ACE) is involved in the conversion of angiotensin I to angiotensin II (ATII). ATII mediates its hemodynamic effects through signalling via angiotensin type 1 (AT1) and type 2 (AT2) receptors. Vascular remodelling and proliferation occurs mainly via the AT1 receptor[181]. All components of the RAS have been shown to be expressed in the retina[182]. The physiologic effects of ATII in the eye include the regulation of intraocular blood flow and pressure, promotion of capillary growth, enhancing vascular permeability, increasing oxidative stress and the regulation of cell growth via the expression of various growth factors including vascular endothelial growth factor (VEGF), insulin like growth factor (IGF) and platelet derived growth factor (PDGF)[181].

Clinical evidence also supports the role for the RAS system in DR pathogenesis. The EURODIAB Controlled Trial of Lisinopril in Insulin-dependent Diabetes (EUCLID) trial provided evidence for lisinopril, an ACE inhibitor, decreasing the progression of DR by 50%[183]. In addition, animal studies have shown that ACE inhibitors and AT1 receptor blockers can prevent retinal neovascularization [184].

The *ACE* INS/DEL polymorphism in intron 16 had the largest number of subjects to be genotyped for any polymorphism and the largest number of studies to include participants of Caucasian ancestry. Three quarters of the studies included participants of Caucasian ancestry. There was no statistically significant association of the

insertion/deletion polymorphism of the ACE gene with DR or DR subtypes in T1DM, T2DM or combined diabetes. This is consistent with the findings of the majority of the included studies examining this polymorphism in this meta-analysis. Two other meta-analyses have examined specifically the association of the insertion-deletion of the *ACE* gene with DR development. Fujisawa *et al*.[185] examined 12 studies, including T1DM and T2DM subjects[185]. Wiwanitkit[186] examined 4 studies and included T2DM subjects only. Both analyses also found no statistically significant association with this polymorphism and the development of DR.

Polyol Pathway

Aldose reductase *(ALR)* is a rate-limiting enzyme of the polyol pathway, which catalyses NADPH-dependent reduction of glucose to sorbitol. This pathway leads to the intracellular accumulation of sorbitol and is primarily active under hyperglycemic conditions[187]. Several mechanisms have been proposed to explain the pathogenesis of diabetic microvascular complications, including the induction of osmotic stress and the activation of protein kinase C as well as pathogenic vascular and hemodynamic alterations[58]. ALR has been identified in human pericytes, which exhibit an active polyol pathway[188]. Animal studies have shown that cultured mural cells from the retinal capillaries of adult rhesus monkeys undergo cellular degeneration after exposure to high glucose levels, with ALR being isolated from these mural cells[189]. The sorbitol pathway is also biologically plausible as it is involved in the selective degeneration of human mural cells in NPDR[190]. Induced hyperglycemia in dogs with galactosemia has similarly shown retinal vascular changes including microaneurysm formation, degeneration of retinal pericytes, retinal hemorrhages and non-perfused or acellular vessels[191].

The *AKR1B1* gene had the largest number of studies examining the relationship of its polymorphisms to DR development, regardless of ethnicity. The z-2 alleles of the $(CA)_n$ microsatellite showed the most significant association with DR, especially in T2DM, conferring risk also in PDR and NPDR subtypes. The z+2 and z alleles conferred protection against overall DR and NPDR respectively and both were protective against PDR when compared to NPDR in T2DM. The majority of studies included in this meta-analysis have individually reported a risk for DR with the z-2 allele, however the z allele was not found to be statistically significant by most studies, with only a fifth of the studies individually reporting z+2 to be protective against DR. The vast majority of studies have included participants of non Caucasian ancestry; however those of Caucasian ancestry found the z-2 allele to confer risk and z+2 to confer protection against overall DR in combined diabetes.

The T allele of the *AKR1B1* promoter SNP rs759853 conferred protection against DR in T1DM of any ancestry and also of Caucasian ancestry alone. This protection against DR in T1DM has also been found in the individual studies examining this polymorphism.

Vascular Endothelial Growth Factor

VEGF is a multifunctional cytokine which promotes angiogenesis and is a potent mediator of microvascular permeability. Diabetic microvascular changes in the retina lead to hypoxia, a stimulator of VEGF production[192]. VEGF has been found to have a significant role in the development of DR by inducing hyperpermeability of retinal vessels, breakdown of the blood-retinal barrier, and neovascularization in PDR[193-195].

Complications such as retinal edema and blinding vitreous hemorrhage arise as a result of the abnormal barrier function of vessels, and the growth of new vessels, which are fragile and prone to rupture.

VEGF protein expression has been shown to be influenced by genetic variation in the *VEGF* gene[196]. VEGF is alternatively spliced to form the angiogenic VEGFxxx and anti-angiogenic VEGFxxxb isoforms and studies have found the VEGFxxxb isoform to be more predominant in normal controls and reduced in diabetic eyes[197]. VEGF levels in the vitreous of patients with PDR are significantly elevated when compared to the vitreous of diabetic eyes without PDR and controls without diabetes[198, 199]. VEGF inhibition has been shown to result in a marked reduction in retinal neovascularization[200] and prevention of the blood retinal barrier breakdown[201], further supporting its role in DR development. Importantly, VEGF inhibition is increasingly being utilised for treatment of retinal complications of not only DR but age related macular degeneration and retinal vein occlusion.

The *VEGF* gene had the largest number of individual SNPs examined in relation to DR. Of these six polymorphisms, the G variant of the rs2010963 polymorphism was found to significantly protect against the development of NPDR in T2DM, but not with overall DR development. In keeping with the findings of this meta-analysis, the majority of studies reported no statistically significant association of this polymorphism with any DR development in T2DM, regardless of ethnicity.

Nitric Oxide Pathway

Endothelial nitric oxide synthase (eNOS) is an enzyme produced by endothelial cells. Nitric oxide (NO) derived from eNOS is a key endogenous vasodilator[202]. It is also believed to be important in the promotion of angiogenesis and regulation of VEGF expression[203]. A low concentration of eNOS is believed to be necessary to maintain endothelial function[204] with experimental deficiency of eNOS shown to significantly decrease retinal neovascularization in a mouse model[205]. Aqueous NO levels have also been found to play an important role in the progression of DR, with levels significantly higher in active PDR[206]. Similarly, NO levels have been found to be significantly elevated in PDR vitreous when compared to non-diabetic participants[207], making the endothelial nitric oxide synthase gene (*NOS3)* a biologically plausible candidate for susceptibility to DR development.

The rs3138808 polymorphism was the most studied polymorphism of *NOS3*, and the majority of studies included participants of non-Caucasian ancestry. No statistically significant association was found with DR development regardless of ethnicity, or for Caucasians alone, consistent with the findings of the majority of the included studies. All other *NOS3* polymorphisms did not show statistically significant associations with the development of DR.

Data on the remaining 27 SNPs examined by a minimum of 2 and maximum of 5 studies revealed the rs2910964 SNP of the *ITGA2* gene and rs13306430 of the *ICAM1* gene to be significantly associated with DR. Both of these variants were examined in T2DM only and by the minimum allowable two studies each. Unless further replication studies are undertaken, the importance of these genes in DR may

remain unclear. Zintzaras et al.[208] performed a meta-analysis of 5 studies examining the link between the C677T polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene and development of DR in T2DM. In contrast with our results, a borderline association between C677T transition and the risk of development DR (OR 1.39 [95% CI 1.05-1.83], p=0.08) was reported.

The definition of DR requires at the minimum microaneurysms to be present[40], however several definitions and criteria exist for the sub-classification of DR and preferences have been variable amongst included studies, with many studies defining DR without the use of standardised scales. Additionally, confounding factors such as glycemic control have not been adjusted for in calculation of the ORs of polymorphisms included in this meta-analysis, as this information provided by included studies was either absent, incomplete, or non-standardised definitions used. These are accepted limitations of this meta-analysis, implicit in the meta-analysis concept when dealing with a large number of studies with different design and reporting style.

In conclusion, this meta-analysis found that sequence variation within the *AKR1B1* gene was the most significantly associated with DR development amongst those genes qualifying for inclusion. This result supports more detailed research for genes found to be positively associated with DR under meta-analysis, particularly *AKR1B1* in order to determine which variants are causally associated and their mechanism of action. Future genetic studies should also include genome-wide association studies to ascertain the relative strength of association of identified genes in comparison to

others with DR susceptibility loci that have not been previously considered as candidates.

CHAPTER 3

Materials and Methods

Participant recruitment

Patients were recruited from Ophthalmology and Endocrinology clinics of the Flinders Medical Centre and Ophthalmology clinics of the Royal Adelaide and Queen Elizabeth Hospitals in metropolitan Adelaide, South Australia. Approval was obtained from the Human Research Ethics Committees of each hospital. All participants were over 18 years of age and were required to have either T1DM of any disease duration or T2DM of at least 5 years duration. Patients with T2DM were required to be on oral hypoglycemic medication or insulin therapy for DM.

Informed consent was obtained from all participants. Retinopathy status was graded by a trained ophthalmologist using slit lamp biomicroscopy following pupil dilation according to the simplified ETDRS (Wisconsin Level) classification as recommended for clinical classification by the NHMRC[40]. Details of this classification are provided in chapter 1 (introduction), however in brief, participants with retinal microaneurysms only were classified as having minimal non-proliferative DR (NPDR). Those with dot haemorrhages/microaneurysms in all 4 retinal quadrants or intra-retinal microvascular abnormalities in one or more quadrants or venous beading in two or more quadrants were classified as severe NPDR. The presence of new retinal blood vessels on the optic disc or elsewhere were required for proliferative DR (PDR) classification. The presence of microaneurysms, dot haemorrhages, hard exudates, retinal nerve layer infarcts / cotton wool spots, intra-retinal microvascular abnormalities or venous beading without the presence of

new vessels were classified as NPDR. For the purpose of this thesis, those meeting requirements for macular edema or clinically significant macular edema (CSME) – ie if retinal thickening was present within 2 disc diameters of the macular centre or hard exudates within 500µm of the macular centre with adjacent thickening, were collectively classified as having CSME.

Retinopathy status for subsequent analyses for a given individual was considered to be that of the *worst eye*. If a participant had received laser treatment for blinding complications such as PDR or CSME, the retinopathy status prior to laser treatment was used in the analyses. Participants were included in the study as diabetic controls without retinopathy if they had either no DR or minimal NPDR. Blinding retinopathy was defined as the presence of either severe NPDR, PDR or CSME. Individuals could be categorised into CSME as well as any other group as CSME can co-occur with any of the other DR gradings. If either eye had CSME irrespective of other DR gradings, the patient was classified as having CSME.

A detailed questionnaire containing information regarding sex, age, ethnicity, age at diagnosis of diabetes, family diabetic history, co-existing risk factors, systemic complications of diabetes, ocular complications as a result of diabetic retinopathy, past ocular history, smoking history and alcohol intake was conducted (Appendix 3). For the purpose of this thesis 'Caucasian ancestry' was defined as being of European descent

Blood pressure and body mass index (BMI) were measured. Renal function tests (serum creatinine, urine albumin and albumin:creatinine ratio), blood cholesterol and

hemoglobin A1c (HbA1c) levels were obtained from a state-wide database (OASIS). Three consecutive HbA1c levels prior to recruitment were averaged for each participant. For those cases diagnosed with blinding DR, HbA1c levels *at the time of the ocular complication* were used, and for controls with DM, HbA1c levels immediately prior to recruitment were averaged. Patients were classified as hypertensive if they were on treatment for hypertension or they had a blood pressure reading greater than or equal to 140/90 mmHg at the time of recruitment. Hypercholesterolemia was defined as a total cholesterol equal or greater than 5.5 mmol/L, or current use of lipid lowering medication. Nephropathy was defined as the presence of microalbuminuria (30-300mg/day) or macroalbuminuria (>300mg/day). DNA was extracted from peripheral blood samples using the QiaAmp Blood Maxi Kit (Qiagen).

As all participants were recruited as part of this PhD project, the number of available subjects for each chapter included in the various studies varies, and reflects the available numbers recruited up to the time of each analysis. It is important to note that the same cohort was included in the *vascular endothelial growth factor A* (*VEGFA)* and *carbonic anhydrase 1* (*CA1*) analyses (chapters 4 and 5). This same cohort was also included in the *erythropoietin* (*EPO*) analyses (chapter 6) with the exclusion of those with end stage renal disease. The majority (but not all) of participants included in this study also provided serum samples, and those available to us at the time of the protein study were included in chapter 8.

SNP selection and genotyping for candidate gene studies

Candidate genes were selected based on interesting results of the meta-analysis or of recent novel findings in the literature relevant to DR. Using the tagger program implemented in Haploview 4.0[209], tag SNPs relevant to the gene examined were selected on the basis of linkage disequilibrium patterns observed in the Caucasian (CEU) samples genotyped as part of the International HapMap Project[210]. A previous study has shown that this population is a suitable surrogate for the selection of tag SNPs to be used in Australian samples with predominantly North Western European descent[211]. *VEGFA*, *CA1*, *EPO* and *aldose reductase* (*AKR1B1*) studies were designed to have at least 80% power to detect SNP associations with odds ratios of approximately 1.5, assuming a disease prevalence of 60% amongst individuals with diabetes, a marker allele frequency of 0.15 and moderate LD between the marker and disease locus. Only SNPs with minor allele frequency greater than 5% in HapMap were considered. SNPs were genotyped on the Sequenom iPLEX GOLD chemistry on an Autoflex Mass Spectrometer at the Australian Genome Research Facility, Brisbane, Australia with subsequent primer designs, PCR optimisation and testing out-sourced to this facility.

Statistical analyses

Baseline characteristics of cases and controls were compared with the use of t-test for continuous variables and chi-square test for discrete traits. Pearson correlation was undertaken for associations of clinical covariates with outcomes of interest in SPSS (v15.0 SPSS Inc, Chicago, IL). SNP genotyping was checked for compliance with Hardy-Weinberg equilibrium using a chi-square test. Linkage Disequilibrium between markers was calculated using Haploview 4.0. Genotypic associations were

assessed in SNPstats[212] and PLINK(v1.06)[213]. Dominant, additive, recessive and genotypic models were considered with respect to the minor allele. Odds ratios were calculated in PLINK(v1.06)[213]. Haplotype associations were undertaken in HaploStats (version 1.2.1,[214]) and PLINK(v1.06)[213].

The threshold for statistical significance was determined for each candidate gene study based on the number of tests performed. Multiple testing of individual SNPs was adjusted for using the Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD) method of Nyholt[215] modified by Li and Ji[216]. Haplotype tests were adjusted with a simple Bonferroni test for the number of haplotypes in the block examined.

Methods specific to individual studies are provided separately in each chapter.

CHAPTER 4

Common sequence variation in the *VEGFA* gene predicts risk of diabetic retinopathy.

The work presented in this chapter has been published in the peer reviewed literature: S Abhary, KP Burdon, A Gupta, S Lake, N Petrovsky, JE Craig Common sequence variation in the VEGFA gene predicts risk of diabetic retinopathy. Invest Ophthal Vis Sci Dec;50(12)2009:5552-8[217]. Dr Abhary's contributions include conception and design of the study, participant recruitment, analysis and interpretation of data and writing of the manuscript.

Introduction

Diabetic microvascular changes in the retina lead to hypoxia, which stimulates production of VEGF, a multifunctional cytokine which promotes angiogenesis and is a potent mediator of microvascular permeability[218]. VEGF is believed to play a significant role in the development of DR by inducing hyperpermeability of retinal vessels, breakdown of the blood-retinal barrier and neovascularization[193-195]. Complications can arise as a result of abnormal barrier function of new vessels, leading to intraretinal hemorrhage and exudation. New blood vessels have increased fragility leading to sudden severe loss of vision due to vitreous hemorrhage.

Evidence for a role of VEGF as an angiogenic factor in PDR has been obtained from both *in vitro* studies and animal models, where VEGF levels have been reported to be elevated up to 30-fold in hypoxic retinas[201, 219] with a resultant increase in vascular

permeability[200, 201]. In ischemic retinas, VEGF inhibition has been shown to cause almost a 100% reduction in retinal neovascularization[200]. Experimental retinal ischemia in primates induces an elevation in aqueous VEGF levels and upregulation of *VEGF* mRNA[220]. Prevention of the blood-retinal barrier breakdown has been reported with VEGF inhibition[201].

VEGF is alternatively spliced to form the angiogenic VEGFxxx and anti-angiogenic VEGFxxxb isoforms and studies have found the VEGFxxxb isoform to be more predominant in normal controls and reduced in diabetic eyes[197]. VEGF levels in the vitreous of patients with PDR have been found to be significantly elevated when compared to the vitreous of controls[198, 199, 221-226]. This is also true in the vitreous of diabetic mice when compared to non-diabetic controls[227]. VEGF protein expression is influenced by genetic variation in the *VEGFA* gene[121, 196, 228].

The human *VEGFA* gene is located on chromosome 6p12[180]. Twelve previous studies have examined the effects of more than 25 single nucleotide polymorphisms (SNPs) in the *VEGFA* gene and the development of DR[119, 121-126, 178, 179, 229-231]. The *VEGFA* gene has had the largest number of individual SNPs examined in relation to DR when compared to any other published gene. The rs2010963 polymorphism has been the most frequently studied SNP, with a significant association with NPDR found when compared to those with no DR(OR 0.62 [95% CI 0.48−0.81], $p=5.0x10^{-4}$)in the meta-analysis[92], as described in chapter 2 of this thesis.

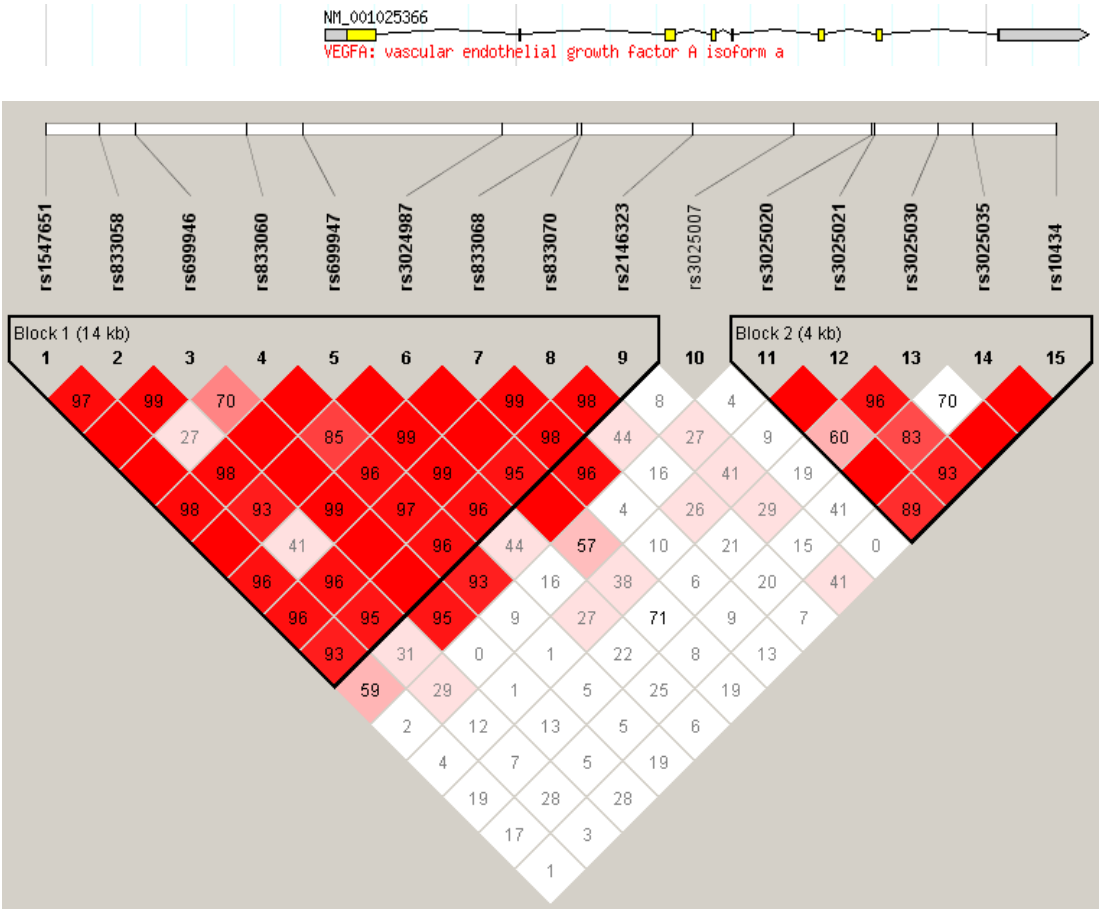
This study describes the association of multiple tag SNPs in the *VEGFA* gene with blinding DR in this Australian cohort of individuals with T1DM and T2DM.

Methods

This study was undertaken following year 1 of recruitment. Thus, the first 554 participants recruited were included in this analysis. Of these, 190 had T1DM and 364 had T2DM. Two hundred and eighty one subjects had no DR, and 273 had any level of DR. Fifteen tag SNPs (rs1547651, rs833058, rs699946, rs833060, rs699947, rs3024987, rs833068, rs833070, rs2146323, rs3025007, rs3025020, rs3025021, rs3025030, rs3025035, rs10434), which captured all alleles with an r^2 of at least 0.8 (mean r^2=0.973), were genotyped in all 554 individuals.

Both individual SNPs and haplotypes were assessed. Haplotype tests were undertaken in two blocks of linkage disequilibrium (LD) observed in this data (Figure 4.1). Block 1 consisted of the first 9 SNPs and block 2 the remaining 6 SNPs. Although SNPs 10 and 11 are not in strong LD with other SNPs they were included in block 2 for completeness of the haplotypes observed.

Figure 4.1 - Linkage disequilibrium between SNPs in and around the *VEGFA* gene in this Australian cohort, generated in solid spine format in Haploview (4.0)[209]. The D' value for each pair of SNPs is given, multiplied by 100. A blank cell indicates D'=1.0. The intensity of the red colour indicates the level of linkage disequilibrium.



Results

Of the participants with DR, 215 were classified as having blinding DR, consisting of 23 participants with severe NPDR, 132 with PDR and 93 with CSME. Subjects with T1DM and no DR had a significantly younger age, shorter disease duration, lower HbA1c levels and lower nephropathy and hypertension rates when compared to the T1DM cases with blinding DR. Subjects with T2DM and no DR were significantly more likely to be female and have shorter disease duration, lower HbA1c levels,

lower body mass index (BMI) readings and less nephropathy rates when compared to subjects with T2DM and blinding DR (Table 4.1).

Table 4.1: Clinical characteristics of participants with no DR compared to blinding DR in T1DM and T2DM

	T1DM			T2DM		
Clinical Characteristics	No DR (n=94)	Blinding DR (n=76)	P value	No DR (n=187)	Blinding DR (n=139)	P value
Sex (female)	48 (51%)	29 (45%)	0.478	100 (53%)	45 (35%)	0.001
Age (years)	38.4 ± 14.9	49.8 ± 15.5	<0.001	64.3 ± 14.5	64.0 ± 10.7	0.853
Disease duration (years)	15.4 ± 9.1	30.9 ± 13.4	<0.001	12.9 ± 8.7	17.5 ± 8.6	<0.001
HbA1c (%)	7.6 ± 2.5	8.8 ± 2.3	0.004	6.6 ± 2.9	7.5 ± 3.4	0.014
BMI (kg/m2)	25.9 ± 7.1	25.9 ± 9.8	0.958	32.2 ± 9.1	29.6 ± 11.5	0.026
Hypercholesterolemia (%)	33 (35%)	32 (50%)	0.062	120 (64%)	81 (63%)	0.802
Nephropathy (%)	40 (15%)	29 (45%)	<0.001	43 (23%)	46 (36%)	0.014
Smoker (%)	44 (47%)	35 (55%)	0.331	99 (53%)	69 (53%)	0.924
Hypertension (%)	37 (39%)	46 (72%)	<0.001	153 (82%)	107 (83%)	0.796

Results are presented as n (%) or mean±standard deviation.

All SNPs were in Hardy-Weinberg Equilibrium (HWE) in all four groups. Genotype frequencies (Table 4.2) are similar between the two types of diabetes.

Table 4.2 – Genotype frequencies for each SNP [results are presented as n(%)] in no DR and blinding DR by type of diabetes.

			T1DM		T2DM	
	SNP	Genotype	No DR n(%)	Blinding DR n(%)	No DR n(%)	Blinding DR n(%)
1	rs1547651	AA	66 (70%)	52 (68%)	142 (77%)	99 (71%)
		AT	22 (23%)	22 (29%)	37 (20%)	37 (27%)
		TT	6 (6%)	2 (3%)	5 (3%)	3 (2%)
2	rs833058	CC	40 (43%)	36 (47%)	69 (38%)	49 (35%)
		CT	35 (38%)	29 (38%)	82 (45%)	74 (53%)
		TT	18 (19%)	11 (14%)	31 (17%)	16 (12%)
3	rs699946	AA	56 (60%)	59 (79%)	117 (64%)	86 (62%)
		AG	33 (35%)	14 (19%)	56 (31%)	50 (36%)
		GG	4 (4%)	2 (3%)	9 (5%)	3 (2%)
4	rs833060	GG	41 (44%)	49 (65%)	98 (54%)	74 (53%)
		GT	46 (49%)	22 (29%)	69 (38%)	51 (37%)
		TT	6 (6%)	4 (5%)	15 (8%)	14 (10%)
5	rs699947	AA	24 (26%)	23 (31%)	45 (25%)	31 (23%)
		AC	43 (46%)	35 (47%)	91 (50%)	74 (54%)
		CC	26 (28 %)	17 (23%)	45 (25%)	31 (23%)
6	rs3024987	CC	75 (81%)	55 (73%)	139 (76%)	108 (78%)
		CT	18 (19%)	17 (23%)	39 (21%)	29 (21%)
		TT	0 (0%)	3 (4%)	4 (2%)	2 (1%)
7	rs833068	GG	33 (35%)	44 (59%)	88 (48%)	61 (44%)
		GA	52 (56%)	26 (35%)	74 (41%)	61 (44%)
		AA	8 (9%)	5 (7%)	20 (11%)	17 (12%)
8	rs833070	TT	24 (26%)	24 (32%)	45 (24%)	33 (24%)
		TC	44 (47%)	35 (46%)	92 (50%)	73 (53%)
		CC	26 (28%)	17 (22%)	47 (26%)	33 (24%)
9	rs2146323	CC	44 (47%)	23 (31%)	85 (47%)	54 (39%)
		CA	35 (38%)	44 (59%)	78 (43%)	69 (50%)
		AA	14 (15%)	8 (11%)	19 (10%)	14 (10%)
10	rs3025007	CC	26 (28%)	19 (25%)	44 (24%)	37 (27%)
		CT	54 (57%)	35 (46%)	94 (51%)	74 (530
		TT	14 (15%)	22 (29%)	46 (25%)	28 (20%)
11	rs3025020	CC	45 (48%)	35 (47%)	94 (52%)	55 (40%)
		CT	43 (46%)	34 (46%)	67 (37%)	74 (53%)
		TT	5 (5%)	5 (7%)	21 (12%)	10 (7%)
12	rs3025021	CC	37 (39%)	30 (39%)	88 (48%)	70 (50%)
		CT	45 (48%)	38 (50%)	70 (38%)	63 (45%)
		TT	12 (13%)	8 (11%)	26 (14%)	6 (4%)
13	rs3025030	GG	71 (77%)	51 (68%)	131 (72%)	94 (68%)
		GC	19 (21%)	23 (31%)	48 (26%)	39 (28%)
		CC	2 (2%)	1 (1%)	3 (2%)	5 (4%)
14	rs3025035	CC	81 (87%)	65 (87%)	163 (90%)	120 (86%)
		CT	12 (13%)	9 (12%)	19 (10%)	17 (12%)
		TT	0 (0%)	1 (1%)	0 (0%)	2 (1%)
15	rs10434	GG	26 (28%)	24 (32%)	56 (31%)	48 (35%)
		GA	44 (47%)	37 (49%)	83 (45%)	76 (55%)
		AA	23 (25%)	15 (20%)	44 (24%)	13 (9%)

Type 1 Diabetes Mellitus

In the multivariate analyses, after controlling for sex, HbA1c and duration of disease, SNP rs699946 was most significantly associated with blinding DR in T1DM under a recessive model (ie AG+GG genotypes, p=0.007, Table 4.3). These genotypes were found less commonly in the blinding cases compared with those with no DR, thus the AA genotype at SNP rs699946 is associated with an increased risk of blinding DR (OR 4.1 [95% CI 1.5-11.4]). The GG genotype of rs833068 was similarly associated (OR 3.1 [95% CI 1.3-7.2], p=0.017) with blinding DR. Both SNPs remained significant following further adjustments for smoking, hypercholesterolemia, hypertension, BMI and nephropathy (p=0.006 and p=0.047 respectively).

Table 4.3 – P-values for association of *VEGFA* tag SNPs with blinding DR in T1DM

		T1DM unadjusted p value		T1DM adjusted p value	
	SNP	Dominant	Recessive	Dominant	Recessive
1	rs1547651	0.800	0.240	0.670	0.150
2	rs833058	0.570	0.400	0.530	0.410
3	rs699946	0.570	**0.010**	0.770	**0.007**
4	rs833060	**0.006**	0.760	0.260	0.420
5	rs699947	0.490	0.430	0.580	0.980
6	rs3024987	0.260	**0.027**	0.820	**0.012**
7	rs833068	0.640**0**	**.003**	0.620**0**	**.017**
8	rs833070	0.380	0.430	0.540	0.980
9	rs2146323	**0.028**	0.400	0.220	0.430
10	rs3025007	0.700	**0.026**	0.800	0.062
11	rs3025020	0.890	0.710	0.680	0.390
12	rs3025021	0.990	0.650	0.320	0.940
13	rs3025030	0.180	0.680	0.390	0.800
14	rs3025035	0.930	0.200	0.840	0.260
15	rs10434	0.610	0.440	0.260	0.660

Both unadjusted and adjusted p-values are given for the two genetic models. SNPs with p values <0.05 are shown in bold.
Adjusted= p values after controlling for sex, duration of disease, type of diabetes and HbA1c.

The subsets of patients with severe NPDR, PDR and CSME were also considered individually for these associated SNPs. Rs699946 was found to be associated with CSME (OR 5.7 [95% CI 1.1-29.3], p=0.039), while rs833068 was associated with

both CSME (OR 5.1 [95% CI 1.3-19.5], p=0.017) and PDR (OR 4.2 [95% CI 1.2-15.1], p=0.029). These two SNPs are in tight linkage disequilibrium (D'=0.99, Figure 4.1). Rs3024987 was also found to be significantly associated with blinding DR in the recessive model (Table 4.3). However only 3 participants with blinding DR and 0 participants with no DR carried this genotype, and with numbers too small to draw conclusions, the importance of this result is unclear.

The SNPSpD method for multiple testing correction in SNP association studies estimated a total of 10 independent tests. After adjusting for these 10 tests in the multivariate analyses, SNP associations did not remain significant for blinding DR in T1DM.

Type 2 Diabetes Mellitus

In T2DM, rs3025021 and rs10434 were both significantly associated with blinding DR after controlling for sex, HbA1c and duration of disease (p=0.002 and 0.001, respectively, Table 4.4) in the multivariate analyses.

Table 4.4: P-values for association of *VEGFA* tag SNPs with blinding DR in T2DM.

		T2DM unadjusted p value		T2DM adjusted p value	
	SNP	Dominant	Recessive	Dominant	Recessive
1	rs1547651	0.220	0.750	0.710	0.300
2	rs833058	0.620	0.160	0.300	0.160
3	rs699946	0.660	0.180	0.640	0.170
4	rs833060	0.910	0.570	0.660	0.330
5	rs699947	0.690	0.660	0.840	0.140
6	rs3024987	0.780	0.610	0.960	0.550
7	rs833068	0.430	0.730	0.280	0.470
8	rs833070	0.710	0.880	0.830	0.290
9	rs2146323	0.190	0.950	0.610	0.520
10	rs3025007	0.580	0.300	0.690	0.800
11	rs3025020	**0.031**	0.190	0.120	0.320
12	rs3025021	0.650	**0.002**	0.400	**0.002***
13	rs3025030	0.450	0.260	0.890	0.240
14	rs3025035	0.380	0.067	1.00	0.046
15	rs10434	0.400	**0.001**	0.990	**0.002***

Both unadjusted and adjusted p-values are given for the two genetic models. SNPs with p values <0.05 are shown in bold and * if they remain statistically significant post correction for multiple SNP testing in the multivariate analysis.
Adjusted= p values after controlling for sex, duration of disease, type of diabetes and HbA1c.

Again, the minor allele for each SNP appears to be protective for blinding DR with the recessive genotype being less frequent in cases (Table 4.2). Thus, the genotypes containing the major allele (ie CC+CT for rs3025021 and GG+GA for rs10434) appear to confer the greatest risk for blinding DR (OR 3.8 [95% CI 1.5-10.0], and OR 2.6 [95% CI 1.3-5.3] respectively). Both SNPs remained significant after adjustment for additional covariates including smoking, hypercholesterolemia, hypertension, BMI and nephropathy (p=0.019 and 0.015 respectively). In the sub-analysis by DR classification, rs3025021 was also associated with PDR (OR 5.8 [95% CI 1.3-26.7], p=0.024) and rs10434 with both CSME (OR 2.9, [95% CI 1.1-7.6], p=0.027) and PDR (OR 3.0 [95% CI 1.2-7.8], p=0.021). After correction for multiple testing, rs3025021 (p=0.02) and rs10434 (p=0.01) both remained significantly associated with blinding DR in T2DM.

Combined DM

When T1DM and T2DM were combined, rs2146323 (p=0.019), rs3025021 (p=0.009), rs3025035 (p=0.026) and rs10434 (p=0.002) were significantly associated with blinding DR in the univariate analyses (Table 4.5). After controlling for sex, disease type, HbA1c and duration of disease in the multivariate analyses, rs3025021 (p=0.014) and rs10434 (p=0.009) and rs3025035 (p=0.018) remained significantly associated with blinding DR (Table 4.5) and rs10434 was also significantly associated with CSME (p=0.003).

Table 4.5 - P-values for association of *VEGFA* tag SNPs with blinding DR in combined DM.

		Combined DM unadjusted p value		Combined DM adjusted p value	
	SNP	Dominant	Recessive	Dominant	Recessive
1	rs1547651	0.260	0.300	0.960	0.093
2	rs833058	0.980	0.110	0.630	0.096
3	rs699946	0.260	0.150	0.270	0.210
4	rs833060	0.130	0.750	0.760	0.20
5	rs699947	0.920	0.440	0.420	0.860
6	rs3024987	0.670	0.470	0.880	0.054
7	rs833068	0.270	0.970	0.590	0.340
8	rs833070	0.670	0.440	0.650	0.850
9	rs2146323	**0.019**	0.570	0.270	0.310
10	rs3025007	0.830	0.660	0.600	0.370
11	rs3025020	0.068	0.340	0.140	0.740
12	rs3025021	0.370	**0.009**	0.890	**0.014**
13	rs3025030	0.170	0.470	0.530	0.350
14	rs3025035	0.450	**0.026**	0.960	**0.018**
15	rs10434	0.330	**0.002**	0.510	**0.009**

Both unadjusted and adjusted p-values are given for the two genetic models. SNPs with p values <0.05 are shown in bold and * if they remain statistically significant post correction for multiple SNP testing.
Adjusted= p values after controlling for sex, duration of disease, type of diabetes and HbA1c.

After correction for multiple testing, rs10434 remained significantly associated with CSME (p=0.03).

Haplotype analyses

Haplotype analyses revealed no significant association with blinding DR in T1DM. In T2DM, after controlling for age, HbA1c and duration of disease, haplotype 1 of Block 1 (ATGGCCACC) was significantly associated with blinding DR (p=0.039) and haplotype 1 of Block 2 (TCCGCG) was most significantly associated with blinding DR under an additive model (p=0.0004, Table 4.6). This haplotype was also significantly associated with CSME (p=0.0007). After correcting for multiple testing, TCCGCG remained significant in the analysis of T2DM patients (blinding DR p=0.004, CSME p=0.007).

Table 4.6 – Association of haplotypes with blinding DR in T1DM (a) and T2DM (b).

(a) T1DM

Block 1	1	2	3	4	5	6	7	8	9	Haplotype frequency	Frequency in no DR	Frequency in blinding DR	P value
1	A	T	A	G	C	T	G	C	C	0.116	0.104	0.196	0.116
2	A	C	A	T	C	C	A	C	C	0.124	0.119	0.152	0.453
3	A	T	G	T	C	C	A	C	C	0.134	0.143	0.087	0.553
4	A	C	A	G	A	C	G	T	C	0.152	0.166	0.065	0.131
5	T	C	A	G	A	C	G	T	A	0.162	0.16	0.174	0.915
6	A	C	A	G	A	C	G	T	A	0.192	0.181	0.261	0.173
Block 2	10	11	12	13	14	15				Haplotype frequency	Frequency in no DR	Frequency in blinding DR	P value
1	T	C	C	C	C	G				0.098	0.098	0.097	0.987
2	T	T	C	G	C	G				0.106	0.104	0.117	0.329
3	T	C	T	G	C	A				0.151	0.149	0.156	0.99
4	C	T	C	G	C	G				0.16	0.148	0.231	0.177
5	C	C	T	G	C	A				0.204	0.207	0.192	0.656

(b) T2DM

Block 1	1	2	3	4	5	6	7	8	9	Haplotype frequency	Frequency in no DR	Frequency in blinding DR	P value
1	A	T	G	G	C	C	A	C	C	0.039	0.037	0.047	**0.039**
2	A	T	A	G	C	C	G	C	C	0.05	0.053	0.039	0.05
3	A	C	A	T	C	C	A	C	C	0.111	0.1	0.156	0.111
4	A	T	A	G	C	T	G	C	C	0.117	0.109	0.148	0.117
5	T	C	A	G	A	C	G	T	A	0.131	0.138	0.109	0.131
6	A	C	A	G	A	C	G	T	C	0.161	0.173	0.109	0.161
7	A	T	G	T	C	C	A	C	C	0.161	0.166	0.14	0.161
8	A	C	A	G	A	C	G	T	A	0.195	0.192	0.203	0.195
Block 2	10	11	12	13	14	15				Haplotype frequency	Frequency in no DR	Frequency in blinding DR	P value
1	T	C	C	G	C	G				0.041	0.034	0.095	**4x10-4***
2	C	C	C	G	C	A				0.048	0.045	0.063	0.652
3	C	C	C	C	C	G				0.069	0.071	0.049	0.505
4	T	C	C	C	C	G				0.071	0.067	0.106	0.668
5	T	C	C	G	C	A				0.081	0.096	0	0.019
6	T	C	T	G	C	A				0.128	0.129	0.126	0.841
7	T	T	C	G	C	G				0.13	0.131	0.091	0.692
8	C	C	T	G	C	A				0.161	0.161	0.163	0.754
9	C	T	C	G	C	G				0.162	0.163	0.181	0.693

The overall haplotype frequency as well as the frequency in No DR and blinding DR along with the p-value for all haplotypes with a frequency >2% in two linkage disequilibrium blocks are given. SNPs are numbered as in Table 4.2.
Haplotypes with p values <0.05 are shown in bold and * if they remain statistically significant post correction for multiple haplotype testing.

Discussion

VEGF is an important cytokine that plays a role in angiogenesis and mediates microvascular permeability, making polymorphisms in the *VEGFA* gene potential candidate contributors to the pathogenesis of DR[192, 227].

There is conflicting evidence for the association of *VEGFA* variants with DR development, with inter-study variability including participant ethnicity, study design, retinopathy grading scales, statistical analytical methods and study power, playing major roles. The most investigated *VEGFA* SNP has been rs2010963, located in the 5' untranslated region of the gene, with most studies showing no significant association between the polymorphism and the presence of DR, regardless of participant ethnicity[121-126, 229-231]. The majority of these studies examined this SNP in participants with T2DM, with Churchill *et al* being the only study to make this comparison in a combined DM cohort and no significant association was found[230]. All studies were of a cross-sectional design, with the exception of two studies examining this SNP in a T1DM cohort[229, 231]. Al-Kateb *et al* (2007) was the largest longitudinal study to investigate *VEGFA* SNPs and DR development, whereby 1369 Caucasian subjects were genotyped from the Diabetes Control and Complications Trial. No association of DR in T1DM with the rs2010963 polymorphism was found in this cohort[229]. However, after controlling for covariate risk factors, they did find eight other SNPs which showed significant association ($p<0.05$) with severe DR (severe NPDR, PDR or scatter laser treatment), with rs3025021 having the most significant association ($p=0.0017$). No associations of *VEGFA* SNPs with CSME were found. However, in another longitudinal study (in a cohort of Japanese ethnicity), after controlling for associated risk factors (including HbA1c) in

multivariate analyses, Nakanishi *et al* did not replicate the association of these SNPs with the progression of DR in 175 Japanese participants with T1DM[231].

This study investigated the association of 15 tag SNPs in the *VEGFA* gene with blinding DR in T1DM and T2DM. After adjustment for known clinical covariates, SNPs rs699946 and rs833068 were most significantly associated with blinding DR in T1DM and rs3025021 and rs10434 in T2DM, although only the T2DM result survived correction for multiple testing. The rs10434 SNP was also significantly associated with CSME in combined DM after correction for multiple testing. To our knowledge this is a novel finding.

This study revealed the C allele of rs3025021 to be a risk allele for DR in T2DM. Although it was not found to be significantly associated with T1DM in this study, it was found to be the most significant SNP for severity of DR in the study of Al-Kateb *et al* (2007), where only T1DM patients were considered[229]. The lack of association in T1DM in the current study may well be due to the smaller cohort size, or different genetic backgrounds between the two studies. Rs3025021 remained significantly associated in the combined T1DM and T2DM analyses, providing additional evidence of its important role. It remains to be determined which of the *VEGFA* variants are functionally related to the DR susceptibility.

Haplotype analysis suggested an important role of the TCCGCG haplotype in blinding DR and CSME in T2DM. This haplotype was not observed at >2% frequency in the smaller T1DM cohort. Although it is relatively rare (overall frequency of ~4%), this haplotype contains the risk allele of the two individually

associated SNPs (C for rs3025021 and G for rs10434). Several other haplotypes also contain these alleles, indicating that the true risk allele that is tagged by these SNPs is probably on the background of this particular haplotype, rather than being these SNPs themselves. To our knowledge this is the first study to report this association.

It is acknowledged that participants with DR in this study had the presence of increased risk factors (including longer diabetes duration, higher HbA1c levels and higher rates of nephropathy) when compared to those without DR. However standard statistical measures to control for their effects on DR development were made in the multivariate analyses.

VEGF protein expression has been shown to be influenced by SNPs in the *VEGFA* gene, particularly in the promoter region[121, 196, 228], and VEGF protein levels have been shown to be elevated in the vitreous of humans and mice with PDR and diabetes [198, 199, 221, 227]. SNPs in the *VEGF* may affect splicing and possibly the decrease in the VEGF anti-angiogenic isoform levels in diabetic when compared to normal eyes (Perrin et al 2005). The associated SNPs in T1DM are in linkage disequilibrium with the promoter region of *VEGFA* and may well be tagging such SNPs, directly influencing protein expression and therefore vitreous concentrations of VEGF. It is less clear how SNPs in block 2 would affect the regulation of VEGF levels, although this is now the second report of significantly associated SNPs in this region, suggesting this is a true association. Associated SNPs could be tagging functional intronic sequence variation or downstream enhancer elements.

In conclusion, several *VEGFA* SNPs are associated with increased risk of developing blinding DR in both T1DM and T2DM, independent of duration of diabetes and degree of glycemic control. Further functional studies are necessary to examine the link between specific *VEGFA* SNPs and haplotypes in DR.

CHAPTER 5

Diabetic retinopathy is not associated with *carbonic anhydrase* gene polymorphisms.

The work presented in this chapter has been published in the peer reviewed literature: S Abhary, KP Burdon, A Gupta, N Petrovsky, JE Craig Diabetic retinopathy is not associated with carbonic anhydrase gene polymorphisms. Molecular Vision 2009 15:1179-1184[232]. Dr Abhary's contributions include conception and design of the study, participant recruitment, analysis and interpretation of data and writing of the manuscript.

Introduction

Carbonic anhydrase (CA) is a widely expressed enzyme in humans that catalyses the conversion of carbon dioxide to bicarbonate and protons and thereby plays an important role in acid-base balance[233]. Several isoforms of CA exist and CA1 has been isolated in the human retinal endothelial cells. Vitreous humor from PDR patients has been found to contain up to 50 different proteins[234, 235] and Gao *et al*. recently undertook a proteomic analysis of vitreous from individuals with diabetes and found the CA1 concentration in individuals with PDR to be significantly higher than in control subjects without DM or individuals with DM but without DR[236]. In support of a pathological role for CA in DR, intravitreal injection of CA in rats induced retinal fluorescein leakage and retinal edema that was inhibited by co-injection of acetazolamide (a specific CA inhibitor). Intravitreal CA1 injection

increased retinal vascular permeability through increasing vitreous pH, leading to activation of the kallikrein-kinin system. Thus, the CA pathway is potentially important in the development of DR (and especially macular edema), which is characterized in part by increased vascular permeability and retinal edema.

Given the evidence indicating a potential role of CA1 in DR pathogenesis, the aim of this study was to determine if common polymorphisms in the *CA1* gene might contribute to DR susceptibility. To our knowledge, this is the first study to examine this potential association.

Methods

Ten tag SNPs spanning the *CA1* gene on chromosome 8 which captured all alleles with an r^2 of at least 0.8 (mean r^2=0.963), were genotyped in 554 individuals with DM, consisting of 190 participants with T1DM and 364 with T2DM.

Results

Of the 554 participants included in this study, 281 subjects had no DR and 273 had DR. Two hundred and fifteen participants were classified as having blinding DR, consisting of 23 participants with severe NPDR, 132 with PDR and 93 with CSME.

Subjects with T1DM and no DR had a significantly lower age, shorter disease duration, lower HbA1c, and lower rates of nephropathy and hypertension compared to the T1DM cases with blinding DR. Subjects with T2DM and no DR were significantly more likely to be female and have shorter disease duration, lower

HbA1c levels, lower BMI readings and lower rates of nephropathy when compared to subjects with T2DM and blinding DR (Table 5.1).

Table 5.1 – Comparison of clinical characteristics of participants by type of diabetes and DR status.

	T1DM			T2DM		
Clinical Characteristics	No DR (n=94)	Blinding DR (n=76)	P value	No DR (n=187)	Blinding DR (n=139)	P value
Sex (female)	48 (51%)	29 (45%)	0.478	100 (53%)	45 (35%)	0.001
Age (years)	38.4 ± 14.9	49.8 ± 15.5	<0.001	64.3 ± 14.5	64.0 ± 10.7	0.853
Disease duration (years)	15.4 ± 9.1	30.9 ± 13.4	<0.001	12.9 ± 8.7	17.5 ± 8.6	<0.001
HbA1c (%)	7.6 ± 2.5	8.8 ± 2.3	0.004	6.6 ± 2.9	7.5 ± 3.4	0.014
BMI (kg/m2)	25.9 ± 7.1	25.9 ± 9.8	0.958	32.2 ± 9.1	29.6 ± 11.5	0.026
Hypercholesterolemia (%)	33 (35%)	32 (50%)	0.062	120 (64%)	81 (63%)	0.802
Nephropathy (%)	40 (15%)	29 (45%)	<0.001	43 (23%)	46 (36%)	0.014
Smoker (%)	44 (47%)	35 (55%)	0.331	99 (53%)	69 (53%)	0.924
Hypertension (%)	37 (39%)	46 (72%)	<0.001	153 (82%)	107 (83%)	0.796

Results are presented as number of subjects (%) or mean±standard deviation.

SNP Analysis

All SNPs were in Hardy Weinberg Equilibrium in all groups. Genotype counts of individuals with DM and no DR were compared to patients with each type of DR. Genotype frequencies for blinding DR are given in Table 5.2 and were similar between T1DM and T2DM.

Table 5.2 - Genotype frequencies by type of diabetes and DR status.

	SNP	Genotype	T1DM No DR n(%)	T1DM Blinding DR n(%)	T2DM No DR n(%)	T2DM Blinding DR n(%)
1	rs2403104	TT	48 (53%)	36 (57%)	78 (43%)	60 (47%)
		TG	34 (37%)	21 (33%)	88 (48%)	54 (42%)
		GG	9 (10%)	6 (10%)	16 (9%)	15 (12%)
2	rs17741410	AA	60 (65%)	42 (67%)	131 (72%)	91 (70%)
		AG	32 (34%)	18 (29%)	46 (25%)	35 (27%)
		GG	1 (1%)	3 (5%)	5 (3%)	4 (3%)
3	rs1496533	TT	22 (24%)	12 (19%)	44 (24%)	34 (26%)
		TC	47 (51%)	32 (52%)	93 (51%)	62 (48%)
		CC	24 (26%)	18 (29%)	44 (24%)	34 (26%)
4	rs17814594	TT	58 (65%)	45 (73%)	131 (72%)	89 (70%)
		TC	30 (34%)	15 (24%)	46 (25%)	36 (28%)
		CC	1 (1%)	2 (3%)	4 (2%)	3 (2%)
5	rs12544332	AA	29 (31%)	23 (37%)	60 (33%)	43 (33%)
		AC	45 (48%)	30 (48%)	89 (49%)	60 (47%)
		CC	19 (20%)	9 (15%)	33 (18%)	26 (20%)
6	rs1496529	AA	56 (60%)	41 (65%)	98 (53%)	69 (53%)
		AG	30 (32%)	18 (29%)	77 (42%)	52 (40%)
		GG	8 (9%)	4 (6%)	9 (5%)	9 (7%)
7	rs725605	TT	24 (26%)	20 (31%)	49 (27%)	41 (32%)
		TC	47 (50%)	31 (48%)	93 (51%)	62 (48%)
		CC	23 (24%)	13 (20%)	40 (22%)	27 (21%)
8	rs2645050	AA	60 (65%)	44 (71%)	131 (72%)	90 (70%)
		AG	32 (34%)	16 (26%)	45 (25%)	36 (28%)
		GG	1 (1%)	2 (3%)	5 (3%)	3 (2%)
9	rs2645049	CC	54 (58%)	37 (59%)	93 (51%)	70 (54%)
		CT	27 (29%)	19 (30%)	80 (44%)	51 (40%)
		TT	12 (13%)	7 (11%)	9 (5%)	8 (6%)
10	rs13278559	CC	78 (84%)	54 (86%)	147 (81%)	105 (81%)
		CT	15 (16%)	8 (13%)	33 (18%)	24 (18%)
		TT	0 (0%)	1 (2%)	2 (1%)	1 (1%)

Results are presented as n (%)

No association was found between any *CA1* SNP and blinding DR (Table 5.3), nor the other DR sub-categories (severe NPDR, PDR or CSME) in combined DM, and for T1DM or T2DM analysed independently. The results remained non-significant in the multivariate analyses after adjusting for disease type, sex, duration of disease or HbA1c.

Table 5.3 – P-values for association of *CA1* tag SNPs with blinding DR by type of diabetes under dominant and recessive genetic models.

		Combined DM		T1DM		T2DM	
	SNP	Dominant	Recessive	Dominant	Recessive	Dominant	Recessive
1	rs2403104	0.46	0.31	0.73	0.92	0.54	0.36
2	rs17741410	0.74	0.79	0.99	0.54	0.7	0.89
3	rs1496533	0.77	0.75	0.68	0.93	0.85	0.85
4	rs17814594	0.83	0.91	0.74	0.8	0.65	0.99
5	rs12544332	0.87	0.73	0.48	0.44	0.82	0.94
6	rs1496529	0.82	0.99	0.36	0.6	0.74	0.8
7	rs725605	0.23	0.68	0.51	0.33	0.26	0.98
8	rs2645050	0.93	0.98	0.95	0.76	0.92	0.85
9	rs2645049	0.83	0.78	0.87	0.45	0.93	0.84
10	rs13278559	0.84	0.45	0.51	0.23	0.55	0.89

P values have been adjusted for sex, diabetes type, duration of disease and HbA1c

Haplotype analyses were undertaken for *CA1* polymorphisms and blinding DR (Table 5.4), NPDR, PDR or CSME and no significant associations were found in the combined DM or for separate T1DM or T2DM analyses with any DR classification.

Table 5.4 – Frequency of common (frequency>2%) haplotypes and association with blinding DR.

T1DM														
Haplotype	1	2	3	4	5	6	7	8	9	10	Haplotype frequency	No DR frequency	Blinding DR frequency	P value
1	T	G	T	C	C	A	C	G	C	C	0.165	0.175	0.151	0.333
2	G	A	T	T	C	G	C	A	T	C	0.205	0.218	0.184	0.616
3	T	A	C	T	A	A	T	A	C	C	0.428	0.410	0.459	0.228

T2DM														
Haplotype	1	2	3	4	5	6	7	8	9	10	Haplotype frequency	No DR frequency	Blinding DR frequency	P value
1	G	A	T	T	A	A	T	A	C	T	0.041	0.045	0.034	0.818
2	T	A	C	T	A	A	T	A	C	T	0.050	0.046	0.051	0.502
3	T	G	T	C	C	A	C	G	C	C	0.151	0.143	0.162	0.466
4	G	A	T	T	C	G	C	A	T	C	0.224	0.220	0.229	0.540
5	T	A	C	T	A	A	T	A	C	C	0.397	0.392	0.410	0.941

SNPs are numbered as in Table 5.2 and the p-value for all haplotypes with a frequency >2% are given.
P values have been adjusted for sex, diabetes type, duration of disease and HbA1c.

Discussion

The pathogenesis of DR is complex, with few independent risk factors identified other than duration and extent of hyperglycemia and systemic hypertension. Increased carbonic anhydrase levels have been described in the vitreous of patients with PDR[236]. Functional evidence from rat models supports a potential role for CA in the pathogenesis of DR[236]. It has been postulated that CA is released from lysed blood cells as a result of retinal and vitreous haemorrhage leading to an increase of vitreous pH, which leads to an increase in vascular permeability through activation of the kallikrein-kinin system[236]. This increase in vascular permeability results in edema and subsequent damage to the retina, contributing to DR (particularly macular edema) and vision loss.

A pilot study of macular edema treatment with acetazolamide showed significant improvement in fluorescein-angiographic findings and visual fields, further supporting the role of carbonic anhydrase in DR development[237]. In the study of Gao *et al* [236], vitreous proteins were assayed at a late time point in the disease process, as vitreous was removed from patients during vitrectomy as a treatment for advanced disease[236]. In addition, the role of CA in these studies has been studied in the context of sight threatening DR. It is therefore possible that CA is involved only in end stage damage to the retina, such as that of PDR and CSME and not in NPDR. Thus it is difficult to know if the CA in vitreous is increased as a consequence of DR or is a late factor exacerbating the disease.

To our knowledge, this study was the first to investigate *CA1* sequence variation as a risk factor for DR. Our results suggest that common sequence variation in the *CA1* gene is not a major risk factor for the development of DR in either T1DM or T2DM. While this study was powered to detect a modest effect size (OR=1.5), the study design does not allow us to exclude a rare *CA1* polymorphism, or a series of separate uncommon pathogenic mutations as a contributor to DR development in a small subset of DR subjects. It is also possible that other factors, perhaps including different genetic loci, may play a role in the regulation of CA. It remains to be determined by clinical studies whether CA represents a valid target for the treatment or prevention of DR.

CHAPTER 6

Association between *erythropoietin* gene polymorphisms and diabetic retinopathy.

The work presented in this chapter has been accepted for publication in the peer reviewed literature: S Abhary, KP Burdon, RJ Casson, N Petrovsky, JE Craig Association between erythropoietin gene polymorphisms and diabetic retinopathy. Archives of Ophthalmology 2010 Jan;128(1):102-6[238]. Dr Abhary's contributions include conception and design of the study, participant recruitment, analysis and interpretation of data and writing of the manuscript.

Introduction

Although the mechanisms underlying DR are still incompletely understood, current models suggest that damage to retinal blood vessels by longstanding hyperglycemia leads to retinal hypoxia, which stimulates the DNA binding activity of hypoxia-inducible factor (HIF-1). HIF-1 upregulates a large number of hypoxia-inducible genes, including *VEGFA* and erythropoietin *(EPO)*[239]. Consequent increased expression of these cytokines may then contribute to DR progression.

EPO is a glycoprotein that plays a major role in stimulation of bone marrow stem cells and erythropoiesis. It has also been shown to stimulate proliferation, migration and angiogenesis in vascular endothelial cells exposed to hypoxia[240, 241]. *EPO* mRNA is expressed in the human retina[242, 243]. Expression of EPO receptors in vascular

endothelial cells[244] and the retina[243, 245] has also been demonstrated. Many studies have reported a higher concentration of EPO in the vitreous of diabetic and PDR patients when compared to controls[198, 199, 242, 246]. Animal studies have shown increased EPO concentrations in the ischemic retina[247, 248], and that EPO inhibitors prevent neovascularization, further supporting a role for EPO in the pathogenesis of PDR[248].

EPO protein expression is influenced by SNPs in the *EPO* gene[247]. Interestingly, there is no correlation between the vitreous and plasma levels of EPO, suggesting increased vitreous EPO is due to increased local production of EPO in the retina[199, 246], rather than due to increased systemic EPO production by the kidney.

The human *EPO* gene is located on chromosome 7q21[180]. Recently, Tong *et al* genotyped 613 T2DM subjects [374 with PDR and end stage renal disease (ESRD), and 239 DM complication-free diabetic controls] for 19 SNPs in 11 genes involved in angiogenesis, including *EPO*. The only significant association with DR was found at SNP rs1617640 in the promoter of the *EPO* gene, where the T allele was significantly associated with PDR and ESRD (OR 2.01 [95% CI 1.23-2.09], p=0.00191). This finding was replicated in two T1DM cohorts (1244 PDR with or without ESRD and 715 DM complication-free controls) with all subjects being of European American descent (overall OR 2.17 [95% CI 1.70-2.76], p=2.76E-11). The TTA haplotype of SNPs rs1617640, rs507392 and rs551238, were also disease-associated (p=0.0005)[247]. We aimed to determine whether the same *EPO* sequence variation was associated with DR development in Australian subjects with DM and without ESRD.

Methods

This cohort consisted of 173 participants with T1DM and 345 with T2DM. Subjects were excluded if they had ESRD, defined as stage V chronic kidney disease on dialysis or renal transplantation. The same three *EPO* SNPs (rs1617640, rs507392 and rs551238) found to be significantly associated with PDR and ESRD in Tong *et al* were genotyped in all individuals. Ninety three percent were of Caucasian European descent, with the remainder of Asian and Middle Eastern descent. In order to test for any population stratification, allele frequencies of rs1617640, rs507392 and rs551238 between Caucasians and non-Caucasians were compared. As no difference was detected (chi-square=0.43, p=0.81), all individuals were analysed together. Three individuals for each genotype at each SNP were sequenced to confirm the integrity of the genotyping assay.

Results

The participants included in this study are those previously included for the VEGF and CA analyses, however with ESRD excluded. Of the 518 subjects genotyped, 233 (67 T1DM and 166 T2DM) subjects had no DR, 122 (50 T1DM and 72 T2DM) NPDR, 126 (46 T1DM and 80 T2DM) PDR and 90 (24 T1DM and 66 T2DM) had CSME.

Comparisons of those with no DR to any DR led to the most significant results and details of these results are subsequently shown with reference to DR subtype analyses made. Subjects with T1DM and no DR had a significantly lower age, shorter disease duration, lower cholesterol and less hypertension rates when compared to subjects with T1DM and DR. Subjects with T2DM and no DR were

more likely to be female, have shorter disease duration, lower HbA1c and higher BMI when compared to subjects with T2DM and DR (Table 6.1).

Table 6.1 – Clinical characteristics of participants with no DR compared to DR cases in T1DM and T2DM.

	T1DM			T2DM		
Clinical characteristics	No DR (n=67)	DR (n=106)	P value	No DR (n=166)	DR (n=179)	P value
Sex (female)	32 (48)	52 (49)	0.868	93 (56)	65 (37)	<0.001
Age (years)	36.3±14.6	48.6±16.0	<0.001	64.5±15.1	64.2±11.1	0.818
Disease duration (years)	12.43±8.1	28.1±11.7	<0.001	12.6±8.9	17.3±8.4	<0.001
HbA1c (%)	7.5±2.0	9.2±7.5	0.074	6.5±3.1	8.5±8.6	0.005
BMI (kg/m2)	25.9±6.7	25.4±10.1	0.726	32.5±9.1	29.7±10.9	0.010
Hypercholesterolemia (%)	22 (33)	51 (48)	0.047	106 (64)	113 (61)	0.889
Nephropathy (%)	11 (16)	30 (28)	0.073	37 (22)	53 (30)	0.122
Smoker (%)	34 (51)	54 (51)	0.930	86 (52)	99 (55)	0.515
Hypertension (%)	23 (34)	68 (64)	<0.001	135 (81)	150 (84)	0.545

Results are presented as number of subjects (%) or mean±standard deviation.

All three SNPs were in complete linkage disequilibrium (D'=1.0, r^2=1.0 for all comparisons) and were in Hardy-Weinberg Equilibrium (HWE) when assessed in the total cohort (p>0.05). When analysed by type of diabetes and DR status, participants with T2DM and no DR deviated from HWE (p=0.009) for all three SNPs but all other groups were in equilibrium. Genotype frequencies of all SNPs in both DM subgroups are shown in Table 6.2.

Table 6.2 – Genotype frequencies for each SNP in participants with no DR and DR cases by type of diabetes.

		T1DM		T2DM	
SNP	Genotype	No DR (n=67)	DR (n=106)	No DR (n=166)	DR (n=179)
rs1617640	TT	24 (37%)	40 (39%)	64 (39%)	65 (38%)
	TG	30 (46%)	44 (43%)	88 (54%)	78 (46%)
	GG	11 (17%)	18 (18%)	11 (7%)	27 (16%)
rs507392	TT	24 (37%)	40 (39%)	63 (39%)	65 (38%)
	TC	30 (46%)	44 (43%)	88 (54%)	78 (46%)
	CC	11 (17%)	18 (18%)	11 (7%)	27 (16%)
rs551238	AA	24 (37%)	40 (39%)	64 (39%)	65 (38%)
	AC	30 (46%)	44 (43%)	88 (54%)	78 (46%)
	CC	11 (17%)	18 (18%)	11 (7%)	27 (16%)

Values are shown as n (%).

Due to the complete linkage disequilibrium observed, all three SNPs gave identical association results. All three SNPs in the *EPO* gene were associated with DR status in the combined DM group (T1DM and T2DM combined), and T2DM alone under a recessive model. The GG genotype of rs1617640, CC genotype of rs507392and CC genotype of rs551238 were all significantly associated with the presence of DR after adjustment for age, sex, HbA1c, duration of disease and nephropathy in combined DM (OR 2.47, 95% CI [1.23-4.94], p=0.008). In the sub-analysis for type of DM, these genotypes were significantly associated with DR in the T2DM group (OR 3.35 [95% CI 1.36-8.25], p=0.006, Table 6.3) but not the T1DM group, although the total number of T1DM subjects was fewer, reducing the statistical power to detect an association in this group.

Table 6.3: P values for association of *EPO* SNPs with DR in T1DM and T2DM.

	rs507392		rs1617640		rs551238	
DM type	Dominant	Recessive	Dominant	Recessive	Dominant	Recessive
Combined	0.950	**0.024**	1.000	**0.023**	1.000	**0.023**
Combined adjusted[1]	0.630	**0.009**	0.590	**0.008**	0.590	**0.008**
Combined adjusted[2]	0.670	**0.017**	0.730	**0.019**	0.670	**0.017**
T1DM	0.770	0.900	0.770	0.900	0.770	0.900
T1DM adjusted1	0.760	0.130	0.760	0.130	0.760	0.130
T1DM adjusted[2]	0.240	0.390	0.240	0.390	0.240	0.390
T2DM	0.900	**0.008**	0.850	**0.008**	0.850	**0.008**
T2DM adjusted[1]	0.570	**0.006**	0.530	**0.006**	0.530	**0.006**
T2DM adjusted[2]	0.540	**0.019**	0.590	**0.020**	0.540	**0.019**

Adjusted[1]= p values are adjusted for sex, age, duration of disease, type of diabetes (only for combined DM analyses), HbA1c and nephropathy.
Adjusted[2]= p values are adjusted for sex, age, duration of disease, type of diabetes (only for combined DM analyses), HbA1c, nephropathy hypertension, hypercholesterolemia, BMI and smoking.
Statistically significant p values are highlighted in bold font.

In the multivariate analyses, further sub-classification for the type of DR found all three SNPs to be associated with PDR, CSME and blinding DR in combined DM (OR 2.64 [95% CI 1.10-6.34], p=0.030, OR 2.52 [95% CI 1.06-6.00], p=0.040 and OR 2.24 [95% CI 1.07-4.69], p=0.033 respectively), and T2DM alone (OR 3.62 [1.22-10.76], p=0.020, OR 3.73 [95% CI 1.26-11.01], p=0.018 and OR 3.28 [95% CI 1.24-8.69], p=0.016 respectively). In addition to controlling for age, sex, HbA1c, duration of disease and nephropathy, further multivariate analyses were undertaken, also controlling for hypertension, hypercholesterolemia, BMI and smoking (Table 6.3). All three SNPs were significantly associated with the presence of any DR, PDR and blinding DR in combined DM and T2DM alone ($p<0.05$). No associations were found in an allelic association model for any SNP and DR (or its subtypes) in combined DM, T1DM or T2DM alone ($p>0.05$).

The GCC haplotype was found at a higher frequency in the DR group than in those with no DR in the combined DM and T2DM alone. This association was significant under a recessive model after adjusting for sex, HbA1c, duration of disease, and

nephropathy (p=0.008, Table 6.4). This haplotype was also associated with PDR, CSME and blinding DR in combined DM (p=0.030, p=0.040, p=0.008 respectively), and T2DM alone (p=0.027, p=0.031 and p=0.009 respectively). No significant association was observed for T1DM alone.

Table 6.4: Association of haplotypes with blinding DR by type of DM.

Haplotype		Combined DM	T1DM	T2DM
TTA	Overall frequency	0.63	0.60	0.64
	Frequency in no DR	0.65	0.60	0.67
	Frequency in DR	0.61	0.60	0.61
	P value	0.652	0.758	0.652
GCC	Overall frequency	0.37	0.40	0.36
	Frequency in no DR	0.36	0.40	0.34
	Frequency in DR	0.39	0.40	0.39
	P value	0.008	0.103	0.008

TTA and GCC consist of consecutive alleles from rs1617640, rs507392 and rs551238 . P values are adjusted for sex, age, duration of disease, type of diabetes (only for combined DM analyses), HbA1c and nephropathy.

Discussion

EPO is an important cytokine that stimulates proliferation, migration and angiogenesis in vascular endothelial cells[240, 244]. EPO protein expression has been shown to be influenced by SNPs in the *EPO* gene and is elevated in the vitreous of subjects with PDR[198, 199, 242, 246]. The *EPO* gene is thus a biologically plausible candidate gene with potential to influence susceptibility to develop DR and this study has investigated the association of *EPO* gene variation with DR development.

This study found an association between 3 *EPO* SNPs and DR in a cohort of DM subjects, with the GCC haplotype having an increased frequency in T2DM patients with DR. In contrast to our study, Tong *et al* identified the T allele of rs1617640 to be the risk associated allele with PDR. It was also reported that the same allele in the *EPO* promoter region had a major effect on *EPO* mRNA transcription levels in an *in*

vitro model. The opposite haplotype (TTA) was reported as the risk haplotype for PDR in their study compared with our cohort[247]. Similar overall allele frequencies (across combined cases and controls) were observed in the two studies. However, a major and important difference between the two cohorts is the complete lack of ESRD in the current study, compared with the majority of cases having ESRD in the study of Tong *et al*. As opposed to Tong *et al*, this study did not find an association of the *EPO* SNPs with DR in T1DM. The substantially larger T1DM cohort of Tong *et al* had additional power, which may explain the lack of association observed in the current study.

ESRD leads to anemia through reduced EPO production. Although similar vascular processes may be involved in the pathogenesis of both diabetic nephropathy and DR, it is possible that different genetic factors play a role in their susceptibility. The development of DR[86-88, 90, 249-251]and nephropathy[252-257], have each been shown to have a strong genetic component. However, the majority of the linkage regions reportedly involved in the inheritance of retinopathy[91, 250, 251] are not shared with those detected for nephropathy[255, 257]. It is therefore possible that different variations within the EPO gene play a role in DR and ESRD development, and therefore possible that Tong *et al* have identified variations in the EPO gene responsible only for ESRD, accounting for the differences between their results and our findings.

Previous studies have suggested no association of plasma EPO levels with increased vitreous EPO, but rather increased vitreous levels to be due to local production of EPO in the retina[199, 246]. Further studies investigating factors influencing local and

systemic production of EPO and tissue specific effects of *EPO* gene regulation are also required to further understand the role of EPO in DR and ESRD pathogenesis.

This study is not the first to report an opposite allele/genotype association of the same SNP to be associated with a disease. For example, the G and C allele of SNP rs3741916 of the *glyceraldehyde-3-phosphate dehydrogenase* gene have been reported by different studies to be significantly associated with Alzheimer's disease in Caucasian subjects[258, 259]. This "flip flop" phenomenon may occur when a single locus association is found in the presence of multi-locus effects and this single locus association may be confounded by other loci. Another reason can include sampling variation amongst the studies, whereby the magnitude of an association between an allele and risk of disease varies across different (especially ethnic) populations due to the presence of different linkage disequilibrium patterns. Finally, associations of opposite alleles of the same SNP may occur because of differences in its relationship with other causal variants, including environmental factors[260].

Another important consideration is the deviation from Hardy-Weinberg equilibrium (HWE) observed in the T2DM no DR group. This group is a highly selected group of controls, and thus the deviation is not necessarily unexpected in the presence of a true association. However, there is a chance that this deviation is due to population stratification. The T1DM groups conform to HWE as does the T2DM with DR group, indicating that recruitment bias and genotyping errors are unlikely to be the cause. However, the reported association does depend on the group that does not conform to HWE.

It is acknowledged that subjects with no DR in our study had shorter duration of DM and fewer associated vascular risk factors when compared to those with DR. We accept this as a limitation of our study. However, an attempt to overcome these influences on the outcome of results has been made by adjusting for these factors in the multivariate analyses.

In conclusion, our results show that in an Australian Caucasian population, variation in the *EPO* gene predicts the risk of developing DR, independent of duration of DM. There is clearly a need for further independent association studies to further explore the role of EPO sequence variation in DR susceptibility. Also further functional characterisation is required to better elucidate the role of *EPO* in DR and ESRD development. If confirmed, this finding leads to the possibility of developing treatments or preventative therapies for DR based on a clearer understanding of the mechanism of involvement of EPO in DR development.

CHAPTER 7

Aldose reductase gene polymorphisms and diabetic retinopathy

The work presented in this chapter has been submitted for publication to *Diabetes Care* and has been favourably reviewed with revisions submitted: Abhary S, Burdon KP, Laurie KJ, Lake S, Thorpe S, Petrovsky N, Craig JE. Aldose reductase gene polymorphisms and diabetic retinopathy. Dr Abhary's contributions include conception and design of the study, participant recruitment, analysis and interpretation of data and writing of the manuscript.

Introduction

The aldo-keto reductase family 1 member B1 (*AKR1B1*) gene (also known as aldose reductase, *ALR*) is located on chromosome 7q35. As described in chapter 1, our meta-analysis investigating the association of 34 genetic polymorphisms with the development of DR revealed the z-2 allele of a microsatellite marker upstream of the *AKR1B1* gene to be associated with increased risk (OR 2.33 [95% CI 1.49–3.64], $p=2x10^{-4}$) for DR in DM, and the z+2 allele with reduced risk (OR 0.58 [95% CI 0.36–0.93], p=0.02) for DR in T2DM. The T allele of the *AKR1B1* promoter rs759853 (C-106T) variant was also associated with reduced risk of DR in T1DM (OR 0.50 [95% CI 0.35−0.71], $p=1.00x10^{-4}$).

Several biochemical pathways, including the polyol pathway, are involved in microvascular damage from chronic hyperglycemia. Aldose reductase (ALR) is the first and rate limiting enzyme in the polyol pathway, reducing glucose to sorbitol

with NADPH as a cofactor. Under hyperglycemic conditions, increased flux leads to sorbitol accumulation and subsequent increase in osmotic pressure and oxidative damage to cells[261]. Pathological changes also occur as a result of the activation of protein kinase C by the oxidation of sorbitol, leading to pathogenic vascular and hemodynamic changes including blood flow abnormalities, increased vascular permeability, neovascularization and vascular occlusion[58, 262].

ALR has been isolated from human retinal endothelial cells and pericytes[188, 190, 263]. Support for the role of ALR in DR development has been provided by several studies. Increased expression of ALR has been demonstrated in the retina of diabetic mice and shown to be involved in DR development by leading to blood-retinal barrier breakdown, loss of pericytes and neovascularization[264]. Randomised controlled trials of ALR inhibition in human subjects with DM are conflicting, with some showing a significant reduction in DR[265-268].

ALR levels have been shown to be higher in erythrocytes of DR patients when compared to those without DR or non-diabetic controls[269]. Similarly, ALR levels have been shown to be higher in those with active PDR than in those with NPDR or quiescent PDR[270]. Increased ALR expression in diabetic subjects with microvascular complications has been shown to be influenced by variation in the *AKR1B1* gene[271, 272].

This study sought to determine whether genetic variation within the *AKR1B1* gene plays a role in DR development in a large Australian cohort of subjects with T1DM and T2DM.

Methods

Recruitment and genotyping

Eight hundred and eighty three individuals (263 T1DM and 620 T2DM) were genotyped for the (CA)n microsatellite. The microsatellite was PCR amplified using primers published by Ko *et al*[141] and alleles determined by separation of fluorescently labeled PCR products on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Fourteen tag SNPs which captured all alleles with an r^2 of at least 0.8 (mean r^2=0.959) were also genotyped in 909 individuals (271 T1DM and 638 T2DM) at the Australian Genome Research Facility, as described in Chapter 3.

Statistical analyses

Allelic and genotypic associations of the (CA)n microsatellite were assessed in SPSS using the Chi-square test and the binary logistic test was applied for multivariate analyses. Genotypic associations for all other SNPs were assessed in PLINK (v1.06)[213]. Dominant and recessive models were considered with respect to the minor allele. To consider the microsatellite in the haplotypic analyses, CLUMPHAP[273] was used. Multivariate haplotypic analyses and also haplotypic analyses of SNPs other than the microsatellites were undertaken in PLINK(v1.06)[213].

Bonferroni correction was applied to microsatellite and haplotypic analyses. Multiple testing of all other individual SNPs was adjusted for using the Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD) method of Nyholt[215], modified by Li and Ji[216].

Results

A total of 514 participants had DR, of which 311 had NPDR (95 with T1DM and 216 T2DM), 188 had PDR (71 T1DM and 117 T2DM), and 150 had CSME (36 T1DM and 114 T2DM). Clinical characteristics of all participants are shown in Table 7.1. Participants with T1DM and DR were found to be significantly older, have longer disease duration, higher rates of hypercholesterolemia, nephropathy and hypertension when compared to those without DR. Participants with T2DM and DR were found to be more likely to be of male sex, have longer disease duration, higher HbA1c and BMI levels and higher rates of nephropathy and hypertension.

Table 7.1 – Clinical characteristics of participants with no DR compared to DR by type of diabetes.

	T1DM			T2DM		
Clinical characteristics	No DR (n=103)	DR (n=168)	P value	No DR (n=294)	DR (n=344)	P value
Female (%)	46 (45%)	86 (51%)	0.296	154 (53%)	145 (42%)	0.009
Age (years)	35.5±13.6	49.4±16.1	<0.001	64.4±16.2	64.7±12.9	0.806
Disease duration (years)	12.91±9.8	28.8±12.2	<0.001	12.0±8.14	17.8±9.7	<0.001
HbA1c (%)	7.3±3.6	7.9±4.0	0.179	5.5±5.8	6.4±5.8	0.017
BMI (kg/m2)	23.0±10.9	21.7±13.7	0.414	28.4±13.2	25.8±15.0	0.022
Hypercholesterolemia (%)	30 (29%)	85 (51%)	0.001	203 (69%)	228 (66%)	0.419
Smoker (%)	45 (44%)	87 (52%)	0.196	163 (56%)	179 (52%)	0.364
Nephropathy (%)	14 (14%)	56 (33%)	<0.001	70 (24%)	109 (32%)	0.029
Hypertension (%)	32 (31%)	103 (61%)	<0.001	233 (80%)	294 (86%)	0.048

Results are shown as n (%) or mean ± standard deviation.

(CA)n microsatellite

Thirteen alleles ranging from 120-144 bp for the *AKR1B1* microsatellite were found in our cohort. The 132 bp allele was the most common, but in order to make comparisons with previous study populations, the 138 bp allele (24 repeats) was

designated as the z allele as defined by Ko *et al*[141]. After adjustment for relevant covariates, the z-4/z-4 genotype was nominally associated with DR in T2DM ($p=0.015$) and the z-10/z-8 genotype with DR in T1DM ($p=0.048$, Table 7.2). These results did not remain significant after Bonferroni correction for multiple testing ($p>0.05$). Sub-analyses for PDR, NPDR and CSME did not reveal any significant associations.

Table 7.2 – Association analysis of (CA)n microsatellite with *any DR* by type of diabetes. Allele and genotype counts are shown along with both unadjusted p-values and p-values after adjustment for relevant covariates.

Allele or genotype	Size of allele	T1DM No DR	T1DM DR	Unadjusted p value	Adjusted p value[1]	T2DM No DR	T2DM DR	Unadjusted p value	Adjusted p value[2]
z-18	120	0	1	0.436	1.000	0	0	NA	NA
z-16	122	0	1	0.436	1.000	0	1	0.352	1.000
z-14	124	1	0	0.197	1.000	1	0	0.282	1.000
z-12	126	1	0	0.197	1.000	2	2	0.884	0.243
z-10	128	5	15	0.225	0.057	26	31	0.905	0.645
z-8	130	65	97	0.293	0.114	158	191	0.586	0.406
z-6	132	67	120	0.341	0.925	180	232	0.072	0.245
z-4	134	23	37	0.900	0.302	91	87	0.131	0.690
z-2	136	10	11	0.325	0.670	32	28	0.255	0.374
z	138	6	6	0.366	0.231	9	9	0.754	0.912
z+2	140	0	1	0.436	1.000	2	2	0.884	0.999
z+4	142	0	0	NA	NA	1	0	0.282	1.000
z+6	144	0	0	NA	NA	1	0	0.282	1.000
z-10/z-8	128/130	1	9	0.066	0.048	11	11	0.728	0.196
z-10/z-6	128/132	1	5	0.283	0.221	8	13	0.439	0.466
z-8/z-8	130/130	8	16	0.648	0.288	28	30	0.759	0.971
z-8/z-6	130/132	38	59	0.695	0.081	72	105	0.086	0.207
z-8/z-4	130/134	12	7	**0.017**	0.521	29	32	0.846	0.400
z-8/z-2	130/136	2	2	0.607	0.510	12	10	0.433	0.933
z-8/z	130/138	4	2	0.138	0.341	3	1	0.249	0.546
z-6/z-6	132/132	10	20	0.605	0.311	33	50	0.195	0.366
z-6/z-4	132/134	8	24	0.115	0.152	44	45	0.530	0.872
z-6/z-2	132/136	7	7	0.327	0.244	16	12	0.242	0.191
z-6/z	132/138	1	4	0.411	0.789	4	4	0.836	0.624
z-4/z-4	134/134	1	3	0.599	0.470	8	2	0.599	**0.015**
z-4/z-2	134/136	1	2	0.877	0.785	3	2	0.539	0.483

[1]= adjusted for age, disease duration, hypertension, nephropathy and high cholesterol.
[2]= adjusted for sex, disease duration, hypertension and HbA1c.
The 138 bp allele (24 repeats) is designated as the z allele[141]. Genotypes have been shown only if carried by 5 or more participants and p-values have not been corrected for multiple testing. Significant p values are shown in bold type.

The z-10/z-8 genotype was significantly associated with blinding DR (Table 7.3) in T1DM (p=0.008) and remained significant after adjustment for associated variables (p=0.001), and after correction for multiple testing (p=0.007). However, only 2 and 6 participants (both with T1DM) with no DR and blinding DR respectively were carrying this genotype and the significance of this result is unclear. All other multivariate analyses revealed no statistically significant associations of the (CA)n

alleles or genotypes with blinding DR in the combined diabetes group or individually in T1DM or T2DM.

Table 7.3 - Association analysis of (CA)n microsatellite with *blinding DR* (severe NPDR, PDR or CSME) by type of diabetes. Allele and genotype counts are shown along with both unadjusted p-values and p-values after adjustment for relevant covariates.

Allele	Base pairs	T1DM No DR	T1DM Blinding DR	Unadjusted p value	Adjusted p value[1]	T2DM No DR	T2DM Blinding DR	Unadjusted p value	Adjusted p value[2]
z-18	120	0	0	NA	NA	0	0	NA	NA
z-16	122	0	0	NA	NA	1	0	0.5042	0.9996
z-14	124	1	0	0.4922	0.9996	1	0	0.5042	0.9996
z-12	126	1	0	0.4922	0.9996	2	1	0.9244	0.6496
z-10	128	10	6	0.6235	0.2746	32	13	0.7708	0.2271
z-8	130	103	51	0.6145	0.2934	212	103	0.2699	0.1228
z-6	132	117	56	0.8332	0.7011	243	118	0.1916	0.6239
z-4	134	44	12	0.0540	0.2331	117	45	0.3204	0.7169
z-2	136	13	5	0.6921	0.6411	38	16	0.8432	0.5714
z	138	8	2	0.4073	0.0880	10	7	0.3471	0.0957
z+2	140	1	0	0.4922	0.9996	2	1	0.9244	0.9994
z+4	142	0	0	NA	NA	1	0	0.5042	0.9996
z+6	144	0	0	NA	NA	1	0	0.5042	0.9996
z-10/z-8	128/130	2	6	**0.0080***	**0.0010***	13	5	0.7769	0.1428
z-10/z-6	128/132	4	0	0.1669	0.9990	9	6	0.4345	0.5470
z-8/z-8	130/130	12	11	0.0866	0.4045	43	13	0.1941	0.7188
z-8/z-6	130/132	62	31	0.7195	0.8360	98	59	**0.0301**	0.0919
z-8/z-4	130/134	18	1	**0.0093**	0.1682	40	20	0.6541	0.1090
z-8/z-2	130/136	3	1	0.7632	0.5210	12	4	0.6095	0.8138
z-8/z	130/138	5	1	0.2565	0.2211	3	1	0.5463	0.9410
z-6/z-6	132/132	18	11	0.4632	0.6622	52	23	0.9768	0.3899
z-6/z-4	132/134	20	9	0.9087	0.9635	58	18	0.1475	0.5745
z-6/z-2	132/136	9	4	0.9242	0.7554	18	9	0.7710	0.5718
z-6/z	132/138	1	2	0.3219	0.5619	4	4	0.5488	0.7612
z-4/z-4	134/134	2	2	0.6479	0.9214	8	2	0.1986	0.0622
z-4/z-2	134/136	1	1	0.7478	0.9480	3	1	0.5463	0.4918

[1]= adjusted for age, disease duration, hypertension, nephropathy and high cholesterol.
[2]= adjusted for sex, disease duration, hypertension and HbA1c.
Genotypes have been shown only if it was carried by 5 or more participants and p-values have not been corrected for multiple testing. Significant p values are shown in bold type.
* = significant p value survives correction for multiple testing.

The frequency of alleles and genotypes did not differ significantly in the sub-analyses for PDR, NPDR and CSME in participants with T1DM or T2DM ($p>0.05$).

Tag SNPs

The genotype frequencies of all 14 tag SNPs are shown in Table 7.4. The A allele of rs9640883 was significantly associated with DR development in combined diabetes (OR 1.42 [95% CI 1.14-1.77], p=0.0015) and T2DM (OR 1.46 [95% CI 1.13-1.88], p=0.0040). The AA or AG genotype of rs9640883 was significantly associated with DR in combined DM (OR 1.62 [95%CI 1.24-2.13], p=0.0005) and T2DM (OR 5.73 [95% CI 4.26-7.69] p=0.0020) under the dominant model. The SNPSpD method for multiple testing correction in SNP association studies estimated a total of 10 independent tests, with the stated associations remaining significant ($p<0.005$) after correction.

Table 7.4 – Genotype frequencies of *AKR1B1* tag SNPs in participants with no DR and DR by type of diabetes.

			T1DM		T2DM	
	SNP	Genotype	No DR (n%)	DR (n%)	No DR (n%)	DR (n%)
1	rs17773344	CC	8 (8%)	15 (9%)	30 (10%)	28 (8%)
		CG	46 (46%)	69 (41%)	120 (42%)	124 (37%)
		GG	47 (47%)	81 (50%)	138 (48%)	181 (54%)
2	rs9640883*	AA	6 (6%)	11 (7%)	17 (6%)	27 (8%)
		AG	28 (29%)	67 (41%)	96 (34%)	147 (44%)
		GG	61 (64%)	85 (52%)	169 (60%)	158 (48%)
3	rs12666691	CC	66 (66%)	116 (70%)	204 (71%)	235 (71%)
		GC	33 (33%)	42 (25%)	74 (26%)	88 (26%)
		GG	1 (1%)	7 (4%)	9 (3%)	10 (3%)
4	rs782054	AA	55 (54%)	112 (68%)	179 (62%)	214 (64%)
		GA	42 (42%)	46 (28%)	85 (30%)	106 (32%)
		GG	4 (4%)	7 (4%)	23 (8%)	15 (4%)
5	rs1708414	AA	58 (57%)	107 (64%)	182 (63%)	218 (65%)
		GA	39 (38%)	51 (31%)	100 (35%)	107 (32%)
		GG	5 (5%)	9 (5%)	7 (2%)	12 (4%)
6	rs1791001	CC	70 (69%)	107 (64%)	191 (67%)	231 (69%)
		GC	28 (28%)	56 (34%)	85 (30%)	97 (29%)
		GG	3 (3%)	3 (2%)	10 (3%)	8 (3%)
7	rs2259458	GG	44 (44%)	89 (54%)	133 (47%)	173 (52%)
		GT	50 (50%)	61 (37%)	123 (43%)	138 (41%)
		TT	7 (7%)	15 (9%)	30 (10%)	24 (7%)
8	rs3896278	CC	35 (35%)	60 (36%)	104 (36%)	126 (38%)
		CT	53 (52%)	82 (50%)	140 (49%)	161 (48%)
		TT	13 (13%)	23 (14%)	44 (15%)	47 (14%)
9	rs17188118	AA	82 (80%)	141 (84%)	247 (86%)	287 (85%)
		CA	19 (19%)	23 (14%)	38 (13%)	48 (15%)
		CC	1 (1%)	3 (2%)	3 (1%)	1 (1%)
10	rs1424426	CC	16 (16%)	29 (18%)	60 (21%)	62 (19%)
		CT	54 (53%)	83 (50%)	146 (51%)	169 (50%)
		TT	32 (31%)	54 (33%)	82 (28%)	104 (31%)
11	rs759853	AA	14 (14%)	22 (13%)	41 (14%)	45 (13%)
		AG	51 (50%)	80 (49%)	138 (48%)	160 (48%)
		GG	36 (36%)	63 (38%)	108 (38%)	129 (39%)
12	rs1708403	CC	24 (24%)	43 (26%)	59 (20%)	68 (20%)
		TC	55 (54%)	83 (50%)	149 (51%)	184 (55%)
		TT	23 (23%)	40 (24%)	82 (28%)	84 (25%)
13	rs1553976	CC	52 (51%)	89 (54%)	161 (56%)	184 (55%)
		CT	45 (45%)	62 (37%)	107 (37%)	130 (39%)
		TT	4 (4%)	15 (9%)	19 (7%)	21 (6%)
14	rs4728326	AA	6 (6%)	15 (9%)	17 (6%)	17 (5%)
		GA	44 (44%)	69 (42%)	109 (38%)	158 (47%)
		GG	51 (50%)	82 (50%)	162 (56%)	158 (47%)

Note: Results are presented as n (%).

*=The AA or AG genotype of rs9640883 was significantly associated with DR in T2DM (OR 5.73 [95%CI 4.26-7.69] p=0.0020 under the dominant model.

Importantly, after adjustment for associated variables in the multivariate analyses, there were no statistically significant associations with DR and any *AKR1B1* SNP in combined DM, T1DM or T2DM (Table 7.5).

Table 7.5 – Associations of *AKR1B1* tag SNPs with any DR by type of diabetes.

		T1DM adjusted p values[1]			T2DM adjusted p values[2]		
	SNP	Genotypic	Dominant	Recessive	Genotypic	Dominant	Recessive
1	rs17773344	0.9811	0.8839	0.9336	0.2914	0.1180	0.6861
2	rs9640883	0.3535	0.1694	0.9912	0.3103	0.1320	0.4681
3	rs12666691	0.3940	0.2484	0.6514	0.5757	0.3234	0.5455
4	rs782054	0.8583	0.5892	0.8072	0.3702	0.6078	0.1589
5	rs1708414	0.6800	0.4559	0.7998	0.2812	0.9537	0.1264
6	rs1791001	0.1219	0.5836	0.0705	0.7547	0.6625	0.4819
7	rs2259458	0.6934	0.4724	0.8101	0.4064	0.2774	0.2825
8	rs3896278	0.9337	0.7111	0.9123	0.9511	0.7548	0.9613
9	rs17188118	0.1979	0.1235	0.6781	0.3417	0.6348	0.2054
10	rs1424426	0.8324	0.6613	0.7891	0.9984	0.9569	0.9972
11	rs759853	0.8230	0.5850	0.6498	0.9903	0.9793	0.9024
12	rs1708403	0.6988	0.4594	0.8782	0.9803	0.9644	0.8423
13	rs1553976	0.2464	0.2269	0.4340	0.9419	0.9304	0.7698
14	rs4728326	0.6326	0.8303	0.3407	0.4734	0.3670	0.5761

[1] = adjusted for age, disease duration, hypertension, nephropathy and high cholesterol.
[2] = adjusted for sex, disease duration, hypertension and HbA1c.
Note: p-values have not been corrected for multiple testing.

The frequency of genotypes also did not differ significantly in the sub-analyses for PDR, NPDR, CSME or blinding DR when compared to no DR in participants with combined DM, T1DM or T2DM.

Haplotype analyses

Haplotype analyses of all *AKR1B1* SNPs (Table 7.6), and (CA)n microsatellites revealed no associations with DR or its subtypes in combined DM, T1DM or T2DM after adjustment for covariates and correction for multiple testing.

Table 7.6 – Associations of *AKR1B1* haplotypes with any DR by type of diabetes undertaken in 2 blocks of linkage disequilibrium.

		T1DM		T2DM	
Block	Haplotype	Frequency	p value	Frequency	p value
1	CGCAACG	0.140	0.993	0.158	0.569
	CGCAAGG	0.149	0.987	0.120	0.345
	GACAACG	0.246	0.409	0.258	0.099
	GACAACT	0.021	0.489	0.021	0.651
	GGCAACT	0.038	0.772	0.046	0.452
	GGCGACT	0.131	0.695	0.140	0.332
	GGCGGCT	0.055	0.728	0.051	0.498
	GGGAGCG	0.126	0.553	0.099	0.559
	GGGAGCT	0.031	0.918	0.022	0.431
	GGGAGGG	NA	NA	0.021	0.492
2	CACGTCG	0.045	0.331	0.068	0.988
	CATGCCA	0.281	0.669	0.257	0.396
	CATGCCG	0.213	0.987	0.195	0.214
	CATGTCG	0.071	0.643	0.086	0.500
	TACATCG	0.105	0.739	0.121	0.711
	TACATTG	0.183	0.858	0.178	0.889
	TCCATTG	0.087	0.322	0.074	0.907

Block 1 consists of rs17773344, rs9640883, rs12666691, rs782054, rs1708414, rs1791001 and rs2259458. Block 2 consists of rs3896278, rs17188118, rs1424426, rs759853, rs1708403, rs1553976, rs4728326.
[1] = adjusted for age, disease duration, hypertension, nephropathy and high cholesterol.
[2] = adjusted for sex, disease duration, hypertension and HbA1c.
Note: only haplotypes of frequency >0.02 are shown.

As the multivariate analysis indicated no independent association of the tag SNPs or haplotypes with DR, we analysed each SNP for association with each covariate. Of note, rs9640883 was associated with duration of diabetes under a dominant model (p=0.014). Sub-analysis by type of diabetes revealed this association to be in T2DM (p=0.002) and no association was found in T1DM (p=0.345).

Discussion

Multiple biochemical pathways are likely to contribute to the pathogenesis of DR, including the polyol pathway. ALR is the first and rate-limiting enzyme in this

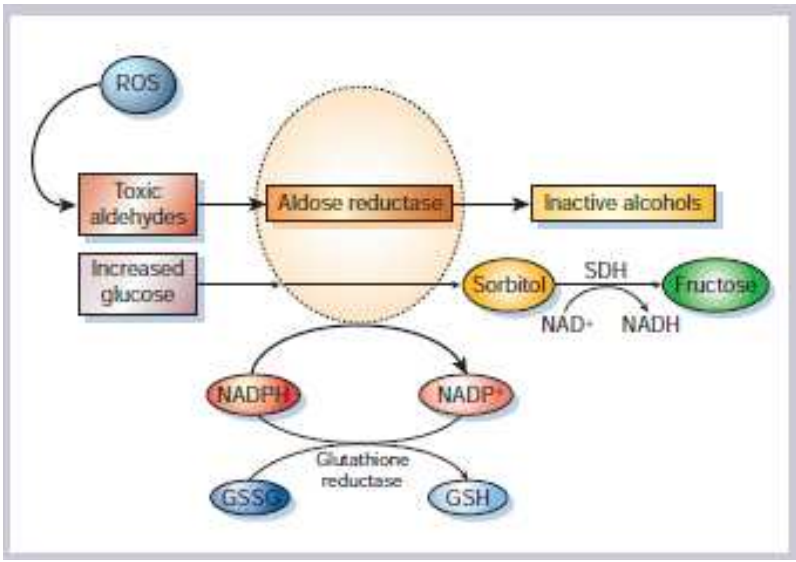
pathway. Pathogenic vascular and hemodynamic changes occur as a result of sorbitol accumulation, oxidative damage and protein kinase C activation[58, 261, 262].

There have been numerous studies assessing polymorphisms of the *AKR1B1* gene and susceptibility to DR in T1DM and T2DM. The (CA)n microsatellite has been the most examined, with 15 studies examining this association[127, 129, 133-145]. The second most commonly studied polymorphism is the rs759853 variant, which has been examined by 7 studies[127-133]. Individual study results have been conflicting. However the meta-analysis (Chapter 2 of this thesis), found the z+2 allele in T2DM and z-2 allele in any type of DM to confer protection from and risk for DR, respectively. Similarly, the C allele of SNP rs759853 was found to confer risk for DR in T1DM.

The present study examined the (CA)n microsatellite, and 14 other SNPs of the *AKR1B1* gene in a large Australian cohort of individuals with T1DM or T2DM. To our knowledge, our study is the largest to investigate the association of the rs759853 SNP with DR in T1DM and the second largest to investigate this SNP in T2DM and also the (CA)n microsatellite in both T1DM or T2DM. Although several tag SNPs were associated with DR, once established risk factors for DR including disease duration, hypertension and HbA1c were considered, no association remained. This suggests particular SNPs may be associated with clinical covariates and well-established risk factors for DR rather than having a direct association with DR itself. We found the DR associated SNP rs9640883 to be associated with duration of diabetes, particularly in T2DM. Using NADPH as a cofactor, aldose reductase reduces toxic aldehydes generated by reactive oxygen species to inactive alcohols and also glucose to sorbitol in the polyol pathway. However, as NADPH is also

required for regenerating reduced glutathione (an antioxidant protective against reactive oxygen species), decreased availability of NADPH due to these reactions could induce or exacerbate intracellular oxidative stress[58] (Figure 7.1).

Figure 7.1 – Aldose reductase and the polyol pathway



ROS=reactive oxygen species, SDH= sorbitol dehydrogenase, GSSG=glutathione disulphide, GSH=reduced glutathione.
Figure reproduced from Brownlee, M., *Biochemistry and molecular cell biology of diabetic complications.* Nature 2001[58].

Chronic hyperglycemia and oxidative stress can result in permanent irreversible damage to the pancreatic beta cell, leading to defective insulin gene expression and secretion as well as apoptosis[274]. Subsequent deterioration of beta cell function over time leads to increased disease severity. Animal studies showing protection of pancreatic islets from hyperglycemia with antioxidant therapy in Zucker diabetic fatty rats provides additional support for this theory, particularly in T2DM[275-277]. In addition, over-expression of *AKR1B1* in an *in vitro* animal study has been shown to induce apoptosis in pancreatic cells[278]. Therefore variation in ALR activity may affect the extent of oxidative stress, and genetic variation in *AKR1B1* may account

for altered ALR activity. Thus, the association observed between SNP rs9640883 and DR may simply reflect the effect this SNP has on diabetes duration (which is an indication of age of onset of disease), in turn influencing DR risk[53, 63-65].

The majority of studies which have previously reported significant associations between the *AKR1B1* gene and DR have not undertaken multivariate analysis for known risk factors, nor corrected for multiple hypothesis testing. In light of the current finding of absence of association, it is possible that the previous studies were influenced by the same confounding effects observed in our study. Although the meta-analysis suggested an association of the *AKR1B1* microsatellite and promoter SNP with DR, the meta-analysis was unable to control for clinical covariates such as duration of diabetes and glycemic control, as the majority of included studies did not provide this information.

In conclusion, the reported association of SNPs within the *AKR1B1* gene with DR is likely to reflect an association between this gene and diabetes duration, which is an indication of age of onset of disease and in turn a major determinant for DR development. To our knowledge, this study is the first to report this association. Our results indicate that future studies, will need to carefully control for known clinical covariates of DR to avoid false positive associations. This will assist not only in increased understanding of pathogenic pathways of DR, but also in the eventual identification of novel preventative treatments for DM or the deterioration of DM, such as antioxidant treatment for the prevention of beta cell deterioration.

CHAPTER 8

Diabetic retinopathy is associated with elevated serum asymmetric and symmetric dimethylarginines (ADMA and SDMA).

The work presented in this chapter has been published in the peer reviewed literature: S Abhary, N Kasmeridis, KP Burdon, A Kuot, MJ Whiting, WP Yew, N Petrovsky, JE Craig Diabetic retinopathy is associated with elevated serum asymmetric and symmetric dimethylarginines Diabetes Care Nov;32(11):2084-6[279]. Dr Abhary's contributions include conception and design of the study, participant recruitment, analysis and interpretation of data and writing of the manuscript. Measurement of serum ADMA has been conducted by Dr Malcolm Whiting and his laboratory staff at the Flinders Medical Centre.

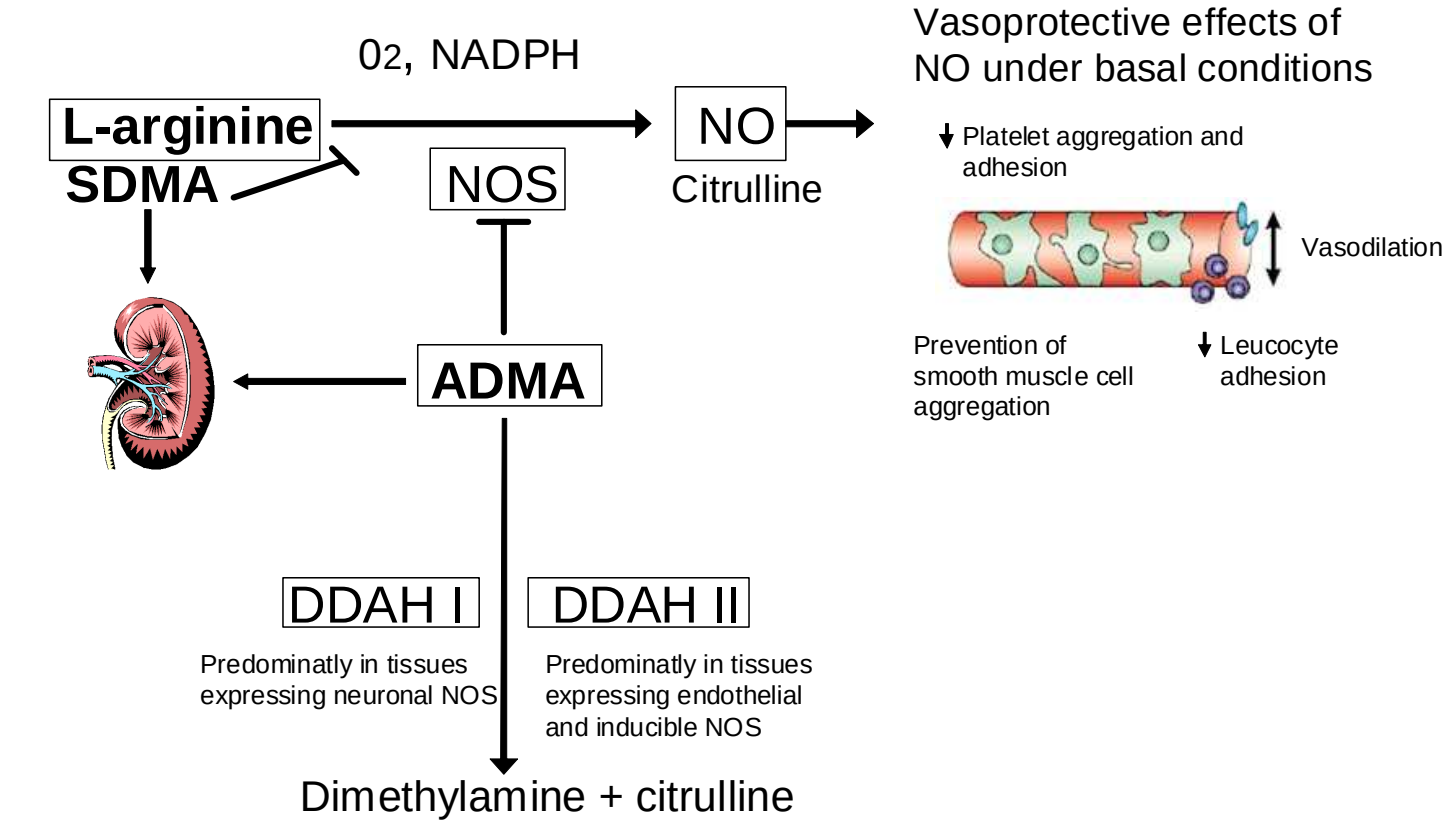
Introduction

Hyperglycemia is a major driver of microvascular complications of DM including DR, and given its critical role in endothelial function, alterations in nitric oxide (NO) bioavailability may play a key downstream role in DR pathogenesis[280]. Endothelium-derived NO helps maintain vascular homeostasis through inducing vasodilatation, suppressing inflammation and the proliferation of vascular smooth muscle cells[281, 282], and preventing platelet adhesion and aggregation[283, 284]. Nitric oxide synthase (NOS) is the key endothelial enzyme that converts L-arginine to L-citrulline and NO. Endothelial dysfunction, such as occurs in hyperglycemia, is associated with decreased NOS activity and NO bioavailability, resulting in vasoconstriction and

increased reactive oxygen species. This leads to impaired ocular hemodynamics underlying DR development[280].

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NOS (Figure 8.1).

Figure 8.1- Nitric oxide pathway



SDMA = symmetric dimethylarginine
NO = nitric oxide
NOS = nitric oxide synthase
ADMA = asymmetric dimethylarginine
DDAH = dimethylarginine dimethylaminohydrolase

Approximately 90% of ADMA clearance occurs via the dimethylarginine dimethylaminohydrolase 1 (DDAH1) and dimethylarginine dimethylaminohydrolase 2 (DDAH2) enzymes, and the remainder via renal clearance[285]. ADMA levels are increased in renal impairment[286], atherosclerosis and other conditions associated with endothelial dysfunction including cardiovascular disease, diabetes, hypertension and hypercholesterolemia[287-289]. ADMA elevation in diabetes may be the result of hyperglycemia-induced inhibition of DDAH enzymes[290]. ADMA is present in the aqueous humor of the human eye and in a recent proteomic study with relatively small numbers, aqueous humor and serum ADMA levels were significantly higher in subjects with diabetes and those with severe retinopathy when compared to non-diabetic controls[291]. Long term adverse effects of ADMA have been investigated in animal studies of wild type and endothelial NOS (eNOS) knockout mice. ADMA infusions in these studies resulted in upregulation of angiotensin-converting enzyme, increased oxidative stress and formation of microvascular lesions [292].

Symmetric dimethylarginine (SDMA) is a stereoisomer of ADMA and its main elimination route is via the kidneys[293] (Figure 8.1). Elevation of serum SDMA has been reported under conditions of decreased renal function[294]. SDMA has no direct inhibitory effect on NOS[295, 296]. However, in conjunction with ADMA, it inhibits the production of NO by competing with L-arginine for cellular uptake, thereby limiting NOS substrate availability[297, 298]. SDMA has been shown to inhibit NO synthesis in a dose-dependent manner in endothelial cells with a resultant increase in reactive oxygen species production[298].

The aim of this study was to investigate the association between DR and serum levels of ADMA, SDMA and L-arginine in a large Australian cohort of subjects with either T1DM or T2DM.

Methods

In total, 505 subjects with available stored serum were included in this study. This cohort consisted of 162 subjects with T1DM and 343 with T2DM. Serum was prepared from venous blood samples and stored at -80 degrees C prior to being analysed for ADMA, SDMA and L-arginine.

Measurement of Serum Concentrations of ADMA, SDMA and Arginine

The concentrations of arginine and its di-methylated metabolites were determined in serum by liquid chromatography-tandem mass spectrometry of the butyl esters on an Applied Biosystems 3200 Q-Trap instrument (Applied Biosystems, Scoresby, Victoria), as described by Schwedhelm *et al*[299]. ®Deuterated internal standards (98 atom% ^{2}H isotopic purity) were purchased from Cambridge Isotope Laboratories (Andover, MA) and L-[$^{2}H_{7}$]-arginine was used for arginine quantitation and 2,3,3,4,4,5,5-[$^{2}H_{7}$]-ADMA for ADMA and SDMA analyses. The between-run coefficients of variation for L-arginine, ADMA and SDMA were determined as 3.3, 4.0 and 11.9% at concentrations of 102, 0.48 and 0.50 µmol/L, respectively. These assays were conducted in SA Pathology (Flinders Medical Centre, SA) by Mr Wai Ping Yew under the supervision of Dr Malcolm Whiting.

Statistical Analysis

Serum ADMA, SDMA and L-arginine concentrations were log transformed before analysis to improve the normality of the distributions. The Mann Whitney test was used to assess crude associations between DR and L-arginine, ADMA and SDMA, followed by multivariate analyses. A hierarchical multiple regression procedure was used and variables (sex, age, type of diabetes, duration of disease, HbA1c, BMI, hypertension, hypercholesterolemia, nephropathy and smoking) significantly correlated with L-arginine, ADMA and SDMA were identified and controlled for in the multivariate analyses. Sub-analyses were performed for PDR and CSME. All analyses were undertaken for all diabetes cases combined, and also individually for T1DM and T2DM cohorts.

Results

Of the 505 participants recruited for this study 329 subjects had no DR and 176 were classified as having blinding DR. In the latter group, 27 had severe NPDR, 101 PDR and 107 CSME. Subjects with T1DM diabetes and no DR had a significantly lower age, shorter disease duration, lower HbA1c levels, and lower rates of nephropathy, hypertension and hypercholesterolemia compared to the T1DM diabetes cases with blinding DR. Subjects with T2DM and no DR had significantly lower HbA1c levels and lower rates of nephropathy and hypertension when compared to subjects with T2DM and blinding DR (Table 8.1).

Table 8.1: Clinical characteristics of no DR compared to blinding DR cases in individuals with T1DM and T2DM.

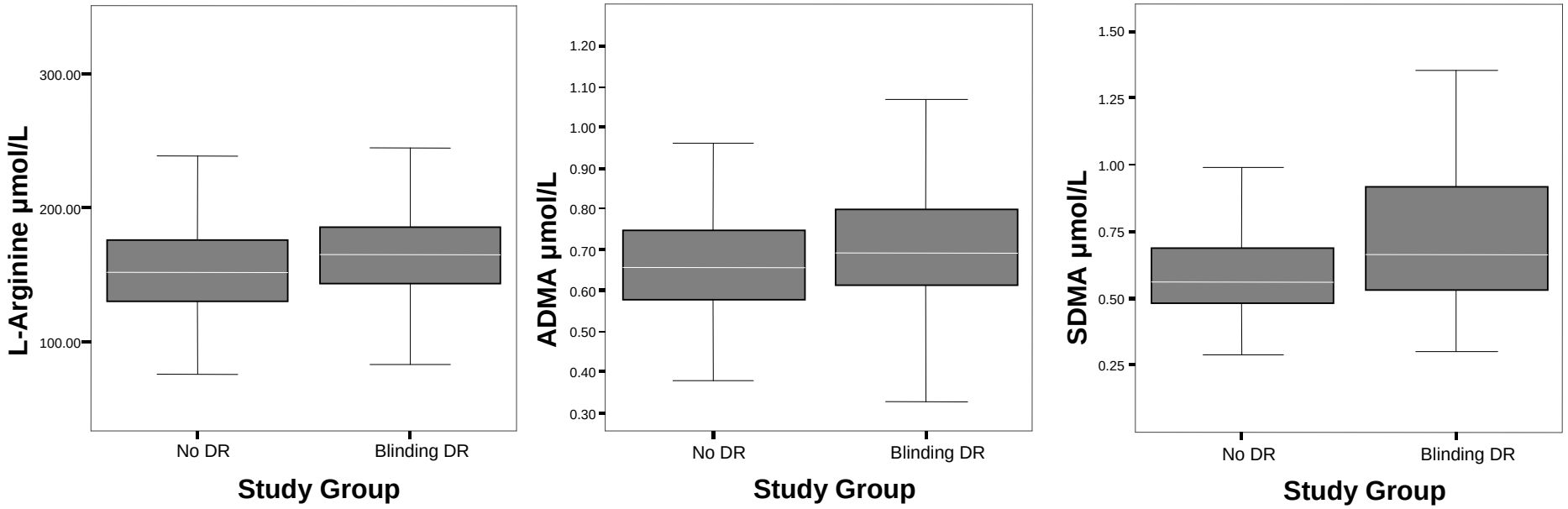
	T1DM			T2DM		
Clinical characteristics	No DR (n=105)	Blinding DR (n=57)	P value	No DR (n=224)	Blinding DR (n=119)	P value
Female (%)	51 (49%)	23 (40%)	0.316	111 (50%)	51 (43%)	0.265
Age (years)	39.8±14.9	54.1±16.0	<0.001	64.5±13.2	61.7±13.4	0.425
Disease duration (years)	17.7±11.3	32.9±14.0	<0.001	13.1±8.6	18.2±8.4	0.11
HbA1c (%)	8.1±1.9	9.0±1.8	0.008	7.5±1.8	8.8±1.8	<0. 001
BMI (kg/m2)	27.9±4.9	28.8±6.2	0.349	32.2±7.4	32.4±6 .7	0.756
Hypercholesterolemia (%)	40 (38%)	34 (60%)	0.009	154 (69%)	86 (73%)	0.499
Smoker (%)	49 (47%)	29 (51%)	0.609	124 (55%)	54 (46%)	0.091
Nephropathy (%)	14 (13%)	23 (40%)	<0.001	53 (24%)	42 (36%)	0.019
Hypertension (%)	39 (37%)	37 (65%)	0.001	178 (80%)	107 (90%)	0.014

Results are presented as number of participants(%) or mean±standard deviation.

In the univariate analyses, blinding DR and PDR was associated with significantly increased serum ADMA (p=0.001), SDMA (p=0.001) and L-arginine (p<0.001) when compared to no DR. Serum ADMA (p=0.002), SDMA (p=0.005) and L-arginine (p=0.03) levels were also significantly increased in the CSME patients when compared to no DR, as were the levels in patients with nephropathy compared to those without nephropathy (ADMA p=0.002, SDMA p<0.001and L-arginine p=0.004).

Disease duration, age, hypertension, hypercholesterolemia, and nephropathy were significantly correlated with blinding DR. Disease duration, age, hypertension, hypercholesterolemia and nephropathy were significantly correlated with PDR (p<0.05). These covariates were subsequently controlled for in the multivariate analyses. Blinding DR (Figure 8.2) and PDR (Figure 8.3) remained strongly associated with elevated serum ADMA (p<0.001), SDMA (p<0.001) and L-arginine (p=0.001) after adjustment for co-variates.

Figure 8.2: Boxplots of untransformed concentrations of L-arginine, ADMA and SDMA (μmol/L) in all subjects with no DR (n=329) and blinding DR (n=176) are shown, regardless of type of diabetes. Data are shown as the 25, 50 and 75 percentiles (represented by grey boxes), range (shown as whiskers, outliers have been removed), and the median (white horizontal line). Details of the mean, standard deviation and adjusted p values for each analyte are provided under its corresponding boxplot.



Note: p values have been adjusted for disease type, disease duration, age, hypertension, hypercholesterolemia and nephropathy.

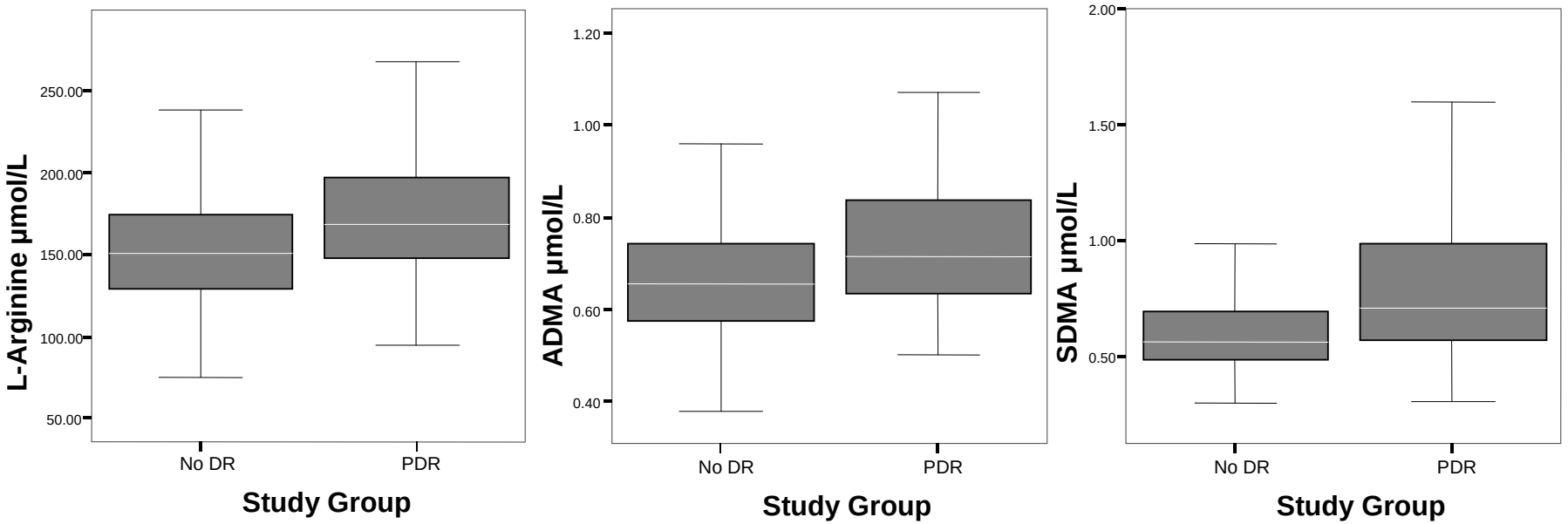
In a sub-analysis by type of diabetes, in T1DM, blinding DR remained associated with significantly increased ADMA (p=0.001) and SDMA (p<0.001) (Table 8.2). In T2DM, blinding DR was significantly associated with increased ADMA (p=0.013) and SDMA (p<0.001, Table 8.2).

Table 8.2 – Multivariate associations of L-arginine, ADMA and SDMA with DR and nephropathy by type of diabetes.

Comparison	Metabolite	T1DM unadjusted	T1DM adjusted	T2DM unadjusted	T2DM adjusted
No DR vs Blinding DR	L-arginine	0.006	0.051	0.007	0.078
	ADMA	0.001	0.001	0.056	0.013
	SDMA	<0.001	<0.001	0.001	<0.001
No DR vs PDR	L-arginine	0.006	0.040	0.001	0.007
	ADMA	<0.001	<0.001	0.017	0.014
	SDMA	<0.001	<0.001	0.002	<0.001
No DR vs CSME	L-arginine	0.097	0.052	0.138	0.135
	ADMA	0.074	0.054	0.008	0.114
	SDMA	0.002	<0.001	0.135	0.078
No Nephropathy vs Nephropathy	L-arginine	0.197	0.034	0.004	0.078
	ADMA	0.011	<0.001	0.105	0.013
	SDMA	<0.001	<0.001	<0.001	<0.001

Note: p values have been adjusted for associated clinical covariates for each comparison.

Figure 8.3: Boxplots of untransformed concentrations of L-arginine, ADMA and SDMA (μmol/L) in all subjects with no DR (n=329) and PDR (n=101) are shown, regardless of type of diabetes. Data are shown as the 25, 50 and 75 percentiles (represented by grey boxes), range (shown as whiskers, outliers have been removed) and the median (white horizontal line). Details of the mean, standard deviation and adjusted p values for each analyte are provided under its corresponding boxplot.

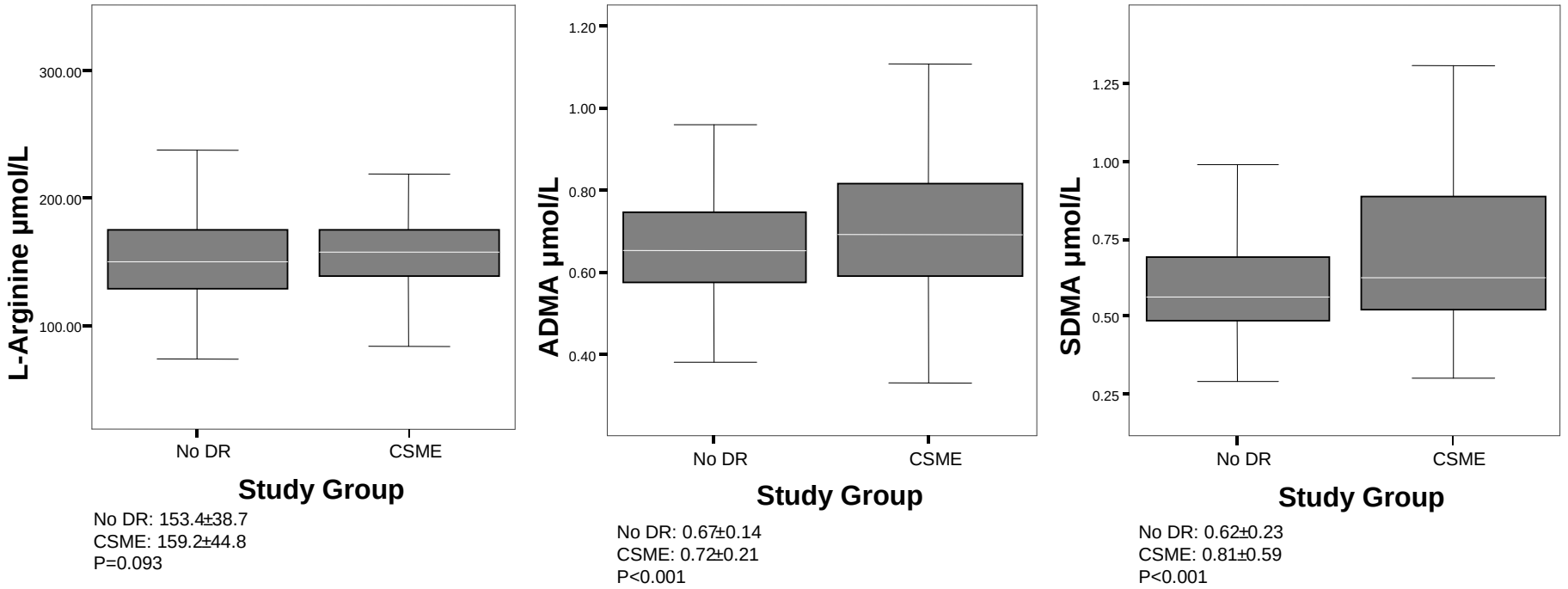


Note: p values have been adjusted for disease type, disease duration, sex, age, hypertension, hypercholesterolemia, nephropathy and BMI.

In the PDR multivariate analyses, ADMA ($p<0.001$) and SDMA ($p<0.001$) serum concentrations of those with T1DM diabetes showed a strong association, and L-arginine a borderline association ($p=0.04$, Figure 8.3). In the T2DM sub-analysis, blinding DR and PDR remained associated with significantly elevated ADMA and SDMA ($p=0.013$ and $p<0.001$ respectively) and ($p=0.014$ and $p<0.001$ respectively). L-arginine also remained associated with blinding DR in T2DM ($p=0.007$, Table 8.2).

Disease duration, age, hypertension, nephropathy and BMI were significantly associated with CSME. In the multivariate analyses of both types of diabetes combined, elevated ADMA ($p<0.001$) and SDMA ($p<0.001$) were significantly associated with presence of CSME (Figure 8.4) after adjusting for associated covariates. However, only SDMA showed a significant elevation in the T1DM diabetes cohort ($p<0.001$). No association was found between CSME and levels of L-arginine, ADMA and SDMA in the T2DM cohort (Table 8.2).

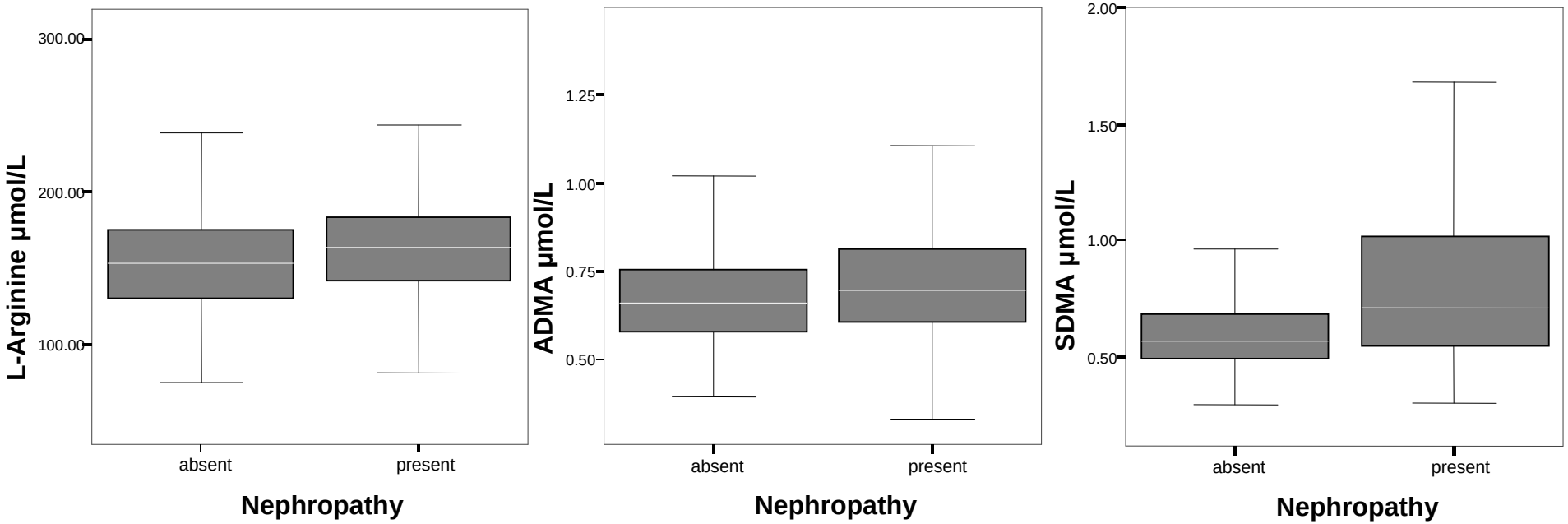
Figure 8.4: Boxplots of untransformed concentrations of L-arginine, ADMA and SDMA (μmol/L) in all subjects with no DR (n=329) and CSME (n=107) are shown, regardless of type of diabetes. Data are shown as the 25, 50 and 75 percentiles (represented by grey boxes), range (shown as whiskers, outliers have been removed) and the median (white horizontal line). Details of the mean, standard deviation and adjusted p values for each analyte are provided under its corresponding boxplot.



Note: p values have been adjusted for disease type, disease duration, age, hypertension, nephropathy and BMI.

Age, disease duration, hypertension, BMI, hypercholesterolemia, smoking and DR were found to be significantly correlated with nephropathy ($p<0.05$). Nephropathy was significantly associated with ADMA ($p<0.001$), SDMA ($p<0.001$) and L-arginine ($p=0.001$) in the multivariate analyses after adjustment for associated covariates (Figure 8.5). All three analytes were significantly associated with nephropathy in T1DM diabetes (ADMA $p<0.001$ and SDMA $p<0.001$, L-arginine $p=0.034$), however only ADMA ($p=0.013$) and SDMA ($p<0.001$) were associated with nephropathy in T2DM (Table 8.2).

Figure 8.5: Boxplots of untransformed concentrations of L-arginine, ADMA and SDMA (µmol/L) in all subjects with nephropathy absent (n=372) and nephropathy present (n=132) are shown, regardless of type of diabetes. Data are shown as the 25, 50 and 75 percentiles (represented by grey boxes), range (shown as whiskers, outliers have been removed) and the median (white horizontal line). Details of the mean, standard deviation and adjusted p values for each analyte are provided under its corresponding boxplot.



Nephropathy absent: 154.8 ±40.0
Nephropathy present: 167.1 ±42.3
P=0.001

Nephropathy absent: 0.67 ±0.13
Nephropathy present: 0.72 ±0.21
P<0.001

Nephropathy absent: 0.62 ±0.21
Nephropathy present: 0.90 ±0.62
P<0.001

Note: p values have been adjusted for disease type, disease duration, age, hypertension, and hypercholesterolemia.

In order to investigate whether true differences of ADMA, SDMA and L-arginine existed between nephropathy and DR, we excluded participants with co-existing nephropathy and DR for the following analysis. The mean levels of all three analytes in participants with blinding DR (n=110 with nephropathy subjects excluded) were compared to the mean levels in nephropathy (n=68 with DR subjects excluded) and no significant differences were found between the two microvascular complications (ADMA p=0.591, SDMA p=0.998 and L-arginine p=0.591).

Discussion

The NO pathway is a key player in the pathogenesis of microvascular damage and DR [280]. This study sought to determine whether in a large Australian cohort of subjects with diabetes, elevated serum levels of ADMA, SDMA or L-arginine were associated with blinding DR, PDR or CSME. The findings confirm that ADMA, SDMA and L-arginine are all significantly elevated in patients with blinding DR and PDR irrespective of underlying diabetes type. This is potentially consistent with elevated levels being a consequence of diabetes-associated hyperglycemia. Overall, there was a more significant relationship between elevated levels of L-arginine, ADMA and SDMA with DR in patients with T1DM diabetes. Of the three analytes, SDMA showed the most significant association with DR. To our knowledge, this is the first study to report an association between elevated levels of ADMA and SDMA and CSME.

Four other studies have investigated serum ADMA levels in DR [291, 300-302]. In keeping with our findings, three of these studies (two with T2DM participants only, and one

with a combined diabetes cohort) have reported elevation of ADMA in patients with DR[291, 300, 301]. Only one of these studies assessed the association of both SDMA and L-arginine with DR in T2DM[301]. In keeping with our results, Malecki *et al* found elevated SDMA in 182 DR subjects with T2DM. However, in contrast to our results, they found no association between levels of L-arginine and PDR, and overall results showed ADMA to be more strongly associated than SDMA with DR. The largest study was undertaken by Tarnow *et al*, where 600 subjects with T1DM diabetes were assessed for DR and nephropathy. In contrast to our study, ADMA levels were not found to be significantly increased in any form of DR or nephropathy. Our study design was deliberately enriched with subjects with potentially blinding DR; severe NPDR, PDR and CSME and the differences in DR phenotype and consequently study power may explain the variation in the above findings.

The relationship between nephropathy and increased risk for DR has been previously reported, especially in association with severe DR[71, 303]. The effect of nephropathy on DR could potentially be mediated by elevated dimethylarginines as they are renally cleared and ADMA and SDMA are elevated in conditions of reduced renal clearance[294, 302, 304-306]. In our cohort, we observed a significant association of all three analytes with nephropathy. Serum levels of ADMA in patients with renal disease, especially end-stage, have also been found to be markedly lower than those of SDMA[305, 307, 308]. Similarly, this trend of higher levels of SDMA compared to ADMA was observed amongst our participants with nephropathy, as well as with retinopathy. A possibility is that decreased renal clearance of these analytes may lead to elevated serum concentrations, directly impacting the development of DR. A prospective study found elevated ADMA and SDMA to be predictive of and risk

factors for the progression of renal disease[305]. It is also a possibility that elevated dimethylarginines may be predictive of and risk factors for the progression of DR.

Major differences in the metabolism of ADMA and SDMA also exist. ADMA is predominantly metabolised by DDAH enzymes[285], whereas SDMA is almost completely renally cleared[293]. A variety of factors are believed to be suppress *DDAH* expression and activity and subsequently increase serum ADMA levels. These factors include hyperglycemia[290], oxidative stress and inflammation[309, 310]. Genetic influences may also play a role in serum ADMA levels, with variation in the promoter region of *DDAH2* found to influence its expression[311]. Genetic determination and environmental influences on these analytes may therefore also in part explain differences in susceptibility to DR.

In conclusion, this study found elevated ADMA, SDMA and L-arginine serum levels to be associated with blinding DR. Further prospective and functional studies investigating the pathological significance of elevated serum ADMA, SDMA and L-arginine in development of DR and its relationship to nephropathy are still required. Future studies investigating the metabolic pathways of ADMA, SDMA and L-arginine, including functional studies of their metabolising enzymes and their genetic influences are also required to increase understanding of the complicated NO pathway and microvascular damage.

CHAPTER 9

A genome wide association study identifies novel susceptibility genes for blinding diabetic retinopathy.

Introduction

DR has a complex and multifactorial pathogenesis and no individual gene thus far has been directly implicated in the pathogenesis of this potentially sight threatening complication in a consistent and replicated manner. A large proportion of investigators' time is dedicated to hypothesis driven exploration of candidate genes coding for relevant molecules in biochemical pathways already established to be of some importance in the pathogenesis of DR, many of which result in negative findings.

Genome-wide studies are not hypothesis driven and as such have increased our understanding of the genetic patterns of complex traits, including the genetic susceptibility to T1DM and T2DM. When followed by individual genotyping, equimolar DNA pooling is a cost effective and rapid alternative phase 1 method to individual genome-wide genotyping for identification of disease susceptibility genes[312]. Novel disease susceptibility genes for complex diseases including Alzheimer's disease[313] and melanoma[314] have been identified in genome-wide studies utilising the DNA pooling technique. This technique requires pool construction to be performed in an accurate manner with the utilisation of quality control steps which take into consideration pooling allele frequency and appropriate SNP array selection. When these factors have been taken into consideration, the

DNA pooling method has been shown to be a valid technique for identifying genetic susceptibility loci for complex diseases when compared to individual genotyping[312, 315, 316].

Genome-wide studies of DR susceptibility have to date been limited to microsatellite linkage studies. One such study of 105 affected sib-pairs in Pima Indians reported suggestive linkage on chromosomes 3 and 9, overlapping with linkage peaks for diabetic nephropathy[250]. A recent expansion of this study utilising 725 sib-pairs and a quantitative approach to retinopathy grading failed to identify these two regions, but did find significant evidence of linkage to 1p36[251]. The gene within this linkage region is yet to be identified.

We aimed to identify novel risk susceptibility genes in the development of DR by undertaking a genome-wide association study (GWAS) and at this time to our knowledge, this is the first GWAS to be undertaken for DR.

Methods

Sample selection

Six hundred and fifty Caucasian participants were included in the equimolar DNA pooling analysis, consisting of 215 participants with T1DM and 435 with T2DM (Table 9.1). The development of minimal NPDR consists of micro-aneurysms only and as the first stage of DR, is not sight threatening. Large longitudinal prospective studies have shown the majority of participants with T1DM[317] and up to 41% with T2DM to develop at least minimal NPDR during the course of their illness[67]. Therefore in order to maximise statistical power, all Caucasian participants with

either no DR or minimal NPDR were classified as controls. Criteria for inclusion as blinding DR cases in this analysis required the diagnosis of severe NPDR, PDR or macular edema. 1102 participants were included in the individual verification genotyping, including the original DNA pooling analyses cohort, additional participants with intermediate DR phenotypes and any participants recruited after the completion of the DNA pools. The total cohort thus consisted of 296 with T1DM, and 806 with T2DM and details of DR phenotype are provided in Table 9.1.

Table 9.1: Participants included in DNA pools and those individually genotyped by type of diabetes and DR subtype.

	Pooled samples			All participants		
DR subtype	T1DM	T2DM	Total	T1DM	T2DM	Total
No DR	94	227	321	112	356	468
NPDR	4*	30*	34*	114	161	275
PDR	60	98	158	79	166	245
CSME	31	106	137	39	186	225
Controls	137	268	405	159	410	569
Blinding	77	168	245	102	296	398
Total	214	436	650	296	806	1102

* = participants have severe NPDR only

Blinding DR consists of severe NPDR, PDR and CSME participants

Power calculations were performed using Purcell and Sham's genetic power calculator[318]. For participants with T1DM in the pooling analysis, this study had a power of 0.74 to detect an allele with a population frequency of 50%, which confers a relative risk of 3.5 in the heterozygous state at the $5x10^{-08}$ level. For participants with T2DM in the pooling analysis, this study had a power of 0.86 to detect an allele with a population frequency of 20%, conferring a relative risk of 2.5 in the heterozygous state at the $5x10^{-08}$ level.

Generation of DNA pools and analysis of array data

The concentration of stock DNA samples was estimated using Smartspec Plus spectrophotometer (Bio-Rad, Sydney, Australia,). Samples were then diluted into the range 100-300 ng/µL in a 96 well plate. A 1/200 dilution of each sample was then quantitated in duplicate using the PicoGreen reagent (Invitrogen) by comparison of fluorescence emission at 520 nm to a standard curve and the concentration of the sample in the 96 well plate calculated. All fluorescence readings were taken using the Fluoroskan (Thermo Corporation, Waltham, USA). The minimum volume of DNA pipetted at any step was 5 µL. Each sample was then diluted to 75 ng/µL based on the first reading, and the quantitation repeated. Samples measuring 50-90 ng/µL were then included in the pool. Samples outside this range were re-diluted appropriately and re-quantitated. To assemble the pool, the amount of DNA in 5 µL of the most concentrated sample was calculated (e.g. for a sample at 90 ng/µL = 450ng). This amount of DNA was then added to the pool for all samples in that pool. All quantitation and pool construction was undertaken by Ms Kate Laurie.

Case and control pools for each type of DM were constructed, making a total of 4 pools (one pool of blinding cases and one pool of controls for each type of diabetes). The T1DM pools consisted of 77 blinding cases and 137 controls and the T2DM pools consisted of 168 blinding cases and 268 controls. Each pool was hybridized to multiple Illumina 1M arrays in the laboratory of Prof Grant Montgomery at Queensland Institute of Medical Research (QIMR), according to the manufacturer's protocols. The number of arrays per pool was dependent on the number of samples in the pool. T1DM and T2DM cases were hybridized to 2 and 3 arrays respectively.

T1DM and T2DM controls were hybridized to 3 and 4 arrays respectively.
Each array slide has the capacity to genotype two independent samples and was allocated to receive a case and a control pool. All analysis of array data was designed and conducted by Dr Stuart Macgregor at QIMR. Briefly, bead-level intensity data were analysed and SNPs with beadscore values ≤50 were excluded. Copy number variants and SNPs from the sex chromosomes were not analyzed. SNPs were also removed in the initial quality control if they had a minor allele frequency <1% in the HapMap CEU reference samples. Beadscores required calibration because green beadscores tended to be larger than red beadscores. Illumina 1M arrays had 20 stripes per array, each with 50,000 SNPs. Within each stripe, half of the SNPs were from the Illumina ‘‘TOP’’ strand (A/C and A/G SNPs) and half were from the Illumina ‘‘BOTTOM’’ strand (T/C and T/G SNPs). The pooling allele frequency (PAF) was computed as the corrected red intensity divided by the total (corrected red plus green) intensity. Normalization was performed within stripe, separately for each strand by rescaling the red beadscore to make the mean PAF value = 0.5 (for all SNPs on that stripe/strand). A very small number of SNPs had <5 PAF estimates available and were dropped. SNPs were removed from the analysis if they had a -log10(P) quality control value >6.

In the T1DM pools 83.7% of SNPs and in the T2DM pools 81.4% of SNPs passed quality control and were taken forward. After correction for green/red ratio variability, a linear model based approach was used. In the linear model the response variable is the set of PAF estimates for each SNP. The predictor variable is case/control status. The linear model was used to estimate the pooling error across all

SNPs [312, 319]. Files containing SNPs ranked by p-value were the final outputs for analysis.

Meta-analysis of T1DM and T2DM pools

A meta-analysis of all SNPs passing quality control checks for T1DM and T2DM was undertaken. A fixed effects model approach was utilised. Binomial sampling variance based on the difference between blinding case and control allele frequencies was calculated and corrected for pooling error. As multiple independent pools were combined together, the relative sizes of the pools and the relative amounts of pooling error were taken into account[319]. As for the array analysis described above, the meta-analysis of the T1DM and T2DM pools were designed and undertaken by Dr Stuart Macgregor at the QIMR.

SNP selection and individual genotyping

The top 10,000 SNPs for each comparison were supplied by Dr Macgregor for data mining. Literature reviews of genes for the ten most significantly associated SNPs by type of diabetes and for genes containing clusters of 3 or more SNPs of significance $p<0.0001$ within 100,000 base pairs were undertaken. Literature reviews were undertaken in order to prioritise SNP selection for individual genotyping. A SNP was given a priority of 1 if it met any of the following criteria:

- Had $p<1x10^{-08}$ in any of the analyses.

- Was a coding variant in a gene found to be highly relevant to DR pathogenesis on literature review and was ranked in the top 100 SNPs for any type of DM.
- Was in a gene that was found to be significantly associated in the other DM groups at $p<0.0001$.
- Was ranked in the top 10 SNPs in either type of DM or the meta-analysis.
- If the SNP was part of a cluster of significantly associated SNPs.

A priority of 2 was given to a SNP if it did not meet priority 1 inclusion criteria or it was a lesser ranked SNP in a gene containing a priority 1 SNP. One hundred and three SNPs were ranked this way and submitted to the Australian Genome Research Facility for assay design. Three multiplexes were designed with a theoretical maximum of 36 SNPs per multiplex. All priority 1 SNPs were assigned to a multiplex, then priority 2 SNPs until no more suitable SNPs could be found. The ability for a SNP to be included in a multiplex is dependent on the base change of the SNP and the flanking sequence. In total, 96 SNPs were included in the assay design and were genotyped in 296 participants with T1DM, and 806 with T2DM using the Sequenom iPLEX GOLD chemistry on an Autoflex Mass Spectrometer at the Australia Genome Research Facility, Brisbane, Australia. SNP associations were considered as validated if $p<0.001$ in the allelic model of the univariate analyses in the original pooled cohort or full participant cohort (including those in the original pooled samples and all subsequent participants recruited).

Statistical analyses

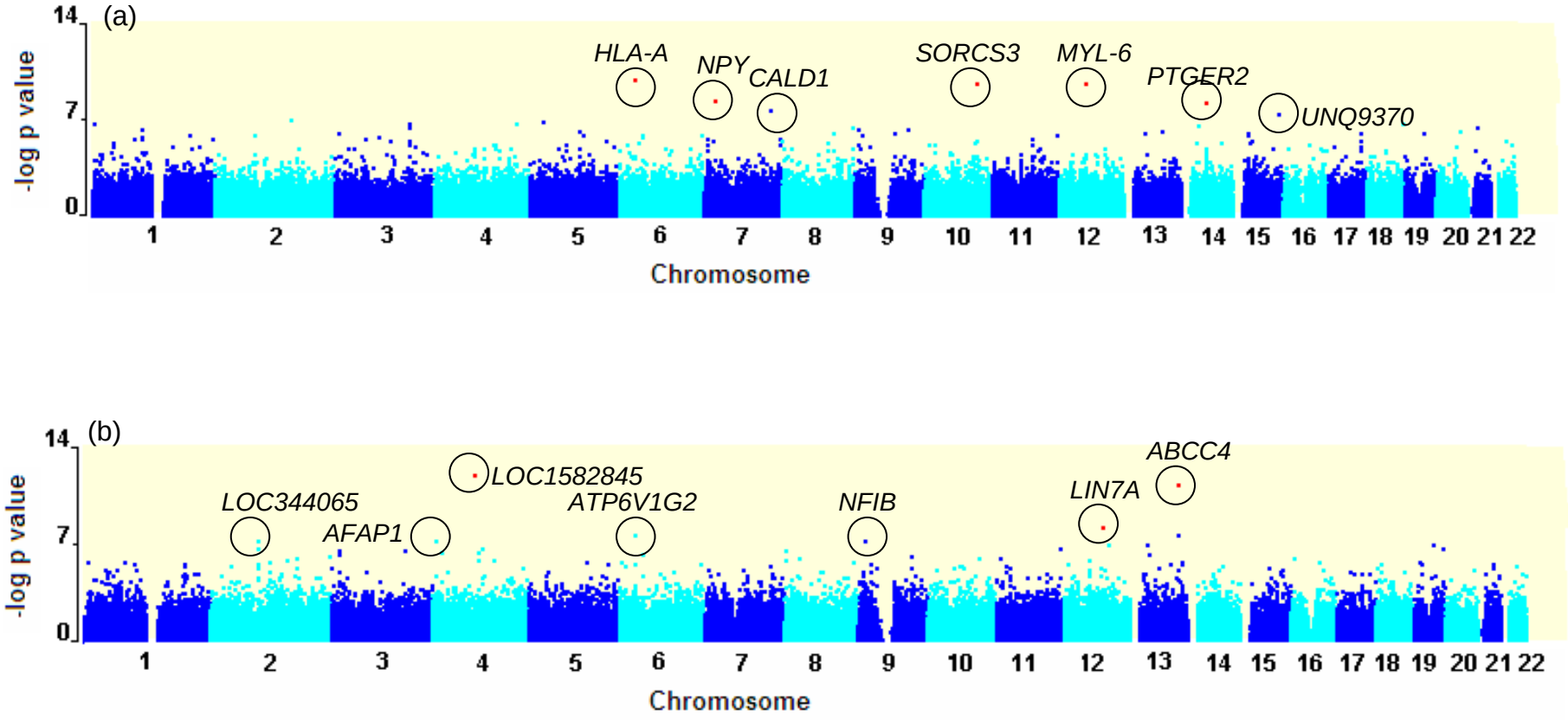
All analyses of individual genotyping were conducted in PLINK(v1.06)[213]. Fisher exact tests were undertaken for the univariate analyses. Logistic regression was undertaken for multivariate analysis and clinical covariates associated with DR were subsequently controlled for.

Results

DNA pooling

In total 24 SNPs reached genome-wide significance ($p<1.0x10^{-7}$) for DR, consisting of seven SNPs for each type of DM and 10 SNPs in the combined DM meta-analysis (Figure 9.1).

Figure 9.1 – Manhattan plots for the top 10,000 SNPs for T1DM (a) and T2DM (b). Each SNP is represented as a dot at its chromosomal location and by its –log p value. Genome-wide significant SNPs are circled and genes in which they are situated or closest to are provided.



Fifty two genes met inclusion criteria for further investigation by literature review. A summary of these genes are presented in Figure 9.2. In total, 16 genes were further investigated for DR in T1DM, 24 genes for T2DM and 18 genes for combined DM. After literature review, seven of the 52 genes were reported to play a role in angiogenesis. Eighteen genes were found to play a role in various cancers and tumor invasion, of which six were shown to independently play a role in angiogenesis and another four in cell adhesion and migration. Eighteen of the 52 genes were found to have unknown functions.

Figure 9.2 – Selected genes from DNA pool that met inclusion criteria for further investigation. Genes are shown by type of diabetes.

T1DM

Gene Symbol	Chromosome	Number of SNPs with p<0.0001	Most significant SNP p value
HLA-A	6	1	1.52E-10
SORCS3	10	5	2.81E-10
MYL6	12	1	3.05E-10
NPY	7	1	4.93E-09
PTGER2	14	6	7.24E-09
CALD1	7	1	2.79E-08
UNQ9370	15	1	4.90E-08
FMNL2	2	1	1.19E-07
LOC729862	5	1	1.98E-07
AJAP1	1	1	2.28E-07
FLJ30375	3	6	3.06E-07
COL22A1	8	3	5.32E-07
SLCO4C1	5	3	1.10E-06
hCG_1644301	17	4	1.44E-06
DLG2	11	3	3.58E-06
LOC344741	3	1	5.13E-06

= associated with angiogenesis

= associated with cell adhesion and migration

= has unknown function

= associated with various cancers

T2DM

Gene Symbol	Chromosome	Number of SNPs with p<0.0001	Most significant SNP p value
LOC152845	4	1	1.11E-12
ABCC4	13	4	6.68E-12
LIN7A	12	1	7.68E-09
ATP6V1G2	6	1	2.52E-08
NFIB	9	2	7.02E-08
AFAP1	4	1	7.98E-08
LOC344065	2	2	7.99E-08
BTG1	12	1	1.24E-07
ZNF302	19	1	1.29E-07
MAL	2	1	2.48E-07
ADH7	4	3	2.58E-07
FGD5	3	4	3.69E-07
TMEM38B	9	3	1.09E-06
KRT76	12	3	1.92E-06
KRT3	12	1	1.94E-06
FER1L6	2	1	3.17E-06
ATP6V1G3	1	4	5.31E-06
LOC729560	11	4	6.19E-06
TIAM1	21	3	6.79E-06
ZNF2	2	1	7.25E-06
CHCHD3	7	3	1.81E-05
tcag7.1130	7	3	2.32E-05
PROM2	2	1	5.08E-05
LOC729923	4	3	5.76E-05

Combined DM

Gene Symbol	Chromosome	Number of SNPs with p<0.0001	Most significant SNP p value
LOC152845	4	1	4.30E-11
UNQ9370	15	1	1.09E-09
MTMR7	8	1	1.58E-08
NBEA	13	3	1.73E-08
LENG1	19	1	2.69E-08
ADH7	4	3	2.85E-08
CHCHD3	7	11	3.68E-08
LOC283551	14	1	4.56E-08
SLU7	5	1	5.66E-08
LOC646982	13	1	6.36E-08
FLJ30375	3	3	3.78E-07
FGD5	3	3	2.20E-06
SKP2	5	3	6.86E-06
SPRY4	5	3	8.27E-06
LOC729923	4	3	9.45E-06
LOC729802	9	3	2.20E-05
TTC13	1	1	3.65E-05
FAM89A	1	2	6.78E-05

Exactly 318 SNPs were common to the top 10,000 hits in T1DM and T2DM, with *CHCHD3* having the most number of SNPs shared for any gene between the two types of DM. SNPs in the regions of genes previously studied in five or more reports as in the literature meta-analysis (*ACE, AKR1B1, ACE, NOS3* and *VEGFA*) and other candidate genes studied in this thesis (*VEGFA, CA1*, *EPO* and *AKR1B1*) were investigated in the top 10,000 hits in all types of DM. In the T1DM data, SNPs rs6900017 and rs3025035 near *VEGFA* (p=0.0153 and p=0.0042 respectively) were nominally associated as was rs7809570 near *AKR1B1* in T2DM (p=0.0068). In comparison to our candidate gene studies, an association of rs3025035 of *VEGFA* with blinding DR in combined DM (p=0.026) and a borderline association in T2DM (p=0.046) was found in our *VEGFA* candidate gene study (chapter 4).

In the DNA pooling analyses, 44 SNPs of 16 genes met the above inclusion criteria for further evaluation in the T1DM pool, 61 SNPs of 26 genes in the T2DM pool and 28 SNPs of 18 genes for the combined DM meta-analysis (Table 9.2).

Table 9.2 – Selected SNPs from top 10,000 hits of T1DM and T2DM DNA pools and meta-analysis for further investigation and individual genotyping, shown in order of chromosomal location.

Diabetes Type	SNP in DNA pool	Equivalent SNP for individual genotyping	Chromosome	Coordinate (bp)	Gene Symbol	Location	P value	Priority	Individual genotyping status
1	rs10915601	NA	1	4,757,006	AJAP1	flanking_3UTR	0.0023	2	Not typed
1	rs2345873	NA	2	152,956,650	FMNL2	intron	5.96E-04	2	Typed
1	rs2346205	NA	2	153,066,251	FMNL2	intron	1.19E-07	1	Typed
1	rs11922072	NA	3	149,019,164	LOC344741	flanking_3UTR	5.13E-06	1	Typed
1	rs28735811	NA	3	148,627,008	FLJ30375	flanking_3UTR	5.49E-07	2	Not typed
1	rs9836676	NA	3	148,665,397	FLJ30375	flanking_3UTR	8.40E-06	2	Typed
1	rs9875987	NA	3	148,643,966	FLJ30375	flanking_3UTR	6.32E-05	2	Typed
1	rs9876193	NA	3	148,683,182	FLJ30375	flanking_3UTR	3.06E-07	1	Typed
1	rs11746764	NA	5	29,520,848	LOC729862	flanking_3UTR	2.00E-04	2	Typed
1	rs17256662	NA	5	101,026,961	SLCO4C1	flanking_3UTR	2.00E-05	2	Not typed
1	rs6885006	NA	5	101,056,643	SLCO4C1	flanking_3UTR	7.22E-05	2	Not typed
1	rs727176	NA	5	101,067,734	SLCO4C1	flanking_3UTR	1.10E-06	1	Typed
1	rs9885405	NA	5	29,556,407	LOC729862	flanking_3UTR	1.98E-07	1	Typed
1	rs2523940	NA	6	30,033,796	HLA-A	flanking_3UTR	0.0014	2	Not typed
1	rs9260575	rs397099	6	30,030,278	HLA-A	flanking_3UTR	1.52E-10	1	Typed
1	rs16166	NA	7	24,268,745	NPY	flanking_5UTR	0.0088	2	Not typed
1	rs2245657	NA	7	24,346,910	NPY	flanking_3UTR	4.93E-09	1	Typed
1	rs4732067	NA	7	134,234,914	CALD1	intron	0.0078	2	Typed
1	rs7777356	NA	7	134,170,505	CALD1	intron	2.79E-08	1	Typed
1	rs11166859	NA	8	140,014,175	COL22A1	flanking_5UTR	1.57E-05	2	Typed

The ‘equivalent SNP for individual genotyping column’ shows SNPs for individual genotyping that have replaced priority 1 SNPs from the DNA pooling analysis that were unable to have appropriate assays designed for the Sequenom platform. P values provided are from the DNA pooling analysis.

Table 9.2 Continued

Diabetes Type	SNP in DNA pool	Equivalent SNP for individual genotyping	Chromosome	Coordinate (bp)	Gene Symbol	Location	P value	Priority	Individual genotyping status
1	rs6984075	rs6992859 and rs6997968	8	140,008,288	COL22A1	flanking_5UTR	5.32E-07	1	Typed
1	rs7017816	NA	8	140,008,194	COL22A1	flanking_5UTR	8.48E-05	2	Typed
1	rs10884162	NA	10	107,259,887	SORCS3	flanking_3UTR	5.79E-05	2	Typed
1	rs11192557	NA	10	107,442,673	SORCS3	flanking_3UTR	2.81E-10	1	Typed
1	rs11819719	NA	10	107,470,509	SORCS3	flanking_3UTR	5.48E-05	2	Not typed
1	rs12259354	NA	10	107,207,371	SORCS3	flanking_3UTR	4.15E-06	2	Not typed
1	rs12264818	NA	10	107,184,914	SORCS3	flanking_3UTR	9.70E-05	2	Typed
1	rs10501541	NA	11	82,867,048	DLG2	intron	5.52E-05	2	Typed
1	rs12271855	NA	11	82,919,096	DLG2	intron	8.13E-06	2	Not typed
1	rs7946060	NA	11	83,111,615	DLG2	intron	3.58E-06	1	Typed
1	GA029022	rs61938990	12	54,840,682	MYL6	coding	3.05E-10	1	Typed
1	rs12431401	NA	14	51,852,891	PTGER2	intron	7.73E-05	1	Typed
1	rs1495785	NA	14	51,863,517	PTGER2	intron	3.01E-05	1	Typed
1	rs17197	NA	14	51,864,131	PTGER2	3UTR	1.51E-05	2	Typed
1	rs1992139	NA	14	51,876,366	PTGER2	flanking_3UTR	7.24E-09	1	Typed
1	rs34337770	NA	14	51,866,690	PTGER2	flanking_3UTR	6.18E-05	2	Not typed
1	rs8008292	NA	14	51,838,314	PTGER2	flanking_5UTR	5.28E-05	2	Typed
1	rs4777642	NA	15	91,628,980	UNQ9370	flanking_3UTR	0.0027	2	Typed
1	rs17766100	NA	17	66,823,722	hCG_1644301	flanking_5UTR	4.47E-06	2	Not typed
1	rs2024070	NA	17	66,931,526	hCG_1644301	flanking_5UTR	1.24E-05	2	Not typed
1	rs2159042	NA	17	66,920,924	hCG_1644301	flanking_5UTR	2.29E-05	2	Typed
1	rs3922318	NA	17	66,947,636	hCG_1644301	flanking_5UTR	1.44E-06	1	Not typed

The ‘equivalent SNP for individual genotyping column’ shows SNPs for individual genotyping that have replaced priority 1 SNPs from the DNA pooling analysis that were unable to have appropriate assays designed for the Sequenom platform. P values provided are from the DNA pooling analysis.

Table 9.2 Continued

Diabetes Type	SNP in DNA pool	Equivalent SNP for individual genotyping	Chromosome	Coordinate (bp)	Gene Symbol	Location	P value	Priority	Individual genotyping status
2	rs10494777	NA	1	196,756,080	ATP6V1G3	flanking_3UTR	7.02E-05	2	Not typed
2	rs10922438	NA	1	196,735,785	ATP6V1G3	flanking_3UTR	5.31E-06	1	Typed
2	rs12725073	NA	1	196,753,425	ATP6V1G3	flanking_3UTR	1.75E-05	2	Typed
2	rs4915292	NA	1	196,727,687	ATP6V1G3	flanking_3UTR	7.06E-05	2	Not typed
2	rs13007001	NA	2	95,249,471	LOC344065	flanking_3UTR	3.51E-05	2	Not typed
2	rs13007001	NA	2	95,249,471	LOC344065	flanking_3UTR	3.51E-05	2	Not typed
2	rs13033750	NA	2	95,242,989	LOC344065	intron	7.99E-08	1	Typed
2	rs13033750	NA	2	95,242,989	LOC344065	intron	7.99E-08	1	Not typed
2	rs35034822	NA	2	95,308,359	PROM2	intron	5.08E-05	2	Typed
2	rs35391246	NA	2	95,222,046	ZNF2	flanking_3UTR	7.25E-06	1	Not typed
2	rs1687291	NA	3	14,887,406	FGD5	intron	7.24E-05	2	Not typed
2	rs2729696	NA	3	14,890,536	FGD5	intron	7.29E-07	2	Not typed
2	rs10516189	NA	4	7,834,186	AFAP1	intron	0.0078	2	Not typed
2	rs17034315	NA	4	157,405,364	LOC729923	flanking_3UTR	9.07E-05	2	Not typed
2	rs4440278	NA	4	157,390,528	LOC729923	flanking_3UTR	5.76E-05	2	Not typed
2	rs4690859	NA	4	157,416,803	LOC729923	flanking_3UTR	5.86E-05	2	Typed
2	rs757242	NA	4	7,820,216	AFAP1	intron	7.98E-08	1	Typed
2	rs2071593	rs3219184 and rs2239709	6	31,620,778	ATP6V1G2	3UTR	2.52E-08	1	Typed
2	rs2463484	NA	7	84,893,602	tcag7.1130	intron	5.25E-05	2	Typed
2	rs2498552	NA	7	84,918,179	tcag7.1130	intron	2.32E-05	2	Not typed
2	rs2498556	NA	7	84,930,126	tcag7.1130	intron	5.46E-05	2	Not typed
2	rs10759135	NA	9	107,561,514	TMEM38B	intron	6.79E-06	2	Typed
2	rs10978240	NA	9	107,575,093	TMEM38B	intron	1.09E-06	2	Not typed

The ‘equivalent SNP for individual genotyping column’ shows SNPs for individual genotyping that have replaced priority 1 SNPs from the DNA pooling analysis that were unable to have appropriate assays designed for the Sequenom platform. P values provided are from the DNA pooling analysis.

Table 9.2 Continued

Diabetes Type	SNP in DNA pool	Equivalent SNP for individual genotyping	Chromosome	Coordinate (bp)	Gene Symbol	Location	P value	Priority	Individual genotyping status
2	rs1322987	NA	9	14,234,753	NFIB	intron	8.04E-05	2	Not typed
2	rs1323351	NA	9	14,432,265	NFIB	flanking_5UTR	7.02E-08	1	Typed
2	rs7872125	NA	9	107,635,863	TMEM38B	flanking_3UTR	8.33E-05	2	Typed
2	rs1157171	NA	10	106,306,466	SORCS3	flanking_5UTR	0.0057	1	Typed
2	rs10835549	NA	11	29,603,523	LOC729560	flanking_5UTR	4.34E-05	2	Typed
2	rs10835550	NA	11	29,610,645	LOC729560	flanking_5UTR	2.57E-05	2	Typed
2	rs326765	NA	11	29,562,842	LOC729560	intron	4.42E-05	2	Not typed
2	rs986705	NA	11	29,608,424	LOC729560	flanking_5UTR	6.19E-06	2	Not typed
2	rs10876339	NA	12	51,448,035	KRT76	flanking_3UTR	1.92E-06	2	Not typed
2	rs11830274	NA	12	90,597,139	BTG1	flanking_3UTR	1.92E-04	3	Typed
2	rs11832716	NA	12	90,597,192	BTG1	flanking_3UTR	1.24E-07	1	Not typed
2	rs1402351	NA	12	79,875,989	LIN7A	flanking_5UTR	0.0041	2	Not typed
2	rs1402589	NA	12	51,446,904	KRT76	flanking_3UTR	2.56E-05	2	Not typed
2	rs17118412	NA	12	51,407,777	LOC643878	flanking_3UTR	1.11E-05	2	Typed
2	rs17738862	NA	12	51,469,295	KRT3	flanking_3UTR	1.94E-06	2	Not typed
2	rs2280479	NA	12	51,448,905	KRT76	coding	7.92E-06	2	Typed
2	rs7312724	NA	12	79,911,181	LIN7A	flanking_5UTR	7.68E-09	1	Typed
2	rs4148435	NA	13	94,697,717	ABCC4	intron	6.68E-12	1	Typed
2	rs4148445	NA	13	94,695,360	ABCC4	intron	9.55E-06	2	Typed
2	rs4148448	NA	13	94,695,191	ABCC4	intron	4.56E-05	2	Not typed
2	rs6492772	NA	13	94,694,322	ABCC4	intron	2.60E-08	1	Not typed
2	rs17831718	NA	14	51,869,786	PTGER2	flanking_3UTR	0.0014	1	Typed
2	rs6510444	NA	19	39,891,021	ZNF302	flanking_3UTR	1.29E-07	1	Typed
2	rs2070415	NA	21	31,519,716	TIAM1	intron	3.62E-06	2	Not typed

The ‘equivalent SNP for individual genotyping column’ shows SNPs for individual genotyping that have replaced priority 1 SNPs from the DNA pooling analysis that were unable to have appropriate assays designed for the Sequenom platform. P values provided are from the DNA pooling analysis.

Table 9.2 Continued

Diabetes Type	SNP in DNA pool	Equivalent SNP for individual genotyping	Chromosome	Coordinate (bp)	Gene Symbol	Location	P value	Priority	Individual genotyping status
2	rs2262256	NA	21	31,487,663	TIAM1	intron	6.79E-06	2	Not typed
2	rs2833335	NA	21	31,497,276	TIAM1	intron	9.08E-05	2	Typed
1 and 2	rs12410445	NA	1	4,767,710	AJAP1	flanking_3UTR	2.28E-07	1	Typed
1 and 2	rs5016773	NA	15	91,627,456	UNQ9370	flanking_3UTR	4.90E-08	1	Typed
2 and combined	rs293924	NA	3	14,895,109	FGD5	intron	3.69E-07	1	Typed
2 and combined	rs9822464	NA	3	14,891,052	FGD5	intron	1.25E-05	2	Typed
2 and combined	rs10516441	NA	4	100,526,190	ADH7	flanking_3UTR	8.81E-05	1	Typed
2 and combined	rs1847819	NA	4	85,114,015	LOC152845	flanking_5UTR	1.11E-12	1	Typed
2 and combined	rs3805331	NA	4	100,552,955	ADH7	3UTR	3.27E-05	1	Typed
2 and combined	rs4147553	NA	4	100,553,966	ADH7	intron	2.58E-07	1	Typed
2 and combined	rs10488210	NA	7	132,203,307	CHCHD3	intron	1.81E-05	1	Typed
2 and combined	rs6967574	NA	7	132,210,695	CHCHD3	intron	3.50E-05	1	Typed
2 and combined	rs7799350	NA	7	132,301,965	CHCHD3	intron	1.81E-05	1	Typed
Combined	rs12032403	NA	1	229,215,921	FAM89A	flanking_3UTR	6.78E-05	2	Typed
Combined	rs12133304	NA	1	229,217,862	FAM89A	flanking_3UTR	4.83E-05	2	Not typed
Combined	rs13374343	NA	1	229,179,417	TTC13	intron	3.65E-05	2	Typed
Combined	rs11960031	NA	5	36,207,094	SKP2	intron	6.88E-05	2	Typed
Combined	rs1198963	NA	5	141,703,049	SPRY4	flanking_5UTR	8.27E-06	2	Typed
Combined	rs1198966	NA	5	141,729,781	SPRY4	flanking_5UTR	8.45E-05	2	Not typed
Combined	rs155543	NA	5	36,196,964	SKP2	intron	7.24E-05	2	Typed
Combined	rs2961944	NA	5	159,768,236	SLU7	coding	5.66E-08	1	Typed
Combined	rs3804437	NA	5	36,216,179	SKP2	intron	6.86E-06	2	Typed
Combined	rs6872619	NA	5	141,666,251	SPRY4	flanking_3UTR	5.59E-05	2	Not typed
Combined	rs10236831	NA	7	132,133,920	CHCHD3	intron	1.00E-05	1	Typed

The ‘equivalent SNP for individual genotyping column’ shows SNPs for individual genotyping that have replaced priority 1 SNPs from the DNA pooling analysis that were unable to have appropriate assays designed for the Sequenom platform. P values provided are from the DNA pooling analysis.

Table 9.2 Continued

Diabetes Type	SNP in DNA pool	Equivalent SNP for individual genotyping	Chromosome	Coordinate (bp)	Gene Symbol	Location	P value	Priority	Individual genotyping status
Combined	rs11982852	NA	7	132,218,795	CHCHD3	intron	6.63E-06	1	Typed
Combined	rs4731905	NA	7	132,175,783	CHCHD3	intron	1.27E-06	1	Not typed
Combined	rs4731912	NA	7	132,180,770	CHCHD3	intron	2.50E-05	1	Typed
Combined	rs6467450	NA	7	132,252,679	CHCHD3	intron	4.45E-06	1	Typed
Combined	rs6951454	NA	7	132,295,443	CHCHD3	intron	5.15E-05	1	Typed
Combined	rs6956407	NA	7	132,327,897	CHCHD3	intron	7.54E-05	1	Typed
Combined	rs6962471	NA	7	132,267,333	CHCHD3	intron	8.67E-07	1	Typed
Combined	rs6981038	NA	8	17,319,080	MTMR7	flanking_5UTR	1.58E-08	1	Typed
Combined	rs1322163	NA	9	10,177,598	LOC729802	flanking_3UTR	2.20E-05	2	Typed
Combined	rs7862769	NA	9	9,895,178	LOC729802	flanking_3UTR	2.43E-05	2	Not typed
Combined	rs7874380	NA	9	9,885,814	LOC729802	flanking_3UTR	1.61E-05	2	Typed
Combined	rs11838873	NA	13	39,849,888	LOC646982	flanking_3UTR	6.36E-08	1	Typed
Combined	rs17758589	NA	13	34,853,999	NBEA	intron	7.63E-05	2	Typed
Combined	rs1853572	NA	13	34,421,614	NBEA	intron	7.13E-05	2	Not typed
Combined	rs9600357	NA	13	34,702,493	NBEA	intron	1.73E-08	1	Typed
Combined	rs17830933	NA	14	49,508,910	LOC283551	flanking_3UTR	4.56E-08	1	Not typed
Combined	rs35089861	NA	19	59,353,855	LENG1	coding	2.69E-08	1	Typed

The ‘equivalent SNP for individual genotyping column’ shows SNPs for individual genotyping that have replaced priority 1 SNPs from the DNA pooling analysis that were unable to have appropriate assays designed for the Sequenom platform. P values provided are from the DNA pooling analysis

After SNP selection from the DNA pooling analyses, eleven (rs2071593, rs6984075, rs9260575, GA029022, rs6492772, rs11832716, rs4731905, rs3922318, rs17830933, rs13033750, rs35391246) priority 1 SNPs (marked "not typed" in Table 9.2), although passing the design phase were not typed due to technical failures. Four of these SNPs (rs2071593, rs6984075, rs9260575, GA029022) were replaced with SNPs with r^2=1.0 within 30kb of the original SNP (rs3219184, rs2239709, rs6992859, rs6997968, rs397099 and rs61938990). A total of 87 SNPs were subsequently individually genotyped in the full cohort (Table 9.2)

Univariate analyses

Allelic analyses of each individually genotyped SNP were undertaken using a Fisher's exact test. This analysis was undertaken separately for participants in the original pooled samples (n=650) and in full participant cohort (n=1102). In total, 10 SNPs (of 6 genes) in T1DM, 15 SNPs (of 11 genes) in T2DM, and 14 SNPs (of 5 genes) in combined DM were considered validated ($p<0.001$) in the pooled samples (Table 9.3) and taken through to the next phase of analyses.

Table 9.3 – Individual genotyping results of DNA pool cohort for blinding DR. Results are shown by type of diabetes in which the original association in the DNA pool was identified. If p<0.001 in the individual genotyping column, SNPs are considered as validated and highlighted in bold.

			DNA Pool	Individual genotyping
Diabetes type	SNP	Gene	P value	P value
1	rs10501541	DLG2	5.5E-05	0.0045
1	rs10884162	SORCS3	5.8E-05	**0.0008**
1	rs11166859	COL22A1	1.6E-05	0.0023
1	rs11192557	SORCS3	2.8E-10	**2.00E-06**
1	rs11746764	LOC729862	0.0002	0.0126
1	rs11922072	LOC344741	5.1E-06	0.0129
1	rs12264818	SORCS3	9.7E-05	0.0013
1	rs12410445	AJAP1	2.3E-07	0.0348
1	rs12431401	PTGER2	7.7E-05	**0.0003**
1	rs1495785	PTGER2	3E-05	**0.0007**
1	rs17197	PTGER2	1.5E-05	**0.0007**
1	rs1992139	PTGER2	7.2E-09	**6.00E-06**
1	rs2159042	hCG_1644301	2.3E-05	0.0226
1	rs2245657	NPY	4.9E-09	**2.00E-05**
1	rs2345873	FMNL2	0.0006	0.0693
1	rs2346205	FMNL2	1.2E-07	0.0113
1	rs397099	HLA-A	1.5E-10	0.0054
1	rs4732067	CALD1	0.00777	0.003
1	rs4777642	UNQ9370	0.00275	0.0693
1	rs5016773	UNQ9370	4.9E-08	0.2205
1	rs61938990	MYL6	3.1E-10	0.0518
1	rs6992859	COL22A1	5.3E-07	0.0122
1	rs6997968	COL22A1	5.3E-07	0.0126
1	rs7017816	COL22A1	8.5E-05	0.0121
1	rs727176	SLCO4C1	1.1E-06	**0.0004**
1	rs7312724	LIN7A	7.7E-09	1
1	rs7777356	CALD1	2.8E-08	**0.0003**
1	rs7946060	DLG2	3.6E-06	**2.00E-05**
1	rs8008292	PTGER2	5.3E-05	0.0067
1	rs9836676	FLJ30375	8.4E-06	0.0019
1	rs9875987	FLJ30375	6.3E-05	0.003
1	rs9876193	FLJ30375	3.1E-07	0.0018
1	rs9885405	LOC729862	2E-07	0.004

Table 9.3 Continued

			DNA Pool	Individual genotyping
Diabetes type	SNP	Gene	P value	P value
2	rs10488210	CHCHD3	1.8E-05	**0.0002**
2	rs10516441	ADH7	8.8E-05	0.3679
2	rs10759135	TMEM38B	6.8E-06	0.0439
2	rs10835549	LOC729560	4.3E-05	**0.0004**
2	rs10835550	LOC729560	2.6E-05	**0.0003**
2	rs10922438	ATP6V1G3	5.3E-06	0.0402
2	rs1157171	SORCS3	0.00574	0.0391
2	rs11830274	BTG1	0.00019	**0.0004**
2	rs12410445	AJAP1	2.3E-07	0.2876
2	rs12725073	ATP6V1G3	1.8E-05	0.0694
2	rs13033750	LOC344065	8E-08	**8.00E-05**
2	rs1323351	NFIB	7E-08	0.1037
2	rs17118412	LOC643878	1.1E-05	**0.0004**
2	rs17831718	PTGER2	0.0014	0.0026
2	rs1847819	LOC152845	1.1E-12	0.0042
2	rs2239709	ATP6V1G2	2.5E-08	0.0077
2	rs2244187	TIAM1	9.2E-05	0.078
2	rs2280479	KRT76	7.9E-06	**4.00E-06**
2	rs2463484	tcag7.1130	5.3E-05	0.1596
2	rs2833335	TIAM1	9.1E-05	0.0574
2	rs293924	FGD5	3.7E-07	**0.0008**
2	rs3219184	ATP6V1G2	2.5E-08	0.0077
2	rs35034822	PROM2	5.1E-05	**0.0001**
2	rs3805331	ADH7	3.3E-05	0.0405
2	rs4147553	ADH7	2.6E-07	0.0163
2	rs4148435	ABCC4	6.7E-12	**0.0004**
2	rs4148445	ABCC4	9.6E-06	**0.0005**
2	rs4690859	LOC729923	5.9E-05	**0.0004**
2	rs5016773	UNQ9370	4.9E-08	0.167
2	rs6510444	ZNF302	1.3E-07	0.0802
2	rs6967574	CHCHD3	3.5E-05	**0.0006**
2	rs757242	AFAP1	8E-08	**6.00E-07**
2	rs7799350	CHCHD3	1.8E-05	**0.0004**
2	rs7872125	TMEM38B	8.3E-05	0.0398
2	rs9822464	FGD5	1.3E-05	0.0013

Table 9.3 continued

			DNA Pool	Individual genotyping
Diabetes type	SNP	Gene	P value	P value
combined	rs10236831	CHCHD3	1E-05	**0.0002**
combined	rs10488210	CHCHD3	1.8E-05	**1.00E-05**
combined	rs10516441	ADH7	8.8E-05	0.1030
combined	rs11838873	LOC646982	6.4E-08	0.0123
combined	rs11960031	SKP2	6.9E-05	0.1266
combined	rs11982852	CHCHD3	6.6E-06	**2.00E-05**
combined	rs1198963	SPRY4	8.3E-06	**5.00E-05**
combined	rs12032403	FAM89A	6.8E-05	**0.0006**
combined	rs1322163	LOC729802	2.2E-05	0.0051
combined	rs13374343	TTC13	3.6E-05	**0.0005**
combined	rs155543	SKP2	7.2E-05	0.0117
combined	rs17758589	NBEA	7.6E-05	0.0277
combined	rs1847819	LOC152845	1.1E-12	0.0041
combined	rs293924	FGD5	3.7E-07	0.0048
combined	rs2961944	SLU7	5.7E-08	0.0019
combined	rs35089861	LENG1	2.7E-08	0.0013
combined	rs3804437	SKP2	6.9E-06	0.0082
combined	rs3805331	ADH7	3.3E-05	0.0019
combined	rs4147553	ADH7	2.6E-07	0.0013
combined	rs4731912	CHCHD3	2.5E-05	**1.00E-05**
combined	rs5016773	UNQ9370	4.9E-08	0.0453
combined	rs6467450	CHCHD3	4.4E-06	**0.0001**
combined	rs6951454	CHCHD3	5.2E-05	**0.0004**
combined	rs6956407	CHCHD3	7.5E-05	**0.0002**
combined	rs6962471	CHCHD3	8.7E-07	**0.0001**
combined	rs6967574	CHCHD3	3.5E-05	**1.00E-05**
combined	rs6981038	MTMR7	1.6E-08	0.0087
combined	rs7799350	CHCHD3	1.8E-05	**1.00E-05**
combined	rs7874380	LOC729802	1.6E-05	**0.0003**
combined	rs9600357	NBEA	1.7E-08	0.0107
combined	rs9822464	FGD5	1.3E-05	0.0038

In T1DM, rs11192557 of sortilin-related VPS10 domain containing receptor 3 (*SORCS3)* (C allele, OR 0.13 [95% CI 0.04-0.36], $p=2.06x10^{-06}$) was the most significantly associated SNP with blinding DR and prostaglandin E receptor 2 (*PTGER2*) had the most number of SNPs (4) associated with DR. In T2DM, the rs757242 *actin filament associated protein 1(AFAP1)* was the most significantly

associated SNP with blinding DR in the Caucasian cohort (A allele, OR 3.3 [95% CI: 2.04-9.33], p=6.14x10^{-07}). Coiled-coil-helix-coiled-coil-helix domain containing 3 (*CHCHD3)* had 3 significantly associated SNP with DR, being the most number of significantly associated SNPs per gene with blinding DR in T2DM. In the combined DM group, rs10488210 of *CHCHD3* was the most significantly associated with blinding DR in pool samples (T allele, OR 0.54 [95% CI 0.41-0.71], p=1.00x10^{-05}).

Rs10488210, rs6967574 and rs7799350 of *CHCHD3* were associated with blinding DR in both T2DM and combined DM, with the *CHCHD3* gene having the most number of associated SNPs with blinding DR (3 SNPs in T2DM and 10 SNPs in combined DM) in the DNA pool cohort.

In the full cohort, rs7946060 of discs large homolog 2 *(DLG2)* (T allele, OR 0.30 [95% CI 0.17-0.53], p=9.48x10^{-06}) was found to be most significantly associated with blinding DR in T1DM (Table 9.4). In T2DM, rs757242 of *AFAP1* reached genome-wide significance in all participants and was the most significantly associated SNP with blinding DR (A allele, OR 2.81 [95% CI: 1.92-4.11], p=5.41x10^{-08}). Rs10488210 of *CHCHD3* was the most significantly associated SNP with combined DM in all participants (T allele, OR 0.57 [95% CI 0.45-0.71], p=6.45x10^{-07}).

Table 9.4 - Individual genotyping results of individually validated SNPs in all participants for blinding DR. Results are shown by type of diabetes. Statistically significant SNPs are highlighted in bold font.

DM type	SNP	Gene	Minor Allele	P value	Allele frequency affected	Allele frequency unaffected	OR	Lower 95% CI	Lower 95% CI
1	rs7777356	CALD1	A	**0.0007**	0.11	0.03	3.64	1.69	7.87
1	rs7946060	DLG2	T	**9.5E-06**	0.08	0.23	0.30	0.17	0.53
1	rs2245657	NPY	G	**0.0003**	0.03	0.11	0.20	0.08	0.53
1	rs12431401	PTGER2	T	**0.0003**	0.06	0.16	0.31	0.16	0.61
1	rs1495785	PTGER2	T	**0.0003**	0.05	0.15	0.29	0.14	0.60
1	rs17197	PTGER2	C	**0.0003**	0.05	0.15	0.29	0.14	0.60
1	rs1992139	PTGER2	A	**0.0002**	0.10	0.23	0.37	0.22	0.63
1	rs727176	SLCO4C1	A	**0.0047**	0.12	0.05	2.73	1.37	5.45
1	rs10884162	SORCS3	C	**0.0009**	0.04	0.12	0.27	0.12	0.61
1	rs11192557	SORCS3	C	**4.6E-05**	0.05	0.16	0.25	0.12	0.51
2	rs4148435	ABCC4	G	0.1362	0.11	0.09	1.32	0.92	1.90
2	rs4148445	ABCC4	G	0.0574	0.11	0.08	1.44	1.00	2.09
2	rs757242	AFAP1	A	**5.4E-08**	0.14	0.06	2.81	1.92	4.11
2	rs11830274	BTG1	T	0.1133	0.03	0.02	1.78	0.90	3.53
2	rs10488210	CHCHD3	T	**8.1E-06**	0.18	0.29	0.55	0.42	0.72
2	rs6967574	CHCHD3	T	**2.8E-05**	0.17	0.27	0.56	0.43	0.74
2	rs7799350	CHCHD3	C	**5.6E-05**	0.16	0.26	0.57	0.43	0.75
2	rs293924	FGD5	T	**4.4E-05**	0.19	0.11	1.90	1.40	2.58
2	rs2280479	KRT76	T	**1.1E-05**	0.09	0.03	2.85	1.77	4.59
2	rs13033750	LOC344065	A	**0.0026**	0.02	0.05	0.35	0.18	0.72
2	rs17118412	LOC643878	T	**0.0381**	0.05	0.03	1.88	1.06	3.35
2	rs10835549	LOC729560	T	0.1279	0.11	0.14	0.76	0.54	1.06
2	rs10835550	LOC729560	C	0.1157	0.11	0.14	0.76	0.54	1.06
2	rs4690859	LOC729923	C	**4.5E-05**	0.13	0.06	2.22	1.52	3.24
2	rs35034822	PROM2	G	**0.0056**	0.02	0.05	0.37	0.18	0.76
combined	rs10236831	CHCHD3	T	**2.2E-06**	0.09	0.17	0.50	0.38	0.68
combined	rs10488210	CHCHD3	T	**6.5E-07**	0.19	0.29	0.57	0.45	0.71
combined	rs11982852	CHCHD3	T	**7.5E-06**	0.17	0.26	0.59	0.47	0.74
combined	rs4731912	CHCHD3	T	**8.9E-07**	0.18	0.28	0.57	0.45	0.71
combined	rs6467450	CHCHD3	A	**3E-05**	0.18	0.26	0.62	0.49	0.78
combined	rs6951454	CHCHD3	T	**0.0002**	0.19	0.27	0.66	0.53	0.82
combined	rs6956407	CHCHD3	C	**0.0001**	0.18	0.26	0.64	0.51	0.81
combined	rs6962471	CHCHD3	A	**4.8E-05**	0.18	0.26	0.63	0.50	0.79
combined	rs6967574	CHCHD3	T	**1.1E-06**	0.18	0.27	0.57	0.45	0.71
combined	rs7799350	CHCHD3	C	**2.7E-06**	0.17	0.26	0.58	0.46	0.73
combined	rs12032403	FAM89A	G	**0.0068**	0.10	0.07	1.60	1.14	2.24
combined	rs7874380	LOC729802	C	**0.0411**	0.06	0.08	0.67	0.46	0.98
combined	rs1198963	SPRY4	C	**0.0003**	0.26	0.19	1.50	1.20	1.87
combined	rs13374343	TTC13	T	**0.0054**	0.10	0.07	1.62	1.16	2.26

OR=odds ratio and CI=confidence interval

All SNPs that were validated from the DNA pool cohort in the univariate analyses remained associated in the full participant cohort in those with T1DM and combined DM (Table 9.4). In T2DM, rs4148435 of ATP-binding cassette, sub-family C, member 4 (*ABCC4)*, rs11830274 of B-cell translocation gene 1 protein (*BTG1*), rs10835549 and rs10835550 of *LOC725960* became non-significant ($p>0.05$) and rs4148445 of *ABCC4* was of borderline significance ($p=0.057$). All other SNPs in the T2DM cohort with all participants included remained significantly associated with blinding DR.

Multivariate analyses

In the T1DM cohort, higher HbA1c levels, older age, increased rates of hypertension, nephropathy and hypercholesterolemia and increased duration of disease were associated with blinding DR ($p<0.05$, Table 9.5). In the T2DM cohort, higher HbA1c levels, increased rates of hypertension and nephropathy and increased duration of disease were associated with blinding DR ($p<0.05$, Table 9.5). In the combined DM cohort, female sex, higher HbA1c levels, increased rates of hypertension, nephropathy and hypercholesterolemia and increased duration of disease were associated with blinding DR ($p<0.05$, Table 9.5). These variables were subsequently controlled for in the multivariate analyses.

Table 9.5 – Associations of clinical characteristics with blinding DR in all participants.

	Combined DM		T1DM		T2DM	
Clinical Characteristic	Pearson correlation	P value	Pearson correlation	P value	Pearson correlation	P value
Diabetes type	0.026	0.412	NA	NA	NA	NA
Sex	-0.065	0.044	-0.067	0.283	-0.067	0.074
HbA1c	0.307	<0.001	0.252	<0.001	0.335	<0.001
Hypertension	0.145	<0.001	0.279	<0.001	0.079	0.035
Nephropathy	0.21	<0.001	0.357	<0.001	0.156	<0.001
Age	0.055	0.085	0.331	<0.001	-0.05	0.188
BMI	-0.002	0.947	0.075	0.24	-0.027	0.49
Hypercholesterolemia	0.071	0.027	0.238	<0.001	-0.004	0.924
Duration of disease	0.345	<0.001	0.51	<0.001	0.305	<0.001
Smoking	-0.028	0.386	0.092	0.138	-0.074	0.049

In the multivariate analyses, rs7946060 of *DLG2* remained significantly associated with blinding DR (OR 0.36 [95% CI 0.17-0.75], p=0.00064, Table 9.6) in all participants with T1DM. Further sub-analysis by DR type showed this SNP to be significantly associated with CSME (OR 0.21 [95% CI 0.07-0.67], p=0.0079). For those with T2DM, rs757242 of *AFAP1* remained as the most significantly associated SNP with blinding DR (OR 2.80 [95%CI 1.83-4.28], $p=2.0x10^{-6}$, Table 9.6). Sub-analysis by DR type revealed an association with PDR (OR 2.61 [95%CI 1.56-4.38], p=0.0003) and CSME (OR 2.99 [95% CI 1.84-4.86], $p= 9.0x10^{-06}$) when compared to no DR, and also in those with PDR when compared to NPDR (OR 2.62 [95% CI 1.42-4.8] p=0.0019). In the combined DM cohort, rs10488210 of *CHCHD3* also remained significantly associated with blinding DR in the multivariate analyses (OR 0.49 [95% CI 0.36-0.66], $p=3.2 x10^{-6}$, Table 9.6). In the DR subtype analysis, rs10488210 was significantly associated with PDR (OR 0.45 [95% CI 0.30-0.67] $p=7.6x10^{-05}$) and CSME (OR 0.53 [95% CI 0.37-0.77], p=0.0008) when compared to no DR, and an increased risk for PDR when compared to NPDR (OR 0.45 [95% CI 0.31-0.69], p=0.0002). Overall, most of the significantly associated SNPs from the

univariate analyses remained significantly associated with blinding DR in all participants after controlling for associated variables (Table 9.6).

Table 9.6 – Associations of validated SNPs in the full participant cohort by type of diabetes and DR. Results have been adjusted for associated clinical variables (as shown in Table 9.5) and associations of p<0.01 are highlighted in bold font.

				Controls vs Blinding				No DR vs any DR				No DR vs PDR			
DM type	SNP	Gene	Minor Allele	Additive	Genotypic	Dominant	Recessive	Additive	Genotypic	Dominant	Recessive	Additive	Genotypic	Dominant	Recessive
1	rs7777356	CALD1	A	0.9993	0.1728	0.0487	0.9993	0.9994	0.9999	0.9998	0.9994	0.9994	0.9532	0.693	0.9994
1	rs7946060	DLG2	T	0.9981	0.0475	**0.0064**	0.9981	0.1311	0.0346	0.0124	0.1857	0.9981	0.0669	0.0103	0.9981
1	rs2245657	NPY	G	0.9991	0.1326	0.0348	0.9991	0.9342	0.8182	0.5609	0.9082	0.9994	0.5358	0.2556	0.9994
1	rs12431401	PTGER2	T	0.9988	0.4526	0.1127	0.9988	0.2866	0.4768	0.8163	0.2681	0.9991	0.9992	0.8268	0.9991
1	rs1495785	PTGER2	T	0.9988	0.5768	0.1683	0.9988	0.3133	0.4638	0.707	0.2903	0.9991	0.9843	0.9348	0.9991
1	rs17197	PTGER2	C	0.9988	0.5768	0.1683	0.9988	0.3224	0.3897	0.5459	0.2903	0.9991	0.9843	0.9348	0.9991
1	rs1992139	PTGER2	A	0.4626	**0.0095**	**0.0027**	0.7438	0.3198	0.4502	0.7173	0.2784	0.2983	0.3818	0.1903	0.3562
1	rs727176	SLCO4C1	A	0.9994	**0.0008**	**0.0002**	0.9994	0.9994	0.3294	0.1603	0.9994	0.9994	0.076	0.0254	0.9994
1	rs10884162	SORCS3	C	NA	NA	**0.0071**	NA	NA	NA	0.1149	NA	NA	NA	0.0491	NA
1	rs11192557	SORCS3	C	0.9986	**0.0057**	**0.0005**	0.9986	0.9985	0.1342	0.012	0.9986	0.9985	0.0191	**0.0013**	0.9985
2	rs4148435	ABCC4	G	0.1144	**0.2835**	**0.5151**	0.1169	0.2847	0.5582	0.6422	0.2886	0.2564	0.2181	**0.1119**	0.2877
2	rs4148445	ABCC4	G	0.0535	**0.1352**	**0.2534**	0.0569	0.1678	0.3535	0.3969	0.1746	0.2409	0.1802	**0.088**	0.2724
2	rs757242	AFAP1	A	0.9987	**4.00E-05**	**2.00E-06**	0.9987	0.9984	0.011	0.0015	0.9984	0.9993	0.002	**0.0003**	0.9993
2	rs11830274	BTG1	T	0.9993	**0.2061**	**0.0501**	0.9993	0.9993	0.2664	0.0816	0.9993	NA	NA	**0.0436**	NA
2	rs10488210	CHCHD3	T	0.0123	**0.0005**	**0.0002**	0.0459	0.0062	0.0032	0.0024	0.02	0.0557	0.0073	**0.002**	0.1368
2	rs6967574	CHCHD3	T	0.0126	**0.002**	**0.001**	0.0318	0.0039	0.0067	0.017	0.0079	0.0643	0.0337	**0.0141**	0.1108
2	rs7799350	CHCHD3	C	0.0192	**0.0024**	**0.001**	0.0454	0.0069	0.0061	0.0079	0.015	0.0742	0.0279	**0.0103**	0.129
2	rs293924	FGD5	T	0.0151	**0.0047**	**0.0023**	0.0276	0.1397	0.0619	0.0218	0.1916	0.5766	0.149	**0.0538**	0.7082
2	rs2280479	KRT76	T	0.1608	**2.00E-05**	**4.00E-06**	0.1993	0.1797	0.0014	0.0003	0.2086	0.1054	0.0017	**0.0004**	0.1273
2	rs13033750	LOC344065	A	NA	**NA**	**0.0073**	NA	NA	NA	0.2477	NA	NA	NA	**0.3931**	NA
2	rs17118412	LOC643878	T	NA	**NA**	**0.0203**	NA	0.9994	0.2152	0.0758	0.9994	NA	NA	**0.0353**	NA
2	rs10835549	LOC729560	T	0.8489	**0.0372**	**0.0221**	0.68	0.8996	0.0043	0.0024	0.6988	0.2296	0.1473	**0.3603**	0.1715
2	rs10835550	LOC729560	C	0.9196	**0.0604**	**0.025**	0.9179	0.927	0.0058	0.0023	0.8726	0.3702	0.3255	**0.444**	0.3062
2	rs4690859	LOC729923	C	0.0757	**0.0021**	**0.0006**	0.1016	0.1795	0.0987	0.0427	0.2036	0.1466	0.0203	**0.0066**	0.1834

Table 9.6 Continued

				Controls vs Blinding				No DR vs any DR				No DR vs PDR			
DM type	**SNP**	**Gene**	**Minor Allele**	**Additive**	**Genotypic**	**Dominant**	**Recessive**	**Additive**	**Genotypic**	**Dominant**	**Recessive**	**Additive**	**Genotypic**	**Dominant**	**Recessive**
2	rs35034822	PROM2	G	NA	**NA**	**0.023**	NA	NA	NA	0.3179	NA	NA	NA	**0.7584**	NA
combined	rs10236831	CHCHD3	T	0.1371	**2.00E-05**	**3.1E-06**	0.2289	0.6506	0.0005	0.0001	0.854	0.7029	0.0005	**0.0001**	0.8696
combined	rs10488210	CHCHD3	T	0.0052	**1.00E-05**	**3.2E-06**	0.0322	0.0185	0.0055	0.0024	0.0589	0.0429	0.0004	**7.6E-05**	0.1414
combined	rs11982852	CHCHD3	T	0.0055	**0.0002**	**8.00E-05**	0.0182	0.023	0.0244	0.0212	0.0447	0.0664	0.0037	**0.001**	0.1431
combined	rs4731912	CHCHD3	T	0.003	**2.00E-05**	**8.00E-06**	0.0137	0.0136	0.0078	0.006	0.0342	0.0482	0.0016	**0.0004**	0.1188
combined	rs6467450	CHCHD3	A	0.0043	**0.0004**	**0.0003**	0.0135	0.0332	0.0229	0.0134	0.0687	0.0603	0.0045	**0.0012**	0.1264
combined	rs6951454	CHCHD3	T	0.0057	**0.001**	**0.0006**	0.0175	0.0394	0.0411	0.0275	0.0747	0.0615	0.0107	**0.0035**	0.1203
combined	rs6956407	CHCHD3	C	0.0055	**0.0009**	**0.0006**	0.0156	0.0697	0.1073	0.0808	0.1067	0.053	0.019	**0.0083**	0.09217
combined	rs6962471	CHCHD3	A	0.0061	**0.0006**	**0.0003**	0.0187	0.0486	0.0303	0.0153	0.0963	0.0818	0.0051	**0.0013**	0.1658
combined	rs6967574	CHCHD3	T	0.003	**3.00E-05**	**1.00E-05**	0.0129	0.0126	0.0084	0.0069	0.0305	0.0492	0.002	**0.0005**	0.1182
combined	rs7799350	CHCHD3	C	0.0027	**4.00E-05**	**2.00E-05**	0.0108	0.0405	0.0121	0.0049	0.0937	0.0673	0.0016	**0.0004**	0.1548
combined	rs12032403	FAM89A	G	0.0318	**0.0335**	**0.0315**	0.0364	0.1038	0.1467	0.1237	0.1098	0.1564	0.2457	**0.2019**	0.1642
combined	rs7874380	LOC729802	C	0.7732	**0.0199**	**0.0079**	0.7076	0.6239	0.8666	0.9173	0.6197	0.3727	0.5735	**0.7676**	0.3618
combined	rs1198963	SPRY4	C	0.0257	**0.0036**	**0.0012**	0.0749	0.0105	0.0088	0.0072	0.0246	0.4091	0.6108	**0.3972**	0.4641
combined	rs13374343	TTC13	T	0.021	**0.0246**	**0.0282**	0.0233	0.9976	0.5663	0.1123	0.9976	0.9985	0.6842	**0.1821**	0.9985

Table 9.6 Continued

				No DR vs CSME				NPDR vs PDR			
DM type	**SNP**	**Gene**	**Minor Allele**	**Additive**	**Genotypic**	**Dominant**	**Recessive**	**Additive**	**Genotypic**	**Dominant**	**Recessive**
1	rs7777356	CALD1	A	NA	NA	0.0778	NA	0.9993	0.9233	0.5378	0.9993
1	rs7946060	DLG2	T	0.9982	0.0534	**0.0079**	0.9982	0.9994	0.471	0.2061	0.9994
1	rs2245657	NPY	G	0.9993	0.5522	0.2416	0.9993	0.9993	0.16	0.0475	0.9993
1	rs12431401	PTGER2	T	0.9991	0.9998	0.7321	0.9991	0.999	0.1977	0.0445	0.999
1	rs1495785	PTGER2	T	0.9991	0.9875	0.8798	0.9991	0.999	0.1644	0.0335	0.999
1	rs17197	PTGER2	C	0.9991	0.9875	0.8798	0.9991	0.9989	0.0918	0.0164	0.999
1	rs1992139	PTGER2	A	0.5642	0.8242	0.6411	0.5781	0.7228	**0.0002**	**5.90E-06**	0.72
1	rs727176	SLCO4C1	A	0.9994	0.1788	0.0708	0.9994	NA	NA	**0.0046**	NA
1	rs10884162	SORCS3	C	NA	NA	0.109	NA	NA	NA	0.0345	NA
1	rs11192557	SORCS3	C	0.9986	0.188	0.0251	0.9986	NA	NA	0.0112	NA
2	rs4148435	ABCC4	G	0.0899	0.2276	0.8014	0.0875	0.7665	0.8145	0.5282	0.7955
2	rs4148445	ABCC4	G	0.0314	0.0977	0.3778	0.0323	0.7909	0.8066	0.5146	0.8201
2	rs757242	AFAP1	A	0.9987	**0.0002**	**9.00E-06**	0.9987	0.8265	**0.0059**	**0.0019**	0.6963
2	rs11830274	BTG1	T	0.9993	0.2366	0.0527	0.9993	NA	NA	0.395	NA
2	rs10488210	CHCHD3	T	0.0566	0.0196	**0.0069**	0.1259	0.7515	0.0804	0.0528	0.5255
2	rs6967574	CHCHD3	T	0.0696	0.0443	0.0196	0.1229	0.6476	0.1414	0.0912	0.4977
2	rs7799350	CHCHD3	C	0.0887	0.0206	**0.0065**	0.168	0.6247	0.1947	0.132	0.5021
2	rs293924	FGD5	T	**0.0069**	**0.0072**	**0.0063**	0.0122	0.1125	0.0538	0.0202	0.1531
2	rs2280479	KRT76	T	0.9993	**0.0001**	**4.10E-06**	0.9993	0.4523	0.1972	0.0717	0.5022
2	rs13033750	LOC344065	A	NA	NA	0.0448	NA	NA	NA	0.4355	NA
2	rs17118412	LOC643878	T	NA	NA	0.0112	NA	0.9993	0.3068	0.1907	0.9993
2	rs10835549	LOC729560	T	0.1505	**0.0026**	**0.0006**	0.2015	0.1243	0.1879	0.1175	0.1437
2	rs10835550	LOC729560	C	0.1404	**0.0041**	**0.0011**	0.1871	0.1772	0.1168	0.0478	0.2175
2	rs4690859	LOC729923	C	0.3342	**0.0071**	**0.0017**	0.4169	0.4199	0.0177	**0.0045**	0.5076
2	rs35034822	PROM2	G	NA	NA	0.1077	NA	NA	NA	0.6312	NA

Table 9.6 Continued

				No DR vs CSME				NPDR vs PDR			
DM type	**SNP**	**Gene**	**Minor Allele**	**Additive**	**Genotypic**	**Dominant**	**Recessive**	**Additive**	**Genotypic**	**Dominant**	**Recessive**
combined	rs10236831	CHCHD3	T	0.7885	**0.0003**	**8.60E-05**	0.9997	0.159	0.0189	**0.0062**	0.2041
combined	rs10488210	CHCHD3	T	0.0583	**0.0032**	**0.0008**	0.1696	0.3213	**0.0008**	**0.0002**	0.6579
combined	rs11982852	CHCHD3	T	0.0696	0.0116	**0.0036**	0.1429	0.2849	**0.0009**	**0.0002**	0.5283
combined	rs4731912	CHCHD3	T	0.0516	**0.0039**	**0.0011**	0.1268	0.291	**0.0023**	**0.0005**	0.5283
combined	rs6467450	CHCHD3	A	0.1034	0.0314	0.0104	0.193	0.1565	**0.0082**	**0.0021**	0.2864
combined	rs6951454	CHCHD3	T	0.1338	0.0346	0.0105	0.2504	0.1731	0.0324	0.0102	0.2864
combined	rs6956407	CHCHD3	C	0.1152	0.0227	**0.0067**	0.2175	0.0795	**0.0095**	**0.0032**	0.1485
combined	rs6962471	CHCHD3	A	0.1298	0.0357	0.0113	0.2358	0.1565	**0.0082**	**0.0021**	0.2864
combined	rs6967574	CHCHD3	T	0.0502	**0.0053**	**0.0015**	0.1185	0.2979	**0.004**	**0.0009**	0.5204
combined	rs7799350	CHCHD3	C	0.0708	**0.0025**	**0.0006**	0.1667	0.1599	**0.0039**	**0.0009**	0.3041
combined	rs12032403	FAM89A	G	0.0803	0.0791	0.0519	0.0882	0.5611	0.8301	0.9763	0.5561
combined	rs7874380	LOC729802	C	0.6003	0.5743	0.4625	0.5762	0.999	0.0119	**0.0069**	0.999
combined	rs1198963	SPRY4	C	0.059	**0.0015**	**0.0003**	0.1869	0.7279	0.4084	0.3422	0.5572
combined	rs13374343	TTC13	T	0.9982	0.3285	0.0397	0.9982	0.582	0.8408	0.998	0.576

Discussion

DR has a complex multifactorial pathogenesis. Despite 8% of all individuals with diabetes suffering from sight-threatening DR[46], there are limited treatment options and its pathogenesis remains poorly understood.

This GWAS was the first to be undertaken for DR and identified several novel putative disease susceptibility genes for blinding DR in T1DM and T2DM. Markers that showed significant association in the pooled analysis were followed up by individual genotyping, which validated the association of 39 SNPs within 21 genes with sight-threatening DR. Only validated genes by individual genotyping are further discussed and several of these genes were found to play plausible roles in angiogenesis and microvascular damage. *Neuropeptide Y* (*NPY)*[164, 320], *prostaglandin E receptor 2* (*PTGER2)*[321-323], *caldesmon* (*CALD1)*[324], *ABCC4* [325] , *BTG1*[326] and *sprouty 4* (*SPRY4)*[327, 328] have been reported to play a role in angiogenesis in various animal and human studies and each is discussed in detail below.

NPY is a neurotransmitter with numerous functions, including the augmentation of vasoconstrictor effects of noradrenergic neurons[329]. Animal studies have shown endothelial cells to have a 4 fold increased migration *in vivo* when pre-incubated with NPY, with aortic rings stimulated by NPY leading to capillary sprouting and angiogenesis[320]. NPY has also been associated with retinal neovascularization in mice with neonatal hyperoxia, which has been shown to be prevented in NPY receptor 2 knockout mice[164].

PTGER2 is a receptor of prostaglandin E2 (PGE2) and has been found to be expressed in the eye, including in retinal blood vessel linings and optic nerve blood vessels[330]. Increased PGE2 production has been shown to stimulate VEGF, a cytokine known to play a role in the angiogenic pathway[322, 323]. PGE2 has been shown to be significantly increased in the serum of people suffering from PDR, compared to those with no DR[331]. In contrast, PGE2 has been shown to be significantly lower in the vitreous of PDR when compared to non-diabetic eyes[332, 333], indicating a difference in the control of local release of prostaglandins in the eye compared to serum. COX1 is responsible for the baseline levels of prostaglandins and COX2 produces prostaglandins as a consequence of inflammation and are located in blood vessels[334]. Intravitreal neovascularization in murine and rat models of PDR have been prevented by COX2 but not COX1 inhibitors[321]. Therefore prevention of neovascularization via COX2 inhibitors may be due to inhibition of PGE2 stimulation of VEGF, indicating potential for future human therapeutic studies involving COX2 inhibitors for DR.

ABCC4 is a transmembrane protein/energy dependent efflux pump that is involved in the active transport of a wide variety of substrates including organic ions, and is an independent regulator of intracellular cyclic nucleotide levels and is involved in cAMP-dependent signal transduction to the nucleus by degrading second messengers cAMP and cGMP[325]. *ABCC4* has been found to be upregulated in proliferating smooth muscle cells of human coronary arteries and injured rat carotid arteries. Inhibition of the ABCC4 pump blocked proliferation of smooth muscle cells and prevented neointimal (pathological proliferative layer) growth in injured rat carotid arteries, providing evidence for a role of ABCC4 in neovascularization[325].

BTG1 is a member of an anti-proliferative gene family that regulates cell growth and differentiation. Its mRNA has been found to be abundantly expressed in quiescent bovine aortic endothelial cells and human umbilical vein endothelial cells[326]. Addition of angiogenic growth factors was shown to decrease *BTG1* mRNA levels in normal endothelial cells and endothelial cells over-expressing *BTG1* displayed increased cell migration, indicating that *BTG1* may play an important role in the process of angiogenesis.[326]

CALD1 is abundantly present in endothelial cells and has been found to be critically involved in the regulation of the actin cytoskeleton and migration of endothelial cells[335]. It has been found that an intranuclear translocation involving *CALD1* serves as an additional regulatory step in the control of mitotic initiation in angiogenesis of human tumors[324].

An *in vitro* murine study has shown SPRY4 to inhibit FGF and VEGF-mediated endothelial cell proliferation and migration[336]. Fibroblast growth factor (FGF) is important for endothelial cell growth, migration, and morphogenesis and is critical for angiogenesis in both normal and disease states[337]. VEGF is a multifunctional cytokine which promotes angiogenesis[218]. Transplanted tumor cells have been shown to grow significantly faster in *spry4* knockout mice than in wild-type mice, which were associated with enhanced neovascularization in the tumors transplanted into knockout mice. Angiogenesis induced by VEGFA was also enhanced in *spry4* knockout mice compared with wild type mice[327]. Similarly, *ex vivo* angiogenesis induced by VEGFA and basic fibroblast growth factor (bFGF) was enhanced in the aortas of *spry4* knockout mice[327]. *Spry4* knockout mice have also been shown to be

more resistant to hind limb and soft tissue ischemia due to accelerated neovascularization[328].

ABCC4 and *SPRY4* have also been shown to be involved in abnormal blood clotting. Along with *PTGER2,* they have also been associated with increased vascular permeability, a vital component of macular edema development. The ABCC4 pump has been isolated in the luminal membrane of brain capillary endothelial cells where it is believed to play a role in the transport of organic ions, affecting the permeability of the blood brain barrier[338-340]. Tachikawa *et al.* investigated expression of the ABCC family in the mouse retina and found *ABCC4* transcript levels in the retinal vascular endothelial cell fraction (RVEC) to be 251 times higher than in the non-RVEC fraction, being the highest expression out of the ABCC family[341]. It was also found to be predominantly expressed in the inner blood-retina barrier (BRB), appearing to play a major role in the efflux transport of their substrates at the inner BRB. It is therefore possible that ABCC4 plays a major role in the permeability of the BRB, which plays an important role in DR pathogenesis.

Altered ABCC4 distribution in platelets of a patient with Hermanksy-Pudlak syndrome (involving defective delta granules) has also been reported. ATP-cGMP transport was also found to be co-distributed with ABCC4 in sub-cellular fractions and this transport was inhibited by non steroidal anti-inflammatory drugs (NSAIDs)[342]. These results indicate a function of ABCC4 in platelet mediator storage. ABCC4 has also been found to interact with prostaglandin E1 and E2 in vivo, catalysing their time- and ATP-dependent uptake. PGF1alpha, PGF2alpha, PGA1 and thromboxane A2 were high-affinity inhibitors (and therefore presumably

substrates of) ABCC4. Also, several NSAIDs were found to be potent inhibitors of ABCC4[343]. This evidence shows that ABCC4 may play a pathophysiological role in abnormal blood clotting, which could be relevant to DR pathogenesis. Inhibition of ABCC4 by NSAIDs may represent a novel mechanism for inhibition of platelet function and as a treatment in disorders involving abnormal blood clotting, including DR.

Via PTGER2, PGE2 stimulation of VEGF occurs[322], and SPRY4 has been found to be an inhibitor of VEGF[336], which is a potent mediator of microvascular permeability[218]. *In vivo* induction of VEGFA induced vascular permeability has been shown to be enhanced in *SPRY4* knockout mice when compared with wild type mice [327]. SPRY4 has also been shown to inhibit protein kinase C[344], which is believed to be involved in pathogenic vascular and hemodynamic changes including blood flow abnormalities, increased vascular permeability and vascular occlusion[58, 262].

ABCC4[345, 346], *PTGER2*[322, 347-349], *BTG1*[326, 350, 351], *AFAP1*[352] and *CHCHD3*[353] were validated in the individual genotyping for blinding DR and they have also been reported to be associated with various cancers. Of these genes, *ABCC4*[325], *PTGER2*[321-323], and *BTG1*[326] have been reported to play a role in angiogenesis. In the DNA pool cohort and all participants, the rs757242 of *AFAP1* was validated and the most significantly associated SNP with blinding DR, placing an individual with T2DM at over 3 times higher risk of developing DR, particularly PDR and CSME. *AFAP1* has also been associated with cell-matrix adhesions and migration of cells[352], which is essential for the ability of smooth muscle cells to proliferate and lead to neovascularization. A substantial number of cancer causing genes appear to be

associated with DR, and it is interesting to speculate that this may be due to a shared pathway of angiogenesis required for both carcinogenesis and DR development.

Over 300 SNPs in the top 10,000 hits were common to T1DM and T2DM including all of the validated *CHCHD3* SNPs. Also, 3 validated SNPs of *CHCHD3* that were associated with blinding DR were common to T2DM and combined DM groups. Some of these SNPs showed an increase in significance in the combined DM group. It is therefore logical to assume that there are possible shared pathogenic pathways for DR regardless of diabetes type. A potential explanation for the lack of detection of these genes post validation of SNPs in both T1DM and T2DM is due to insufficient power, especially in the T1DM cohort.

Interestingly, many of our validated genes have been reported in various human and animal studies to play a role in the development of DM, DR and its risk factors. For example, inducible expression of murine *SPRY4* in pancreatic beta cells has been shown to provoke a significant reduction in islet size, an increased number of alpha cells per islet area, and impaired islet cell type segregation[354]. Functional analysis of islet cell differentiation in cultured pancreatic cells showed SPRY4 repressed adhesion and migration of differentiating pancreatic endocrine cells. It is therefore possible that *SPRY4* may contribute to pancreatic cell dysfunction and play a role in the development of diabetes. The most number of SNPs associated with DR of any given gene was of *CHCHD3*. The function of the CHCHD3 protein is poorly understood and recently it was found to be a mitochondrial protein[355] and a novel protein kinase A (PKA) substrate[356]. PKA has several functions in the cell, including regulation of glycogen, sugar and lipid metabolism. RIIβ is a regulatory subunit of

PKA and *RIIβ* knockout mice have shown protection against diet-induced obesity, insulin resistance, and dyslipidemia[357]. PKA is also involved in regulation of the cell cycle and cell proliferation[358], which is important for angiogenesis. Collectively these findings indicate that some genes that are known to play a role in DM development and DM risk factors are also likely to be associated with DR development. In support of this theory, several genes previously detected in other GWAS studies for DM, such as *TCF7L2*, *SLC30A8* and *CDKAL1*[24-28] in T2DM, were also found to be in the top 10,000 hits for DR in our pooled T1DM, T2DM and combined DM cohorts. On another note, *NPY* was found to be associated with blinding DR in our T1DM participants. Studies have shown NPY to be an angiogenic factor[164, 320] and to be associated with DR[164, 165, 359]. Genetic variation in *NPY* has also been associated with established DR risk factors such as hyperglycemia[360, 361], hypertension[362] and hypercholesterolemia[361-363]. *CALD1* has been found to have glucose regulated expression[364] and PTGER2 exerts its vascular effects via PGE2, which has been found to be significantly elevated in humans[331, 365], mice[366] and rats[321] with diabetes and shown to be reversed with insulin treatment [366]. Therefore another reason for detection of these genes in our participants with DR may be due to the link of increased severity of DM and or increased severity of DR risk factors with DR development itself.

In the multivariate analyses, our most significant results were from the controls vs blinding comparison. When participants with intermediate NPDR subtypes were added to the analyses and participants with no DR were compared to those with any level of DR, overall weaker associations were found. When broken down by DR subtype, associations were most significant in the PDR and CSME subtypes,

particularly in the larger T2DM and combined DM subsets, with many of these SNPs also found to be significantly associated with PDR when compared to those with NPDR. It is possible that associations were found to be stronger in T2DM and combined DM due to increased power in these groups when compared to T1DM.

A large proportion of the genes associated with DR in the pooling method were of unknown function and many other genes found to be associated with DR have poorly understood functions. It is hoped that the findings of this study will form the basis of future research into novel pathogenic pathways for DR. Also, traditionally investigated genes in the pathogenesis of DR, such as *VEGFA* and *AKR1B1*, although found to be in the top 10,000 hits for DR, were of far less effect size in the GWAS in comparison to many other SNPs, indicating that we have potentially identified with the GWAS approach more important pathways involved in DR susceptibility than those previously established.

Our comprehensive GWAS investigated over 800,000 SNPs in T1DM and T2DM participants. Previous genome-wide studies for DR have been linkage scans for DR in T2DM participants, a limited method with low resolution for detecting genetic association. One such study has been undertaken in Pima Indians[250, 251], whereby 516 microsatellite markers were scanned in up to 322 sib-ships with T2DM. Significant evidence of linkage to 1p36 in the larger study was found[251]. In our pooling cohort, several genes were found to be present in the 1p36 region. Specifically *ACTL8, ALDH41, PAX7, ARHGEF10L, IGSF21, KLHDC7A*, and *PRDM2* were found to be in the top 10,000 hits for blinding DR. Literature review of these genes revealed no plausible relationships of these genes with DR. However like many of our validated

genes, *ACTL8*[367], *ARHGEF10L*[368] and *PAX7*[369, 370] have been associated with various cancers. *PAX7* acts as a transcriptional repressor and has a proliferative effect on cancer cells[369, 370]. As angiogenesis also requires the ability of endothelial cells to proliferate, the detection of cancer related genes again may reflect their possible involvement in angiogenesis.

Another genome-wide linkage study was undertaken in 282 affected Mexican-American sibling pairs with T2DM[88]. In this study 360 markers were investigated and suggestive linkage to chromosomes 3, 5, 6, 12, 19 and 20 were found. Genes in regions under linkage peaks of several of these chromosomes were also detected in the top 10,000 hits of our pooled cohort for blinding DR, including *IMPG2 of* chromosome 1, *ROBO2* and *PROS1* on chromosome 3, *KIAA0947* on chromosome 5, *EDN1* on chromosome 6, *GNB3* and *WNK1*on chromosome 12 and *IGF1R* on chromosome 15 and *PRPF31* on chromosome 19. Literature review revealed *EDN1* and *IGF1R* to be of particular relevance to DR. *EDN1* codes for endothelin 1, which is a potent vasoconstrictor and mitogen, possibly contributing to the development of endothelial dysfunction[371]. Endothelin-1 has been shown to lead to retinal vasoconstriction and to have mitogenic effects on retinal pericytes in rats[372]. It has been shown to be significantly elevated in PDR vitreous[373, 374] and plasma[375]when compared to non-diabetic controls and those with no DR respectively. Increased retinal expression of endothelin-1 and its receptors has been shown in diabetic rats[376-378] and elevated levels in plasma of diabetic patients with DR have been reported when compared to healthy controls[379]. A candidate gene study in Chinese patients with T2DM reported the Asn/Asn genotype of EDN1 to be associated with a reduced risk of DR (OR=0.19 [95% CI 0.07-0.53], p=0.002)[380].

IGF1R codes for insulin like growth factor 1 receptor, which has been isolated in the rat retinal blood vessels[381, 382] and increased expression in diabetic rat retinas has been found[383]. Transgenic mice with retinal elevations of IGF-1 have been shown to have increased IGF1R content and signalling, leading to VEGF accumulation. IGF-I elevation was also shown to be sufficient to trigger processes leading to blood-retinal barrier breakdown and increased retinal vascular permeability[384]. In an oxygen induced retinopathy model, *IGF1R* knockout mice have been shown to have a significant reduction in neovascularization[385]. Similarly, IGF-1 receptor antagonists have been shown to suppress retinal neovascularization *in vivo*[386]. In high glucose treated human retinal endothelial cells, activation of IGF-IR contributed to disruption of tight junctions by decreasing occludin expression[387]. This mechanism may be important in the pathogenesis of blood-retinal barrier dysfunction in DR. As with other genes detected in our GWAS for DR, *IGF-1R* has also been associated with T2DM development, with polymorphisms of *IGF1R* being associated with modified weight change responses to lifestyle interventions[388]. Many cancer associated genes were detected in our GWAS, and similarly *IGFR1* has also been found to be highly over-expressed in many malignant tissues, where it functions as an anti-apoptotic agent by enhancing cell survival[389-391]. Although these genes seem to be of high relevance to DR, further comprehensive candidate gene studies are required to confirm DR associations with their genetic variations.

This study utilised equimolar DNA pooling as an alternative method to individual genome-wide genotyping for identification of disease susceptibility genes for blinding DR. In comparison to individual genotyping, the ability of DNA pooling to detect true associations is additionally reliant on the precision of allele frequency

measurements made by the SNP genotyping microarray, accuracy of pool construction by quantitation and pipetting techniques, the integrity of the pooled genomic DNA and the number of individuals pooled[316]. Strengths of this study include stringent protocols undertaken for our pooling methods and in order to additionally minimise pooling error, the case and control pools were deliberately enriched with participants who had DR phenotypes of similar severity and all participants were of Caucasian ancestry. Several limitations of this study are accepted. Due to resource constraints, numbers of SNPs selected for individual genotyping was limited in this study, with other potentially important SNPs unable to be included. The inability to follow up some SNPs that were significant in the DNA pool due to failed assay design on the Sequenom platform is another limitation and alternative technologies will need to be utilised to genotype these SNPs. Also, experimental noise as a result of the DNA pooling design can lead to pooling error and possible imprecision in allele frequency estimation. It is also of note that the significance of most associations detected in the DNA pooling was reduced on individual typing, perhaps suggesting inflation of their result in the pool. Similarly, other significant SNPs may have not been detected in the pooling due to a false reduction in associations. Another limitation of this study includes suboptimal power in the DNA pooling, particularly in T1DM. Finally the inability to undertake multivariate and DR subtype analyses in the DNA pooling method is a further limitation as it does not allow for solid associations to be made without individual genotyping. It is accepted that replication of these results are required in further large independent cohorts to confirm the findings of this study.

In summary, this GWAS study identified several novel putative susceptibility genes for blinding DR, many coding for proteins with plausible functions in the angiogenic pathway, microvascular damage, diabetes development and risk factors for DR. Future larger GWAS studies with individual genotyping are required to replicate these findings in Caucasian and other ethnic cohorts. Upon confirmation of disease susceptibility, further investigation of unknown genes will be required to better understanding their role in DR pathogenesis. This has potential to lead to novel therapeutic targets for DR prevention and treatment. Additionally, further understanding of the role of these genes in pathogenesis of angiogenesis and microvascular damage may not only benefit management of DR, but could also assist in identifying targets for the treatment of many other conditions including retinopathy of prematurity and cancer.

CHAPTER 10

Discussion and Conclusions

Diabetic retinopathy, a microvascular complication of DM, is one of the top 5 global causes of blindness[44] and is the leading cause of blindness in working age adults[45]. Approximately 40% of individuals with diabetes develop DR, of which 8% have sight threatening disease[46]. DR pathogenesis remains poorly understood and current established treatments for DR do not reverse pathological changes, at best delaying the progression of DR and vision loss. With the prevalence of DM being predicted to more than double over the next two decades[31], the subsequent increase in DR and blindness is inevitable, and the burden of disease will be a major public health and economic problem.

Diabetic ocular screening is a major public health cost and a significant amount of eye care providers' time is devoted to the clinical screening of patients with DM who have no DR and who may never develop significant visual loss. Clinical experience has also shown the development of blinding DR complications may occur at a relatively early stage in some individuals who presumably have a high susceptibility to DR. Genetic factors are believed to be responsible in part for these different clinical scenarios and this thesis aimed to explore these risk factors for the development of DR, with a particular focus on sight threatening DR.

Over 160 candidate gene studies have been undertaken for DR to date. Fundamental differences in study design have led to difficulty in establishing specific genetic risk

factors for the development of DR. Our meta-analysis of all relevant candidate gene studies for DR revealed the aldose reductase (*AKR1B1)* gene to be the most significantly associated of the previously studied genes in the meta-analysis, with the z-2 microsatellite and rs759853 most significantly associated with DR. As aldose reductase is a rate limiting enzyme in the polyol pathway, which is an established pathway for microvascular damage in DM, *AKR1B1* is a plausible candidate gene for DR. We were unable to replicate this finding in a large cohort of Australian participants with T1DM or T2DM, but found another SNP of *AKR1B1* (rs9640883) to be associated with DR. However, after adjusting for clinical parameters known to be associated with DR, this SNP was associated with DR development independently of other risk factors. Further analysis showed a direct association of this SNP with duration of diabetes, reflecting the age of onset of diabetes. This finding suggested that particular genes may be associated with clinical risk factors for diabetes pathogenesis (rather than having a direct association with DR itself), thus highlighting the complexity of genetic influence in DR pathogenesis and in itself making a significant novel contribution to understanding age of onset of type 2 diabetes.

VEGF is a cytokine that plays a major role in vascular permeability and angiogenesis in response to hypoxia[218]. In the DR meta-analysis, sequence variation in the gene for the A isoform of VEGF (*VEGFA)* was shown to be significantly associated with PDR development when compared to those with NPDR. Our candidate gene study also found variation in the *VEGFA* gene to be associated with DR, in particular sight-threatening forms of DR, and replication of associated SNPs from a large prospective study[229] suggests that these findings represent a true association. Future candidate

gene studies should also focus on genes coding for VEGF receptors and whether genetic variation in their respective genes influences DR development. Anti-VEGFA clinical trials for treatment of diabetic retinopathy are currently underway[83, 84], and it may prove to be fundamentally important to consider the genotype of patients at the level of VEGFA and its receptors when interpreting the results of these trials in the future.

Carbonic anhydrase 1 (CA1) is an enzyme that is involved in retinal vascular permeability. This enzyme has been found at higher levels in PDR vitreous when compared to controls and injection of CA1 into rat vitreous induced macular edema [236]. These findings formed the basis of our novel candidate gene study for sequence variation in *CA1* in DR susceptibility. This study failed to show an association of genetic variation in the *CA1* gene with DR or its sight-threatening subtypes. Further studies are required to assess *CA* genetic variation with serum and vitreous CA levels in order to explore direct genetic associations with CA function. The lack of common genetic sequence variation in *CA1* being associated with the clinical outcome of DR susceptibility does not rule out an important role of carbonic anhydrase in DR pathogenesis.

The association of diabetic nephropathy and DR have been reported in many studies[71, 72, 392]. As an end result of hyperglycemia, the involvement of various cytokines and growth factors, such as protein kinase C, in pathways involved in DR and nephropathy development may be shared[262, 393]. EPO is an important cytokine that stimulates proliferation, migration and angiogenesis in vascular endothelial cells[240, 241]. Increased expression of EPO has been reported in the diabetic rat kidney

and retina[247]. Our *EPO* candidate gene study revealed variation in *EPO* to be significantly associated with DR. However, our findings showed an opposite direction of association to larger American cohorts with DR and end stage renal disease (ESRD)[247]. In examining genetic variation of the *EPO* gene for an association with DR, our study deliberately excluded participants with ESRD. Another fundamental difference between the study of Tong *et al*[247] and our study was their failure to undertake multivariate analyses and control for factors that influence DR and ESRD. In particular, we controlled for nephropathy in our multivariate analyses for DR. Since nephropathy and DR have been reported to be associated with each other, it is possible that genetic associations with DR are confounded by diabetic ESRD in the cohorts of Tong *et al*[247]. Solid conclusions from their results for genetic influences in DR are therefore not possible, as they have been potentially confounded by their associations with nephropathy. Our findings highlight the importance of undertaking multivariate analyses in studying genetic influences in complex traits. Clearly, more detailed further investigation in large cohorts is required to further dissect what may be an important pathway in diabetic complications with therapeutic ramifications.

ADMA, SDMA and L-arginine are involved in the NO pathway and our serum protein study revealed their significant elevation in blinding forms of DR. It remains unclear whether these analytes lead to DR development or are elevated as a consequence of factors such as diabetic nephropathy, and further explorative studies are required. If a causal or predictive relationship can be established with DR in future prospective studies, then this could lead to design of therapeutic and or

preventative strategies to correct NO levels in the ocular environment and thereby retard or prevent the development of DR.

The first GWAS for DR was undertaken as a part of this thesis. Over 800,000 SNPs were investigated for each type of DM by equimolar DNA pooling, followed by individual genotyping of highly associated SNPs. Several novel genes were found to be strongly associated with DR and validated in by individual genotyping in a larger cohort. Although future replication studies are required, many of the strongly associated genes found in our GWAS have known roles in angiogenesis and vascular permeability. Another group of genes associated in our study have also been reported to be associated with various cancers. This raises the possibility that these genes may share a role in angiogenic pathways required for PDR and carcinogenesis. Many genes associated with DR in our GWAS have also been previously associated with DM pathogenesis and DR risk factors. Studies like this one therefore not only help to increase our understanding of DR, but help to also further understand DM and DR risk factor pathogenesis and identify other treatment targets for these conditions. Future directions should be focused on larger GWAS studies with individual genotyping in Caucasians and other ethnic cohorts, followed by candidate gene studies for confirmation of disease susceptibility genes and involvement of specific loci.

Our candidate gene studies and genome-wide association studies revealed many SNPs and genes associated with DR to be different between T1DM and T2DM. In many instances, the same SNPs identified only in one particular DM subtype showed association with DR in the combined DM analyses. In fact some of these SNPs often

showed stronger associations in the combined DM analyses, indicating the lack of association with individual types of diabetes to be due to insufficient power, particularly in the smaller T1DM cohort. Another possible explanation is that different SNPs and genes may be associated with DR in T1DM compared to T2DM.

A large proportion of individuals with diabetes develop DR at some stage of their illness, but unless it is of the more severe sight-threatening subtypes, the condition does not usually affect visual outcomes. It is therefore interesting that the strongest candidate gene and GWAS associations identified in this thesis were generally in the blinding DR comparisons and weakest in the 'any DR' comparisons. In the sub-analyses by DR type, PDR and CSME showed the overall strongest associations and were of similar strength. Many associated SNPs were common to these subtypes of DR, indicating that the genetic components of these subtypes are at least in part shared. Although PDR is commonly sub-analysed in genetic studies, very few studies have investigated genetic associations with the common subtype of CSME and this blinding complication requires a particular focus and further exploration in future genetic studies.

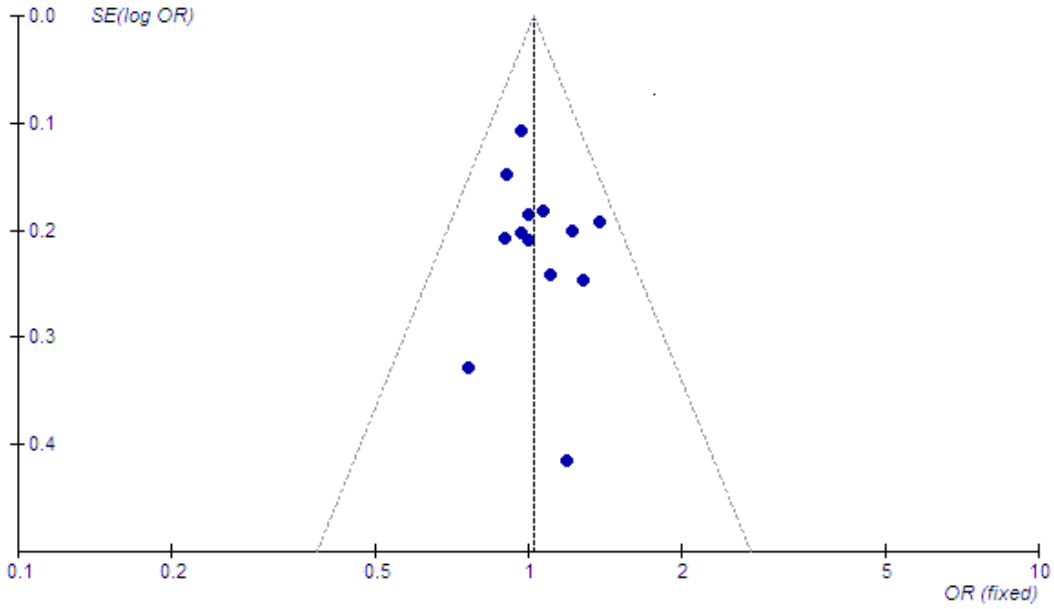
In summary, this thesis has identified and refined plausible candidate genes and serum markers associated with DR, particularly sight threatening DR. Given the findings of previous candidate gene studies and those of this thesis, it is most likely that a complex polygenic influence on DR exists. Many of these genes are likely to have a particular involvement in the angiogenic and vascular permeability pathway. Other genes may indirectly exert their effects on DR by their direct influence on associated risk factors and severity of diabetes.

Australia-wide collaborations have begun for the Genetic Study of Diabetic Retinopathy. Research for this project will be therefore ongoing after the completion of this thesis with the aim for replication studies in larger cohorts of DM participants and an individual genotyping GWAS to be undertaken. Genetic research into DR susceptibility has the potential for a direct impact on patient management by improving DR screening regimes in an individualised manner. Identifying specific genetic markers for those at high-risk of developing sight threatening DR could allow for more refined DR screening algorithms and earlier intervention. Additionally, this may allow for the development of novel DR treatments targeting these genetic pathways, thereby helping to reduce the global burden of this common and debilitating disease.

Appendices

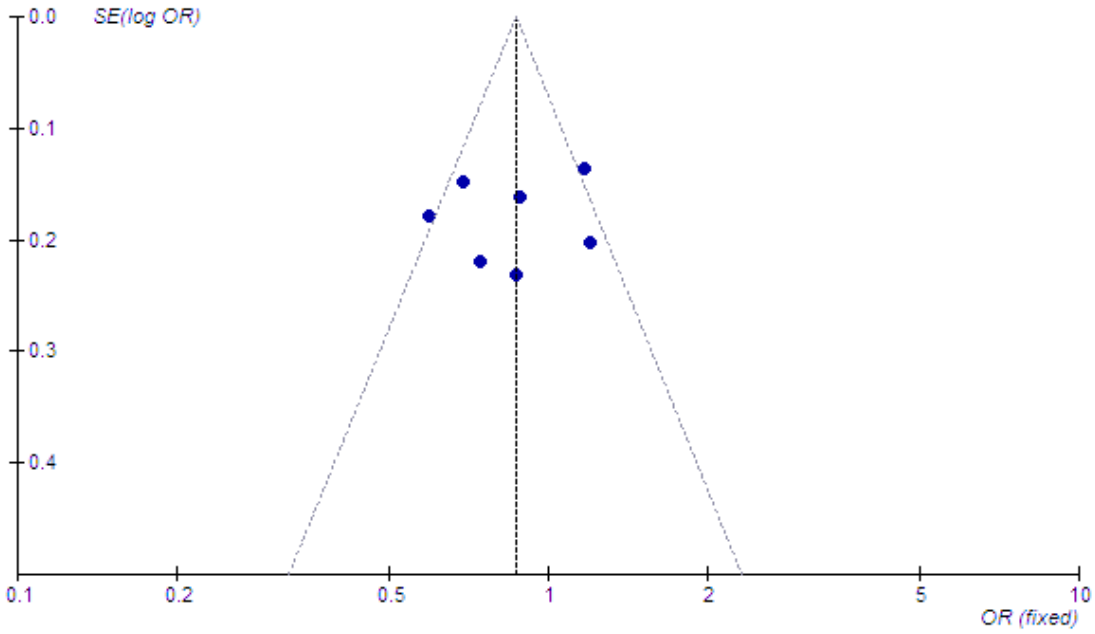
Appendix 1 - Funnel plots for studies of variants examined by a minimum of five studies. The horizontal axes represent the odds ratio and the vertical axes standard error (log(odds ratio)). The dotted lines represent the 95% confidence limits, individual studies are marked by a dot and arrows indicate studies lying outside of the graph. Egger's test has been applied for each variant and a p value provided.

(a) ACE - INS/DEL



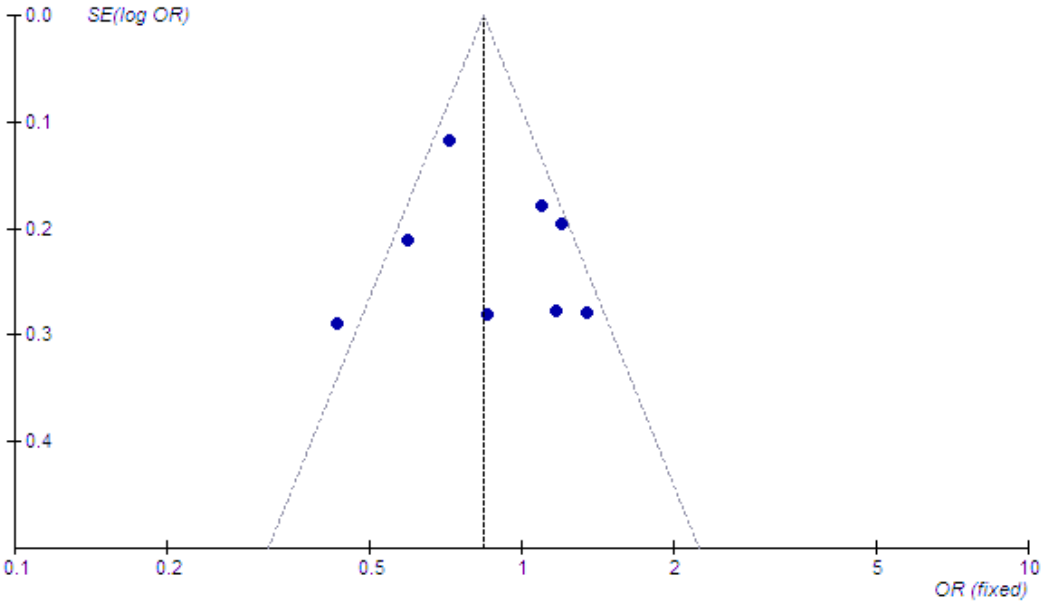
Egger's test p=0.412

(b) VEGF- 2010963



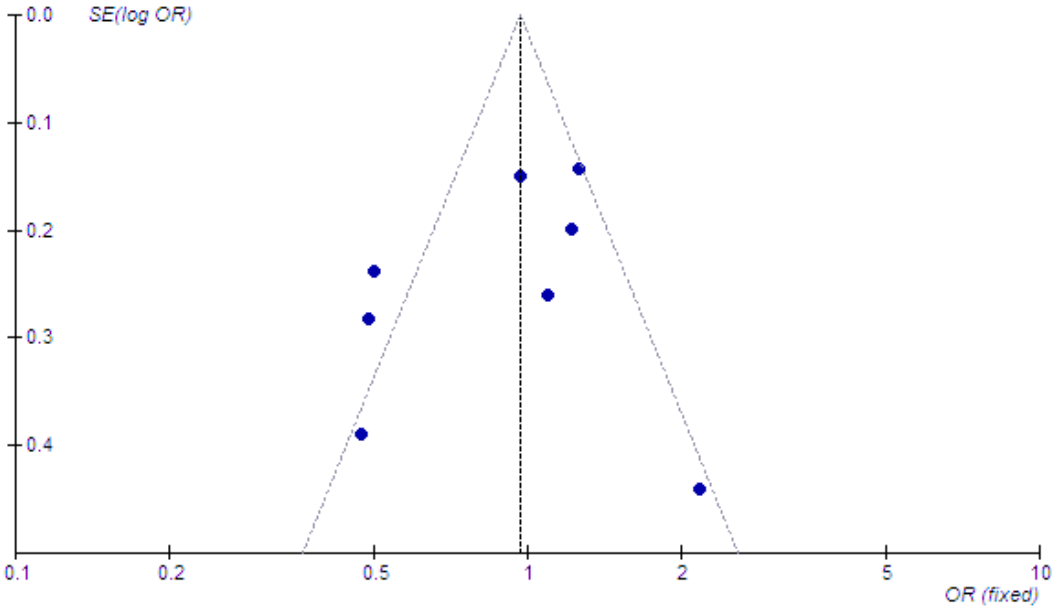
Egger's test p=0.694

(c) NOS3 - rs3138808



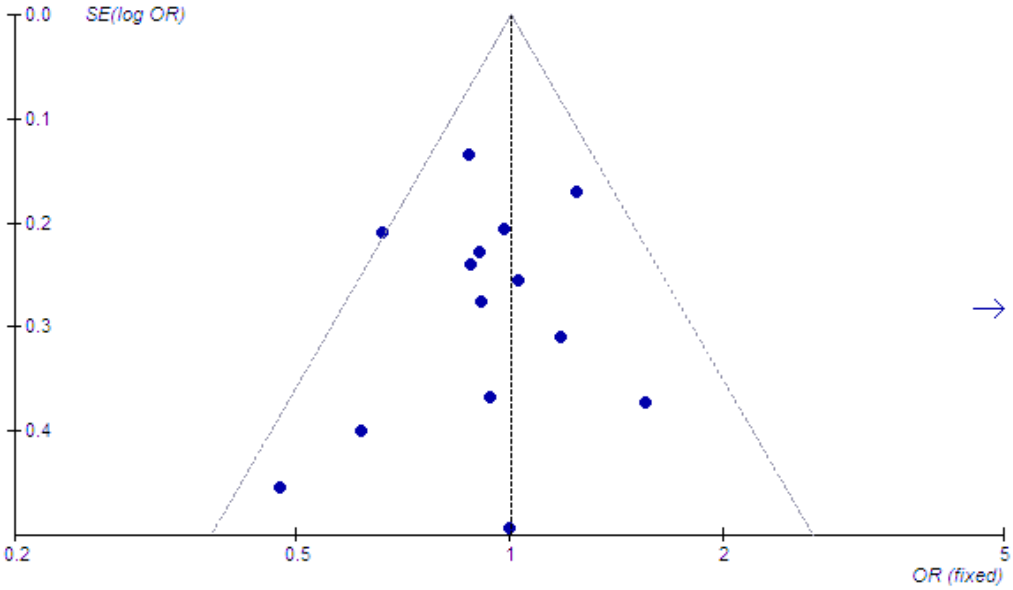
Egger's test p=0.649

(d) AKR1B1- rs759853



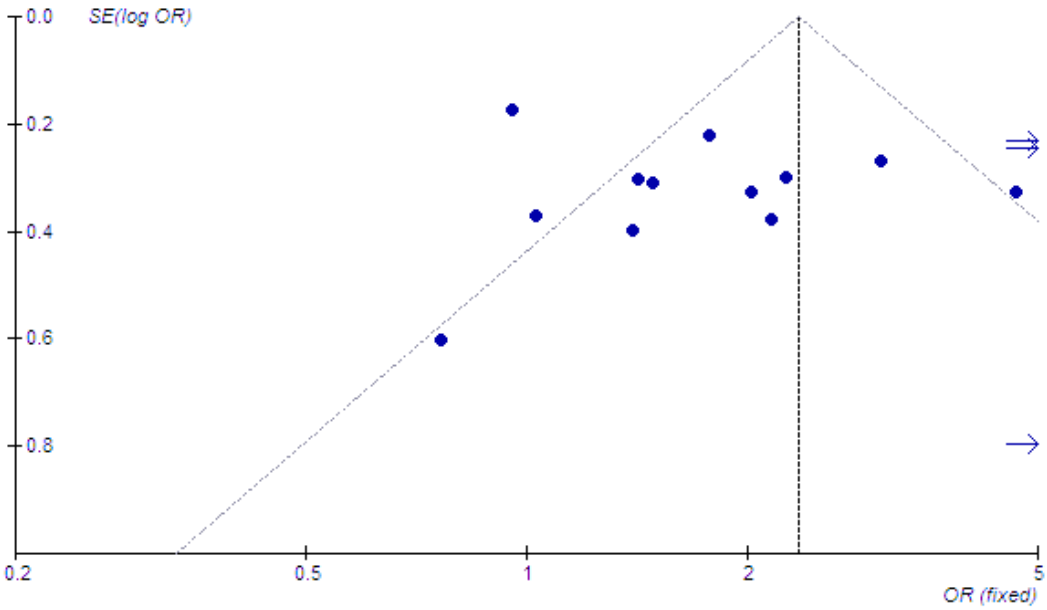
Egger's test p=0.415

(e) AKR1B1 z microsatellite



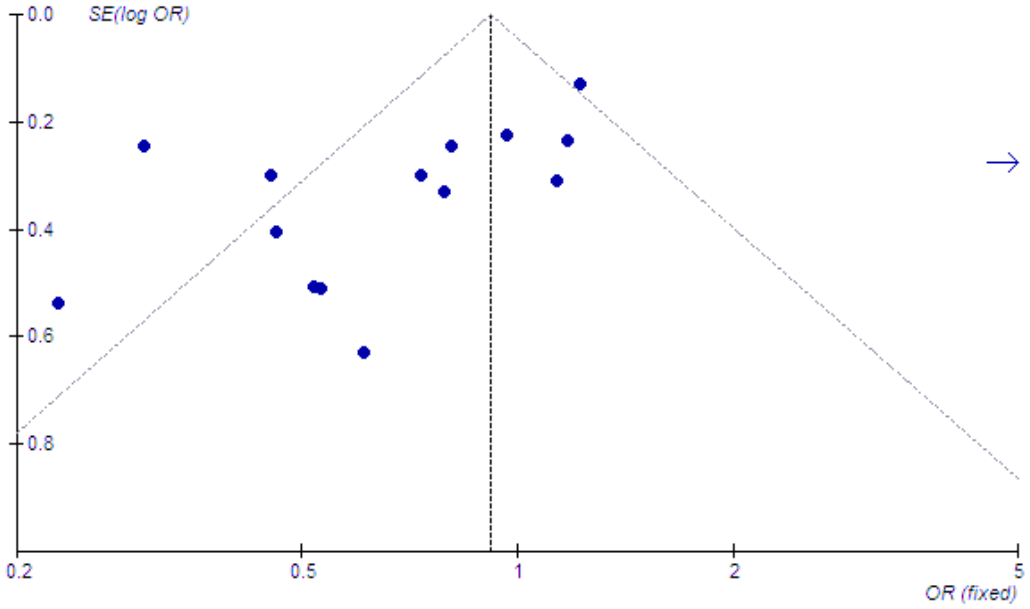
Egger's test p=0.779

(f) AKR1B1 z-2 microsatellite



Egger's test p=0.913

(g) AKR1B1 z+2 microsatellite



Egger's test p=0.208

Appendix 2

(a) Design details of studies examining INS/DEL (*ACE*), rs3138808 (*NOS3*), rs2010963 (*VEGF*), and rs759853 (*AKR1B1*)

Gene	Variant	Risk allele	Study	Ethnicity	Study Design	DM type	Retinopathy Grading	Grading method	Grader	Standardised Scale	Total Subjects	Non-risk allele (%)	Risk allele (%)	OR (95% CI)	HWE deviation	Factors adjusted for in multivariate analyses	Reference
ACE	INS/DEL	287 base pair deletion	Agardh et al. 2004	Caucasian (Swedish)	Case:control	1	NPDR	Fundus photography	Not specified	ETDRS	48	40 (42)	56 (59)	1.19 (0.53−2.68)	Data not provided	Nil	97
			Marre et al. 1994	Caucasian (French)	Case:control, cross-sectional	1	PDR	Not specified	Ophthalmologist	Not specified	84	67 (40)	101 (61)	0.77 (0.41−1.46)	Data not provided for all subjects	Nil	98
			Nagi et al. 1995	Caucasian (British)	Case:control	1	DR (no breakdown)	Ophthalmoscopy	Ophthalmologist	Nil	186	179 (48)	193 (52)	0.90 (0.60−1.35)	Data not provided	Nil	99
			Rabensteiner et al. 1999	Caucasian (Austrian)	Case:control	1	NPDR, PDR	Not specified	Not specified	Modified Airlie House	255	243 (48)	267 (53)	1.38 (0.95−2.01)	No	Disease duration and HbA1c	100
			Tarnow et al. 1995	Caucasian (Danish)	Case:control	1	PDR	Fundus photography	Not specified	Nil	222	189 (43)	255 (58)	1.00 (0.67−1.51)	No	Nil	101
			Van Ittersumet al. 2000	Caucasian (Dutch)	Cross-sectional	1	DR (no breakdown)	Not specified	Ophthalmologist	Nil	252	267 (53)	237 (48)	1.07 (0.75−1.53)	Data not provided	Not specified	102
			Araz et al. 2001	Turkish	Case:control, cross-sectional	2	DR (no breakdown)	Direct ophthalmoscopy, biomicroscopy and fluroescein angiography	Not specified	Nil	239	203 (42)	275 (58)	1.00 (0.70−1.44)	No	Nil	103
			Degirmenci et al. 2005	Turkish	Case:control, cross-sectional	2	DR (no breakdown)	Fundus examination and fluroescein angiography	Not specified	Nil	140	123 (44)	157 (57)	1.28 (0.79−2.08)	Data not provided	Nil	104
			Fujisawa et al. 1995	Japanese	Cross-sectional	2	NPDR, PDR	Not specified	Not specified	Not specified	267	334 (63)	200 (38)	1.10 (0.69−1.77)	Data not provided	Nil	105
			Globocnik-Petrovic et al. 2003	Caucasian (Slovenian)	Case:control	2	NPDR, PDR	Direct ophthalmoscopy and fundus photography	Not specified	ETDRS	204	211 (52)	197 (49)	0.97 (0.66−1.45)	No	Age	106
			Matsumoto et al. 2000	Japanese	Case:control, cross-sectional	2	DR (no breakdown)	Funduscopy and fluorescein angiography	Ophthalmologist	Nil	210	245 (58)	175 (42)	1.22 (0.83−1.82)	No	Nil	107

Appendix 2 (a) continued

Gene	Variant	Risk allele	Study	Ethnicity	Study Design	DM type	Retinopathy Grading	Grading method	Grader	Standardised Scale	Total Subjects	Non-risk allele (%)	Risk allele (%)	OR (95% CI)	HWE deviation	Factors adjusted for in multivariate analyses	Reference
ACE	INS/DEL	287 base pair deletion	Nagi et al. 1995	Caucasian (British)	Case:control	2	DR (no breakdown)	Ophthalmoscopy	Ophthalmologist	Nil	363	350 (48)	376 (52)	0.91 (0.68−1.22)	Data not provided	Nil	99
			Thomas et al. 2003	Chinese	Case:control	2	NPDR, PDR	Not specified	Not specified	Not specified	826	1115 (68)	537 (33)	0.97 (0.79−1.20)	Data not provided	Nil	108
			Crook et al. 2003	Mixed (black and caucasian American)	Cross-sectional	2	NPDR, PDR	Case notes	Not specified	Not specified	46	37 (40)	55 (60)	0.72 (0.28−1.85)	Data not provided	Nil	110
			Isotani et al. 1999	Japanese	Case:control, cross-sectional	2	NPDR, PDR	Not specified	Not specified	Not specified	28	35 (63)	21 (38)	1.37 (0.46−4.13)	No	Nil	112
			Kankova et al. 2001	Caucasian (Czech)	Case:control	2	NPDR, PDR	Direct ophthalmoscopy	Not specified	ETDRS	246	231 (47)	261 (54)	1.10 (0.75−1.63)	No	Nil	111
NOS3	rs3138808	393 base pair insertion	Taverna et al. 2002	Caucasian (French)	Case:control, randomly recruited	1	NPDR	Fundoscopy and fluorescein angiogram	Ophthalmologist	Modified Airlie House	200	337 (84)	63 (16)	0.43 (0.25−0.76)	No	Nil	113
			Awata et al. 2004	Japanese	Case:control	2	NPDR, PDR	Fundoscopy, fundus photography, fluoroscein angiography	Ophthalmologist	Nil	132	100 (49)	104 (51)	0.86 (0.50−1.49)	Data not provided	Sex, age at onset, disease duration, systolic BP, HbA1c, cholesterol, insulin therapy	114
			Chen et alet al. 2007	African	Case:control	2	DR (no breakdown)	Fundoscopy	Ophthalmologist	Nil	369	469 (64)	269 (37)	0.60 (0.40−0.90)	Data not provided	Nil	115
			de Syllos et al. 2006	Brazilian	Case:control	2	DR (no breakdown)	Fundoscopy and fundus photography	Ophthalmologist	Nil	200	338 (85)	62 (16)	1.17 (0.68−2.01)	No	Nil	116

Appendix 2 (a) continued

Gene	Variant	Risk allele	Study	Ethnicity	Study Design	DM type	Retinopathy Grading	Grading method	Grader	Standardised Scale	Total Subjects	Non-risk allele (%)	Risk allele (%)	OR (95% CI)	HWE deviation	Factors adjusted for in multivariate analyses	Reference
NOS3	rs3138808	393 base pair insertion	Ezzidi et al. 2008	Tunisian	Retrospective, case-control	2	DR (no breakdown)	Fundoscopy, fundus photography and fluorescein angiography	Ophthalmologist	Nil	872	1349 (77)	395 (23)	0.72 (0.58−0.91)	No	Age, gender, age of disease onset, HbA1c, hypertension, total cholesterol	117
			Petrovic et al. 2008	Caucasian (Slovenian)	Case:control, cross-sectional	2	DR (no breakdown)	Fundoscopy and fundus photography	Ophthalmologist	ETDRS	426	886 (78)	246 (22)	1.00 (0.76−1.33)	No	Nil	118
			Suganthalakshmi et al. 2006	Indian	Cross-sectional	2	DR (no breakdown)	Fundoscopy	Not specified	Nil	210	355 (85)	65 (16)	1.34 (0.78−2.32)	Data not provided	Nil	119
			Uthra et al. 2007	Indian	Population based, cross-sectional	2	NPDR, PDR	Fundoscopy and fundus photography	Not specified	Modified Airlie House	375	623 (83)	127 (17)	1.19 (0.82−1.76)	Data not provided	Age, gender, disease duration, smoking, HbA1c, BMI, insulin therapy, BP	120
VEGF	rs2010963	G	Awata et al. 2002	Japanese	Case:control	2	PDR	Fundus photography	Ophthalmologist	Nil	268	226 (42)	310 (58)	0.60 (0.42−0.85)	No	Disease duration, systolic BP, insulin therapy	121
			Awata et al. 2005	Japanese	Cross-sectional	2	DR (no breakdown)	Fundoscopy, photography and fluoroscein angiography	Ophthalmologist	International Clinical Diabetic Retinopathy and Macular Edema Disease Severity Scales	375	322 (43)	428 (58)	0.69 (0.52−0.92)	Data not provided	Sex, age, age at onset, disease duration, BP, HbA1c, cholesterol, insulin therapy, BMI	122
			Nakamura et al. 2008	Japanese	Cross-sectional	2	PDR	Fundoscopy and fundus photography	Ophthalmologist	Nil	465	411 (44)	519 (56)	1.17 (0.90-1.53)	No	Age	123
			Petrovic et al. 2008	Caucasian (Slovenian)	Case:control, cross-sectional	2	DR (no breakdown)	Fundoscopy and fundus photography	Ophthalmologist	ETDRS	349	248 (36)	450 (65)	0.89 (0.65-1.22)	No	Diabetes duration, age of onset, insulin therapy	124

Appendix 2 (a) continued

Gene	Variant	Risk allele	Study	Ethnicity	Study Design	DM type	Retinopathy Grading	Grading method	Grader	Standardised Scale	Total Subjects	Non-risk allele (%)	Risk allele (%)	OR (95% CI)	HWE deviation	Factors adjusted for in multivariate analyses	Reference
VEGF	rs2010963	G	Suganthalakshmi et al. 2006	Indian	Cross-sectional	2	DR (no breakdown)	Fundoscopy	Not specified	Nil	210	251 (60)	169 (41)	1.20 (0.81−1.78)	Data not provided	Age	119
			Szaflik et al. 2008	Caucasian (Polish)	Cross-sectional	2	NPDR, PDR	Fundoscopic examination and fluorescein angiography	Not specified	Nil	206	228 (55)	184 (45)	0.74 (0.48-1.14)	Data not provided	Nil	125
			Uthra et al. 2008	Indian	Population based, cross-sectional	2	NPDR, PDR	Fundoscopy and fundus photography	Not specified	Modified Airlie House	199	110 (28)	288 (73)	0.87 (0.55-1.37)	Data not provided	Nil	126
AKR1B1	rs759853	T	Demaine et al. 2000	Caucasian (British)	Case:control	1	DR (no breakdown)	Fundoscopy	Ophthalmologist or endocrinologist	Nil	141	191 (68)	91 (33)	0.49 (0.29−0.85)	No	Nil	127
			Kao et al. 1999	Unspecified	Case:control	1	DR (no breakdown)	Fundus photography	Not specified	Nil	164	199 (61)	129 (40)	0.50 (0.32−0.80)	Data not provided	Nil	128
			Richeti et al. 2007	Brazilian	Case:control	1	NPDR, PDR	Fundoscopy and retinography	Not specified	EDTRS	62	81 (65)	43 (35)	0.47 (0.23−1.02)	No	Nil	129
			Dos Santos et al. 2006	Caucasian-Brazilian	Case:control	2	NPDR,PDR	Ophthalmoscopy and fluorescein angiogram	Ophthalmologist	Nil	424	497 (59)	351 (42)	0.97 (0.73−1.30)	Data not provided	Gender, systolic BP, insulin use, serum creatinine	130
			Dos Santos et al. 2006	African-Brazilian	Case:control	2	NPDR,PDR	Ophthalmoscopy and fluorescein angiogram	Ophthalmologist	Nil	155	216 (70)	94 (31)	1.10 (0.66−1.83)	Yes	Gender, systolic BP, insulin use, serum creatinine	130
			Li et al. 2002	Chinese	Case:control	2	DR (no breakdown)	Not specified	Not specified	Not specified	133	226 (85)	40 (16)	2.16 (0.92−5.12)	Data not provided	Nil	131
			Santos et al. 2003	Euro-Brazilian	Cross-sectional	2	DR (no breakdown)	Ophthalmoscopy and/or biomicroscopy	Ophthalmologist	EDTRS	209	243 (58)	175 (42)	1.22 (0.83−1.81)	Yes	Nil	132
			Wang et al. 2003	Chinese	Cross-sectional	2	DR (no breakdown)	Direct ophthalmoscopy	Ophthalmologist or endocrinologist	Nil	738	1168 (79)	308 (21)	1.26 (0.96−1.67)	Data not provided	Age and duration of disease	133

(b) Design details of studies investigating z, z-2 and z+2 microsatellites of *AKR1B1*

Gene	Variant	Risk allele	Study	Ethnicity	Study Design	DM type	Retinopathy Grading	Grading method	Grader	Standardised Scale	Reference
AKR1B1	(CA)n dinucleotide repeat	z, z-2, z+2	Demaine et al. 2000	Caucasian (british)	Case:control	1	DR (no breakdown)	Fundoscopy	Ophthalmologist or endocrinologist	Nil	127
			Heesom et al. 1997	Caucasian (british)	Case:control	1	DR (no breakdown)	Fundoscopy	Ophthalmologist or endocrinologist	Nil	134
			Kao et al. 1999	Unspecified	Case:control	1	DR (no breakdown)	Fundus photography	Ophthalmologist	Nil	135
			Lajer et al. 2004	Caucasian (Danish)	Case:control	1	PDR	Fundus photography	Not specified	Nil	136
			Richeti et al. 2007	Brazilian	Case:control	1	NPDR, PDR	Fundoscopy and retinography	Not specified	EDTRS	129
			Yamamoto et al. 2003	Japanese	Longitudinal	1	DR (no breakdown)	Fundoscopy	Ophthalmologist	Nil	137
			Kumaramanickavel et al. 2003	Indian	Case:control, cross-sectional	2	DR (no breakdown)	Fundus photography	Not specified	Nil	142
			Fujisawa et al. 1999	Japanese	Case:control	2	PDR	Fundus photography	Ophthalmologist	Nil	138
			Petrovic et al. 2005	Caucasian (Slovenian)	Cross-sectional	2	NPDR, PDR	Fundus photography	Ophthalmologist	Nil	139
			Ikegishi et al. 1999	Japanese	Case:control	2	PDR	Fundus photography	Not specified	Nil	140
			Ko et al. 1995	Chinese	Case:control	2	DR (no breakdown)	Fundus photography	Not specified	Nil	141
			Lee et al. 2001	Chinese	Cross-sectional	2	DR (no breakdown)	Not explained	Not specified	Nil	143
			Park et al. 2002	Korean	Cross-sectional	2	DR (no breakdown)	Not explained	Not specified	Nil	144
			Wang et al. 2003	Chinese	Cross-sectional	2	DR (no breakdown)	Direct ophthalmoscopy	Ophthalmologist or endocrinologist	Nil	133
			Ichikawa et al. 1999	Japanese	Case:control	2	NPDR, PDR	Not specified	Ophthalmologist	Nil	145

Appendix 2 (b) - continued

Gene	Variant	Risk allele	Study	Total subjects	z [n(%)]	OR (95% CI)	z+2 [n(%)]	OR (95% CI)	z-2 [n(%)]	OR (95% CI)	HWE deviation	Factors adjusted for in multivariate analyses	Reference
AKR1B1	(CA)n dinucleotide repeat	z, z-2, z+2	Demaine et al. 2000	229	185 (40)	0.98 (0.65−1.47)	318 (69)	0.30 (0.19−0.49)	160 (35)	3.04 (1.80−5.16)	No	Nil	127
			Heesom et al. 1997	134	105 (39)	1.03 (0.62−1.70)	106 (40)	0.45 (0.25−0.81)	188 (70)	2.26 (1.25−4.08)	Data not provided	Nil	134
			Kao et al. 1999	163	27 (8)	0.47 (0.19−1.16)	134 (41)	0.46 (0.21−1.02)	251 (77)	5.49 (3.39−8.90)	Data not provided	Nil	135
			Lajer et al. 2004	294	234 (40)	1.24 (0.89−1.74)	250 (43)	0.97 (0.62−1.50)	459 (78)	0.96 (0.68−1.35)	Data not provided	Nil	136
			Richeti et al. 2007	64	45 (35)	1.55 (0.75−3.23)	62 (48)	0.52 (0.19−1.40)	89 (70)	1.03 (0.50−2.13)	No	Nil	129
			Yamamoto et al. 2003	101	61 (30)	1.18 (0.65−2.17)	110 (54)	1.14 (0.62−2.09)	104 (51)	1.39 (0.64−3.04)	Data not provided	Nil	137
			Kumaramanickavel et al. 2003	170	119 (35)	0.91 (0.58−1.42)	174 (51)	0.81 (0.50−1.31)	188 (55)	1.42 (0.78−2.57)	Data not provided	Nil	142
			Fujisawa et al. 1999	205	146 (36)	0.66 (0.44−1.00)	248 (60)	0.73 (0.41−1.31)	205 (50)	1.78 (1.15−2.74)	Data not provided	Nil	138
			Petrovic et al. 2005	61	53 (43)	0.94 (0.46−1.93)	54 (44)	0.23 (0.08−0.66)	76 (62)	0.77 (0.24−2.49)	Data not provided	Nil	139
			Ikegishi et al. 1999	44	22 (25)	1.00 (0.38−2.62)	44 (50)	0.53 (0.19−1.45)	46 (52)	8.81 (1.85−41.88)	Data not provided	Nil	140
			Ko et al. 1995	214	88 (21)	0.88 (0.55−1.41)	210 (49)	1.17 (0.74−1.86)	234 (55)	2.02 (1.06−3.85)	No	Nil	141
			Lee et al. 2001	384	59 (8)	5.96 (3.43−10.35)	128 (17)	7.35 (4.30−12.58)	663 (86)	4.68 (2.46−8.88)	Data not provided	Nil	143
			Park et al. 2002	127	113 (44)	0.91 (0.53−1.57)	178 (70)	0.79 (0.41−1.50)	95 (37)	1.49 (0.81−2.73)	Data not provided	Nil	144
			Wang et al. 2003	738	416 (28)	0.88 (0.67−1.14)	374 (25)	1.22 (0.95−1.57)	1128 (76)	13.5 (8.57−21.27)	Data not provided	Age and duration of disease	133
			Ichikawa et al. 1999	87	32 (18)	0.62 (0.28−1.35)	114 (66)	0.61 (0.18−2.09)	72 (41)	2.16 (1.03−4.53)	Data not provided	Nil	145

Appendix 3

Participant questionnaire administered and clinical data obtained for the Genetic Study of Diabetic Retinopathy

The Genetic Study of Diabetic Retinopathy

Please affix patient's sticker here

Date

FirstName | Surname | Sex

DOB | FMC UR | Group | Group No

Address

HomeTel | Patient's country of birth

WorkTel | Mother's country of birth

Mobile | Father's country of birth

Who is your GP? | ... your Endocrinologist? | ... your Eye Dr?:

Diabetes Type | Year of Diagnosis

Please tick if yes

Does anyone in your family have diabetes? ☐ If yes please list their relationship to you:

Do you have high blood pressure? ☐

Do you have high cholesterol? ☐

Do you have any kidney disease ? ☐

Have you lost any sensation in your legs (peripheral neuropathy)? ☐

Do you have any peripheral vascular disease? ☐

Have you had a heart attack or angina? ☐

Have you ever had a stroke or TIA (mini stroke)? ☐

Have you ever smoked? ☐

Have you stopped smoking? ☐

when did you cease smoking?

For how many years did (or have) you smoke(d)?

How many cigarettes do / did you smoke each day?

Do you drink any alcohol? ☐

How many standard drinks do you have: Per week / Per month / Per year

Have you had cataracts or cataract surgery? ☐

Do you have macular degeneration? ☐

Do you have glaucoma? ☐

Have you had a retinal detachment? ☐

Have you had a vitreous hemorrhage ☐

Have you had rubeosis/iris neovascularisation ☐

Have you had any operations or laser treatment to your eyes? (please specify)

Please leave blank

Recent Laboratory Results

Most recent total Cholesterol

Most recent HDL level

Most recent LDL level

Most recent triglycerides

Most recent urinary Albumin

Most recent Alb:Creat Ratio

Most recent serum Creatinine

Most recent HbA1C

Current weight

Height

Blood pressure /

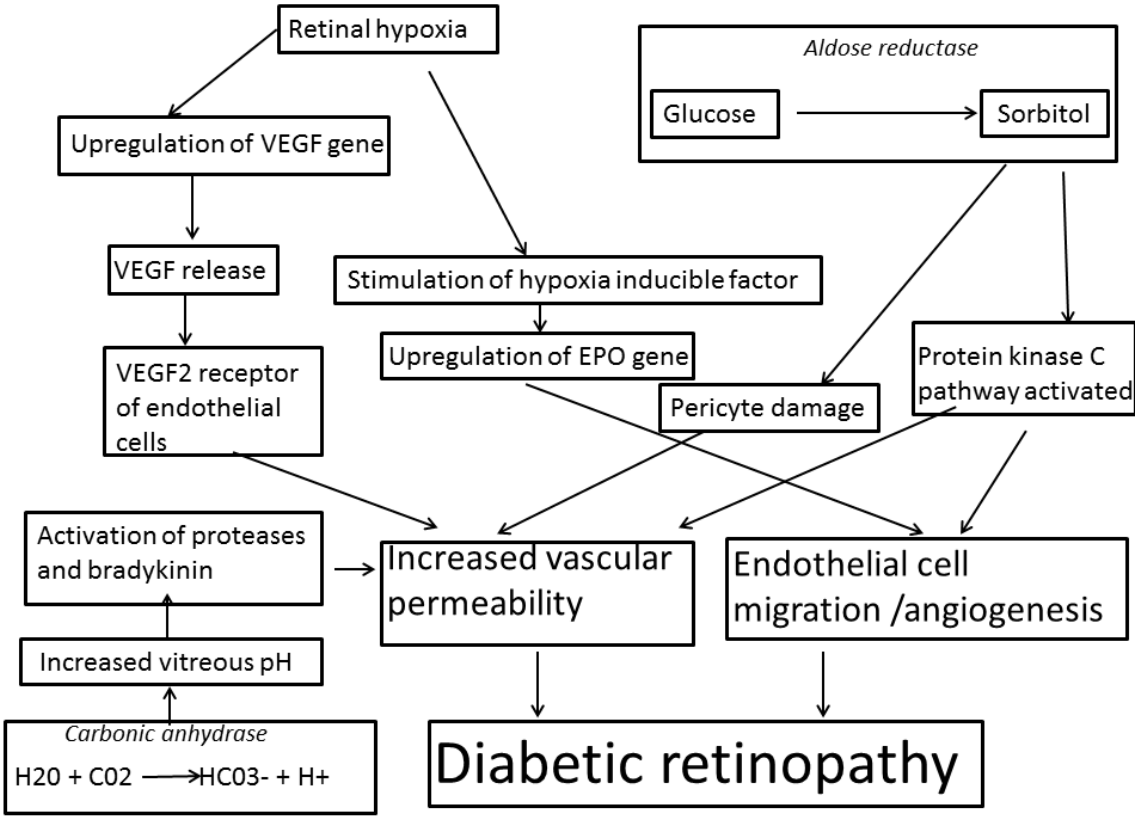
Ocular Diabetic Complications

	R	L	Group
No DR	☐	☐	☐
Minimal NPDR	☐	☐	☐
Mild NPDR	☐	☐	☐
Moderate NPDR	☐	☐	☐
Severe NPDR	☐	☐	☐
PDR	☐	☐	☐
Macular Oedema	☐	☐	☐
CSMO	☐	☐	☐
Focal laser	☐	☐	
Macular grid laser	☐	☐	
PRP	☐	☐	

Year of DR development

Appendix 4

Summary of candidate gene pathways and the development of diabetic retinopathy



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