**Aldose reductase gene polymorphisms and diabetic retinopathy susceptibility**

**Short running title:** Aldose reductase gene and diabetic retinopathy

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**Objective:** Aldose reductase (ALR) is involved in diabetic microvascular damage via the polyol pathway. A recent meta-analysis found genetic variation in the ALR gene (**AKR1B1**) to be significantly associated with diabetic retinopathy (DR). We aimed to investigate the genetic association of **AKR1B1** with DR.

**Research Design and Methods:** 909 individuals with diabetes mellitus were enrolled. Participants were genotyped for an **AKR1B1** (CA)n microsatellite, and 14 tag single nucleotide polymorphisms and ophthalmological assessment performed.

**Results:** 514 individuals were found to have DR. Rs9640883 was significantly associated with DR (p=0.0005). However, **AKR1B1** variation was not independently associated with DR development after adjusting for relevant clinical parameters. Rs9640883 was associated with duration of diabetes (p=0.002).

**Conclusion:** Many previous reports have failed to account for known risk factors for DR. The commonly reported association of **AKR1B1** with DR may be due to an association of the gene with younger age of onset of diabetes.
The polyol pathway is involved in microvascular damage, a hallmark of diabetic retinopathy (DR). Aldose reductase (ALR) is the first and rate limiting enzyme in the polyol pathway and pathogenic vascular and hemodynamic changes contributing to DR can subsequently occur as a result of sorbitol accumulation, oxidative damage and protein kinase C activation(1-3). Over 160 candidate gene studies have been reported for DR, and their recent meta-analysis found genetic variation in the ALR gene (AKR1B1) to be the most significantly associated with DR(4). We aimed to determine whether genetic variation in the AKR1B1 gene was associated with DR in a large cohort of Australian patients with type 1 or 2 diabetes mellitus.

RESEARCH DESIGN AND METHODS
Following approval from relevant Human Research Ethics Committees, participants diagnosed with type 1 diabetes (n=271) or type 2 diabetes (n=638) with a minimum of 5 years duration were recruited from ophthalmology and endocrinology outpatient clinics of three tertiary hospitals in Adelaide, South Australia. Retinopathy status for the worst eye was graded according to the Early Treatment Diabetic Retinopathy Study criteria(5). If either eye had clinically significant macular edema (CSME), irrespective of other DR gradings, the participant was also classified as having CSME. Blinding DR was defined as severe non-proliferative DR (NPDR), or proliferative DR (PDR), or CSME.

Blood pressure (BP), body mass index, renal function tests, serum cholesterol and HbA1c levels were obtained. Hypertension was defined as BP ≥140/90 mmHg, or on antihypertensive medication at recruitment. Hypercholesterolemia was defined as total cholesterol of >5.5 mmol/L, or use of lipid lowering medication. Albuminuria was defined as urine albumin ≥30mg/day.

The AKR1B1 (CA)n microsatellite was PCR amplified using fluorescently labelled primers published by Ko et al(6) in 883 individuals (263 type 1 and 620 type 2 diabetes) and genotyped by electrophoresis on an ABI PRISM 3100 (Applied Biosystems, USA). Using the tagger program implemented in Haplovie 4.0, 14 tag SNPs across the AKR1B1 gene, including the promoter region were selected and genotyped in 909 individuals (271 type 1 and 638 type 2 diabetes) using iPLEX Gold chemistry on an autoflex Mass Spectrometer (Sequenom, USA).

The chi-square test for categorical and univariate binary logistic regression for continuous clinical covariates with DR were calculated in SPSS (v15.0 SPSS Inc, Chicago). Allelic and genotypic associations of the (CA)n microsatellite were calculated using the chi-square test and multivariate analysis with the binary logistic regression controlling for associated variables. Testing for association of all SNPs and haplotypes with DR was undertaken with the chi-square test for univariate analysis and binary logistic for multivariate analysis in PLINK (v1.06)(7) and also CLUMPHAP(8) when microsatellites were incorporated into haplotype analyses.

Bonferroni correction was applied to microsatellite and haplotype analyses. Multiple testing of individual SNPs was adjusted for using the Single Nucleotide Polymorphism Spectral Decomposition method of Nyholt(9), modified by Li and Ji(10), which estimated 10 independent tests.

RESULTS
A total of 514 participants had DR, of which 311 had NPDR (95 type 1 and 216 type 2 diabetes), 188 had PDR (71 type 1 and 117
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4 type 2 diabetes), and 150 had CSME (36 type 1 and 114 type 2 diabetes).

Thirteen alleles (120-144 bp) for the AKR1B1 microsatellite were present in our cohort. The 138 bp allele (24 repeats) was designated as the z allele. The z-10/z-8 genotype was significantly associated with blinding DR in type 1 diabetes (p=0.008) and remained significant in the multivariate analysis (p=0.001), and after correction for multiple testing (p=0.007). However, only 2 and 6 participants with no DR and blinding DR respectively carried this genotype and the significance of this result is unclear. No association of the microsatellite with DR was detected in type 2 diabetes.

Rs9640883 was significantly associated with DR in combined diabetes (p=0.0005 OR:1.62, 95%CI:1.24-2.13), and type 2 diabetes (p=0.002, OR:5.73, 95%CI:4.26-7.69) under the dominant model. This remained significant (p<0.005) after correction for multiple testing. Haplotype analyses revealed no associations with DR. No tag SNP remained associated with DR after adjustment for associated covariates for type 1 diabetes duration, hypertension, albuminuria and hypercholesterolemia) and type 2 diabetes (sex, diabetes duration, hypertension, and HbA1c, Table 1). Each SNP was subsequently tested for association with each individual covariate in a univariate analysis. Of note, rs9640883 was associated with duration of diabetes under a dominant model (p=0.014), particularly in the type 2 diabetes cohort (p=0.002).

DISCUSSION

There have been numerous studies assessing polymorphisms of the AKR1B1 gene and DR susceptibility, with (CA)n microsatellite and rs759853 most commonly studied. A recent meta-analysis found the z+2 allele in type 1, and z-2 allele in any type of diabetes conferred protection from and risk for DR, respectively. whilst the C allele of rs759853 conferred risk for DR in type 1 diabetes(4).

This study examined the (CA)n microsatellite and 14 tag SNPs. Although AKR1B1 variation was associated with DR, once established risk factors, including diabetes duration and HbA1c were considered, no association remained. This suggests particular SNPs may be associated with the clinical covariates rather than having a direct association with DR.

We found the DR associated SNP rs9640883 to also be associated with duration of diabetes. ALR reduces toxic aldehydes generated by reactive oxygen species to inactive alcohols. Decreased availability of the cofactor NADPH could induce or exacerbate intracellular oxidative stress(1). Chronic hyperglycemia and oxidative stress can result in permanent irreversible damage to pancreatic beta cells(11). Subsequent deterioration of beta cell function and increased disease severity results, with animal studies providing support for this hypothesis(12; 13). Variation in ALR activity may affect the extent of oxidative stress, and genetic variation in AKR1B1 may account for altered ALR activity. The association observed between rs9640883 and DR, and those previously reported for this gene may reflect the effect this gene has on age of onset of diabetes and therefore on diabetes duration, in turn influencing DR risk(14; 15).

The majority of previous studies have not undertaken multivariate analysis to consider known risk factors for DR. They may be influenced by the same confounding effect of duration of diabetes observed in this study.

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The authors declare no conflict of interest.

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10. Li J, Ji L: Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity* 95:221-227, 2005
Table 1 – Associations of *AKR1B1* tag SNPs with any DR by type of diabetes.

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

1 = adjusted for age, diabetes duration, hypertension, albuminuria and high cholesterol.

2 = adjusted for sex, diabetes duration, hypertension and HbA1c.

Note: p-values shown have not been corrected for multiple testing.