Decline in Neurophysiological Function After 7 Years in an Adolescent Diabetic Cohort and the Role of Aldose Reductase Gene Polymorphisms

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OBJECTIVE — This 7-year longitudinal study examines the potential impact of aldose reductase gene (AKR1B1) polymorphisms on the decline of nerve function in an adolescent diabetic cohort.

RESEARCH DESIGN AND METHODS — Patients with type 1 diabetes ($n = 262$) were assessed with three cardiovascular autonomic tests (heart rate variation during deep breathing, Valsalva maneuver, and during standing from a lying position) and pupillometry (resting pupil diameter, constriction velocity, and reflex amplitude), thermal, and vibration thresholds on the foot. Genotyping was performed for promoters (C-106T and C-12G), $(CA)_n$ dinucleotide repeats, and intragenic BamH1 polymorphism.

RESULTS — Median time between first and last assessment was 7.0 years (interquartile range 5.1–11.1), with a median of five assessments (four to seven) per individual. At first assessment, median age was 12.7 years (11.7–13.9), median duration was 5.3 years (3.4–8.0), and median HbA_{1c} was 8.5% (7.8–9.3). All tests declined over time except for two cardiovascular autonomic tests and vibration discrimination. Faster decline in maximum constriction velocity was found to associate with the Z-2 allele ($P = 0.045$), Z-2/Z-2 ($P = 0.026$). Slower decline in hot thermal threshold discrimination associated with Z+2 ($P = 0.044$), Z+2/Z+2 ($P < 0.0005$), Z+2/T ($P = 0.038$), and bb ($P = 0.0001$).

CONCLUSIONS — Most autonomic and quantitative sensory nerve testings declined over time. AKR1B1 polymorphisms were strongly associated with the rate of decline of these complications.

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Diabetic neuropathy is a common cause of morbidity and mortality among patients with diabetes (1,2). Peripheral neuropathy predisposes to foot ulceration and limb amputation, whereas autonomic neuropathy has been associated with an increased risk of sudden death and increased mortality (3,4). The duration and level of hyperglycemia are important determinants in the development of neuropathy (5). However, despite cross-sectional studies (6) indicating abnormal function, there are only relatively short longitudinal studies (7,8) of autonomic and peripheral nerve function in adolescents with type 1 diabetes.

Evidence is accumulating to support functional polymorphisms of the aldose reductase gene (AKR1B1) playing a part in individuals developing neuropathy (9–11). Aldose reductase, the first and rate-limiting enzyme of the pathway, catalyzes the NADPH-dependent reduction of glucose to sorbitol. Increased flux through this polyol pathway leads to intracellular accumulation of sorbitol and various metabolic imbalances including enhanced oxidative stress (12–14).

A $(CA)_n$ dinucleotide repeat sequence 2.1 kb upstream of the transcription site (Z-2) and a single nucleotide polymorphism situated in the promoter region (C-106T) have been found to associate with retinopathy, nephropathy, and neuropathy in type 1 diabetic patients (9,10,15–17). Only one association study (11) has examined the role of these polymorphisms in longitudinal analysis in type 2 diabetes with controversial results for the C-106T locus.

This 7-year longitudinal study examines the potential impact of polymorphisms of the AKR1B1 gene on the decline of peripheral sensory and autonomic nerve function in an adolescent type 1 diabetic cohort.

RESEARCH DESIGN AND METHODS

— Patients were Australian adolescents with type 1 diabetes attending their annual complications screening at the Children's Hospital at Westmead. Inclusion criteria were two or more neuropathy assessments performed over a minimum of 4 years, with the median time between the first and last assessment of 7.0 years (interquartile range 5.1–11.1). Two hundred and sixty-two patients satisfied these criteria. Genomic DNA was extracted from white blood cells for the genotyping of the AKR1B1 gene. All patients gave informed consent, and the study was approved by the ethics committee of the Children's Hospital at Westmead, Sydney, Australia.

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Abbreviations: DBHRV, heart rate variation during deep breathing.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—*Correlation coefficients between genotypes and two autonomic and two peripheral nerve function tests and allele frequencies*

		Complication			
Predictor	Frequency	Maximum constriction velocity	DBHRV	Left foot cold threshold	Left foot hot threshold
Z-2/Z-2	37/262	−0.37 (0.026)			
Z-2 allele	152/262	−0.25 (0.045)			
ZZ/−106CC	29/250		2.40 (0.029)		
Z+2/Z+2	6/262			−0.061 (0.047)	−0.36 (<0.0005)
Z+2 allele	62/262				−0.16 (0.044)
Z+2/−106T	43/250				−0.18 (0.038)
bb (BamH1)	107/232				−0.28 (0.001)

P values were indicated in parentheses. All coefficients were adjusted for time effect, and for the outcome of hot threshold, sex adjustment was also applied. Number of subjects with both $(CA)_n$ dinucleotide polymorphism and C-106T available was 250. Intragenic BamH1 polymorphism data was available in 232 patients.

Nerve function assessment

Cardiovascular autonomic function was assessed by the measurement of heart rate variation during deep breathing (DBHRV), the Valsalva maneuver, and during standing from a lying position (30-to-15 ratio). Pupillometry was performed in the left eye after 5 min of dark adaptation with an infrared computerized pupillometer (Pupilscan; Fairville Medical Optics, Amersham, Buckinghamshire, U.K.). The phasic light reflex was measured in response to a light stimulus of 25-foot candles by recording pupillary diameter over the next 3 s. Resting pupil diameter was measured, and the maximum constriction velocity and reflex amplitude were calculated.

Thermal thresholds for hot and cold were determined on the dorsum of the left foot using a thermal threshold tester (Medelec, Old Working, U.K.), and vibration thresholds were determined at the large toe and medial malleolus of the left foot (Biothesiometer; Biomedical Instrument, Newbury, OH), as previously described, using reference ranges derived for 11- to 19-year-old subjects (18,19).

Analysis of AKR1B1 gene

Genomic DNA was extracted from white blood cells for genotyping of the AKR1B1 gene for the following polymorphisms: AKR1B1 promoters (C-106T and C-12G), $(CA)_n$ dinucleotide repeats (microsatellites), and intragenic BamH1 polymorphism.

The 263-bp fragment from the promoter region, containing two single nucleotide polymorphisms (C-106T and C-12G), and the $(CA)_n$ dinucleotide repeat were amplified for all 262 patients as previously described (20,21). The BamH1 polymorphism was identified by using the restriction enzyme BamH1 (NEB). After digestion, the B-allele (wild type) yielded the undigested 252-bp fragment and the b (A–C substitution)-allele yielded 174- and 78-bp fragments (22).

Genotyping for all polymorphisms was repeated on a second occasion to ensure interexperiment consistency. To ensure intersampling consistency, two blood samples were obtained, on two separate occasions from 13 patients, and genotypes were identical.

Statistical analysis

Longitudinal analysis was performed by using generalized estimating equations with all available data points included for each patient in SPIDA software (version 6; Statistical Computing Laboratory, Sydney, Australia). All neurological outcome variables (three pupillary, three cardiovascular, and two peripheral nerve) were analyzed continuously, with the effect modification of genotypes/alleles on changes in nerve function evaluated over time. The effect of genotypes/alleles was assessed with adjustment for duration, time, sex, HbA_{1c} (A1C), and blood pressure percentiles when appropriate. Statistical significance was defined as $P < 0.05$.

RESULTS

Patient characteristics

At first assessment of 262 patients (114 male patients, 45%), the median age was 12.7 years (interquartile range 11.7–13.9), median duration was 5.3 years (3.4–8.0), and median A1C was 8.5% (7.8–9.3). Ethnic background was Caucasian (n = 234, 89%), Middle Eastern (10, 4%), Asian (9, 3%), or other. Over the median follow-up of 7 years, the median number of assessments per patient was five (four to seven) and A1C was 8.5% (7.6–9.3). At last assessment, their median age was 20.1 years (17.9–24.0) and median duration of diabetes was 13.5 years (9.9–17.4). Mild to moderate background retinopathy was present in 126 of 257 (49%) and microalbuminuria in 11 of 245 (4.5%) subjects.

Nerve testing

All three pupillary function tests and two peripheral nerve tests declined over time. Resting pupil diameter reduced from 6.25 to 5.91 mm ($P = 0.003$), reflex amplitude reduced from 2.07 to 1.73 mm ($P < 0.0005$), and maximum constriction velocity decreased from 6.23 to 5.33 mm/s ($P < 0.0005$). Thermal discrimination declined over time, with the hot threshold increasing from 0.31 to 0.74°C ($P < 0.0005$) and cold threshold increasing from 0.12 to 0.21°C ($P < 0.013$). Vibration thresholds did not change over time. Only one cardiovascular autonomic test, DBHRV, declined from 35 to 30 beats/min ($P < 0.0005$). At last assessment, 44% had abnormal pupillary tests, 14% had abnormal cardiovascular autonomic tests, 30% had abnormal thermal threshold tests, and 7% had abnormal vibration thresholds.

Genetic associations

The Z-2 allele of the $(CA)_n$ repeat polymorphic marker was more common in this study population compared with the Z+2 allele (58 vs. 24%, respectively) (Table 1). This is in agreement with published results from other type 1 and type 2 diabetic populations with microvascular complications (11,15,23).

Over time, patients with at least one copy of the Z-2 allele had slower maximum constriction velocity, indicative of worse autonomic nerve function, compared with patients without the Z-2 allele (Fig. 1, $P = 0.045$). Patients who were homozygous for the Z-2 allele had reduced maximum constriction velocity compared with patients without the Z-2 allele ($P = 0.026$).

For peripheral sensory nerve tests, the presence of the Z+2 allele was associated with a smaller increase in thermal threshold for heat, indicating better sen-

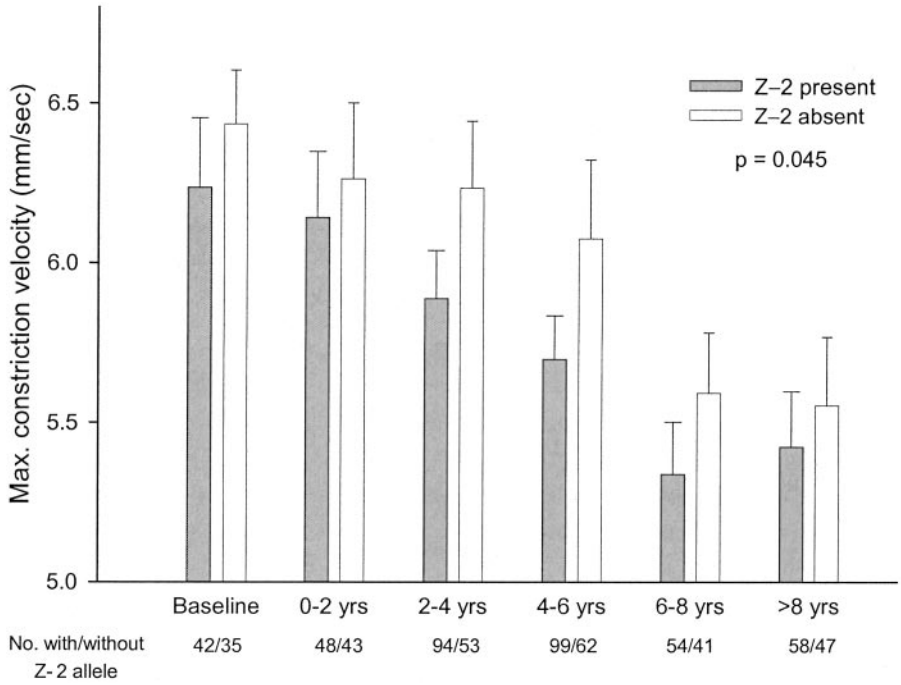

Figure 1—*Maximum constriction velocity of the pupil and the effect of the Z-2 allele. Data are presented as means ± SD. Baseline refers to first assessment for complications. Effects of time and Z-2 were significant (*P *< 0.0005 and* P *= 0.045, respectively).*

sory nerve function, compared with other microsatellite alleles over time (Fig. 2). In addition, a smaller increase in hot thermal threshold was also found significantly associated with Z+2/Z+2, Z+2/-106T, and bb (BamH1) alleles.

The genotype ZZ/-106CC had a smaller reduction in DBHRV, indicating better autonomic nerve function compared with those without the genotype. Similarly, the Z+2/Z+2 genotype was associated with a smaller increase in cold threshold (Table 1). The promoter polymorphism C-12G was not associated with any difference in nerve function test results.

CONCLUSIONS — In this study of 262 adolescents with median diabetes

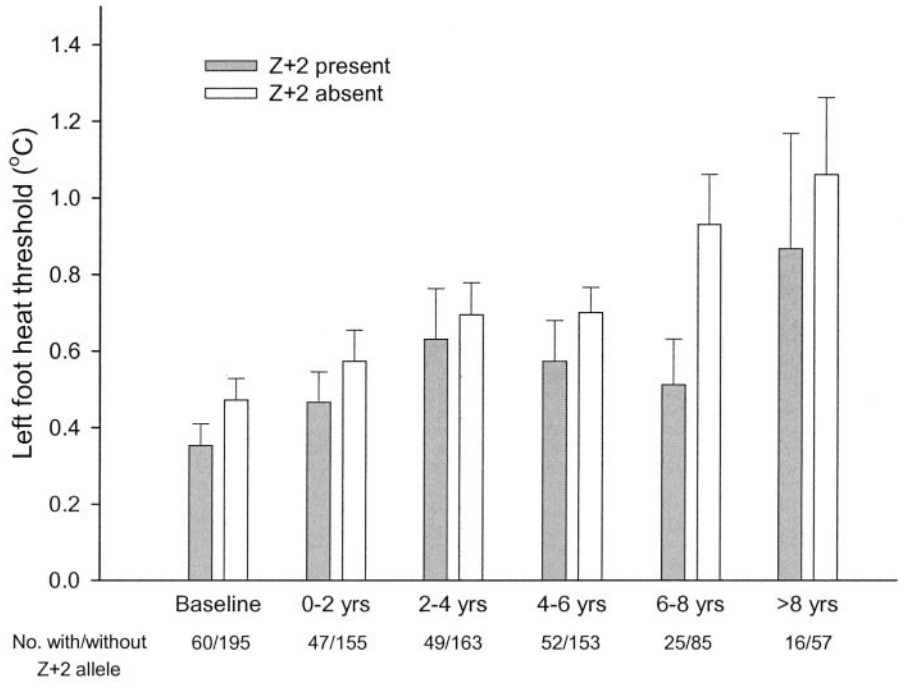

Figure 2—*Left foot heat threshold and the effect of the Z+2 allele. Data are presented as means ± SD. Baseline refers to first assessment for complications. Predictors of increase in thermal heat threshold were time (*P *< 0.0005) and the absence of Z+2 (*P *= 0.044), using generalized estimating equations.*

duration of 6 years at baseline, neurophysiological function declined during follow-up. Both autonomic and quantitative sensory nerve function declined over a median of 7 years. Polymorphisms in the AKR1B1 gene were significantly associated with both increased and decreased rates of decline of neurological complications in these patients.

The nerve function decline may in part be due to an age effect. When reference ranges were derived in our unit in adolescents aged 11–19 years, DBHRV was negatively correlated with age, but no such correlation was found for the other variables measured (18,19). This study does show a decline in nerve function in patients with type 1 diabetes in this age-group (12–24 years), using noninvasive tests, specifically DBHRV, the resting pupil size, and the phasic light response and discrimination thresholds for hot and cold. Vibration discrimination did not decline, but the frequency of abnormalities was low, measured by biothesiometry, suggesting that this test is not as sensitive as thermal discrimination in this young age-group.

From our results, it was evident that pupillometry measurements were more likely to deteriorate over time than the cardiovascular autonomic measurements. Of the cardiovascular tests, DBHRV was more sensitive than the other two measurements. This is in agreement with the Diabetes Control and Complications Trial, in which intensive therapy significantly slowed the decline in DBHRV but not the Valsalva maneuver (5). Others (7,24,25) have found pupillary tests, both resting and dynamic tests, to be more sensitive than cardiovascular tests to a diabetes effect. Our data also could be interpreted as evidence that the cardiovascular autonomic nervous system is affected later than the pupil.

The reported quantitative sensory tests are easier to administer and less invasive than nerve conduction velocity studies. Thermal threshold discrimination tests small unmyelinated and thinly myelinated fibers. These tests are independent of the observer/operator but are limited by the subject's attention, motivation, and cooperation. However, any variation is unlikely to have contributed systematically to the increase in abnormalities over time. Indeed, a learned effect from frequent testing should show improvement and not deterioration of the tests. Furthermore, the operators were blinded to patient's AKR1B1 genotypes.

Patients with the Z-2 allele of the $(CA)_n$ repeat polymorphism exhibited greater decline in maximum constriction velocity of pupil, i.e., worse autonomic nerve function, compared with patients without the Z-2 allele. Conversely, patients with the Z+2 allele or Z+2 with the −106T genotype showed attenuated decline in thermal discrimination, i.e., better nerve function, compared with patients lacking these alleles. In addition, the bb genotype of the intragenic BamH1 polymorphism also significantly associated with slower decline of hot thermal discrimination.

Most of the observed modifications of neurological responses support Z-2 as being the "susceptible" allele and Z+2, Z+2/T, and bb as being the "protective" allele/genotypes. This is in agreement with previous cross-sectional association studies (9,16,22,23) of microvascular complication studies and, more specifically, in neuropathy. Cross-sectional studies, particularly in patients with shorter diabetes duration, can provide only limited information on the association between complications and genes.

This is the first longitudinal genetic association study in relation to diabetic neuropathy in type 1 diabetic patients reported to date. Functional correlates of these polymorphisms are in agreement with the susceptible and protective polymorphisms at the $(CA)_n$ locus shown in this study. The Z-2 allele of the $(CA)_n$ repeat polymorphism has been reported to be associated with higher aldose reductase mRNA levels (26) and with higher aldose reductase activity levels (27). An in vitro study by Hodgkinson et al. (28) found the susceptible Z-2 allele had higher transcriptional activity compared with the protective Z+2 allele. Subsequently, Yang et al. (29) reported that Z-2 with −106C had higher transcriptional activity compared with Z+2 with the −106T haplotype of the AKR1B1 gene. Only the DBHRV protection association with the ZZ/CC genotype is surprising. This genotype appeared protective in the current study but was previously classified by us and others within the susceptible genotype group (9,29). Further functional studies are needed to clarify its role. The precise localization of the polyol pathway in small fibers is uncertain. Aldose reductase immunoreactivity was detected in the Schwann cell cytoplasm of unmyelinated fibers of peripheral rat nerve (30). However, no information is available on localization of sorbitol dehydrogenase in unmyelinated fibers. In myelinated fibers, there appears to be more sorbitol dehydrogenase in axons than in Schwann cells (14).

Our findings are somewhat at variance to a recent longitudinal study of type 2 diabetic patients. Sivenius et al. (11) found that type 2 diabetic patients with the −106T allele had lower sensory response amplitude measurements compared with −106C at baseline examination. However, associations were no longer significant after allowing for physiological and environmental factors, specifically BMI. The observation at baseline can be related to the significantly higher BMI for the patients with the −106T compared with patient with the −106C allele. Moreover, in a larger group, we have recently showed increased BMI and height to be positively associated with peripheral nerve function abnormalities in adolescents with type 1 diabetes (31).

Familial clustering of diabetes complications has been shown for nephropathy and also retinopathy (32,33) but has not been demonstrated for neuropathy. Duration of diabetes before complication onset is important in detecting gene associations (major and minor susceptibility genes [34]). Our cohort of patients was unselected and asymptomatic and was recruited from routine complication assessment visits. Futhermore, the statistical model enables adjustment for duration and glycemic control and allows for varying length of follow-up and number of assessments.

Determining the best methods for measuring neuropathy progression is essential for the design of appropriate, cost-effective clinical trials. In addition, pharmacogenomics can aid in successful design of trials to identify patients with particular risks. Quantitative sensory testings and pupillometry are valuable tools that could be used along with genotyping in interventional studies.

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References

1. Boulton AJ, Malik RA, Arezzo JC, Sosenko JM: Diabetic somatic neuropathies (Review). *Diabetes Care* 27:1458–1486, 2004
2. Vinik AI, Maser RE, Mitchell BD, Freeman R: Diabetic autonomic neuropathy (Review). *Diabetes Care* 26:1553–1579, 2003
3. Boulton AJ, Kirsner RS, Vileikyte L: Clin-

ical practice: neuropathic diabetic foot ulcers. *N Engl J Med* 351:48–55, 2004
4. Maser RE, Mitchell BD, Vinik AI, Freeman R: The association between cardiovascular autonomic neuropathy and mortality in individuals with diabetes: a meta-analysis. *Diabetes Care* 26:1895–1901, 2003
5. The Diabetes Control and Complications Trial Research Group: The effect of intensive diabetes therapy on the development and progression of neuropathy. *Ann Intern Med* 122:561–568, 1995
6. Hyllienmark L, Brismar T, Ludvigsson J: Subclinical nerve dysfunction in children and adolescents with IDDM. *Diabetologia* 38:685–692, 1995
7. Karachaliou F, Karavanaki K, Greenwood R, Baum JD: Consistency of pupillary abnormality in children and adolescents with diabetes. *Diabet Med* 14:849–853, 1997
8. Pena MM, Donaghue KC, Fung AT, Bonney M, Schwingshandl J, Howard NJ, Silink M: The prospective assessment of autonomic nerve function by pupillometry in adolescents with type 1 diabetes mellitus. *Diabet Med* 12:868–873, 1995
9. Donaghue KC, Margan SH, Chan AK, Holloway B, Silink M, Rangel T, Bennetts B: The association of aldose reductase gene (AKR1B1) polymorphisms with diabetic neuropathy in adolescents. *Diabet Med* 22:1315–1320, 2005
10. Heesom AE, Millward A, Demaine AG: Susceptibility to diabetic neuropathy in patients with insulin dependent diabetes mellitus is associated with a polymorphism at the 5′ end of the aldose reductase gene. *J Neurol Neurosurg Psychiatry* 64:213–216, 1998
11. Sivenius K, Pihlajamaki J, Partanen J, Niskanen L, Laakso M, Uusitupa M: Aldose reductase gene polymorphisms and peripheral nerve function in patients with type 2 diabetes. *Diabetes Care* 27:2021–2026, 2004
12. Dent MT, Tebbs SE, Gonzalez AM, Ward JD, Wilson RM: Neutrophil aldose reductase activity and its association with established diabetic microvascular complications. *Diabet Med* 8:439–442, 1991
13. Hamada Y, Kitoh R, Raskin P: Crucial role of aldose reductase activity and plasma glucose level in sorbitol accumulation in erythrocytes from diabetic patients. *Diabetes* 40:1233–1240, 1991
14. Oates PJ: Polyol pathway and diabetic peripheral neuropathy. *Int Rev Neurobiol* 50: 325–392, 2002
15. Demaine A, Cross D, Millward A: Polymorphisms of the aldose reductase gene and susceptibility to retinopathy in type 1 diabetes mellitus. *Invest Ophthalmol Vis Sci* 41:4064–4068, 2000
16. Heesom AE, Hibberd ML, Millward A, Demaine AG: Polymorphism in the 5′-end of the aldose reductase gene is strongly associated with the development of diabetic nephropathy in type I diabetes. *Diabetes* 46:287–291, 1997
17. Moczulski DK, Scott L, Antonellis A, Rogus JJ, Rich SS, Warram JH, Krolewski AS: Aldose reductase gene polymorphisms and susceptibility to diabetic nephropathy in type 1 diabetes mellitus. *Diabet Med* 17:111–118, 2000
18. Donaghue KC, Bonney M, Simpson JM, Schwingshandl J, Fung AT, Howard NJ, Silink M: Autonomic and peripheral nerve function in adolescents with and without diabetes. *Diabet Med* 10:664–671, 1993
19. Schwingshandl J, Simpson JM, Donaghue K, Bonney MA, Howard NJ, Silink M: Pupillary abnormalities in type I diabetes occurring during adolescence: comparisons with cardiovascular reflexes. *Diabetes Care* 16:630–633, 1993
20. Kao YL, Donaghue K, Chan A, Knight J, Silink M: A novel polymorphism in the aldose reductase gene promoter region is strongly associated with diabetic retinopathy in adolescents with type 1 diabetes. *Diabetes* 48:1338–1340, 1999
21. Ko BC, Lam KS, Wat NM, Chung SS: An (A-C)n dinucleotide repeat polymorphic marker at the 5′ end of the aldose reductase gene is associated with early-onset diabetic retinopathy in NIDDM patients. *Diabetes* 44:727–732, 1995
22. Kao YL, Donaghue K, Chan A, Knight J, Silink M: An aldose reductase intragenic polymorphism associated with diabetic retinopathy. *Diabetes Res Clin Pract* 46:155–160, 1999
23. Lajer M, Tarnow L, Fleckner J, Hansen BV, Edwards DG, Parving HH, Boel E: Association of aldose reductase gene Z+2 polymorphism with reduced susceptibility to diabetic nephropathy in Caucasian type 1 diabetic patients. *Diabet Med* 21: 867–873, 2004
24. Nakayama M, Nakamura J, Hamada Y, Chaya S, Mizubayashi R, Yasuda Y, Kamiya H, Koh N, Hotta N: Aldose reductase inhibition ameliorates pupillary light reflex and F-wave latency in patients with mild diabetic neuropathy. *Diabetes Care* 24:1093–1098, 2001
25. Pittasch D, Lobmann R, Behrens-Baumann W, Lehnert H: Pupil signs of sympathetic autonomic neuropathy in patients with type 1 diabetes. *Diabetes Care* 25:1545–1550, 2002
26. Shah VO, Scavini M, Nikolic J, Sun Y, Vai S, Griffith JK, Dorin RI, Stidley C, Yacoub M, Vander Jagt DL, Eaton RP, Zager PG: Z-2 microsatellite allele is linked to increased expression of the aldose reductase gene in diabetic nephropathy. *J Clin Endocrinol Metab* 83:2886–2891, 1998
27. Zou X, Lu J, Pan C: Effect of polymorphism of (AC)n in the 5′ end of aldose reductase gene on erythrocyte aldose reductase activity in patients with type 2 diabetes mellitus. *Chinese J Endocrinol Metab* 16:346–349, 2000
28. Hodgkinson AD, Sondergaard KL, Yang B, Cross DF, Millward BA, Demaine AG: Aldose reductase expression is induced by hyperglycemia in diabetic nephropathy. *Kidney Int* 60:211–218, 2001
29. Yang B, Millward A, Demaine A: Functional differences between the susceptibility Z-2/C-106 and protective Z+2/T-106 promoter region polymorphisms of the aldose reductase gene may account for the association with diabetic microvascular complications. *Biochim Biophys Acta* 1639: 1–7, 2003
30. Powell HC, Garrett RS, Kador PF, Mizisin AP: Fine-structural localization of aldose reductase and ouabain-sensitive, K(+)-dependent p-nitro-phenylphosphatase in rat peripheral nerve. *Acta Neuropathol (Berl)* 81:529–539, 1991
31. Mohsin F, Craig ME, Cusumano J, Chan AK, Hing S, Lee JW, Silink M, Howard NJ, Donaghue KC: Discordant trends in microvascular complications in adolescents with type 1 diabetes from 1990 to 2002. *Diabetes Care* 28:1974–1980, 2005
32. The Diabetes Control and Complications Trial Research Group: Clustering of long-term complications in families with diabetes in the Diabetes Control and Complications Trial. *Diabetes* 46:1829–1839, 1997
33. Seaquist ER, Goetz FC, Rich S, Barbosa J: Familial clustering of diabetic kidney disease: evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 320: 1161–1165, 1989
34. Rogus JJ, Warram JH, Krolewski AS: Genetic studies of late diabetes complications: the overlooked importance of diabetes duration before complication onset. *Diabetes* 51:1655–1662, 2002