

The relationship between *ACE* genotype and risk of severe hypoglycaemia in a large population-based cohort of children and adolescents with type 1 diabetes

M. K. Bulsara · C. D. J. Holman ·
F. M. van Bockxmeer · E. A. Davis · P. H. Gallego ·
J. P. Beilby · L. J. Palmer · C. Choong · T. W. Jones

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Abstract

Aims/hypothesis Genetic factors may account for familial clustering related to diabetes complications. Studies have shown a significant relationship between the presence of the deletion (*D*) allele of the gene encoding ACE and risk of severe hypoglycaemia. This large prospective cohort study assesses this relationship in a large sample of children and adolescents with type 1 diabetes.

Subjects and methods We studied 585 children and adolescents (mean age 11.9±4 years, 48.4% males). The frequency of severe hypoglycaemia (an event leading to loss of consciousness or seizure) was prospectively assessed over the 13-year period 1992–2004. Patients were seen with their parents every 3 months and data recorded at each visit. The *ACE* gene was detected using PCR.

Results In our cohort of 585 children, 186 (31.8%) had at least one episode of severe hypoglycaemia, and of these

28.0% had the *II* genotype, 48.9% had the *ID* genotype and 23.1% had the *DD* genotype. This was in agreement with the Hardy–Weinberg proportion. A total of 477 severe hypoglycaemic episodes was recorded with a total of 3,404 person-years of follow-up, giving a total incidence of 14 per 100 patient-years. No significant increase in risk for *DD* genotype (incidence rate ratio=0.97, 95% CI 0.61–1.55) relative to *II* genotype was observed.

Conclusions/interpretation This large prospective study concludes that the presence of the *D* allele of the *ACE* gene does not predict a significantly higher risk of severe hypoglycaemia in type 1 diabetic children and adolescents.

Keywords ACE · Angiotensin-converting enzyme · *ACE* genotype · Complications · Polymorphism · Severe hypoglycaemia · Type 1 diabetes mellitus

M. K. Bulsara (✉) · C. D. J. Holman
School of Population Health, University of Western Australia,
35 Stirling Highway, Crawley, Nedlands,
Perth, WA 6009, Australia
e-mail: max.bulsara@uwa.edu.au

E. A. Davis · P. H. Gallego · C. Choong · T. W. Jones
Department of Endocrinology, Princess Margaret Hospital,
Perth, WA, Australia

M. K. Bulsara · E. A. Davis · T. W. Jones
Centre for Child Health Research,
Telethon Institute of Child Health Research,
Perth, WA, Australia

J. P. Beilby
Clinical Biochemistry, PathWest,
Queen Elizabeth II Medical Centre,
Perth, WA, Australia

F. M. van Bockxmeer
School of Surgery and Pathology, University of Western Australia,
Perth, WA, Australia

L. J. Palmer
University of Western Australia Centre for Medical Research
and Western Australian Institute for Medical Research,
Perth, WA, Australia

C. Choong
School of Pediatrics and Child Health,
University of Western Australia,
Perth, WA, Australia

Abbreviation

IRR incidence rate ratio

Introduction

An important limiting factor to achieving good metabolic control of type 1 diabetes in children and adolescents is the increased risk and fear of hypoglycaemic episodes and in rare circumstances death [1–4]. Recurrent episodes of hypoglycaemia may result in hypoglycaemia unawareness [5]. Large population-based studies have shown that despite efforts to improve treatment of type 1 diabetes, hypoglycaemia remains a problem [2, 5] and there is need for strategies that will minimise episodes of severe hypoglycaemia. On the other hand, a majority of patients never experience a hypoglycaemic coma or convulsion [2]. It has been shown that the incidence of severe hypoglycaemia within a cohort is highly skewed [6] with variable inter-individual susceptibility. The Diabetes Control and Complications Trial demonstrated familial clustering of diabetes complications [7] suggesting that genetic factors may account for some of this variability [8]. The cause of severe hypoglycaemia remains multifactorial. It has been suggested that genetic factors may predispose to a susceptibility to severe hypoglycaemia. Studies have shown that the use of ACE inhibitors may be a factor in the incidence of hypoglycaemia in adults [9, 10], but this still remains controversial [11–14], as these studies were retrospective in design and were unable to adjust for some important confounding variables.

The *ACE* gene is located in chromosome 17q23 and a common 287-bp Alu element insertion polymorphism exists in intron 16 with an insertion allele frequency of about 0.5 (50%) in most European populations. This polymorphism has been shown to correlate with serum ACE activity, where the *D* allele is associated with higher serum ACE activity [15, 16]. The *D* allele has also been associated with increased risk of hypertension, diabetic renal complications and cardiovascular complications. Some studies have shown an association between *ACE* and physical performance [17–19], and it has been proposed that there may be a similarity in metabolic features between hypoglycaemia and exercise [8, 20, 21], such that patients with type 1 diabetes and the *DD* genotype are at increased risk of severe hypoglycaemia compared with those with the *II* genotype [21].

Our aim in this study was to determine the relationship between this genetic polymorphism and susceptibility to severe hypoglycaemia in a large population-based sample of individuals with type 1 diabetes mellitus, in whom hypoglycaemic events had been closely characterised prospectively from diagnosis [2].

Subjects and methods

Subjects The adolescents and children studied were from a population-based cohort with type 1 diabetes. They were aged ≤ 18 years, and attending the diabetes clinic at Princess Margaret Hospital for Children. Type 1 diabetes was defined in this cohort by a combination of clinical features and by measurement of C-peptide and autoimmune markers. Longitudinal clinical data on these patients were collected prospectively from the time of diagnosis of diabetes. Princess Margaret Hospital is the only paediatric diabetes referral centre for diabetes in Western Australia, and all diagnosed children in the state are registered and treated there, thus achieving a 99% case ascertainment rate [22, 23] with a very low drop-out rate (<4%). Western Australia has an estimated total population of approximately two million. Of this, indigenous Australians account for 3% of the total [23]. The majority (>90%) of the population are white and of European descent, with almost 80% of the total population residing in the urban area [24].

After initial diagnosis each patient was seen by the diabetes care team in the presence of their parents. The diabetes team includes a clinician, a diabetes nurse, an educator, a dietitian and a social worker. The specialist clinician obtains a detailed history from each patient. All patients and their parents have extensive education on how to manage their child's diabetes, which includes details on the identification and treatment of hypoglycaemia, as well as insulin dose adjustment. Parents are provided with a log book to record all treatment and adverse events. All data are collected prospectively at each clinic visit every 3 months.

The study protocol was in accordance with guidelines set out by National Health and Medical Research Council and was approved by the institutional ethics committee with written consent obtained from all parents and patients after explanation of the objectives and details of the study.

Definition of outcome Severe hypoglycaemia was defined as an event leading to a loss of consciousness or seizure. This strict definition was used as it is an unambiguous endpoint rather than the more commonly used definition of severe hypoglycaemia, which is an event requiring help of another individual. This definition was consistently used over the whole follow-up period.

Data were collected at visits every 3 months using a specifically designed data collection form, which was completed by a limited number of physicians following a specific protocol (E. A. Davies, T. W. Jones). Hypoglycaemic events were carefully documented and recorded by the patient or parent when they occurred; both patient and parents were instructed on how to record details of the event, including blood glucose levels and response to treatment. All parents were instructed to obtain a blood

glucose value at each event once the child's safety had been assured. In our cohort, glucose values were obtained more than 98% of the time. This information was subsequently reviewed by the clinician and if validated, recorded on a data collection form. The physician took into account the history of the event, the glucose recording and its timing, and the recovery history before judging the event to be hypoglycaemia-related. In addition to the logbooks, most families phoned the diabetes management team to receive advice on event management after a hypoglycaemic event of this severity. These calls were recorded. We note that there was a close correlation between recall at clinic through logbooks and calls to the diabetes team, providing further evidence that recall was accurate over this time period. Patients and parents were instructed on how to record data at each clinic visit.

HbA_{1c} measurements HbA_{1c} was determined at each three-monthly visit and was measured by agglutination inhibition immunoassay (type 1 diabetes reference <6.2%; Ames DCA 2000; Ames Minilab, Mulgrave, VIC, Australia).

Genotyping Blood was collected into EDTA blood tubes and DNA was extracted from the buffy coats using a salting out method [25]. The 287-bp Alu insertion in *ACE* was genotyped directly by PCR amplification, followed by separation on a 2% agarose gel. The *I/D* polymorphism of the *ACE* gene was detected by PCR according to the method of Rigat et al. [26]. The insertion allele (*I*) was identified by the presence of a 480-bp band, the deletion allele (*D*) by a 191-bp band. Samples identified as being *DD* homozygous were confirmed using an insertion-specific forward primer 5'-TTTGAGACG GAGTCTCGCTC-3'. The *DD* genotype was confirmed by the absence of PCR product. Misclassification of *ID* to *DD* was checked by performing a second independent PCR amplification of *DD* samples, using an insertion-specific primer [27].

Statistical analysis All statistical analysis was conducted using Stata version 9 (Stata Statistical Software, College

Station, TX, USA). Allele frequencies were compared using a chi-squared analysis for categorical variables between groups, whereas continuous variables were compared using a non-parametric test. We tested Hardy–Weinberg equilibrium using a chi-squared test on one degree of freedom. Genotypic models were tested by recoding genotypes with the most common genotype coded as zero for the baseline. Under additive models, heterozygous variants were coded as 1 and homozygous variants for the rarer allele as 2. Under dominant models, both the heterozygous and homozygous variants for the rarer allele were coded as 1, while under recessive models heterozygous variants were coded as zero and homozygous variants for the rarer allele were coded as 1.

The primary outcome measure was the number of episodes of severe hypoglycaemia during the whole follow-up period and was analysed using a negative binomial regression, which is an extension of the log-linear Poisson model and results in a better fit than the Poisson model [6]. This model takes into account the varying length of follow-up period for each patient. Results are reported as incidence rate ratio (IRR) with 95% CIs. A proportional hazard regression model was used to examine factors associated with the risk of the first severe hypoglycaemic event using duration of diabetes as the time variable. Results are reported as hazard ratio with 95% CIs. All variables were tested in a univariate model and those that achieved statistical significance ($p < 0.05$) were considered for the subsequent multivariate model.

Results

A total of 585 children and adolescents (mean age 11.9±4 years, 48.4% males) was studied. The average age at entry into the cohort was 9.3±4.0 years with a median age of 10.0 years. The demographic and clinical characteristics of these patients are shown in Tables 1, 2 and 3.

During the follow-up period of the cohort (Table 1), nearly 20% of cohort members were <6 years old, with

Table 1 Demographic characteristics of patients by age group

Characteristics	Age group (years)		
	<6	6–12	13–18
Patient numbers	112	334	139
Sex (% boys)	50.0	45.5	54.0
Diabetes duration ±SD (years)	7.0±4.8	7.0±4.7	5.2±4.0
Mean HbA _{1c} ±SD (%)	8.3±0.8	8.7±1.1	9.0±1.7
Total SH events	146	270	61
Follow-up (patient-years)	806.85	2,108.29	488.76
SH incidence rate ^a	18.1 (15.5–20.9)	12.8 (11.4–14.3)	12.5 (9.7–15.7)

SH, severe hypoglycaemia
^aper 100 patient-years
 (95% CI)

Table 2 Clinical and demographic characteristics of patients by severe hypoglycaemic status

Characteristics	Number of severe hypoglycaemic events			<i>p</i> value
	0	1	≥2	
Patients (<i>n</i>)	399	82	104	
Age at onset ±SD (years)	9.9±4.0	8.3±3.7	7.8±3.3	<0.0001
Sex (% boys)	47.9	48.8	50.0	0.920
Diabetes duration ±SD (years)	5.4±4.2	7.9±4.9	9.9±4.4	<0.0001
Mean HbA _{1c} ±SD (%)	8.6±1.3	8.8±1.1	8.9±1.0	0.003
ACE genotype (<i>n</i> [%])				
<i>II</i>	95 (23.8)	20 (24.4)	32 (25.1)	
<i>ID</i>	192 (48.1)	42 (51.2)	49 (47.1)	
<i>DD</i>	112 (28.1)	20 (24.4)	23 (22.1)	
Hardy–Weinberg ^a	0.474	0.825	0.606	

^a*p* value

57% between 6 and 12 years of age. The number of patients experiencing at least one severe event was 186 (31.8%) (Table 2). In the youngest age group (<6 years) 26.8% experienced two or more severe events compared with 7.9% for the oldest age group. For children who experienced at least one severe hypoglycaemic episode during the whole follow-up period significant differences were observed for age at onset, duration of diabetes, HbA_{1c} and total daily insulin dose.

The ACE genotype distribution was in Hardy–Weinberg equilibrium, both overall and across the sub-groups of patients with and without severe hypoglycaemia. The proportion of patients with *II* genotype who had at least one severe hypoglycaemic event was higher (35%) than for patients of the *ID* (32%) and *DD* (28%) genotypes. However, this difference was not significant (*p*=0.357).

The clinical characteristics by genotype are given in Table 3. No significant differences were observed between genotypes with respect to age at onset, sex, duration of diabetes, HbA_{1c} and insulin dose. The overall incidence rate of severe hypoglycaemia was 14.0 per 100 patient-years with 3,404 person-years of follow-up. The highest incidence rate was observed in patients with the *ID* genotype.

A multivariate analysis modelling all severe events confirmed the relationship between severe hypoglycaemia

and known predictors (Table 4). However, for the additive model, patients with the *DD* genotype showed no significant increase in risk of severe hypoglycaemia compared with patients with the *II* genotype (IRR=0.97, 95% CI 0.61–1.55, *p*=0.914). For the dominant model, the *DD* genotype showed a non-significant increased risk compared with *ID+II* (IRR=1.10, 95% CI 0.73–1.65, *p*=0.647) and similar results for the recessive model. Analogous results were obtained when modelling just the first severe event using a proportional hazard regression model (Table 5, Fig. 1).

Discussion

It is well known that many chronic diseases are influenced both by environmental and genetic factors and that multiple genes interacting with the environment may add to disease propensity. Specific candidate genes for variants that might predispose to diabetic nephropathy and retinopathy have been examined in detail [28]. Some evidence has been presented to suggest that predisposition to severe hypoglycaemia may also have some genetic basis. Herings et al. [9] and Morris et al. [10] have shown that the use of ACE inhibitors increased the risk of having a severe hypoglycaemic event in adults, but these studies were retrospective

Table 3 Clinical and demographic characteristics of patients by genotype

Characteristics	ACE <i>ID</i> genotype			<i>p</i> value
	<i>II</i>	<i>ID</i>	<i>DD</i>	
Patients (<i>n</i>)	147	283	155	
Age at onset ±SD (years)	9.2±4.25	9.4±3.84	9.2±4.00	0.748
Sex (% boys)	47.6	47.0	51.6	0.639
Diabetes duration ±SD (years)	6.6±4.56	6.6±4.72	6.4±4.64	0.992
Mean HbA _{1c} ±SD (%)	8.6±1.28	8.7±1.25	8.7±1.19	0.487
Total SH events	120	252	105	
Follow-up (patient-years)	884.65	1,650.50	868.76	
SH incidence rate ^a	13.6 (11.4–16.0)	15.3 (13.6–17.1)	12.1 (10.1–14.4)	

^aper 100 patient-years (95% CI)

Table 4 Multivariate analyses of the relationship between *ACE* genotype and other risk factors and risk of severe hypoglycaemia

Variable	IRR	95% CI (IRR)	<i>p</i> value
Age	0.96	0.91–1.01	0.121
Sex (male)	1.28	0.93–1.77	0.128
Duration of diabetes	1.11	1.06–1.17	<0.0001
HbA _{1c}	0.78	0.71–0.86	<0.0001
<i>ACE</i> <i>I/D</i> genotype			
<i>II</i>	1.0	–	–
<i>ID</i>	1.11	0.77–1.60	0.565
<i>DD</i>	0.97	0.61–1.55	0.914
<i>II</i> vs <i>ID+DD</i>	1.07	0.76–1.50	0.715
<i>DD</i> vs <i>ID+II</i>	1.10	0.73–1.65	0.647

IRR, incidence rate ratio

in design and the findings remain controversial [11–14]. The fact that a small proportion of patients experiences severe hypoglycaemia whereas other patients with similar glycaemic control remain spared indicates that genetic factors may contribute to an increased risk, and studies conducted in Denmark and Sweden have supported this notion [8, 20, 21].

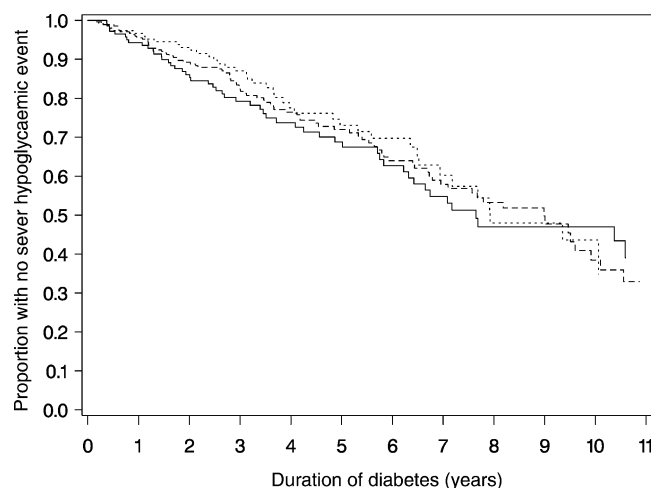
This study was of a large population-based cohort of type 1 diabetic children followed prospectively for 12 years and in whom the phenotype of hypoglycaemic frequency has been carefully documented. The prospective design and strict definition of severe hypoglycaemia, limited to seizures and coma, minimised the chance of false positives and recall bias. Furthermore, the low drop-out rate and near complete ascertainment adds to the analysis. Of the 585 patients, 186 experienced a total of 477 severe episodes with a total follow-up time of approximately 3,500 patient-years. The overall incidence rate of 14.0 per 100 patient-years is similar to the rate we have reported previously [2, 22] and comparable with other studies [1, 29, 30]. A Swedish study reported an overall incidence rate of 17.0 per 100 patient-years [20], which is slightly higher than our study, and a median age of 12.8 years compared with 10 years in our study.

Table 5 Multivariate analyses of the relationship between *ACE* genotype and other risk factors and risk of first severe hypoglycaemic event

Variable	Hazard ratio	95% CI (hazard ratio)	<i>p</i> value
Age	0.92	0.88–0.96	<0.0001
Sex (male)	1.15	0.86–1.55	0.349
HbA _{1c}	0.65	0.59–0.72	<0.0001
<i>ACE</i> <i>I/D</i> genotype			
<i>II</i>	1.0	–	–
<i>ID</i>	0.97	0.68–1.37	0.844
<i>DD</i>	0.93	0.61–1.40	0.712
<i>II</i> vs <i>ID+DD</i>	0.95	0.69–1.32	0.770
<i>DD</i> vs <i>ID+II</i>	1.06	0.74–1.50	0.756

The present report reveals that the frequency of the *D* allele of the *ACE* gene was 47.6% for the children who experienced at least one severe episode compared with 52.1% in those children who never experienced a severe episode. This difference was not significant. In fact the incidence of severe hypoglycaemia for patients with the *D* allele was lower than for patients with the *I* allele. Pedersen-Bjergaard et al. report that in their patients with the *DD* genotype, 54% had at least one severe episode [21], which contrasts to our cohort where this proportion was only 28%. We have demonstrated that there is no increased risk of severe hypoglycaemia with the *D* allele of the *ACEI/D* polymorphism.

Our findings are contrary to those reported recently in adult (sample size of 171 and 207 outpatients) [8, 21] and paediatric populations (sample size of 86 patients) [20]. In light of this several questions arise. First, could the negative results of the present report be due to type 1 error? The sample size in our study is large with an equally large number of severe events and hence unlikely to suffer from type 1 error. In fact, our sample size of 585 children is able to detect an increase in IRR of 13% with 84% power. Second, could there be a diluting effect with our long follow-up time? Our analysis using just the first severe hypoglycaemic events showed no significant increase in risk, hence this is unlikely to explain the difference. It could be due to differences in the study population or it could be due to the large inter-individual variability in serum ACE levels, which can differ by fivefold among subjects [31]. Our population should be genetically similar to the white European population and hence this is unlikely to explain the differences. Most of our patients are of white Anglo-Celtic origin [32] and so racial disparity probably does not explain this difference [33].

**Fig. 1** Kaplan–Meier curve for risk of first severe hypoglycaemic event by *ACE* genotype. Continuous line, *ACE* genotype *II*; dashed line, *ACE* genotype *ID*; dotted line, *ACE* genotype *DD*

The quality and robustness of the endpoint data are of critical importance. This is particularly relevant to hypoglycaemia events because of the potential bias that may be otherwise introduced by both false positive and false negative recording of hypoglycaemic episodes. Variable definitions of hypoglycaemia, retrospective accounts of hypoglycaemic events and small samples sizes all add to the difficulty of interpreting studies that characterise factors predisposing to hypoglycaemia. In our report we have taken a large population-based sample, in whom hypoglycaemia has been consistently recorded over time. We have employed the strict endpoint of hypoglycaemic coma and convulsions, as these events may be categorically defined and are less likely to be misinterpreted than less robust definitions. Indeed, it is tempting to speculate that differences in definitions may explain discrepancies with previous studies.

Relationships between serum ACE activity and *ACE* genotype have been reported [15, 31, 34, 35], where individuals with the *DD* genotype had a 40% higher serum ACE activity than those with the *II* genotype. It has also been reported that the *ACE I/D* polymorphism accounts for 47% of *ACE* phenotypic variance [31]. What is not clearly established is whether the *ACE I/D* polymorphism determines the amount of ACE activity produced at the cellular level. It has been suggested that the *ACE I/D* polymorphism could be a marker for a variant linked to another nearby locus [31, 34, 35] and that the interaction of the two could be responsible for producing ACE activity in light of the fact that the *ACE I/D* polymorphism is intronic. Whether there is a relationship between ACE activity in the *ACE* genotype in children as in adults has not been studied. If the polymorphism is not functionally important in the young, this may explain the discrepancy between our results and those of Nordfeldt [20].

The potential benefits of identifying a marker for patients at risk of severe hypoglycaemia are undisputable. Our study, however, was unable to confirm previous findings. Other recent studies suggest similar conclusions. Geddes et al. recently presented results showing an overall weak correlation between serum ACE and incidence of severe hypoglycaemia, but no significant relationship once serum ACE was divided into quartiles [36]. This was based on 300 randomly selected type 1 diabetic patients in Edinburgh, Scotland; the investigators concluded that they were unable to confirm the results of the Danish studies, noting in addition that both surveys were in white European populations. In addition, a recent study by Holstein et al., based on 231 German type 1 diabetic patients of white European origin, showed no effect of the *ACE I/D* polymorphism on impaired hypoglycaemia awareness [37]. This study used standardised questionnaires to assess

hypoglycaemia awareness and was unable to replicate the results from the Danish study [21].

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Duality of interest The authors of this article are not aware of any duality of interest.

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