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Impact of the *TCF7L2* genotype on risk of hypoglycaemia and glucagon secretion during hypoglycaemia

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Abstract

Introduction: In healthy carriers of the T allele of the transcription factor 7-like 2 (*TCF7L2*), fasting plasma glucagon concentrations are lower compared with those with the C allele. We hypothesised that presence of the T allele is associated with a diminished glucagon response during hypoglycaemia and a higher frequency of severe hypoglycaemia (SH) in type 1 diabetes (T1DM).

Material and methods: This is a *post hoc* study of an earlier prospective observational study of SH and four mechanistic studies of physiological responses to hypoglycaemia. 269 patients with T1DM were followed in a one-year observational study. A log-linear negative binomial model was applied with events of SH as dependent variable and *TCF7L2* alleles as explanatory variable. In four experimental studies including 65 people, *TCF7L2* genotyping was done and plasma glucagon concentration during experimental hypoglycaemia was determined.

Results: Incidences of SH were TT 0.54, TC 0.98 and CC 1.01 episodes per patient-year with no significant difference between groups. During experimental hypoglycaemia, the *TCF7L2* polymorphism did not influence glucagon secretion.

Discussion: Patients with T1DM carrying the T allele of the *TCF7L2* polymorphism do not exhibit diminished glucagon response during hypoglycaemia and are not at increased risk of severe hypoglycaemia compared with carriers of the C allele.

Key Words

- ▶ type 1 diabetes
- ▶ severe hypoglycaemia
- ▶ *TCF7L2*
- ▶ glucagon
- ▶ epidemiology
- ▶ experimental hypoglycaemia

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Introduction

Patients with type 1 diabetes are at daily risk of events of severe hypoglycaemia (1, 2, 3, 4, 5, 6, 7). The distribution of these events is much skewed

with a minority of subjects accounting for the vast majority of episodes (1, 2). Although important risk markers for severe hypoglycaemia like hormonal



counter-regulatory failure (8) and impaired hypoglycaemia awareness (6) have been identified, the highly varying inter-individual susceptibility to severe hypoglycaemia is largely unexplained. We previously speculated whether genetic factors are implicated and reported a high rate of severe hypoglycaemia in type 1 diabetic subjects carrying the common deletion-allele of the angiotensin-converting enzyme (ACE) gene that confers high enzyme activity in blood and tissues (9). This finding has been reproduced in six studies in five different populations by four independent research groups (10, 11, 12, 13, 14, 15). Schouwenberg and coworkers showed in healthy adults that possessing the Arg Arg (vs the Gly Gly) polymorphism of the β 2-adrenergic receptor may to some extent protect against the development of hypoglycaemia unawareness (16, 17), a major risk indicator of severe hypoglycaemia. In contrary Holstein *et al.* found no association between four genetic polymorphisms associated with insulin secretion and risk of severe hypoglycaemia in sulfonylurea-treated persons with type 2 diabetes (18).

Glucagon is a key player during the first-phase hormonal counter-regulation of hypoglycaemia (19). In people with type 1 diabetes, the glucagon secretory response to hypoglycaemia is initially diminished and subsequently lost within a few years of the onset of diabetes (20, 21). In the search for candidate risk genes for type 2 diabetes, the T allele of rs7903146 transcription factor 7-like 2 (*TCF7L2*) has been shown to confer the hitherto strongest association with type 2 diabetes, with a per allele odds ratio of 1.4 (22, 23, 24). During the efforts to elucidate the underlying mechanism, a functional impact of the gene variant on glucagon concentrations has emerged. Thus, fasting glucagon concentrations were reported to be lower in healthy carriers of the T allele (approximately 30% of the population (25)) compared with those with the C allele (26, 27, 28), whereas incremental glucagon secretion after a meal test does not differ between the alleles in normal, glucose-tolerant subjects (29). The extent to which the *TCF7L2* gene polymorphism influences glucagon secretion and/or risk of hypoglycaemia in people with type 1 diabetes is unknown.

We explored whether variants of the *TCF7L2* gene influence the risk of severe hypoglycaemia in people with type 1 diabetes. The hypothesis was tested in a cohort of patients with type 1 diabetes previously followed for one year in an observational prospective study of the

occurrence of severe hypoglycaemia (14). We also explored if variants of the *TCF7L2* gene influence the secretion of glucagon during experimental hypoglycaemia in healthy adults and adult people with type 1 diabetes.

Materials and methods

Epidemiological study

This study is a *post hoc* study of blood samples collected during a prospective study designed to evaluate risk markers for severe hypoglycaemia in type 1 diabetes (14). Consecutive adult (>18 years of age) outpatients with type 1 diabetes for more than two years were recruited by mail. Type 1 diabetes was defined by insulin treatment from the time of diagnosis and non-stimulated C-peptide <300 pmol/L or stimulated (venous blood glucose concentration >12 mmol/L) C-peptide <600 pmol/L. Pregnant women and subjects on haemodialysis or suffering from concomitant malignant disease were excluded from participation. Clinical characteristics of the participating subjects appear from Table 1. Consent to participate was obtained from each subject after full explanation of the purpose and nature of all procedures used, and the study was approved by the Regional Committee on Biomedical Research Ethics. Results regarding the association between renin–angiotensin system components and risk of severe hypoglycaemia have been published elsewhere (30, 14).

At baseline, participants were characterised by their previous experience of hypoglycaemia. Episodes of severe hypoglycaemia were then reported prospectively for one year. The events were reported by telephone within 24 h and validated according to the triad of Whipple as described previously in detail (14). Only definite (all three criteria in Whipple's triad) and probable episodes (two criteria in Whipple's triad) of hypoglycaemia were classified as severe hypoglycaemia and used in the statistical analysis. These episodes were the primary endpoint. Two trained study nurses performed structured telephone interviews to establish the level of documentation, classify severity and explore circumstances of the incidents. Additionally, at the end of the follow-up period, severe hypoglycaemia was reported retrospectively by questionnaire, and in the case of missed reporting by telephone, interviews were carried out. Severe hypoglycaemia was defined as an episode at which the patient needs assistance from another person to restore the blood glucose level.

Table 1 Patient characteristics in the total cohort and divided into *TCF7L2* genotype.

	All	TT	TC	CC	Missing genotype	P
Number of patients (n; %)	269 (100)	13 (4.8)	89 (33.1)	143 (53.2)	24 (8.9)	
Men (%)	160 (59.5)	10 (76.9)	48 (53.9)	85 (59.4)	17 (70.8)	0.27
Women (%)	109 (40.5)	3 (23.1)	41 (46.1)	58 (40.6)	7 (29.2)	
Age (years)	45.1 ± 13.4 19–84	46.6 ± 10.6 30–67	46.2 ± 14.5 20–84	44.2 ± 13.3 19–83	45.3 ± 11.6 20–64	0.55
BMI (kg/m ²)	25.1 ± 3.65 17.7–38.6	25.4 ± 3.83 19.4–33.9	24.6 ± 3.63 17.7–35.6	25.4 ± 3.68 19.5–38.6	25.0 ± 3.52 20.3–34.9	0.32
Duration of diabetes (years)	20.9 ± 11.8 3–56	21.2 ± 9.7 9–38	20.5 ± 12.3 3–55	20.5 ± 11.5 3–56	20.3 ± 13.0 3–50	0.98
Age at diagnosis of diabetes (years)	24.6 ± 14.2 0–70	25.5 ± 13.1 2–43	25.7 ± 15.5 0–60	23.8 ± 13.8 2–70	20.3 ± 13.0 3–50	0.62
HbA1c at entry (%)	8.60 ± 1.22 5.2–14.0	9.1 ± 1.1 7.0–11.1	8.5 ± 1.3 5.2–12.4	8.7 ± 1.2 5.5–14.0	8.38 ± 1.05 7.0–10.8	0.17
C-peptide positive (%) ^a (pmol/L)	59.9 65 ± 91 0–400	8 (62) 48 ± 55 0–189	56 (63) 84 ± 106 0–400	77 (54) 50 ± 77 0–343	20 (83.3) 90 ± 115 0–383	0.38 0.09
Plasma glucagon (pmol/L)	4.8 ± 3.4	6.2 ± 4.8	4.9 ± 3.6	4.7 ± 3.3	4.7 ± 2.9	0.70
BG ≥ 4 (n = 227 (87%))	4.9 ± 3.5	6.6 ± 5.3	4.9 ± 3.5	4.8 ± 3.4	4.3 ± 2.7	0.85
BG < 4 (n = 35 (13%))	4.5 ± 3.2	4.7 ± 3.1	5.6 ± 5.0	4.1 ± 2.3	6.0 ± 3.5	0.64
Plasma glucose at entry (mmol/L)	9.9 ± 4.9 0.9–24	8.8 ± 5.2 1–18	10.2 ± 4.7 0.9–23	9.9 ± 5.0 1–24	10.5 ± 5.9 2–25	0.61
Severe hypoglycaemic events per patient-year (mean, min–max)	0.96, 0–16	0.54, 0–5	0.97, 0–15	0.99, 0–16	0.67, 0–5	
None (n; %)	179 (66.5)	11 (84.6)	55 (61.8)	95 (66.4)	18 (75.0)	
One (n; %)	43 (16.0)	0	20 (22.5)	21 (14.7)	2 (8.3)	0.24
More than one (n; %)	47 (17.5)	2 (14.4)	14 (15.7)	27 (18.9)	4 (16.7)	
Awareness (%)						
Normal	44	25.0	38.6	45.1	66.7	
Impaired	44	50.0	50.5	42.3	29.2	0.15
Unaware	12	25.0	11.4	12.7	4.2	

Values are mean (s.d.) or percentage when indicated. Frequency of severe hypoglycaemia is described by mean, min–max and distribution of zero, one or more episodes per year. We used one-way ANOVA, chi-square test and Kruskal–Wallis test as appropriate. *P* values refer to comparisons between *TCF7L2* genotypes (missing genotype group is not included in the statistical assessment). ^aA subject is classified as C-peptide negative if below the detection limit of 10 pmol/L.

Hypoglycaemia awareness was estimated by a validated method based on self-reported ability to perceive hypoglycaemia (31). This method has previously been validated prospectively (32) and proven clinically relevant across language barriers in different populations (2, 33). The prevalence of unawareness corresponds to the prevalence of impaired awareness according to methods with only two categories (31, 5, 6). Data on history of diabetes were extracted from medical records as described previously (14).

Experimental study

This study is a *post hoc* study of blood samples collected during four previously reported experimental studies. The first three were designed to evaluate the effect of activity in the renin–angiotensin system on hormonal, cardiac and cognitive function parameters during experimental hypoglycaemia (34, 35, 36). The fourth study was a

randomised controlled trial (RCT) designed to evaluate the effect of exogenous erythropoietin on cognitive function during hypoglycaemia in patients prone to severe hypoglycaemia (37). An overview of the four studies is available in Table 2. Specific details about the experimental protocol, including ethical issues and consent to participate, are reported elsewhere (34, 35, 36, 37).

Laboratory analyses

Radioimmunological determination of plasma glucagon concentrations was performed using a specific assay as described (38). The analytical detection limit was 1 pmol/L and intra- and inter-assay coefficients of variation were <6% and <15% respectively. Genotyping for *TCF7L2* rs7903146 was performed using allelic discrimination performed with an ABI 7900 system (KBioscience, Hoddesdon, UK) as described (39). The genotype distribution obeyed Hardy–Weinberg equilibrium

Table 2 Characteristics of the four experimental studies.

	Study 1	Study 2	Study 3	Study 4
Reference	Hoi-Hansen <i>et al.</i> (35)	Due-Andersen <i>et al.</i> (36)	Bie-Olsen <i>et al.</i> (34)	Kristensen <i>et al.</i> (37)
Study objective	To study the effect of high or low activity of RAS on cognitive function and hormonal counter regulation during hypoglycaemia	To study to the effect of high or low activity of RAS on the QT interval and its dynamics during hypoglycaemia and hypoxaemia	To study the effect of high or low activity of RAS on changes in cerebral activity during hypoglycaemia measured with PET	To study the effect of treatment with EPO on cognitive function during hypoglycaemia
Population	Type 1 diabetes: 9 with high and 9 with low activity in RAS	Healthy adults: 10 with high and 10 with low activity in RAS	Healthy adults: 10 with high and 10 with low activity in RAS	Type 1 diabetes: Prone to severe hypoglycaemia
Induction of hypoglycaemia	Standardised intravenous insulin infusion. No glucose was infused	Subcutaneously administered insulin	Standardised intravenous insulin infusion. No glucose was infused	Intravenous hypoglycaemic clamp
<i>N</i>	18	20	20	11
Mean plasma glucose during hypoglycaemia, mean (s.d.) (mmol/L)	2.5 (0.3)	2.8 (0.3)	2.2 (0.4)	2.2 (0.1) EPO-day 2.2 (0.2) placebo-day
TCF7L2 polymorphism, TT/TC/CC/U (%)	0/3/14/1 (0/17/78/5)	3/8/7/2 (19/38/33/10)	3/6/11/0 (15/30/55/0)	3/4/3/1 (27/37/27/9)
Baseline plasma glucagon (normoglycaemia) mean (s.d.) (nmol/L)	7.1 (1.9)	6.9 (2.2)	14 (3.7)	4.2 (0.7)
Peak plasma glucagon (hypoglycaemia) mean (s.d.) (nmol/L)	8.5 (2.9)	28 (11)	51 (21)	8.1 (3.0)

EPO, erythropoietin; PET, positron emission tomography; RAS, renin–angiotensin system; U, undetermined genotyping.

($P > 0.05$). HbA1c was measured spectrophotometrically (DCA-2000, Bayer; normal range 4.1–6.4%). C-peptide levels were measured by radioimmunoassay (Autodelphia, Wallac Oy, Turku, Finland). Subjects were classified as being C-peptide negative if the value is below 10 pmol/L (detection limit of the assay). Measurements of HbA1c, C-peptide and glucagon were done at the time of the primary studies, except for measurement of glucagon in the epidemiological study; genotyping was done *post hoc*. Blood for *post hoc* laboratory analysis was stored at -80°C since the completion of the primary studies.

Statistical analyses

No participant appears twice in the epidemiological study, or the experimental studies, but some participants were included in both the epidemiological study and an experimental study.

Epidemiological study As the distribution of episodes of severe hypoglycaemia (primary endpoint) is very skewed (1, 2), a log-linear negative binomial regression model was applied (SPSS: Analyze > Generalized

Linear Models > Negative Binomial) with events of severe hypoglycaemia as dependent variable. First, we did univariate analyses with plasma glucagon or TCF7L2 genotype as explanatory variables. Secondly, we performed a multivariate analysis with adjustment for

Table 3 Regression model with episodes of severe hypoglycaemia as dependent variable.

Variable	Change in episodes of SH per year per change in explanatory variable			
	Univariate	<i>P</i>	Multivariate	<i>P</i>
Glucagon (pmol/L)	0.0 (–0.0 to 0.0)	0.83	0.0 (0.0–0.0)	0.96
TCF7L2 genotype				
TT	–0.4 (–1.0 to 0.3)	0.26	–0.2 (–0.8 to 0.3)	0.42
TC	–0.0 (–0.2 to 0.2)	0.89	–0.0 (–0.2 to 0.1)	0.71
CC	–	–	–	–

Regression coefficients (95% confidence intervals) for concentrations of plasma glucagon and the TCF7L2 genotype (explanatory variables) in a generalized linear model (negative binomial distribution) with episodes of severe hypoglycaemia (SH) as dependent variable. The multivariate analyses are adjusted for age, duration of diabetes, C-peptide status, hypoglycaemia awareness status, HbA1c and serum ACE activity at baseline. The CC genotype serves as reference and is set to zero. ACE, Angiotensin-converting enzyme.

age, duration of diabetes, C-peptide status, hypoglycaemia awareness status and HbA1c and serum ACE at baseline. Comparisons between the subgroups in Table 1 were done by one-way ANOVA, Kruskal–Wallis test or chi-square tests as appropriate.

Experimental study We report fasting plasma glucagon concentration at baseline (normoglycaemia) and during experimental hypoglycaemia. Baseline plasma glucagon concentration is calculated as the mean of two baseline values. In the RCT (37), baseline values from both arms of the cross-over study were pooled. Only data from the hypoglycaemic (and not normoglycaemic (control) or hypoxic) study days in the three other studies (34, 35, 36) were included. Peak values of glucagon concentration were defined as the highest glucagon concentration measured during the hypoglycaemic clamp. We used a linear mixed model (SPSS: Analyze>Mixed Models>Linear>Continue) to compare the glucagon concentration during the experiments, the primary endpoint, in the three *TCF7L2* polymorphism groups. Person-id was set as a random factor, and experimental condition (hypoglycaemia vs normoglycaemia), diabetes status (type 1 diabetes vs healthy) and *TCF7L2* polymorphism as fixed factors. Potentially, an interaction among diabetes status, experimental condition and *TCF7L2* polymorphism was present. Therefore, interaction terms were constructed and included as fixed factors: experimental condition×diabetes status, experimental condition×*TCF7L2* polymorphism and diabetes status×*TCF7L2* polymorphism.

Calculations were performed with SPSS software package (version 11.0). We considered a two-tailed *P* value of less than 0.05 statistically significant.

Results

Epidemiological study

A total of 235 episodes of severe hypoglycaemia (0.96 episodes per patient-year) were reported by 90 of 269 patients (37%). The *TCF7L2* alleles could be detected in 245 of 269 patients (91%) and were distributed as follows: TT 5%, TC 36% and CC 59%. The mean incidences of severe hypoglycaemic episodes were 0.54 (0–5) in the TT group, 0.97 (0–15) in the TC group and 0.99 (0–16) episodes per patient-year in the CC group (Table 1).

The risk of severe hypoglycaemia was associated neither with the plasma glucagon concentration nor

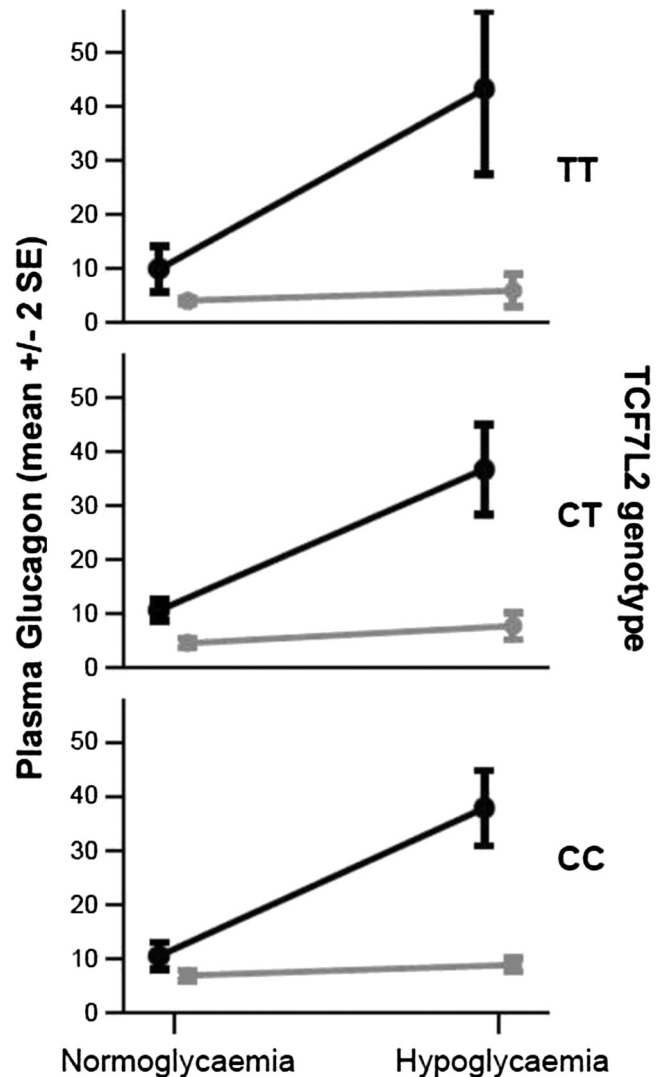


Figure 1 Mean values of plasma glucagon in 38 healthy adults (black lines) and 27 people with type 1 diabetes (grey lines) divided according to the *TCF7L2* genotypes TT, CT and CC before (normoglycaemia) and during experimental hypoglycaemia.

with the *TCF7L2* genotype in univariate or multivariate regression analyses (Table 3). The hypoglycaemia awareness status was not different in the three *TCF7L2* genotype groups ($P=0.15$, chi-square test) (Table 1). Mean plasma glucagon concentrations (± 1 s.d.) were 6.2 ± 4.8 pmol/L, 4.9 ± 3.6 pmol/L and 4.7 ± 3.3 pmol/L in the TT, TC and CC groups, respectively ($P=0.70$; Kruskal–Wallis test) (Table 1). Plasma glucagon concentrations did not differ between subjects with blood glucose concentrations equal to or above vs below 4 mmol/L at the time of glucagon measurement ($P=0.86$), data not shown.

Experimental study

Sixty-nine participants took part in the four studies. Among these, 40 were healthy adults and 29 had type 1 diabetes. The *TCF7L2* alleles could be detected in 65 (38 healthy adults and 27 with type 1 diabetes) participants and were distributed as follows: TT 15%, TC 32% and CC 53%. In the mixed model, there was no effect of *TCF7L2* polymorphism on plasma glucagon concentrations ($P=0.85$ for main effect, $P=0.74$ for the interaction term diabetes status \times *TCF7L2* polymorphism and $P=0.47$ for the interaction term experimental condition and \times *TCF7L2* polymorphism). The interaction term diabetes status \times experimental condition strongly affected glucagon concentration ($F=99.6$, $P=2.0 \times 10^{-14}$). The estimated means of plasma glucagon in healthy adults were 11 (95% C.I. 7.5–14) nmol/L during normoglycaemia and 39 (36–42) nmol/L during hypoglycaemia. The estimated means of glucagon in participants with type 1 diabetes were 5.0 (0.9–9.0) nmol/L during normoglycaemia and 8.3 (4.0–13) nmol/L during hypoglycaemia (Fig. 1).

Discussion

We explored if variants of the *TCF7L2* gene influence the risk of severe hypoglycaemia in a cohort of 245 people with type 1 diabetes followed for one year in an observational prospective study of the occurrence of severe hypoglycaemia and risk markers for the events. In univariate and multivariate analyses, we found no significant relationship between the distribution of *TCF7L2* alleles and the risk of severe hypoglycaemia. Neither did we find any relationship between concentration of random measurements of plasma glucagon on one side and the risk of severe hypoglycaemia on the other.

Healthy carriers of the T allele of the *TCF7L2* gene have been reported to have lower fasting glucagon concentrations compared with carriers of the C allele (26, 27, 28). Therefore, we hypothesised that carriers of the T allele could have a lower glucagon secretory capacity than carriers of the C allele, leading to diminished plasma glucagon responses during hypoglycaemia and thereby an increased risk of SH. We tested this in 38 healthy adults and 27 persons with type 1 diabetes and found no evidence supporting our a priori study hypothesis.

The strength of the epidemiological study is the large number of well-described patients with type 1 diabetes followed prospectively for a relatively long

time. Moreover, thorough gathering of data regarding episodes of severe hypoglycaemia (primary endpoint) and using a statistical model that deals with the much skewed distribution of episodes of severe hypoglycaemia is an advantage. Therefore, we find the results regarding the primary endpoint reliable. It is a possible limitation of the study that episodes of SH may be underreported, especially nocturnal episodes and especially by patients with recurrent episodes (32). Another limitation is the non-standardised measurement of plasma glucagon levels performed in non-fasting blood samples stored for several years. This may lead to both pre-analytical and analytical variation in plasma glucagon concentrations, thereby underestimating any possible role of glucagon. Moreover, our *post hoc* study was done in a population of patients with relatively long-standing type 1 diabetes, which is associated with a reduced secretory glucagon response to hypoglycaemia (20, 21). Potential differences in plasma glucagon levels due to *TCF7L2* gene variations may be blurred by this.

The strength of the experimental studies is the relatively large number of persons studied during controlled hypoglycaemia. However, the methods to obtain hypoglycaemia and the subjects in the four studies were different, making pooling of data questionable. Nevertheless, we believe that the potential problem of using different methods to obtain hypoglycaemia in this *post hoc* study is insignificant as, in all four studies, a robust hypoglycaemic stimulus was obtained. Moreover, a possible influence of the *TCF7L2* polymorphism should not depend on the method of inducing hypoglycaemia. The different subject characteristics of the populations in the four studies probably leads to greater variation in plasma glucagon concentrations rendering possible genotype-related differences in plasma glucagon concentration more difficult to find, increasing the risk of a statistical type 2 error regarding the potential impact of the *TCF7L2* polymorphism on plasma glucagon levels. However, analysing delta values of glucagon separately in participants with or without diabetes did not change the conclusions of this *post hoc* study.

The only hitherto consistently reported biomarkers of severe hypoglycaemia in type 1 diabetes are undetectable C-peptide levels (9, 4), low HbA1c (40, 41, 42) and high serum ACE activity (10, 11, 12, 13, 14, 15). However, these markers only explain a minor proportion of the risk variation between subjects. We conclude that determination of *TCF7L2* gene variations

and random measurements of plasma glucagon do not help predict episodes of severe hypoglycaemia in type 1 diabetes. We also conclude that glucagon secretion during experimental hypoglycaemia in patients with and without type 1 diabetes is not affected by the *TCF7L2* gene polymorphism. These results add up to the complicated picture of glucagon function in glucose homeostasis. Although the *TCF7L2* gene polymorphism seems to affect glucagon secretion during normoglycaemia and hyperglycaemia in normal man and patients with type 2 diabetes, the response to hypoglycaemia both in normal man and patients with type 1 diabetes is unaffected by this polymorphism. However, due to the limitations of the study (pre-analytical and analytical variation in plasma glucagon concentrations), we need studies to validate the effect of *TCF7L2* genotypes on glucagon levels and risk of severe episodes of hypoglycaemia in type 1 diabetes. Finding robust risk factors for the identification of patients at high risk of severe hypoglycaemia would be beneficial in daily diabetes management. Moreover, it could improve the understanding of the physiology of hypoglycaemia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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