CLINICAL STUDY

Activating glucokinase (GCK) mutations as a cause of medically responsive congenital hyperinsulinism: prevalence in children and characterisation of a novel GCK mutation

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Abstract

Objective: Activating glucokinase (GCK) mutations are a rarely reported cause of congenital hyperinsulinism (CHI), but the prevalence of GCK mutations is not known.

Methods: From a pooled cohort of 201 non-syndromic children with CHI from three European referral centres (Denmark, n = 141; Norway, n = 26; UK, n = 34), 108 children had no KATP-channel (ABCC8/KCNJ11) gene abnormalities and were screened for GCK mutations. Novel GCK mutations were kinetically characterised.

Results: In five patients, four heterozygous GCK mutations (S64Y, T65I, W99R and A456V) were identified, out of which S64Y was novel. Two of the mutations arose de novo, three were dominantly inherited. All the five patients were medically responsive. In the combined Danish and Norwegian cohort, the prevalence of GCK-CHI was estimated to be 1.2% (2/167, 95% confidence interval (CI) 0–2.8%) of all the CHI patients. In the three centre combined cohort of 72 medically responsive children without KATP-channel mutations, the prevalence estimate was 6.9% (5/72, 95% CI 1.1–12.8%). All activating GCK mutations mapped to the allosteric activator site. The novel S64Y mutation resulted in an increased affinity for the substrate glucose (S0.5 1.49 ± 0.08 and 7.39 ± 0.05 mmol/l in mutant and wild-type proteins respectively), extrapolating to a relative activity index of ~22 compared with the wild type.

Conclusion: In the largest study performed to date on GCK in children with CHI, GCK mutations were found only in medically responsive children who were negative for ABCC8 and KCNJ11 mutations. The estimated prevalence (~ 7%) suggests that screening for activating GCK mutations is warranted in those patients.

European Journal of Endocrinology 159 27–34

Introduction

Congenital hyperinsulinism (CHI), also known as hyperinsulinaemic hypoglycaemia or persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), is a heterogeneous disease entity with disease-causing mutations in at least eight different genes, two major histological types and a highly variable clinical phenotype with respect to the age of onset, duration and severity (1–4). Mutations are most frequently found in the ABCC8 and KCNJ11 genes coding for the two KATP-channel subunits SUR1 and Kir6.2 respectively (5, 6). Whilst a recessive paternally inherited KATP-channel mutation in combination with maternal loss of heterozygosity is associated with the focal histological type of CHI, dominant or recessive KATP-channel mutations are associated with the diffuse CHI type (7–9). More rarely, dominant mutations in the (GCK) glucokinase, (GLUD1) glutamate dehydrogenase, (INSR) insulin receptor, (HNF4A) hepatocyte nuclear factor 4α genes and, most recently, in the promoter of the SLC16A1 (monocarboxylate transporter 1) gene have been found to cause CHI and similar syndromes featuring hyperinsulinaemic hypoglycaemia (10–14).
Rare recessive mutations of the HADH (formerly SCHAD) gene may also lead to this disease (15). For more than 50% of patients with CHI the genetic aetiology remains unknown (16, 17). This may in part be due to a lack of systematic genetic testing across the known genes involved in CHI.

GCK inactivating mutations cause GCK-maturity-onset diabetes of the young (MODY), which is characterised by a right shift in the enzyme activity glucose dependency curve. By contrast, activating mutations lead to CHI with a left shift of the glucose dependency curve resulting in a lower threshold for glucose-stimulated insulin release (GSIR) (18, 19). The activating mutations described to date have been shown to map to a confined region of the enzyme, which has been termed the allosteric activator site and is remote to the substrate-binding site. Recently, polyubiquitin chains have been suggested to be endogenous ligands for this domain of the enzyme (20). A total of six chains have been suggested to be endogenous ligands to map to a confined region of the enzyme, which has been systematically in large series of CHI cases. Unlike CHI caused by ABCC8/KCNJ11 mutations, GCK-CHI is predicted to be responsive to K<sub>ATP</sub>-channel openers such as diazoxide, as the K<sub>ATP</sub>-channel is intact. However, there is evidence of at least one case where diazoxide treatment was unsuccessful, probably due to a functionally severe mutation (22). Here, we report a large series of patients with transient, persistent or recurrent CHI, with a focus on cases with no K<sub>ATP</sub>-channel abnormalities (n=108) from three European referral laboratories receiving patient DNA samples from several countries. The aim of this study was to estimate the prevalence and describe the phenotype of activating GCK mutations in children with CHI.

**Patients and methods**

**Subjects**

A consecutive pooled cohort of 201 children with an onset of CHI below 15 years of age and having genetic analysis performed in one out of our three genetic analysis centres were included in this study (Table 1). The patients came from different institutions in the UK, Denmark, Sweden, Norway, Portugal, Italy, Holland, Switzerland, Lithuania, Macedonia, Serbia, Turkey, Saudi Arabia, Malaysia, Singapore, Venezuela and Canada. Patient data were shared between the three referral centres to identify any duplicate samples. A diagnosis of CHI was made at local institutions according to the definition of persistent, recurrent or transient hypoglycaemia greater than 1-week duration without any other explanation (e.g. maternal diabetes, intrauterine growth retardation, asphyxia, metabolic disease and medication) and a simultaneous inappropriately elevated p-insulin and/or p-C-peptide and suppressed free fatty acids and ketones. Blood glucose, p-insulin and C-peptide were analysed at the appropriate time points.

**Genetic screening**

In all UK patients, ABCC8, KCNJ11 and GCK (GenBank NM_000352; NM_000525 and NM_000162 respectively) were sequenced directly on either an ABI3700 or ABI3100 capillary sequencer as described previously (21, 28). Sequences were compared with the published sequences using Mutation Surveyor v.3.0 (Biogene, Cambridge, UK). In the Danish centre, mutational screening of the three genes was performed by denaturing high-performance liquid chromatography (DHPLC) analysis of all exons and adjacent intron regions, with direct sequencing of abnormal chromatograms as described previously (18, 29). Sequences were analysed using SeqMan v. 7.2 from the DNASTAR software package (DNASTAR, Inc., WI, USA). In the Norwegian centre, screening was performed by direct sequencing on an ABI3730 capillary sequencer and sequences were analysed using SeqScape Software v.2.1 (Applied Biosystems, Warrington, UK).

GCK analysis was performed in non-syndromic patients with a negative ABCC8 and KCNJ11 mutational screening, or when one K<sub>ATP</sub>-channel gene mutation could not explain the phenotype (e.g. a heterozygous
K\textsubscript{ATP}-channel gene mutation in CHI of uncertain histological type or a heterozygous mutation in CHI of diffuse type.

Prior to compiling prevalence data, patient databases were unblinded to identify duplicate samples referred to multiple testing centres. A total of six patients without K\textsubscript{ATP}-channel mutations had been referred to the UK and Danish centres allowing a quality control check of the two mutation screening approaches in these centres. Out of these six cases, all had been successfully screened by direct sequencing in the UK. In the Danish centre one patient had been excluded due to DNA of poor quality; in the remaining five duplicate samples, the same DNA variations were identified by both the centres.

All GCK mutations were confirmed by PCR amplification of a fresh dilution of patient DNA. The frequency of novel mutations was examined in >400 normal chromosomes and co-segregation with CHI in parental DNA established. Parental relationships were confirmed by microsatellite analysis (details available on request).

**Biochemical characterisation of S64Y GCK**

Recombinant human islet wild-type (WT) enzyme and mutant S64Y GCK were generated and expressed in the form of glutathionyl-S-transferase fusion proteins as previously described (24). A structural analysis was performed using a homology model based on the WT-GCK crystal structure (30). Mathematical modelling was performed as described previously (19).

**Prevalence estimates**

GCK-CHI prevalences were estimated in two cohorts: i) relative to all non-syndromic children with CHI in the Danish–Norwegian international cohort including the five Danish–UK duplicate patients (n = 167); ii) relative to all non-syndromic K\textsubscript{ATP}-channel negative children in the pooled Danish–Norwegian–UK cohort (n = 108) with subdivision into medically responsive (n = 72), and non-responsive, children (n = 21) (Table 1).

**Results**

**Identification of GCK mutations**

A total of 167 non-syndromic children with a diagnosis of CHI were identified in the Danish–Norwegian international cohort. In the pooled Danish–Norwegian–UK cohort, 108 patients were negative for mutations in the K\textsubscript{ATP}-channel genes ABCC8 and KCNJ11 and subjected to GCK mutation screening (Table 1).

GCK screening identified five patients with four different heterozygous mutations (c.191C>A, p.S64Y; c.194 C>T, p.T65I; c.295T>A, p.W99R and c.1367 C>T, p.A456V). The details of three of these patients (T65I, W99R and A456V) have been reported previously (18, 21). A second unrelated proband with the W99R mutation was identified amongst the UK subjects. The sequencing of parental DNA established that the mutation had arisen de novo in the proband. A novel S64Y mutation was identified in a proband from the UK by two of the referral centres (UK and Denmark). This mutation was not present in >400 normal chromosomes and DNA sequencing and microsatellite analysis demonstrated that the mutation had arisen de novo in the proband. In our case series, two mutations arose de novo and vertical transmission was found in three families (18, 21).

**Clinical phenotype of GCK-CHI**

All five GCK-CHI patients had a diagnosis of hypoglycaemia from day 1 or within the neonatal period (Table 2). All the patients were males, born at term with a birth weight in the normal range (mean birth weight of 3382 g). The mean birth weight in patients inheriting a mutation from an affected mother (n = 2) was 2765 g compared with 3793 g in those with unaffected mothers (n = 3). All were medically responsive, although patient 2 (W99R) had hypoglycaemia that was difficult to treat and required a maximal dose of diazoxide (up to 20 mg/kg per d) plus additional medication. This patient at 10 months of age had one documented episode of ketotic hypoglycaemia (blood glucose 1.2 mmol/l and hydroxybutyrate 1.10 mmol/l) with raised p-lactate (2.4 mmol/l) and suppressed p-insulin and p-C-peptide. The investigation for other causes of hypoglycaemia was negative. The patient’s affected asymptomatic father carrying the same mutation had a much milder phenotype and was only diagnosed following his son’s genetic diagnosis at 33 years of age (lowest blood glucose 2.7 mmol/l). Patient 3 carrying the same W99R mutation also had a milder phenotype, requiring lower doses of diazoxide (8–9 mg/kg per d) without the need for additional medication. Patient 1 (A456V) had a period of 10 years without medication, but still required frequent meals to avoid hypoglycaemic symptoms. Both patients 1 (A456V) and 4 (T65I) received no medication throughout childhood, but experienced severe aggravation of their hypoglycaemic symptoms at 14 and 15 years of age respectively when treated with diazoxide. The other patients had a persistent need for diazoxide throughout childhood.

**Functional characterisation of the novel GCK S64Y mutation**

Purified mutant S64Y GCK showed a significant increase in affinity for glucose indicated by approximately a fivefold reduction in glucose S0.5 (1.49 ±0.08 and 7.39 ±0.05 mmol/l for S64Y and WT respectively; Table 3). The km for ATP was increased (1.07 ±0.05 29EUROPEAN JOURNAL OF ENDOCRINOLOGY (2008) 159

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and 0.64 ± 0.04 mmol/l for S64Y and WT respectively. The effective activation of mutant GCK is expressed by the increased activity index of \( w \) compared with WT extrapolating to a marked enhancement of \( b \)-cell glucose usage (19). The structural modelling of S64Y showed that the mutated residue localises to the allosteric activator site. The physiological consequences of the kinetic characteristics of S64Y on GSIR, and therefore glucose homeostasis, were investigated using the published mathematical model that takes into account the impact of blood glucose levels on GCK expression of both alleles (19). Using this model, a predicted threshold of 1.4 mmol/l was obtained.

**Prevalence of activating GCK mutations**

In the largest study performed to date on non-syndromic CHI children without KATP-channel mutations, we have identified five patients with activating GCK mutations. The large sample size allows for the first time an estimation of the prevalence of GCK-CHI. In the Danish–Norwegian international cohort, two GCK mutations were found among a total of 167 non-syndromic CHI patients, with or without \( K_{ATP} \)-channel mutations (Table 1). GCK analysis was performed in 101 out of these patients; that is, in all \( K_{ATP} \)-channel negative children with available DNA (\( n = 74 \)) and in some children with \( K_{ATP} \)-channel mutations in search for additional genetic mutations (\( n = 27 \)). The GCK mutations were only found in \( K_{ATP} \)-channel negative children. Assuming that no patients with a \( K_{ATP} \)-channel mutation also had a GCK mutation, the prevalence of GCK-CHI in all non-syndromic children with CHI is estimated to be 1.2% (2/167; 95% CI 0–2.8%).

In the \( K_{ATP} \)-channel negative pooled cohort of all three centres, the estimated prevalence of GCK mutations was 4.6% (5/108; 95% CI 0.7–8.6%). Out of these patients, 72 were medically treated (including the five with GCK mutations) and 21 underwent pancreatic surgery (Table 1). Accordingly, the estimated prevalence of GCK-CHI in the medically treated group was 6.9% (5/72; 95% CI 1.1–12.8%), and zero in the group treated by surgery.

**Discussion**

**Activating GCK mutations**

Activating GCK mutations are rarely reported in CHI. Including this report, only seven mutations in nine families have been described, in contrast to over 200 inactivating GCK mutations causing the opposite phenotype, GCK-MODY (10, 18, 21–24, 31). In the present study, we have identified a seventh, naturally occurring activating GCK mutation (S64Y), and have functionally characterised the mutant enzyme. Our data...

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<th>Table 2</th>
<th>Clinical characteristics of the five patients with glucokinase (GCK)-congenital hyperinsulinism (CHI).</th>
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<tbody>
<tr>
<td>Patient number</td>
<td>1</td>
</tr>
<tr>
<td><strong>GCK mutation</strong></td>
<td>A456V</td>
</tr>
<tr>
<td><strong>Inheritance</strong></td>
<td>Maternal</td>
</tr>
<tr>
<td><strong>Gestation (weeks)</strong></td>
<td>Term</td>
</tr>
<tr>
<td><strong>Birth weight (g)</strong></td>
<td>2400</td>
</tr>
<tr>
<td><strong>Age at first diagnosis of hypoglycaemia</strong></td>
<td>Day 1</td>
</tr>
<tr>
<td><strong>Lowest blood glucose (mmol/l)</strong></td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Simultaneous blood glucose (mmol/l)</strong></td>
<td>2.6</td>
</tr>
<tr>
<td><strong>Simultaneous p-Insulin (mU/l)</strong></td>
<td>–</td>
</tr>
<tr>
<td><strong>Clinical course</strong></td>
<td>Asymptomatic after 8 months, aggravation with seizures. 14 years of age</td>
</tr>
<tr>
<td><strong>Diazoxide responsive</strong></td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Diazoxide dose (mg/kg per d)</strong></td>
<td>0–12(^{a})</td>
</tr>
</tbody>
</table>

\(^{a}\)In combination with chlorothiazide, change to octreotide long acting release in adulthood.

\(^{b}\)In combination with chlorothiazide and nifedipine, also octreotide responsive.

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<th>Table 3</th>
<th>Functional characterisation of the novel S64Y glucokinase (GCK) mutation.</th>
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</thead>
<tbody>
<tr>
<td><strong>Kinetic parameter</strong></td>
<td>Wild-type GCK</td>
</tr>
<tr>
<td><strong>( K_{cat} ) (S(^{-1}))</strong></td>
<td>61.52 ± 0.69</td>
</tr>
<tr>
<td><strong>Glucose ( S_{0.5} ) (mmol/l)</strong></td>
<td>7.39 ± 0.05</td>
</tr>
<tr>
<td><strong>( nH ) (unitless)</strong></td>
<td>1.58 ± 0.64</td>
</tr>
<tr>
<td><strong>ATP ( k_{m} ) (mmol/l)</strong></td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td><strong>Relative activity index (unitless)</strong></td>
<td>1</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.m. Results are based on the kinetic analysis of three independent expressions of wild-type and mutant GST-GCK.
showed that this mutation, like other CHI-causing GCK mutations, maps to the allosteric activator site. Our kinetic characterisation demonstrated that the mutation was indeed activating and that the increased activity was driven by an increase in affinity for the substrate glucose (Table 3). A comparison with the previously reported and functionally characterised activating mutations demonstrated that S64Y is similar in potency to G68V and A456V (Fig. 1) (18, 24). The importance of performing functional studies is underscored by the limitations of database-based functional prediction in activating GCK mutations. Using the PolyPhen Database (http://genetics.bwh.harvard.edu/pph), the S64Y mutation was predicted to be damaging with a position-specific independent count (PSIC) difference score of 2.153. However, other functionally proven mutations (T65I, W99R and A456V) were predicted to be benign with PSIC scores of 0.187, 0.192 and 1.252 respectively.

With the addition of our novel mutation, the known naturally occurring activating GCK mutations mapping to the allosteric site are: S64Y, T65I, G68V, W99R, Y214C, V455M and A456V (10, 18, 21–24). In addition, the GCK-MODY V62M mutation has been shown to be kinetically activating in vitro. This paradox may be explained by the inability to bind regulatory proteins such as GCK regulatory protein GKRP and/or unidentified endogenous GCK activators (32). The allosteric area is involved in transforming GCK from the closed (active) to the super-open (non-active) state of the enzyme, involving three intermediate stages (30, 33). Proposed natural ligands and activating mutations slow down the conformational changes between the intermediate stages from the closed to the super-open stage. To mimic the activating properties of GCK-CHI mutations, drugs have been designed to target the allosteric activating site in the treatment of T2DM (30, 34, 35). These drugs have been shown to have their effect abolished by diazoxide in accordance with our finding that GCK-CHI was diazoxide-responsive (35).

**Phenotype**

There has been some debate whether all GCK-CHI patients are medically responsive. One historical patient with the Y214C genotype underwent subtotal pancreatectomy after an unsuccessful trial of diazoxide for 6 months at a dose of 2 mg/kg (22). In vitro, the Y214C mutation has very high activating properties with a relative activity index of ~130 compared with ~30 for A456V (Fig. 1). The patient was subjected to subtotal pancreatectomy, although the maximal diazoxide dose given was 5 mg/kg per d, far from the maximally recommended dose of 20 mg/kg per d. It remains possible that the patient may have responded at higher doses and thereby avoided surgery. The treatment in this patient illustrates the problem of categorising GCK-CHI as severe or mild according to whether pancreatic surgery was performed (see below).

In GCK-MODY (caused by inactivating mutations) there is strong evidence that foetal growth is influenced by both the foetal and the maternal GCK genotypes (36). Affected offspring who have paternally inherited their mutation are on average ~500 g lighter than those who have maternally inherited their mutation (36). The changes in birth weight are likely to reflect changes in foetal insulin secretion which in turn is influenced by the foetal genotype and indirectly by maternal hyperglycaemia. It is therefore likely that with activating GCK mutations, the opposite scenario exists; affected offspring who have paternally inherited their mutation would have increased birth weight whilst those who have maternally inherited their mutation would be predicted to have a birth weight within the normal range. Unfortunately, the small number of cases reported to date does not allow us to formally test this hypothesis.

Although all the patients in our study were diagnosed with hypoglycaemia in the neonatal period, several other GCK-CHI patients have been diagnosed at a much later age, even as late as adulthood (10, 18, 21, 23, 24). The highly variable age of diagnosis is likely to be influenced by several factors including the functional severity of the mutations, the genetic background and delay in
diagnosis. The phenotype of GCK-CHI may be highly variable even within families with the same GCK mutation (10, 18, 21, 23, 24). In our study, the W99R GCK mutation was associated with both mild and more difficultly treated hypoglycaemia with occasional ketosis, although functional characterisation of this mutation has demonstrated only mild GCK activation (Fig. 1). Intermittent attacks of ketotic hypoglycaemia have been reported in two other diazoxide-treated CHI patients with unknown genetic aetiology (37). The coincidental occurrence of CHI and ‘idiopathic’ ketotic hypoglycaemia could explain the more severe phenotype of patient 2 and the two reported cases, although a rare side effect of diazoxide treatment cannot be excluded.

Prevalence

The prevalence of activating GCK mutations among children with CHI has hitherto not been systematically studied. The large number of patients included in the present study as a result of three international centres pooling cases allows a robust estimate of the prevalence of GCK-CHI. Among non-syndromic children with CHI, only 1.2% had GCK-CHI. Among non-syndromic children with CHI, only 1.2% had GCK alterations, whilst in non-syndromic children with CHI without K\textsubscript{ATP}-channel mutations, 4.6% had GCK-CHI. The frequency increased to 6.9% when only patients who were medically responsive were considered. GCK-CHI was not found in surgically treated patients, suggesting that medically non-responsive GCK-CHI is exceedingly rare if ever occurring with today’s medication regime.

These estimates may have been influenced by the patient selection for genetic analysis. By recruiting children with CHI diagnosed within the first week, we may inadvertently have included a high proportion of children with transient neonatal CHI, especially in those cases referred from Denmark. If a more restrictive definition had been employed, e.g. a minimum duration of 1 month, the prevalence estimate might have been higher, as the majority of patients with transient CHI do not have any of the known genetic aetiologies. However, in one of our patients (patient 4, T65I), the hypoglycaemia was asymptomatic and untreated in the neonatal period with a clinical relapse at 15 years of age. Mild or undetected hypoglycaemia in the neonatal period and a delayed onset of hypoglycaemic attacks up to adulthood have been seen in many other cases (10, 18, 21, 23, 24). Accordingly, the exclusion of mild and transient neonatal cases could a priori also lead to an underestimation of the true prevalence.

The prevalence estimates may also have been influenced by the inclusion of several patients from countries other than Denmark, UK and Norway. This could lead to a selection bias towards severely affected patients with an expected higher prevalence of K\textsubscript{ATP}-channel mutations. However, the majority of patients from other countries were not subjected to pancreatic surgery at the time of genetic analysis, and only few had a rapid genetic analysis for the prediction of focal versus diffuse disease (38). Moreover, the prevalence estimate of GCK-CHI in medically treated children without ABCC8/KCNJ11 mutations could have been influenced by a different threshold for pancreatic surgery in different referral centres and over time. Finally, the sensitivity of the DHPLC method used by the Danish centre is estimated to be 95–100% in GCK analysis (39), which may result in up to a 5% underestimation of the true prevalence. However, the comparable results from the five duplicate patients studied by both DHPLC and direct sequencing supported that the underestimation was low.

The medical CHI treatment of today includes a number of drugs and dose recommendations (17). Earlier recommendations of prompt subtotal pancreatectomy to reduce the risk of brain damage from hypoglycaemia are no longer supported for a number of reasons: i) focus ablation is sufficient to cure patients with focal disease (8), ii) some patients with diffuse CHI may be treated successfully by medication and eventually undergo clinical remission after months or even several years (40-42) and iii) subtotal pancreatectomy is complicated by an increased risk of subsequent diabetes and mild malabsorption (43). The switch towards a more conservative approach in diffuse CHI (if possible) and a rapid focal resection in focal CHI in many centres implies that a uniform definition of CHI as mild/moderate or severe according to treatment modality may be questioned.

We propose that non-syndromic patients with medically responsive CHI who are negative for K\textsubscript{ATP} mutations should be considered for GCK genetic testing. Due to the low prevalence of GCK-CHI, the first genetic screening performed should in any case be of the K\textsubscript{ATP}-channel genes (ABCC8/KCNJ11) with the rare exception of a known GCK mutation in the family. If an activating GCK mutation is identified, the investigation of family members should be carried out to detect asymptomatic or mild symptomatic CHI.

In conclusion, we have identified a novel S64Y GCK mutation and a second patient with the W99R mutation. We have performed the largest study to date of a pooled cohort of 108 patients from three international referral centres to assess the prevalence of GCK-CHI in subjects in whom K\textsubscript{ATP}-channel mutations have been excluded. Our study suggests that activating GCK mutations are a rare cause of medically responsive CHI with an estimated prevalence of ~7% in this group of patients. In non-syndromic children with CHI, the overall prevalence of GCK-CHI was estimated to only ~1%.

Acknowledgements

The authors thank Sarah Flanagan for her assistance with compiling the clinical data on the UK patients with hypoglycaemia of infancy. This study was funded
in Oxford by Diabetes UK and the University of Oxford John Fell Research Fund, in Denmark by the Danish Diabetes Association and the County of Funen, and in Norway by grants from Helse Vest and the Research Council of Norway. A. G. is a Diabetes UK RD Lawrence Research Fellow. The authors have nothing to disclose.

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Received 18 April 2008

Accepted 22 April 2008