

1 **Opposite clinical phenotypes of “glucokinase disease”:** description of a novel activating
2 **mutation and contiguous inactivating mutations in human glucokinase (*GCK*) gene.**

3
4 Abbreviated title: Biostructural analysis of GCK disease

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6 Fabrizio Barbetti ^{a, b, c}, Nadia Cobo-Vuilleumier ^d, Sonia Toni ^e, Carlo Dionisi-Vici ^a, Paolo
7 Ciampalini ^a, Ornella Massa ^a, Pablo Rodriguez-Bada ^d, Carlo Colombo ^a, Lorenzo Lenzi ^e,
8 María A. Garcia-Gimeno ^f, Francisco J. Bermudez^d, Fernando Rodriguez de Fonseca^d, Patrizia
9 Banin ^g, Juan C. Aledo ^{d,h}, Elena Baixeras ^d, Pascual Sanz ^f and Antonio L. Cuesta-Muñoz ^d.

10
11 ^a Bambino Gesù Pediatric Hospital, IRCCS, Rome, Italy

12 ^b Department of Internal Medicine, University of Rome Tor Vergata, Rome, Italy

13 ^c Laboratory of Molecular Endocrinology and Metabolism, S Raffaele Biomedical Park
14 Foundation, Rome, Italy

15 ^d Center for the Study of Pancreatic beta cell Diseases. IMABIS Foundation and Carlos Haya
16 Hospital, Málaga, Spain.

17 ^e Regional Center for Juvenile Diabetes, Meyer Pediatric Hospital, Florence

18 ^f Institute of Biomedicine of Valencia (CSIC) and CIBERER-ISCIH, Valencia, Spain.

19 ^g Pediatric and Adolescent Unit - S. Anna Hospital - Ferrara, Italy

20 ^h Molecular Biology and Biochemistry Department. University of Málaga, Málaga, Spain.

21
22 **Correspondence and requests for reprints to:**

23 Fabrizio Barbetti, MD, PhD, Tor Vergata University Hospital, Laboratory Medicine, 1st floor,
24 section D, room 118, Viale Oxford 81, 00134 Rome, Italy. E-mail: mody.2@libero.it or
25 fabrizio.barbetti@spr-r.it, and Antonio Cuesta-Munoz, MD, PhD, Center for the Study of
26 Pancreatic beta cell Diseases. IMABIS Foundation and Carlos Haya Hospital. Avda. Carlos
27 Haya 82, Pabellón A-7ª planta 29010 Málaga, Spain. E-mail: alcm@fundacionimabis.org.

29 **Key words:** *Glucokinase, Glucokinase mutations, Glucokinase structural analysis, Monogenic*
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31 **Abbreviations:** *GCK*, glucokinase gene; *GK*, glucokinase protein; *GK-WT*, glucokinase wild-
32 type; *GSIR*, glucose stimulated insulin release; *GSIR-T*, threshold for glucose stimulated insulin
33 release; *FPG*, fasting plasma glucose; *IFG*, impaired fasting glucose; *OGTT*, oral glucose
34 tolerance test; *IVGTT*, intravenous glucose tolerance test; *FPIR*, first phase insulin response.

35

36 **Disclosure Statement**

37 The authors have nothing to disclose.

38

39 **Abstract**

40 Glucokinase (GK) is essential for glucose-stimulated insulin release from pancreatic β -
41 cell, serving as glucose sensor in humans. Inactivating or activating mutations of glucokinase
42 lead to different forms of “Glucokinase Disease”, i.e. Monogenic Diabetes of Youth (GCK-
43 MDY), Permanent Neonatal Diabetes (inactivating mutations) and Congenital Hyperinsulinism,
44 respectively. Here we present a novel *GCK* activating mutation (p.E442K) found in an infant
45 with neonatal hypoglycaemia (1.5 mmol/l) and in two other family members suffering from
46 recurrent hypoglycemic episodes in their childhood or adult life. In contrast to the severe
47 clinical presentation in the index case, functional studies showed only a slight activation of the
48 protein (relative activity index of 3.3). We also report on functional studies of two inactivating
49 mutations of the *GCK* (p.E440G and p.S441W), contiguous to the activating one, that lead to
50 GCK-MDY. Interestingly, adult family members carrying the GK pE440G mutation show a
51 unusually “progressive” diabetic phenotype.

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53

54 INTRODUCTION

55 Glucokinase enzyme (GK) has unique functional and structural properties for acting as
56 the pancreatic β -cell “glucose sensor”. GK plays thus a role in glucose-stimulated insulin
57 release (GSIR) (1), and regulates the threshold for GSIR (GSIR-T) (1). The high control of GK
58 on β -cells function is best illustrated by the profound impact on GSIR-T of mutations of
59 glucokinase gene (*GCK*). Indeed, GSIR-T in carriers of inactivating *GCK* mutations increases,
60 leading to the mild fasting hyperglycemia in subjects with heterozygous mutations or to severe
61 diabetes like in the case of individuals with homozygous or compound heterozygous mutations.
62 The resulting clinical phenotype of partial glucokinase deficiency is GCK-Monogenic Diabetes
63 of Youth (GCK-MDY), also known as Maturity Onset Diabetes of the Young 2 (MODY2) (2),
64 while complete glucokinase deficiency leads to permanent, neonatal diabetes mellitus (GCK-
65 PNDM) (3,4). On the contrary, the GSIR-T in carriers of activating mutations of *GCK*
66 decreases, causing hypoglycemia (GCK-HI) due to inappropriate insulin secretion when plasma
67 glucose is below the normal GSIR-T, featuring mild or severe forms of the disease (5-8). These
68 different GK linked disorders of glucose metabolism (GCK-MDY, GCK-PNDM and GCK-HI)
69 are the three different forms of “glucokinase disease”.

70 “Glucokinase disease” can be caused by missense *GCK* mutations located anywhere in
71 the primary sequence with no major “hot spots” defined (9,10). However GCK-HI mutations
72 cluster in the small domain of GK protein, where the allosteric activator site is located (11)

73 In this manuscript we present a novel *GCK* activating mutation that, in spite of
74 presenting the lowest relative activity index (%AI) and the highest predicted GSIR-T of all
75 naturally occurring GK activating mutations described so far, leads to severe neonatal
76 hypoglycaemia (1.5 mmol/l). Furthermore, we also report functional studies of contiguous
77 inactivating mutations in *GCK* that lead to the hyperglycaemic form of the “glucokinase
78 disease” (GK-MDY).

79

80 RESULTS

81 *Identification of missense mutations in the GCK gene*

82 Family trees of the three patients carrying the *GCK* mutations studied in this report are
83 depicted in figure 1. Denaturing gradient electrophoresis (DGGE) experiments showed
84 abnormal patterns of PCR products of exon 10 of the *GCK* in all affected families' members
85 available for analysis. We identified three missense mutations in the heterozygous state. A novel
86 mutation in codon 442, with lysine substituting for glutamic acid (p.E442K) in the proband of
87 family GCK-HI-1 (subject III-2 in Fig. 1a), as well as in her affected brother and mother
88 (subjects III-1 and II-1 in Fig. 1a). A single nucleotide change resulting in a tryptophan for
89 serine substitution at codon 441 (p.S441W) in the proband of family FE-11 (subject III-1 in Fig.
90 1b), in his younger brother, his mother and maternal grandmother. This mutation had been
91 previously described in another Italian family (10). Finally, a novel mutation resulting in a
92 substitution of glutamic acid by glycine at codon 440 (p.E440G) was found in the proband of
93 family FI-10 (subject III-1 in Fig. 1c), in his younger brother, in his father and in a paternal
94 uncle. None of the mutations were found in 100 healthy controls' chromosomes.

95 *Metabolic features of families with GK diseases*

96 Family GCK-HI-RM-1 (E442K). The proband with hypoglycaemia (Subject III-2 in Fig. 1a)
97 was born at the 41st week of gestation and presented at 1st day of life with plasma glucose (PG)
98 of 1.5 mmol/L. Her birth weight was 2,840 g and the clinical examination unremarkable. Family
99 history disclosed that the maternal grand father, the mother as well as the eldest brother
100 presented with recurrent, symptomatic “hypoglycemic-like” episodes characterized by cold
101 sweating, pallor, fatigue, hunger, and tachycardia; in addition, the mother and the brother of the
102 index case showed fasting plasma glucose of 2.2-3.0 mmol/l in several occasions. At the age of
103 3 weeks (weight: 4,200 g) the proband was referred to the Metabolic Unit of Bambino Gesù
104 Pediatric Hospital for further investigation. At that time her plasma glucose ranged between 1.9
105 and 4.3 mmol/L. She had normal plasma values of ammonia, lactate, triglycerides (122 mg/dl)
106 and total cholesterol (130 mg/dl). The urine excretion of α -ketoglutarate, another marker of HI
107 due to gain-of-function mutations of glutamate dehydrogenase gene (*GLUD1*) (12), was normal,
108 as well as blood acylcarnitines, aminoacids, and serum transferrin IEF. Abdominal and brain
109 ultrasonography were also normal. Following i.m. glucagon (1 mg.), plasma glucose (PG) rose

110 from 2.2 to 6.0 mmol/L; simultaneous baseline evaluation of plasma cortisol (23 µg/dl), IGF1
111 (237 ng/ml) and ACTH (35 pg/ml) were all normal. These clinical investigations combined
112 excluded some causes of hypoglycemia. Low dose diazoxide therapy (2 mg/kg/d) was started in
113 the proband which normalized blood glucose and was continued for 3 months. The patient, now
114 6 years old and on diet therapy only, did not suffer of any other symptomatic hypoglycemia; in
115 contrast the patient's elder brother experienced in the same time interval two unexplained
116 syncopal episodes.

117 Family FE-11 (S441W). All the affected members of this family (Fig. 1b) presented
118 impaired fasting glucose (IFG) (6.5-6.9 mmol/l), and all were treated only with diet. The oral
119 glucose tolerance test (OGTT) carried out in the proband (Fig. 1b, subject III-1) showed IGT
120 and low plasma insulin at 30' (18 µU/ml with corresponding plasma glucose of 10 mmol/l)
121 (Table 1), a result which is typically found in GCK-MDY patients.

122 Family FI-10 (E440G). The proband and his younger brother presented impaired fasting
123 glucose (IFG) (6.1 and 5.8 mmol/l, respectively) (Fig. 1c, subjects III-1 and III-2). Intravenous
124 glucose tolerance test (IVGTT)-derived first-phase insulin response (FPIR) of children carrying
125 the p.E440G mutation showed unexpectedly high figures of 300 and 241 µU/ml. These values
126 respectively exceed the 97th (260.7 µU/ml) and the 90th (223.7 µU/ml) centile of normal Italian
127 children of corresponding pubertal status (13). This high FPIR was observed again in both
128 brothers when the IVGTTs were repeated two years later (308 and 215 µU/ml, respectively)
129 (Table 1). In addition, adult family members carrying the mutation showed a diabetic
130 phenotype, not typical of GCK-MDY, with proband's father presenting high fasting plasma
131 glucose (10.4 mmol/l) and a paternal uncle treated with oral hypoglycemic agents; unfortunately
132 two other paternal uncles, one treated with OHA and another with insulin were not available for
133 genetic analysis (Fig. 1).

134 *Kinetic analysis of recombinant wild type and mutant glucokinase.*

135 Kinetic properties of WT and mutant GK are shown in Table 2. Mutation GK-E442K
136 showed a higher affinity for glucose ($S_{0.5}$ = 4.43 mmol/l) than GK-WT and a relative activity

137 index (%AI) which was over 3 times higher (Table 2), indicating that K442 is an activating
138 mutation. On the contrary, mutations GK-E440G and GK-S441W showed a lower affinity for
139 glucose, ($S_{0.5} = 10.85$ and 15.89 mmol/l, respectively), as compared to GK-WT. The lowest
140 affinity for the second substrate $MgATP^{2-}$, catalytical activity and cooperativity index (Hill
141 number - n_H) were found in mutation GK-S441W (Table 2). The %AI of GK- E440G and GK-
142 S441W were almost 60% and 90% lower than GK-WT. Consequently, the calculated GSIR-T of
143 MDY-causing mutations GK-E440G and GK-S441W was higher than GK-WT (5.82 and 6.63
144 mmol/l vs. 5 mmol/l respectively), while calculated GSIR-T of the activating mutation GK-
145 E442K was lower (4.14 mmol/l) than GK-WT (Fig. 2).

146 *Prediction of structural effects of naturally occurring glucokinase mutations*

147 We introduced the mutated residues into the closed active and super-open inactive GKB
148 (Glucokinase Beta cell-specific) structure models described by Kamata et al (14) and then
149 compared mutated GK-G440, GK-W441 and GK K442 with GK-WT. In Kamata's model the
150 side chain of Glu 442 (E442) is exposed to the solvent in both closed and super-open
151 conformations. The mutant Lys residue in GK-HI-causing K442 would also be exposed to the
152 solvent in both conformations (Fig. 3a). However, the positive charge of the side chain of the
153 Lys 442 residue may interact with the negative charge of Glu 216 residue (E216), stabilizing the
154 closed conformation. In addition, in the super-open conformation (Fig. 3a), the side chain of
155 Lys 442 would be closer to Ala 454 residue (A454), destabilizing the super-open conformation.
156 As a result, the structure of K442 enzyme would favor the closed, active conformation, and
157 would consequently lead to higher glucose affinity.

158 The change of Glu 440 residue (E440) by Gly residue (G440) (Fig. 3b) would
159 destabilize the structure of the protein, since Gly residues are prone to modify the conformation
160 of a particular structure. This interpretation would explain the observed reduction in glucose and
161 $MgATP^{2-}$ affinity. In the case of the S441W mutation (Fig. 3c, W441), the new bulkier
162 tryptophane residue would be projected toward the inside of the protein, destabilizing it because
163 of its interaction with the $\alpha 5$ helix. This would cause a dramatic reduction in glucose affinity, as
164 observed in *in vitro* experiments.

165

166 **DISCUSSION**

167 In this report we present a novel activating *GCK* mutation (p.E442K) in a newborn with
168 severe neonatal hypoglycemia. She is now 6 years old, in good general condition and treated
169 with diet therapy only. Her older brother and mother, also bearing the same *GCK* mutation, did
170 not present hypoglycemia in the neonatal period but repeated episodes of symptomatic
171 hypoglycemia later in life. Functional studies of mutated protein showed an enzyme with almost
172 a two-fold increase in glucose affinity compared to wild type GK. The E442 residue is located
173 in a loop between β 13 and α 13 domains of GK. According to Kamata's model (14), this loop
174 plays an important role in the conformational change of the GK from the super-open to the
175 closed active conformation of the enzyme. Although E442 does not participate directly in the
176 binding to the allosteric activator (compound A in Kamata's model) (14), the K442 mutation
177 favours the transition to the closed conformation of the enzyme, leading to increased glucose
178 affinity (Fig. 3a). The structural analysis along with the calculated %AI and GSIR-T (3.3 and
179 4.14 mmol/l, respectively) obtained from the experimental data, indicate that the activation of
180 GK caused by the E442K mutation is the cause of hypoglycaemia in the proband and in other
181 members of the family carrying the mutation (Figure 1a).

182 This mutation is a good example of the discrepancy one may find between the severity
183 of the clinical phenotype at presentation and the data resulting from the functional analysis "*in*
184 *vitro*" of the mutated enzyme. Indeed, the results of the *kinetic* analysis showed that the
185 mutation E442K presents the lowest %AI as well as the highest GSIR-T of all naturally
186 occurring *GCK* activating mutations described to date (11,15). This is in line with the relatively
187 mild symptoms of hypoglycemia showed in affected members in childhood and adult life, but in
188 contrast with the severe clinical onset presented by the proband. We can only speculate that the
189 proband could have some transient immaturity of glucoregulatory pathways which contributed
190 to lower her plasma glucose levels. However, we do not believe that this may be ascribed to the
191 relatively low birth weight of the proband, which was in line with that reported in other patients
192 with activating *GCK* mutations born from affected mothers (16).

193 We also described two inactivating *GCK* mutations (p.E440G, p.S441W) contiguous to
194 the activating p.E442K. Patients carrying the mutation p.S441W present a phenotype highly
195 characteristic of GCK-MDY patients, i.e. IFG combined with impaired glucose tolerance (IGT)
196 with low values at tests evaluating (early) insulin release (17). In contrast, the two young and
197 lean brothers carrying the mutation p.E440G showed high plasma insulin levels at 30' of OGTT
198 and in two independent IVGTT tests (Table 1). Alike the family previously reported with GCK-
199 MDY (mutation p.L184P) (18), we also observed some metabolic heterogeneity within the
200 different E440G carriers of family FI-10 (Fig. 1). As a matter of fact, though functional and
201 structural studies of GK-E440G (Table 2) fully explain the basic metabolic features of the
202 proband and his brother, they are not sufficient to clarify the severe diabetic phenotype observed
203 in adult family members carrying the mutation. Thus, the co-existence of other unknown genes
204 implicated in glucose metabolism or unrecognized environmental factors should be considered
205 in this kindred.

206

207 **MATERIAL and METHODS**

208 *Subjects*

209 The proband with hypoglycemia (Subject III-2 in Fig. 1a) was referred to the Metabolic
210 Unit of Bambino Gesù Children's Hospital for diagnostic workup. Routine laboratory exams
211 were all normal with the exception of insulin, which was inappropriately high (12 μ U/ml) for
212 the corresponding plasma glucose. Family history disclosed that her elder brother and mother
213 (subjects III-1 and II-1 in Fig. 1a) suffered repeated episodes of symptomatic hypoglycemia and
214 the maternal grandfather (deceased, not tested) had "hypoglycemia-like" symptoms (i.e.
215 sweatiness, headache, hunger and weakness).

216 The diabetic probands studied in this report were identified during the course of an Italian
217 nation-wide project aimed at selecting patients with monogenic diabetes (MD) (subjects III-1 in
218 Fig. 1b and 1c) out of subjects referred to pediatric diabetes clinics for incidental hyperglycemia
219 (10,19).

220 *Metabolic studies*

221 Probands of families with (GCK-MDY) (subjects III-1 in Fig. 1b and 1c) underwent two
222 tests as part of the protocol for studying subjects with incidental hyperglycemia: a standard
223 OGTT with serum insulin determinations and an IVGTT. IVGTT was performed injecting 0.5 g
224 of glucose per kg/body weight. Blood samples were taken at -15, 0, and 1, 3, 5, 10 minutes after
225 the glucose injection for plasma insulin determination. FPIR was calculated as the sum of
226 insulin immunoreactivity at minute 1 and 3 and the result compared to those obtained in normal
227 Italian children according to their pubertal stage (13). Insulin assay was not centralized.
228 However other IVGTT tests performed in the same center of family FI-10, on probands with
229 mutations in the GCK always elicited low FPIR (see Table 1). All tests were approved by the
230 local institutional ethics committee and a written informed consent was obtained from the
231 parents of the probands.

232 *Molecular genetic studies*

233 Genomic DNA was extracted from peripheral lymphocytes. The complete coding sequence of
234 the *GCK* was amplified by the polymerase chain reaction (PCR) and analysed by denaturing
235 gradient electrophoresis (DGGE) as previously described (10). In the proband clinically defined
236 as having hyperinsulinemic hypoglycemia and his relatives, screening of *GCK* gene was
237 performed first, based on the autosomal-dominant mode of inheritance of hypoglycaemia (5,
238 11). We excluded the possibility of mutations of the glutamate dehydrogenase gene (*GDH*)
239 because of the normal plasma ammonia concentrations (20). The PCR products showing
240 abnormal electrophoretic pattern were subjected to direct sequencing by an ABI DNA
241 sequencing apparatus 373A (Perkin Elmer Applied Bio Systems, Foster City, CA). Mutations
242 were confirmed in all affected family members available for analysis.

243 *Site-directed mutagenesis*

244 Mutations p.E440G, p.S441W, p.E442K on *GCK* were introduced into the wild type
245 human pancreatic *GCK* using the Quick Change site-directed mutagenesis kit from Stratagene
246 (La Jolla, CA), (oligonucleotides sequences available upon request). Plasmid pUC-GlkB was
247 used as a template in the PCR reactions. All plasmids were sequenced to confirm that only the
248 desired mutation had been introduced. Mutated plasmids (pUC-GlkB-E440G, S441W and

249 E442K) were digested with *EcoRI* and *Sall* and the insert subcloned into plasmid pGEX-6P-1
250 (Amersham Pharmacia), to allow its expression in *Escherichia coli* as a glutathionyl S-
251 transferase (GST) fusion protein. Purified recombinant GST-GK was routinely screened for
252 purity by SDS-PAGE.

253 *Kinetic and structural analysis of the GK protein*

254 Studies of the kinetic properties of GK wild type (GK-WT) and GK-E440G, GK-S441W
255 and GK-E442K in the presence of 2 mmol of dithiothreitol per liter of reaction mixture were
256 performed spectrophotometrically as described previously (21). At least three different
257 preparations of GK-WT and GK-mutants were made and analysed. We used non-linear kinetics
258 according to the Hill equation to determine the affinity of the enzyme for glucose, and the Hill
259 coefficient that characterizes the sigmoidal glucose dependency of GK. In order to measure the
260 glucose phosphorylation capacity of the enzyme we used the relative activity index, which was
261 calculated according to the formula previously reported (11). The structural analysis of the
262 activating mutation was carried out using the crystal structure of human GK (14).

263 *Mathematical modelling*

264 We used a minimal mathematical model (1,11,21) to assess the impact of GK mutations
265 on GSIR. We determined the impact of blood glucose levels on GK expression for both wild
266 type and mutated alleles by using the expression coefficient for either allele: $e = (S^{nH} \times 2) / (S^{nH} +$
267 $S_{0.5}^{nH})$, where S refers to the glucose level at threshold, nH is the Hill coefficient for
268 cooperativeness with glucose, the numerical value of 2 indicates that half-maximal induction is
269 achieved at glucose $S_{0.5}$, and $S_{0.5}$ refers to the concentration of glucose needed to achieve the
270 half-maximal rate of phosphorylation (1, 11).

271

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278

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348 LEGENDS TO FIGURES.

349

350 **Fig. 1** Family-trees of families GCK-HI-1, FE-11 and FI-10. Patients with no mutations
351 detected in *GCK* are denoted as n/n; those carrying the mutations are denoted as n/m; nt= not
352 tested. Individuals identified by symbols with vertical white lines have IFG. Individuals with
353 symbols filled in black have diabetes. Individuals with horizontal black lines have
354 hyperinsulinism and hypoglycemia. Current treatment is indicated below patients' symbols.

355

356 **Fig. 2** Threshold for glucose stimulated insulin release (t-GSIR) as a function of the relative
357 activity index of glucokinase protein wild type (GK-WT) and naturally occurring mutations
358 GK-E440G, GK-S441W and GK-E442K.

359

360 **Fig. 3** Close-up of the structural model of the GK-E440, GK-S441, GK-E442 (wild type), GK-
361 E440G, GK-S441W (GCK-MDY naturally occurring mutation) and GK-E442K (HI mutation).
362 The key $\alpha 5$ and $\alpha 13$ helices of glucokinase are indicated in the left structures of **Panel a**,
363 (super-open conformation of glucokinase) (14) and **Panels b, c** (closed conformation) (14). An
364 enlargement of the region of interest (dotted square) is shown in each panel. Mutated residues
365 are shown in red. The interacting residues are in green, cyan and magenta. Crystal coordinates
366 from the closed active (1V4S) and super-open inactive (1V4T) conformation of GlkB (14) were
367 visualized using the Pymol Molecular Graphics System version 0.97 (Delano Scientific LLC).

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Table 1: Results of the metabolic studies performed in the probands with GCK-MDY.

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Family/mutation	AGE (years)	BMI (Kg/m ²)	IVGTT		OGTT					
			FPIR (μ U/ml)	FPG (mmol/L)	IRI/bas. (μ U/ml)	IRI/30 min. (μ U/ml)	PG/30 min. (mmol/L)	IRI/120 min. (μ U/ml)	PG/120 min. (mmol/L)	
FI-10/E440G										
Subject III-1										
1997	10	17.5	308	6.1	13.9	180	10	50	7	
1999	12		310							
Subject III-2										
1997	8	17.2	241	5.8	7	70	9.5	11.1	5.1	
1999	10		215							
FE-11/S441W										
Subject III-1	10	n.a	n.a	6.7	5	18	10	20	7.9	
MI-34/S441W										
Proband (9)	9	16.2	96	7.6	13	n.a.	12.8	n.a.	8.2	

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(9); The results of this proband presented in this table have been previously reported in Massa et al. Diabetologia 44:898-905. n.a.; not available.

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The following FPIR values have been obtained in probands with GCK mutation who underwent IVGTT in the same Center of family FI-10: proband FI-2, mutation S383fsdelC1=37 (<1st centile; ref 18); proband FI-22, mutation Y108H=54 (=1st centile); proband FI-14, mutation L276P=56 (<3rd centile); proband FI-18, mutation F150Y=80 (<25th centile); proband FI-4, mutation N231fsdelA=92 (=25th).

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Table 2: Functional characteristics of the recombinants glucokinase proteins wild type (GK-WT), and the naturally occurring mutations GK-E440G, GK-S441W and GK-E442K. Data are means \pm SE from three separate enzyme preparations.

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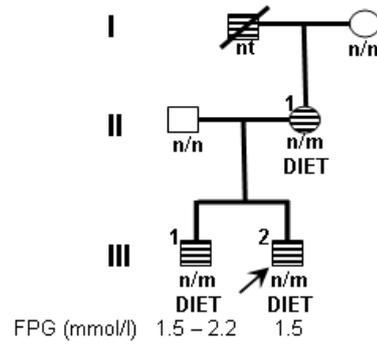
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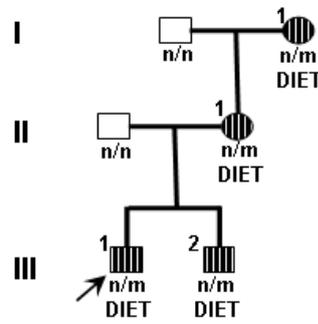
	S_{0.5} (mmol/l)	n_H (unitless)	ATP_{Km} (mmol/l)	K_{cat} (sec ⁻¹)	Relative Activity Index (%AI)
WT	7.97 \pm 0.31	1.71 \pm 0.07	0.45 \pm 0.05	65.57 \pm 7.19	1
E440G	10.85 \pm 0.68	1.52 \pm 0.03	0.74 \pm 0.04	51.55 \pm 4.64	0.41
S441W	15.89 \pm 2.49	1.38 \pm 0.07	1.12 \pm 0.14	20.72 \pm 4.56	0.11
E442K	4.43 \pm 0.17	1.6 \pm 0.05	0.72 \pm 0.1	41.98 \pm 6.20	3.33

Figure 1

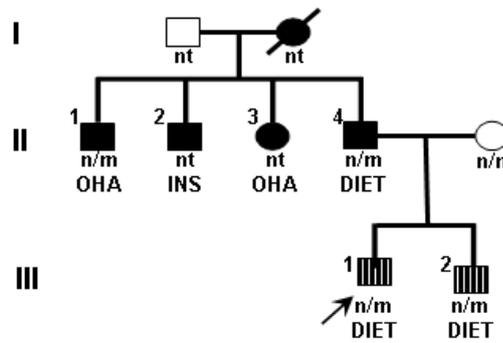
a Family RM-1
(p.E442K)



b Family FE-11
(p.S441W)



c Family FI-10
(p.E440G)



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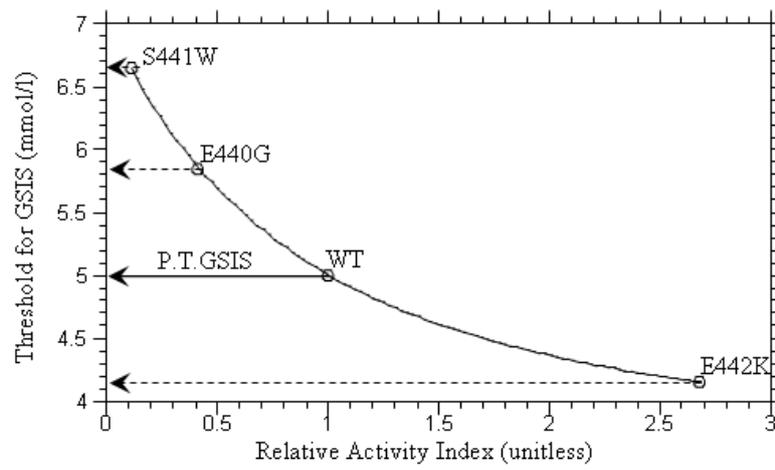
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Figure 2



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Figure 3

