

Permanent diabetes during the first year of life: multiple gene screening in 54 patients

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Abstract

Aims/hypothesis The aim of this study was to investigate the genetic aetiology of permanent diabetes mellitus with onset in the first 12 months of age.

Methods We studied 46 probands with permanent, insulin-requiring diabetes with onset within the first 6 months of life (permanent neonatal diabetes mellitus [PNDM]/monogenic diabetes of infancy [MDI]) (group 1) and eight participants with diabetes diagnosed between 7 and 12 months of age (group 2). *KCNJ11*, *INS* and *ABCC8* genes were sequentially sequenced in all patients. For those

who were negative in the initial screening, we examined *ERN1*, *CHGA*, *CHGB* and *NKX6-1* genes and, in selected probands, *CACNA1C*, *GCK*, *FOXP3*, *NEUROG3* and *CDK4*. The incidence rate for PNDM/MDI was calculated using a database of Italian patients collected from 1995 to 2009.

Results In group 1 we found mutations in *KCNJ11*, *INS* and *ABCC8* genes in 23 (50%), 9 (19.5%) and 4 (8.6%) patients respectively, and a single homozygous mutation in *GCK* (2.1%). In group 2, we identified one incidence of a *KCNJ11* mutation. No genetic defects were detected in

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other loci. The incidence rate of PNDM/MDI in Italy is estimated to be 1:210,287.

Conclusions/interpretation Genetic mutations were identified in ~75% of non-consanguineous probands with PNDM/MDI, using sequential screening of *KCNJ11*, *INS* and *ABCC8* genes in infants diagnosed within the first 6 months of age. This percentage decreased to 12% in those with diabetes diagnosed between 7 and 12 months. Patients belonging to the latter group may either carry mutations in genes different from those commonly found in PNDM/MDI or have developed an early-onset form of autoimmune diabetes.

Keywords *ABCC8* gene · Infancy-onset diabetes mellitus · *INS* gene · *KCNJ11* gene · Monogenic diabetes of infancy · Neonatal diabetes mellitus · Non-syndromic diabetes

Abbreviations

CNV	Copy number variation
DEND	Developmental delay, epilepsy, neonatal diabetes
GADA	Glutamic acid decarboxylase autoantibodies
IA-2A	Tyrosine phosphatase-related proteins islet antigen 2 autoantibodies
IAA	Insulin autoantibodies
ICA	Islet-cell antibodies
iDEND	Intermediate DEND
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
ISPED	Italian Society of Pediatric Endocrinology and Diabetology
K _{ATP}	ATP-sensitive potassium channel
MDI	Monogenic diabetes of infancy
MLPA	Multiplex ligation-dependent probe amplification
PNDM	Permanent neonatal diabetes mellitus
TNDM	Transient neonatal diabetes mellitus
ZnT8A	Zinc transporter 8 autoantibodies

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Introduction

Permanent diabetes with onset within the first 6 months following birth (permanent neonatal diabetes mellitus [PNDM]/monogenic diabetes of infancy [MDI]) is a rare condition associated with defects in genes that play major roles in pancreatic beta cell development and function; mutations in the genes encoding the ATP-sensitive potassium channel (K_{ATP}) subunits, *KCNJ11* (also known as *KIR6.2*), *ABCC8* (also known as *SUR1*) and insulin (*INS*), account for most cases of PNDM/MDI [1–6]. Mutations in both the K_{ATP} channel and *INS* genes can also cause diabetes with onset in childhood and adulthood [7–9]. In addition, K_{ATP} channel mutations are a prominent cause of transient neonatal diabetes mellitus (TNDM), a form of neonatal/infancy-onset diabetes that usually remits within 6 months of its onset [1, 2, 4]. Mutations in K_{ATP} channel genes that cause PNDM/MDI are usually activating, dominant and sporadic, but patients with recessive mutations in *ABCC8* have also been described [10]. Patients with *INS* gene mutations, which can be either dominant [5, 6] or recessive [11], present with non-syndromic diabetes. In contrast, patients carrying mutations to K_{ATP} genes may also show variable degrees of motor/mental developmental delay with epilepsy (developmental delay, epilepsy, neonatal diabetes; DEND) or without epilepsy (intermediate DEND; iDEND). Moreover, individuals with some exceedingly rare forms of syndromic PNDM may bear recessive mutations in several genes, including *PDX1*, *EIF2AK3*, *PTF1A*, *GLIS3*, *RFX6* or *FOXP3* [12–18], while recessive mutations in glucokinase give rise to isolated neonatal diabetes [19]. In 2002 we reported the basis for the currently used definition of PNDM/MDI by providing strong evidence that permanent diabetes with onset within 6 months of life is not autoimmune, but rather genetic, in origin [20]. This conclusion was supported by the absence of type 1 diabetes mellitus autoantibodies in patients presenting with diabetes in the first 180 days of life. In the present investigation we have assessed the power of this temporal cut-off in defining neonatal/infancy-onset diabetes by performing sequential screening of *KCNJ11*, *INS* and *ABCC8* genes in 54 patients with permanent diabetes: 46 patients with onset of the disease within 6 months from birth, and eight patients between 7 months and 1 year of age. In those who were negative for mutations in the initial screening, five other candidate genes (*ERN1*, *CHGA*, *CHGB*, *NKX6-1* and *CACNA1C*) were sequenced. In specific patients, *GCK*, *FOXP3*, *NEUROG3* and *CDK4* genes and gene copy number variations were also evaluated.

Methods

Probands A total of 46 patients with diabetes onset before 6 months of age (17, or 37%, with onset within the first

6 weeks of life) (group 1) and eight patients with diabetes diagnosed between 7 and 12 months (group 2) were included in this study. Of these 54 patients, 22 patients in group 1 and two in group 2 have not been reported previously. Most probands studied in the present investigation were of Italian descent, except for one of Moroccan (nd-VI/1), one of Albanian (nd-MI/3), one of Chinese (nd-MO/3) and one of Masai descent (nd-RM/6; Table 1). Family history disclosed consanguinity only in the Albanian family. In group 1, a male patient (deceased; nd-BR/1) presented

with a phenotype resembling IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome [18]. Two other infants had syndromic diabetes with a phenotype that was different from DEND/iDEND, or any other form caused by known genes [12–17]. One of these (group 1) suffered from episodes of liver insufficiency, with high ammonium and transaminase levels and low albumin, but with a mute liver biopsy; he also presented with anaemia that was successfully treated with erythropoietin (nd-NA/2). The other patient (group 2)

Table 1 Clinical and genetic features of patients with diabetes onset within the first year of life studied in the present investigation

Patient	T1D autoantibodies tested	Age at onset (days)	Gene variant	Other features	SU treatment
Group 1					
nd-VI/1	ICA, GADA, IA-2A	1	<i>KCNJ11/V59A</i>	DEND	Yes
nd-BR/1	None	2	–	Diarrhoea	
nd-RM/4	IAA, GADA, IA-2A	2	<i>KCNJ11/R201S</i>		Yes
nd-MI/3	IAA, GADA, IA-2A, ZnT8A	2	<i>KCNJ11/R201C</i>		Yes
nd-PD/2	None	3	<i>ABCC8/L213P</i>	DEND	Yes
nd-FI/1	None	15	<i>ABCC8/V324M</i> ; <i>ABCC8/W688R</i>		Yes
nd-CT/2	None	27	–		
nd-MI/2	ICA, GADA, IA-2A	38	<i>KCNJ11/K170R</i>		Yes
nd-LE/2	ICA, IAA, GADA, IA-2A	39	–		
nd-PR/2	None	40	<i>ABCC8/L213P</i>	iDEND	Yes
nd-NA/1	None	40	<i>KCNJ11/R201C</i>		Yes + insulin
nd-CT/1	none	60	<i>KCNJ11/V59M</i>	iDEND	Yes
nd-NA/2	ICA, GADA, IA-2A	71	<i>ABCC8/A355T</i>	Anaemia	Yes + insulin
nd-MO/3	ICA, IAA, GADA	73	<i>KCNJ11/H46Y</i>		Yes
nd-RM/4	IAA, GADA, IA-2A	80	–		
nd-TO/3	GADA, IA-2A	82	–		
nd-TS/2	None	120	<i>KCNJ11/V59M</i>	iDEND	Yes
nd-RM/6	None	120	<i>KCNJ11/R195H^a</i>		
nd-RM/5	IAA, GADA, IA-2A	135	<i>KCNJ11/E322K</i>		Yes
nd-PI/1	ICA	141	–		
nd-BG/1	GADA	180	<i>ABCC8/S1054N^a</i>		
nd-CES/3	None	190	–		
Group 2					
mdi-RM/3	None	220	<i>KCNJ11/V59M</i>	iDEND	Yes
mdi-NA-B/1	ICA	251	–		
mdi-PA/1	ICA, IAA, GADA, IA-2A	270	–		
mdi-RM-OBG/1	IAA, GADA	289	–	Muscle hypotrophy	
mdi-CES/1	ICA, IAA	300	–		
mdi-RM-OBG/3	IAA, GADA, IA-2A	330	–		
mdi-RM-OBG/2	IAA, GADA, IA-2A	330	–		
mdi-NA/2	GADA, IA-2A	354	–		

SU treatment denotes complete withdrawal of insulin therapy unless specified. Mutations *KCNJ11/H46Y* and *KCNJ11/V59A* were found in probands with Chinese and Moroccan ancestry, respectively. The Albanian patient carried mutation *KCNJ11/R201C*

T1D type 1 diabetes, SU sulfonylurea

^a Benign gene variants

showed muscle hypotrophy in the lower limbs, delayed puberty and retarded growth (mdi-RM-OBG/1).

Type 1 diabetes autoantibodies Twenty-seven patients in group 1 and all patients in group 2 tested negative for at least one type 1 diabetes autoantibody (i.e. ICA [islet cell autoantibodies], IAA [insulin autoantibodies], GADA [glutamic acid decarboxylase autoantibodies], IA-2A [tyrosine phosphatase-related proteins islet antigen 2 autoantibodies] or ZnT8A [zinc transporter 8 autoantibodies]) evaluated at the time of diagnosis.

Genetic testing Informed consent was obtained at each local paediatric diabetes centre involved in the study. Genomic DNA was extracted from peripheral lymphocytes by DNeasy Tissue Isolation kit (Qiagen, Valencia, CA, USA), amplified by PCR and sequenced using a DNA automated sequencer (Applied Biosystems ABI 3730, Foster City, CA, USA). Based on their frequency, our current routine for new cases is to sequence *KCNJ11* and *INS* genes at the same time and, if no mutation is found, the larger *ABCC8* gene is examined [4, 6, 21]. In those patients where no mutation was identified, the entire coding region of the following candidate genes, *ERN1*, *CHGA*, *CHGB*, *NKX6-1* (in patients in groups 1 and 2) and *CACNA1C* (in group 1 only), were sequenced. *CACNA1C* [22], which encodes voltage-dependent L-type calcium channel subunit α -1C (CAV1.2), *CHGA*, *CHGB* [23] and *ERN1* [24] were chosen for their role in insulin secretion; *NKX6-1* was selected because of its role in endocrine pancreas development [25]. In particular, *ERN1* encodes inositol-requiring protein 1 (IRE1A), which is involved in the unfolded protein response and insulin biosynthesis [24]. *FOXP3* and *NEUROG3* were sequenced in the proband with IPEX-like features [18, 26], *CDK4* in the proband with diabetes and muscle hypotrophy [27], and *GCK* in patients presenting with diabetes onset in the first week of life, together with low birthweight [19]. Primer sequences and PCR conditions for candidate genes are listed in Table 1 of the electronic supplementary material (ESM).

New variants in K_{ATP} genes were searched by DNA direct sequencing of genomic DNA from 50 normal controls.

The multiplex ligation-dependent probe amplification (MLPA) technique (MRC-Holland, Amsterdam, the Netherlands) was applied to search for *ABCC8* gene deletions. In two patients with syndromic diabetes, copy number variations (CNVs) were also assessed. Patients' genomic DNA was analysed with the GeneChip 6.0 microarray (Affymetrix, Santa Clara, CA, USA). Labelled DNA was hybridised for 16–18 h; the chip was washed, stained and scanned using a Scanner 3000 7G (Affymetrix). The generated file, containing a single intensity value

calculated for each probe, was loaded into Genotyping Console 3.0.2 and the SNP 6.0 copy number calls from 270 International HapMap Project control samples (www.hapmap.org/) were used as a reference model file for comparison. CNVs were selected based on variables previously described [28].

PNDM/MDI incidence in Italy Information was obtained from the Italian network database on early-onset diabetes (presentation within the first 24 months of life). The database works under the auspices of the Italian Society of Pediatric Endocrinology and Diabetology (ISPED) and collects clinical data from 45 paediatric diabetes clinics, which are the referral centres for the diagnosis and treatment of patients with neonatal and childhood diabetes in Italy.

Incidence rates per 100,000 live births were computed for cases that developed diabetes in the first 6 months of life and were born between 1995 and 2009. For comparison, we also calculated incidence rates utilising the old (1995) limit of ≤ 42 days of life for the diagnosis of PNDM [29]. Confidence intervals for incidence rates were estimated using the exact Poisson distribution.

Results

Disease-causing mutations in K_{ATP} genes In group 1, heterozygous mutations in the *KCNJ11* gene were identified in 23 patients (50%), including 14 probands previously reported by our group [21, 30–32]. All mutations were considered to be de novo, because parental DNA sequencing disclosed wild-type *KCNJ11* genes. Table 1 shows mutations recently or previously identified in patients with PNDM/MDI, including H46Y (nd-MO/3) [33], V59A (nd-VI/1) [34], V59M, K170R, R201C and E322K (nd-RM/25; Table 1) [35]. We also identified a new substitution, arginine by serine, in codon 201 (R201S, c.601C>A), that we believe is novel (nd-RM/4). In Group 2, we found the previously described *KCNJ11*/V59M mutation [3, 21] in a patient with diabetes diagnosed at 220 days of life (mdi-RM/3). *ABCC8* gene sequencing led to the identification of five heterozygous mutations in four probands from group 1. *ABCC8*/L213P (c.638T>C) was found to be a de novo mutation in a patient with complete DEND syndrome (nd-PD/2), and in a second proband with iDEND, who had inherited the mutation from her father (Fig. 1), who also presented with iDEND (nd-PR/2). A mutation in the same codon, *ABCC8*/L213R, has been previously reported in association with iDEND [36]. Of our patients, one proband was a compound heterozygote for the *ABCC8*/V324M mutation, previously described in patients with the transient form of the disease (TNDM) [36], who usually need insulin therapy for less than 1 year [4], and for the novel W688R

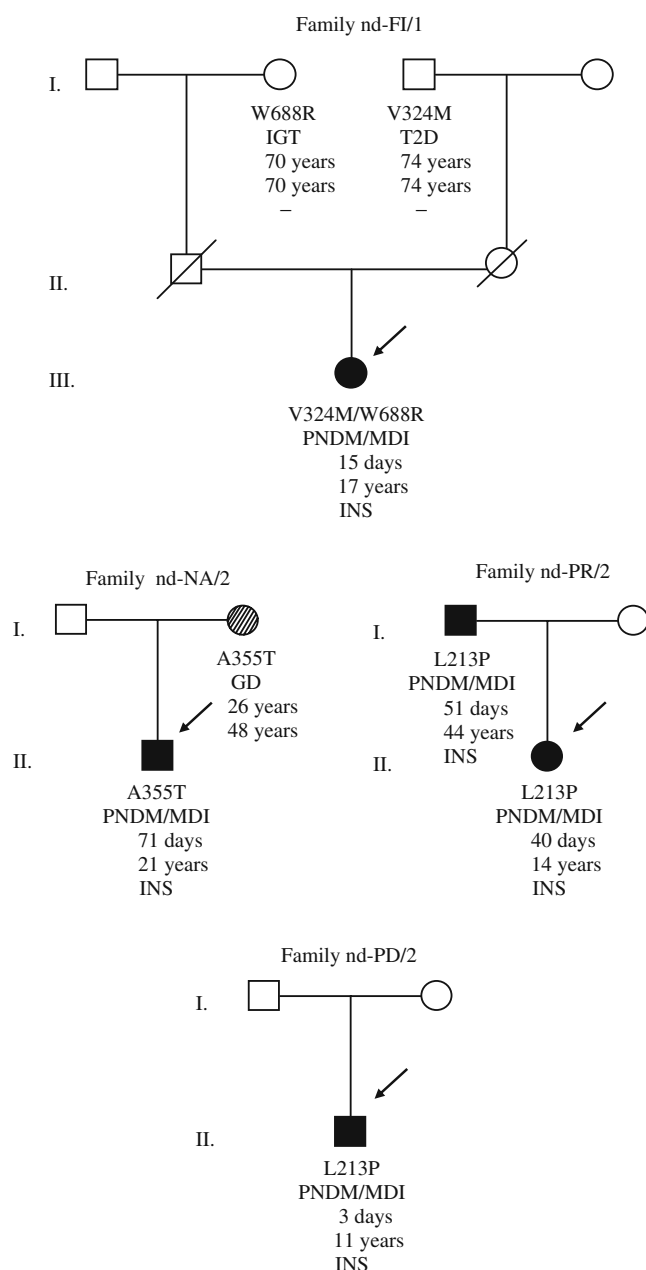


Fig. 1 Pedigrees of four families with mutations in *ABCC8*; from top to bottom: mutation, phenotype, age at presentation/diagnosis of diabetes, current age and initial therapy for diabetes. For the two grandparents of proband nd-FI/1, OGTT tests were performed at 70 (W688R) and 74 (V324M) years of age. GD, gestational diabetes; IGT, impaired glucose tolerance; INS, insulin

(c.2062T>G) mutation (nd-FI/1). Residue W688 is conserved in dog, cow, mouse, chicken and zebra fish. Because both of the patient's parents were deceased, we analysed the grandparents' DNA and confirmed the *ABCC8*/V324M mutation in the maternal grandfather and the *ABCC8*/W688R mutation in the paternal grandmother (Fig. 1). After identifying the mutations, an OGTT was done in both patients; the carrier for V324M was diagnosed with

diabetes (2 h plasma glucose 14 mmol/l) and the carrier for W688R presented with impaired glucose tolerance (2 h plasma glucose 8.4 mmol/l). Finally, a patient carrying the unreported *ABCC8*/A355T (c.1063G>A; nd-NA/2) variant inherited the mutation from his mother, who was diagnosed with gestational diabetes at the age of 26 years. Residue A355 is conserved in dog, cow, rat, mouse and chicken, but not in zebra fish, where it is substituted by glycine. None of the new *KCNJ11* or *ABCC8* mutations was detected in 100 chromosomes from normal controls.

Polymorphisms—*K_{ATP}* genes In the patient of Masai descent (nd-RM/6) we detected the variant *KCNJ11*/R195H (c.584G>A; single nucleotide polymorphism: rs5217; Table 1), while in the proband nd-BG/1 we found the variant *ABCC8*/S1054>N (c.3161G>A, numbered according to [36]; Table 1), inherited from the patient's father, who had normal glucose tolerance. MLPA of the *ABCC8* gene did not disclose any deletion in this patient. Thus, we currently classify *ABCC8*/S1054N as a rare, benign variant, taking into account the fact that residue S1054 is conserved from dog through to mouse, but not in chicken and zebra fish, where is substituted by cysteine and valine, respectively.

No additional mutations were found in *INS* (6), *GCK* (19) or any of the other genes (*FOXP3*, *NEUROG3*, *ERN1*, *CHGA*, *CHGB*, *NKX6-1*, *CACNA1C* and *CDK4*) screened at this time.

Clinical features in patients with diabetes and negative for *KCNJ11*, *INS* and *ABCC8* Patients from group 1, negative in the genetic screening for genes commonly causing PNDM/MDI (hereafter called PNDM/MDI 'X') had a higher birthweight (median 3,164 g) than patients with *KCNJ11* mutations (median 2,460 g; $p<0.0003$), and similar to individuals carrying *INS* mutations (Table 2). Moreover, in patients with PNDM/MDI 'X', diabetes was diagnosed 1 month later (median) than carriers of mutations of *K_{ATP}* genes.

Incidence of PNDM/MDI in Italy The incidence of PNDM/MDI was calculated at 1:210,287 (95% CI 1:300,300–1:151,976) live births for years 1995–2009. Of note, 22 out of 23 patients (95%) included in the database for years 2000–2009 (incidence rate: 1:213,198, 95% CI 1:336,700–1:142,045) have been subjected to genetic screening for *KCNJ11*, *INS* and *ABCC8* and 15 (68%) carried a causative mutation in either *KCNJ11* or *INS*. The incidence rate (years 1995–2009) was found to be 1:473,146 when calculated using the original definition of neonatal diabetes as insulin-requiring hyperglycaemia with onset within the first 6 weeks of life [29].

Table 2 Birthweight and age at diabetes onset of probands with *KCNJ11*, *INS* and *ABCC8* mutations and of unknown genetic origin

Gene	<i>KCNJ11</i>	<i>INS</i>	<i>ABCC8</i>	Unknown
<i>n</i>	22	9	4	8
Birthweight (g)	2,460±394.34	3,050±203.88 [†]	2,825±663.79	3,150±204.57 [‡]
Age at diagnosis (days)	53±59.7	86±45.6	275±30.0	82±68.9

Birthweight and age at diabetes onset are median values±SD

Birthweight was not available for one patient carrying a *KCNJ11* mutation, and for a second patient born in Kenya (nd-RM/6). The patient with IPEX-like features (birthweight 990 g, born at 22 weeks of gestation) was not included in the group of unknown genetic aetiology (PNDM/MDI 'X')

p value vs *KCNJ11*: [†] 0.009, [‡] 0.003

Discussion

In 2002 we suggested that the cause of diabetes in patients with disease onset within 6 months of life might be genetic [20]. At that time only one gene, *GCK* [19], was known to be linked with rare cases of insulin-deficient diabetes, while *IPF1* (also known as *PDX1*), *EIF2AK3* and *FOXP3* had been associated with early onset, syndromic diabetes [12, 13, 18]. More recently, mutations in the *KCNJ11*, *ABCC8* and *INS* genes have been identified, which cause diabetes with onset in the first 6 months of life [3–6] and beyond [7–9, 37]. In the present study, we report the identification, by sequential sequencing of the three latter genes, of a disease-causing mutation in 36 probands with diabetes diagnosed before 6 months of age and in a single patient with diabetes onset after 6 months but before 1 year of age. In Group 1, novel mutations in *KCNJ11*/R201S, *ABCC8*/L213P and *ABCC8*/W688R were identified. The latter was found in a patient who also carried the TNDM-causing *ABCC8*/V324M mutation [36]. PNDM/MDI can result from recessive *ABCC8* mutations, usually one with a mild activating effect and the other with loss-of-function, which are observed in recessive hyperinsulinism [10]. In our case, however, we favour the hypothesis that both *ABCC8*/V324M and *ABCC8*/W688R are mildly activating, based on the fact that W688R is associated with impaired glucose tolerance in the paternal grandmother. In contrast, we cannot explain at this time the extremely different phenotypes we observed in the carriers of the mutation *ABCC8*/A355T, who show either PNDM/MDI or gestational diabetes. Previously, phenotypic variability in *ABCC8* mutation carriers has been described in families in which the proband presents with TNDM and first-degree relatives carry the same mutation showing early-onset type 2 diabetes or even normal glucose tolerance [7, 38]. Because the patient with *ABCC8*/A355T also has a liver-related and haematologic phenotype, it is conceivable that he may carry a mutation in another locus that impacts on glucose metabolism. Functional studies are definitely needed to firmly establish the impact of *ABCC8*/A355T on insulin secretion.

In the present work, the mutation detection rate of *KCNJ11*, *INS* and *ABCC8* genes was 78.2% (36/46) for patients in

group 1 and 12.5% (1/8) for patients in group 2. After sequencing the same three genes, Støy et al. [39] obtained comparable results, with a 63% and 6.6% detection rate in patients for permanent diabetes before or after 6 months of life, respectively. Our results from group 1 were not influenced by type 1 diabetes autoantibody status, which was unknown in 15 (40%) patients who harboured a disease-causing mutation [6, 21, 30–32] (Table 1).

Sequencing of *KCNJ11*, *INS* and *ABCC8* in patients from group 2 was justified by the fact that mutations in these genes have previously been detected in patients with diabetes onset in infancy, childhood and even adulthood [7–9, 37, 38 and F. Barbetti, unpublished observations], eliciting a negative result in >85% of cases examined. Screening of candidate genes in these and other patients was also negative [present study; 40]. Consequently, the autoimmune or genetic aetiology for seven of the remaining patients in group 2 remains open. Four patients tested negative for IAA, which are found at an increased rate in individuals with diabetes onset before 5 years of age, and one of these four was negative for all autoantibodies commonly used as tools for the diagnosis of type 1 diabetes. However, the latter patient may still harbour autoantibodies against ZnT8A, which can be detected as a single autoantibody in patients with type 1 diabetes previously classified as autoantibody-negative on the basis of existing markers (i.e. IAA, GADA, IA-2A and ICA) [9, 41]. Of note, one patient who was negative to GADA at diagnosis (and carrying *HLA-DR3/DR4*) and was initially included in group 2 became weakly GADA positive during the study; this individual was therefore re-classified as having type 1 diabetes and was not investigated further. A limitation/weakness of our study is that the HLA status of most patients in group 2 was unknown, and type 1 diabetes autoantibodies were not thoroughly tested.

Conversely, we think that the nine patients in group 1 who had no disease-causing mutation identified, and possibly the patient carrying the *ABCC8*/A355T mutation, who showed clinical features not associated with mutations to K_{ATP} channel genes, are likely to carry a mutation in a locus that has not, as yet, been found. Seven of these

patients presented with non-syndromic diabetes and birth-weight close to normal (Table 2) and were not good candidates for screening of any of the known genes giving rise to syndromic diabetes [12–17] or hyperglycaemia in the first week of life [42, 43]. We therefore sequenced genes that we considered to be candidates, based on the phenotypic consequences of their manipulation/ablation in mice [22–25], with no success. Because hundreds of genes can have an impact on pancreatic beta cell function [44] we believe that new approaches, such as whole exome/genome sequencing, should be used in the future for PNDM/MDI gene discovery [45, 46].

The incidence rate of PNDM/MDI in Italy, utilising the 6 month limit as a cut-off for diagnosis of neonatal/infancy-onset diabetes, is about 1:210,000 live births. This result is comparable to recent reports from Slovakia, the UK, the Netherlands and Poland [47, 48], showing an incidence of PNDM/MDI of 1:215,417 [47] and 1:260,000 [48], respectively. In summary, a molecular genetic diagnosis can be reached in ~75% of patients with diabetes onset ≤ 6 months of age when *KCNJ11*, *INS* and *ABCC8* genes are sequenced in populations with a low consanguinity rate. Moreover, because the status of type 1 diabetes autoimmunity was unknown in about 40% of patients in this group, we also conclude that knowledge of type 1 diabetes autoantibodies is not a prerequisite to proceeding to genetic screening, which we recommend for all patients with diabetes onset before 6 months of age. The incidence of PNDM/MDI varies, as expected, if different age cut-offs for disease onset are used as criteria for aetiological (genetic vs autoimmune) diagnosis.

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Appendix

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