

Insulin Mutation Screening in 1,044 Patients With Diabetes

Mutations in the *INS* Gene Are a Common Cause of Neonatal Diabetes but a Rare Cause of Diabetes Diagnosed in Childhood or Adulthood

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OBJECTIVE—Insulin gene (*INS*) mutations have recently been described as a cause of permanent neonatal diabetes (PND). We aimed to determine the prevalence, genetics, and clinical phenotype of *INS* mutations in large cohorts of patients with neonatal diabetes and permanent diabetes diagnosed in infancy, childhood, or adulthood.

RESEARCH DESIGN AND METHODS—The *INS* gene was sequenced in 285 patients with diabetes diagnosed before 2 years of age, 296 probands with maturity-onset diabetes of the young (MODY), and 463 patients with young-onset type 2 diabetes (nonobese, diagnosed <45 years). None had a molecular genetic diagnosis of monogenic diabetes.

RESULTS—We identified heterozygous *INS* mutations in 33 of 141 probands diagnosed at <6 months, 2 of 86 between 6 and 12 months, and none of 58 between 12 and 24 months of age. Three known mutations (A24D, F48C, and R89C) account for 46% of cases. There were six novel mutations: H29D, L35P, G84R, C96S, S101C, and Y103C. *INS* mutation carriers were all insulin treated from diagnosis and were diagnosed later than ATP-sensitive K⁺ channel mutation carriers (11 vs. 8 weeks, $P < 0.01$). In 279 patients with PND, the frequency of *KCNJ11*, *ABCC8*, and *INS* gene mutations was 31, 10, and 12%, respectively. A heterozygous R6C mutation cosegregated with diabetes in a MODY family and

is probably pathogenic, but the L68M substitution identified in a patient with young-onset type 2 diabetes may be a rare nonfunctional variant.

CONCLUSIONS—We conclude that *INS* mutations are the second most common cause of PND and a rare cause of MODY. Insulin gene mutation screening is recommended for all diabetic patients diagnosed before 1 year of age. *Diabetes* 57:1034–1042, 2008

An estimated 1–2% of all diabetes is due to a monogenic etiology. HLA studies show that patients diagnosed with diabetes in the first 6 months of life are very likely to have monogenic neonatal diabetes rather than type 1 diabetes (1,2). Neonatal diabetes is a rare disorder with an incidence of 1 in 215,000–500,000 live births (3,4), with ~50% having permanent neonatal diabetes (PND). Heterozygous activating mutations in the *KCNJ11* and *ABCC8* genes, which encode the Kir6.2 and SUR1 subunits of the ATP-sensitive K⁺ channel (K_{ATP} channel), are the most common causes of PND (5–9). A number of other rare genetic aetiologies have been identified (*GCK*, *IPF1*, *PTF1A*, *GLIS3*, *FOXP3*, *EIF2AK3*, *GLUT2*, and *HNF1B* mutations); most of these show autosomal recessive inheritance, and all except for *GCK* mutations result in additional non-pancreatic features.

We have recently described a new monogenic cause of neonatal diabetes: mutations in the insulin (*INS*) gene (10). We reported 16 families with 10 different heterozygous missense *INS* mutations in probands with PND; these mutations are predicted to disrupt the folding of the proinsulin molecule and result in misfolded protein or retention of the protein in the endoplasmic reticulum (ER), resulting in ER stress and β -cell apoptosis (11). Disulphide bonds are crucial for proinsulin folding in the ER, and 60% (6 of 10) of the mutations either abolish or disrupt disulfide bridge formation within the protein either by substitution of a cysteine residue (e.g., C96Y) or the creation of an additional cysteine (e.g., R89C).

The most common forms of monogenic diabetes are subtypes of maturity-onset diabetes of the young (MODY) due to heterozygous mutations in the transcription factor genes *HNF1A/TCF1* or *HNF4A* or the *GCK* gene that

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ER, endoplasmic reticulum; ISPAD, International Society of Pediatric and Adolescent Diabetes; K_{ATP} channel, ATP-sensitive K⁺ channel; MODY, maturity-onset diabetes of the young; PND, permanent neonatal diabetes.

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TABLE 1
Clinical characteristics of the study groups

	PND	Infancy	Early childhood	MODY	Type 2 diabetes	Hyperinsulinism
Age at diabetes diagnosis (weeks and years)	0–26 weeks	27–52 weeks	53–104 weeks	<25 years	<45 years	
<i>n</i>	141	86	58	296	463	49
Sex (% male)	50	58	41	36	48	67
Current age (years)	7 (0–69)	10 (1–51)	13 (2–72)	36 (5–77)	38 (3–87)	3 (0.4–21)
Age at diagnosis (weeks and years)	8 weeks (0–26)	39 weeks (27–52)	97 weeks (57–104)	16 years (3–25)	29 years (3–46)	7 weeks (0–728)
BMI (kg/m ²)	17 (9–40)*	19 (13–30)*	22 (17–25)*	24 (13–30)*	23 (15–30)	NA
Number of patients with an affected first-degree relative	24 (17)	9 (10)	11 (19)	296 (100)	208 (45)	—
Treatment: insulin/OHA + insulin/OHA/diet (% cases)	100/0/0/0	100/0/0/0	100/0/0/0	49/4/26/21	45/2/32/21	—
<i>KCNJ11</i> / <i>ABCC8</i> mutations excluded	141/129	86/21	58/2	0/0	0/0	49/49
<i>HNFL1A</i> / <i>HNF4A</i> / <i>GCK</i> mutations excluded	—	—	—	189/81/56	180/76/87	—

Data are *n* (%) or median (range). Insulin gene (*INS*) mutations were investigated in 1,044 patients with permanent diabetes diagnosed before 6 months of age (PND, 0–26 weeks), between 6–12 months of age (infancy, 27–52 weeks), and 1–2 years of age (early childhood, 53–104 weeks); in MODY diagnosed <25 years of age or type 2 diabetes diagnosed <45 years; and in 49 patients with hyperinsulinism. *Datasets for BMI incomplete. OHA, oral hypoglycemic agent; NA, not available.

encodes the pancreatic glucose sensor, glucokinase (12). *HNFL1A* and *HNF4A* mutations result in progressive β -cell dysfunction, and diabetes is usually diagnosed before 25 years of age. *GCK* mutations cause elevated fasting hyperglycemia from birth but are often not diagnosed until young adulthood (13). Rare mutations in other transcription factor genes (*HNFL1B/TCF2*, *IPF1*, and *NEUROD1*) have been reported in MODY, but at least 11% of U.K. families who fit the clinical criteria for MODY do not have a mutation in any of the known genes (14). The initial report of *INS* mutations includes the father of a child with neonatal diabetes who was diagnosed with type 2 diabetes at the age of 30 years (10). It is not known whether insulin gene mutations can cause MODY.

We now report mutation screening of a large series of patients (*n* = 1,044) with permanent diabetes diagnosed during infancy, childhood, and adulthood (up to 45 years of age) in order to determine the prevalence of *INS* gene mutations. We also studied a series of patients with hyperinsulinism (*n* = 49) where mutations in the K_{ATP} channel genes had been excluded by sequencing; this group was investigated to determine whether a similar situation exists for *INS* as that seen for *GCK* (15), *KCNJ11*, and *ABCC8*, where different mutations result in the opposite phenotypes of diabetes and hyperinsulinism.

RESEARCH DESIGN AND METHODS

We studied five groups of patients with permanent diabetes at the time of referral (Table 1). The PND group consisted of 141 patients with diabetes diagnosed in the first 6 months of life who were on insulin treatment at the time of referral. Mutations in *KCNJ11* had been excluded by sequencing in all 141 patients, and *ABCC8* mutations had been excluded in the 129 cases where there was sufficient DNA. Patients diagnosed outside the first 6 months of life were divided into those diagnosed in infancy (between 27 and 52 weeks of life, *n* = 86) and those diagnosed in early childhood (between 53 and 104 weeks of life, *n* = 58). We also screened 296 cases who met minimal diagnostic criteria for MODY (at least two generations affected and at least one subject diagnosed before 25 years of age). Mutations in the *HNFL1A*, *HNF4A*, and *GCK* genes had been excluded by sequencing in 189, 81, and 56 cases, respectively. Finally we screened 463 nonobese patients with young type 2 diabetes diagnosed before 45 years of age. Of these, 45% had a diabetic first-degree relative. Mutations in *HNFL1A*, *HNF4A*, and *GCK* were excluded by sequencing in 180, 76, and 87 cases, respectively.

Patients diagnosed between 0 and 2 years of age (*n* = 285) were predominantly recruited in Exeter through the International Society of Pediatric and Adolescent Diabetes (ISPAD) group. These were supplemented by 39 patients from the Barts and Oxford (BOX) study and the British Diabetes Association 1972–1981 cohort, as previously described (2). In addition, we also screened 49 patients with isolated hyperinsulinism, diagnosed at a median age of 7 weeks (0–728 weeks), where mutations in the K_{ATP} channel genes (*KCNJ11* or *ABCC8*) had been excluded by sequence analysis.

Clinical characteristics are presented as median (range) and comparative analysis using a Kruskal Wallis or Mann-Whitney *U* test. The summary clinical details of these groups are shown in Table 1.

Mutations were tested for cosegregation with diabetes in family members, and the *INS* gene was sequenced in 111 U.K. population Caucasian control subjects. Clinical characteristics were obtained from hospital notes with assistance from the referring clinician.

Molecular genetic analysis. Genomic DNA was extracted from peripheral leukocytes using standard procedures. Coding exons 2 and 3 of the *INS* gene were amplified by PCR. Sequence-specific primers for each exon (10) were tagged with 5' M13 tails to allow sequencing to be performed with a "universal" M13 primer. A new primer for the first coding exon was used: *INS*_Exon2F-TGTAACACGACGGCCAGTTGGCTGGGCTCGTGAAG and *INS*_Exon2R-CAGGAACACGCTATGACCCCTTCTGCCATGCTG.

Single-strand sequencing was carried out using standard methods on an ABI 3730 (Applied Biosystems, Warrington, U.K.). Sequences were compared with the published sequence (NM_000207) using Mutation Surveyor v2.61. Changes in the sequence were checked against published polymorphisms and mutations and for conservation across species. We used a panel of microsatellites for chromosome 20q (16) or chromosome 11p (17) to confirm family

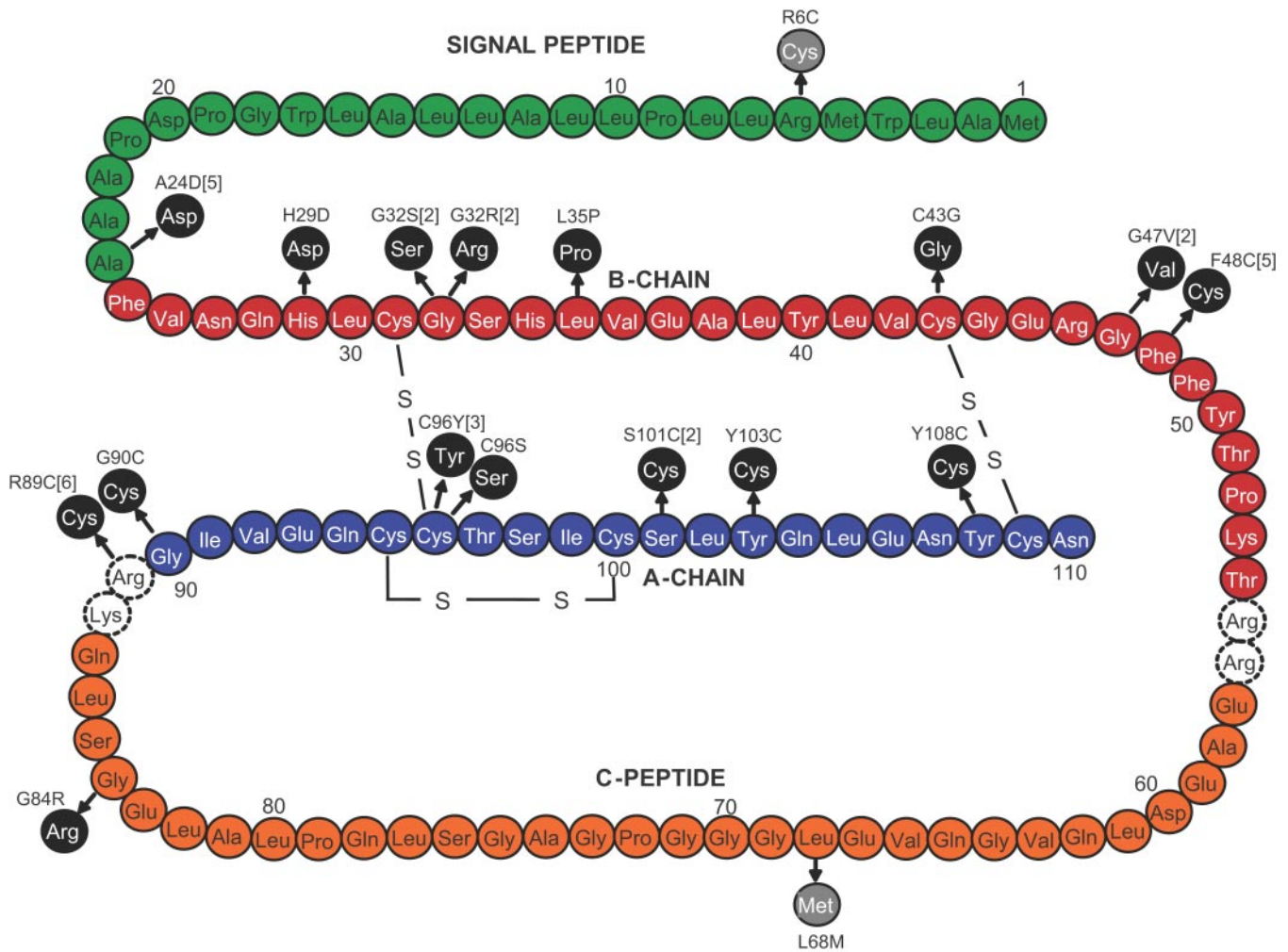


FIG. 1. Mutations identified in the preproinsulin molecule. Black-filled circles represent amino acid changes identified in probands with diabetes diagnosed before 12 months, gray circles represent amino acid changes identified in probands with possible MODY. Where the number of probands with the mutation is greater than one, the total number is indicated in the square brackets.

relationships for sporadic cases of diabetes where neither parent was shown to carry the mutation found in their child.

RESULTS

Prevalence of mutations in the different patient groups tested. Heterozygous *INS* mutations were found in 33 of 141 (23%) probands diagnosed before 6 months, 2 of 86 (2%) between 6 and 12 months, and none of 58 diagnosed between 12–24 months of age. Only 1 of 296 (0.3%) MODY and 1 of 463 (0.2%) young type 2 diabetic probands had possible mutations identified by sequencing. No mutations were found in the 49 patients with hyperinulinism. The details of the mutations found and the associated clinical characteristics are discussed below.

Molecular genetics. We identified 16 different heterozygous mutations in 35 probands with diabetes diagnosed in the first year of life (Fig. 1). Of the 35 families, 12 have been reported previously (10). Of the 16 mutations found, 10 have previously been described (10): A24D (five families), G32S (two families), G32R (two families), C43G, G47V (two families), F48C (five families), R89C (six families), G90C, C96Y (three families), and Y108C. There are six novel mutations: H29D (c.85C>G, p.His29Asp), L35P (c.104T>C, p.Leu35Pro), G84R (c.250G>A, p.Gly84Arg), C96S (c.287G>C, p.Cys96Ser), S101C (c.302C>G,

p.Ser101Cys in two families), and Y103C (c.308A>G, p.Tyr103Cys). These residues are conserved across species (human, mouse, dog, horse, lizard, and *Xenopus*), with the exception of Y103 (F103 in *Xenopus*) and G84 (R84 in platypus). These mutations were not seen in 222 U.K. Caucasian control chromosomes.

Three mutations (A24D, F48C, and R89C) account for 16 of 35 (46%) of cases, but only R89C occurs at a CpG dinucleotide, where the spontaneous deamination of methylcytosine is frequent. Of the 16 mutations, 9 either abolish a cysteine residue or create an additional cysteine.

Six families demonstrated autosomal dominant inheritance of diabetes that cosegregated with the *INS* mutation, including one previously published family ISPAD 155 (10) (Fig. 2). An additional 11 family members were shown to have an *INS* mutation. Six were diagnosed before 6 months, three between 6 and 12 months, 1 at 7 years, and 1 at 22 years of age. In 29 of 35 (83%) of probands, an affected child was born to unaffected parents, representing probable de novo mutations. In 17 families, DNA was available from both unaffected parents, and microsatellite analysis established that these were de novo mutations (including 11 previously published families [10]). In family ISPAD180, two affected brothers had the S101C mutation, but the mutation was not detected in leukocyte DNA from

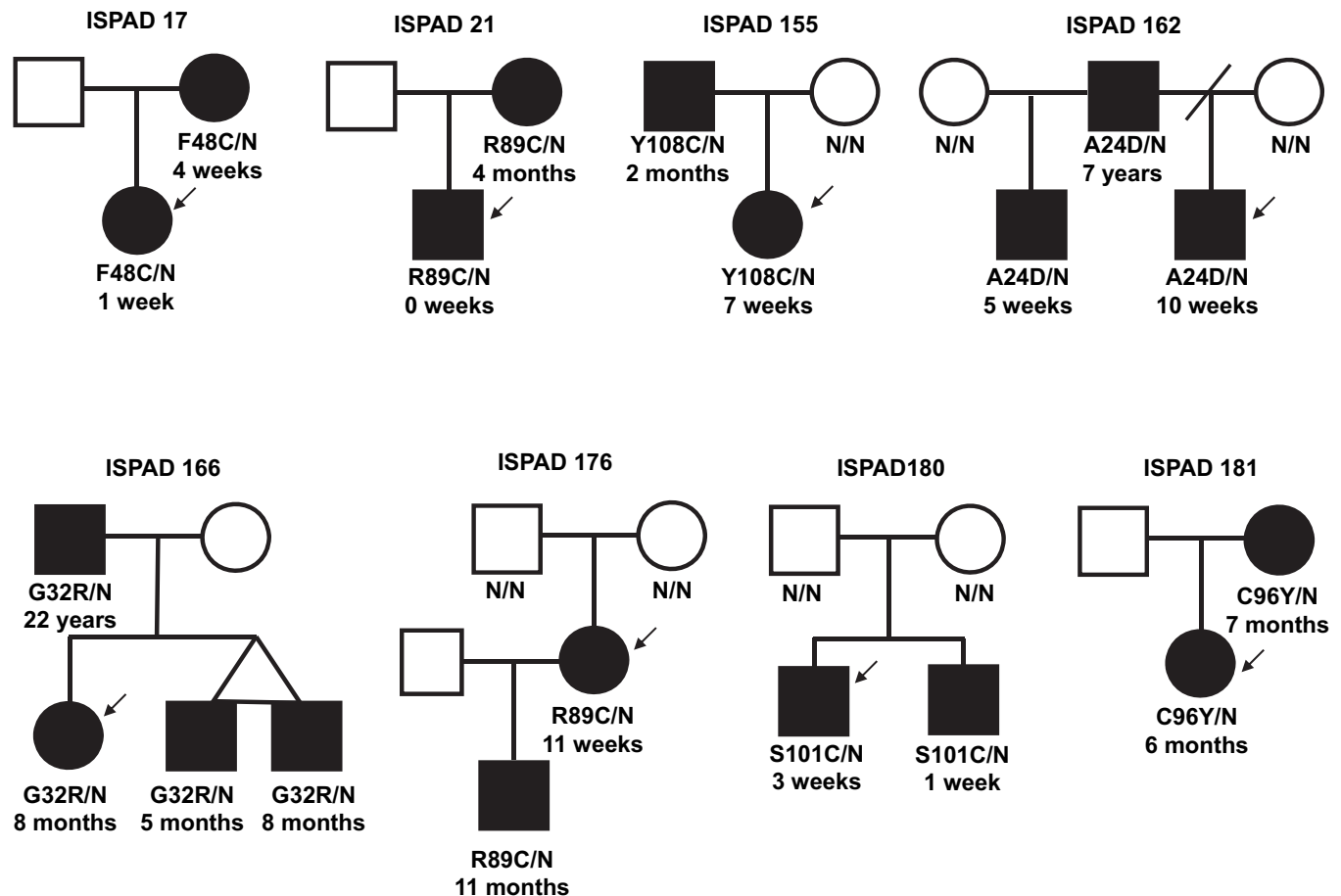


FIG. 2. Partial pedigrees for families with multiple individuals affected with diabetes. Filled symbols represent patients with diabetes. The genotype is shown underneath each symbol. N/N denotes no mutation identified. Below the genotype is the age of diagnosis of diabetes. Arrows indicate the proband.

either parent (Fig. 2). This pattern of inheritance is consistent with germline mosaicism. The clinical features of this family have previously been described (18).

INS mutations: clinical characteristics. Clinical characteristics are provided for all *INS* mutation carrying probands diagnosed under 1 year of age and their affected family members (Table 2). The median age at diagnosis of the *INS* mutation carriers was 11 weeks. Presentation was either with symptomatic hyperglycemia (41%) or diabetic ketoacidosis (59%). All patients

were treated with insulin replacement therapy and most with a full replacement dose (>0.5 units \cdot kg $^{-1}$ \cdot day $^{-1}$) (median 0.7 units \cdot kg $^{-1}$ \cdot day $^{-1}$ [range 0.3–1.9]). Glycemic control was variable (A1C median 7.95% [range 4.6–13.8]). Autoantibodies, when measured, were not detected in any cases, although the two affected siblings from family ISPAD180 were previously reported with autoantibodies to the exocrine pancreas but not the islet at diagnosis (18).

Birth weights were reduced, consistent with in utero

TABLE 2

Clinical characteristics of probands diagnosed in infancy and their family members with an *INS* gene mutation

	Age at diagnosis (weeks and years)			All subjects
	0–26 weeks	27–52 weeks	1–45 years	
<i>n</i>	39	5	2	46
Sex (% male)	19 of 39 (49)	2 of 5 (40%)	2 (100%)	23 of 46 (50%)
Current age (years)	10 (0.5–42)	16 (1–45)	42 (35–48)	14 (0.5–48)
Current BMI (kg/m 2)	17 (11–36)	18 (16–30)	NA	17 (11–36)
Birth weight (kg)	2.6 (1.7–3.8)	3 (2.9–3.9)	2.7 (2.4–2.9)	2.7 (1.7–3.9)
Gestational age (weeks)	40 (35–42)	40 (37–42)	40	40 (35–42)
Corrected birth weight (centile)	3 (<1st–87th)	44 (5th–83rd)	4.5 (<1st–8th)	6 (<1st–87th)
Age at diagnosis (weeks and years)	9 weeks (0–26)	35 weeks (31–48)	14.5 years (7–22)	11 weeks (0–1,144)
Insulin treatment (%)	100	100	100	100
Insulin dose (units \cdot kg $^{-1}$ \cdot day $^{-1}$)	0.73 (0.3–1.9)	0.6 (0.2–0.7)	0.45 (0.3–0.6)	0.7 (0.3–1.9)
A1C	7.9 (4.6–13.8)	7.6 (6.5–9.5)	NA	7.95 (4.6–13.8)
Antibody status: Neg./pos./NA	18/0/21	2/0/3	0/0/2	20/0/26

Data are median (range) unless otherwise indicated. NA, not available.

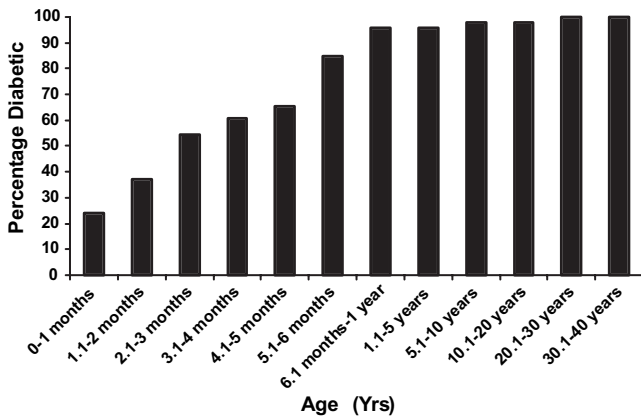


FIG. 3. Penetrance of *INS* gene mutations in 46 individuals from 35 families where the proband was diagnosed in the first year of life. The percentage of patients with an *INS* mutation and diabetes at any given age is shown.

growth retardation due to reduced insulin secretion. The median birth weight was 2.7 kg (range 1.7–3.9), corresponding to the 6th percentile (range <1st–87th). There was no difference in the age at diagnosis for male *INS* mutation carriers compared with female carriers (median 10 weeks [range 0–1,144] for male vs. 13 weeks [1–35] for female carriers; $P = 0.9$), but birth weight was lower for male carriers (median centile birth weight <1 [range <1–87] for male vs. 11th centile [<1–83] for female carriers; $P = 0.048$).

Figure 3 illustrates the penetrance of *INS* mutations in families where the proband was diagnosed in infancy. The percentage of patients with diabetes increases from 24% in the first month to 85% at 6 months and to 96% at 1 year.

Comparison of *INS* and K_{ATP} channel mutations. The clinical characteristics of patients with an *INS*, *KCNJ11*, or *ABCC8* gene mutation were compared (Table 3). No difference in sex, birth weight, or gestational age was observed between the three groups, but patients with an *INS* mutation were diagnosed later (median 11 weeks [range 0–1,144]) than those with a K_{ATP} channel mutation (median 8 weeks [range 0–40], $P < 0.01$).

Frequency of *INS* mutations in PND. The 33 *INS* mutation carriers diagnosed before 6 months of age are

from a cohort of patients with PND referred to Exeter. This cohort includes 87 patients with *KCNJ11* mutations, 29 with *ABCC8* mutations, and 22 with mutations in the *GCK*, *EIF2AK3*, or *FOXP3* genes (10, 8, and 4 cases, respectively). There are also 108 subjects in whom no mutations were found after sequencing the *KCNJ11*, *ABCC8*, and *INS* genes. We were unable to complete sequencing of these three genes in 21 cases due to insufficient DNA. The prevalence of the different genetic subtypes within the 279 fully tested cohort members is shown in Fig. 4. Sequence analysis for *GCK* was performed only for consanguineous pedigrees, and *EIF2AK3/FOXP3* mutation testing was guided by extra-pancreatic features and/or consanguinity; thus, these etiologies may be underestimated.

***INS* mutations in MODY and young type 2 diabetes.** Two different missense mutations were identified in two families: one in a proband with MODY (1 of 296) and one in a proband with young type 2 diabetes (1 of 463). The novel mutation, R6C (c.16C>T, p.Arg6Cys), was found in a U.K. MODY family and was not present in 222 U.K. Caucasian control chromosomes. The novel mutation L68M (c.202C>A, p.Leu68Met) was identified in a family of Turkish origin with young type 2 diabetes and was not seen in 170 Turkish control chromosomes.

The R6C mutation cosegregated with diabetes, as it was found in the proband, the proband’s mother, and the maternal grandmother, who were diagnosed with diabetes at 15, 15, and 65 years of age, respectively. The clinical features in the three affected family members were consistent with a diagnosis of MODY: they were nonobese (BMIs 24.1, 26.9, and 29.3 kg/m²), and their diabetes was noninsulin dependent. The proband had 10 years on diet and then oral agents before starting low-dose insulin (0.2 units · kg⁻¹ · day⁻¹), her mother was treated with diet for 40 years before starting oral agents at the age of 55 years, and the maternal grandmother has been on diet treatment for the 8 years since diagnosis. The L68M mutation was found in a proband diagnosed with diabetes at the age of 30 years (BMI 17 kg/m²). She was treated with diet for 1 year and started oral agents but soon required insulin treatment. Her father was diagnosed at age 31 years and was insulin treated; he died from diabetes complications, and DNA from her unaf-

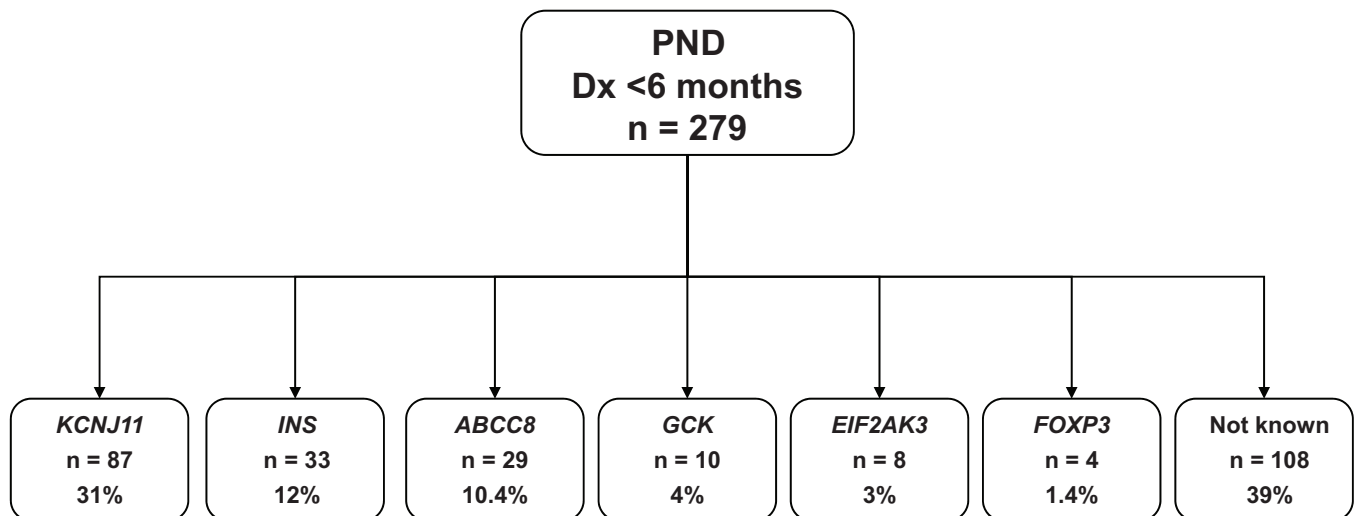


FIG. 4. Etiology of PND in a cohort of 279 patients diagnosed before 6 months. The number of probands with mutations identified is shown, together with the percentages for each etiology. All subjects of unknown etiology were sequenced for *KCNJ11*, *ABCC8*, and *INS*. Dx, diagnosis.

TABLE 3
Comparison of the clinical characteristics of *INS*, *KCNJ11*, and *ABCC8* mutation carriers

	<i>INS</i>	<i>KCNJ11</i>	<i>ABCC8</i>	<i>P</i>
<i>n</i>	46	100	31	
Sex (% male)	23/46 (50)	53/100 (53)	13/31(42)	0.6
Birth weight (kg)	2.7 (1.7–3.9)	2.66 (1.85–3.6)	2.7 (1.51–4.20)	0.8
Gestational age (weeks)	40 (35–42)	40 (33–42)	40 (26–40)	0.2
Corrected birth weight (centile)	6 (<1st–87th)	7 (<1st–91st)	13 (<1st–95th)	0.2
Age at diagnosis (weeks)	11 (0–1,144)	8 (0–33)	8 (0–40)	0.04

Data are median (range).

fectured mother was not available (Fig. 5). Mutations in the GCK, HNF1A, or 4A genes were excluded in both probands by sequence and dosage analysis.

The R6 residue is conserved across mammalian species (Rhesus monkey, mouse, dog, and horse) and *Xenopus* but not conserved in lizard (S6) or platypus (G6). The L68 residue is also conserved in mammals (Rhesus monkey, mouse, dog, and horse) but absent from *Xenopus*, lizard, and platypus. The SIFT analysis software (19) predicts that L68M is tolerated but R6C is not. PolyPhen (20) predicts that the L68M variant is benign. While leucine and methionine are both small hydrophobic amino acids, the R6C substitution creates an additional cysteine residue and the likelihood of disrupted signal peptide localization and/or function.

In addition to these nonsynonymous mutations, we also detected the common intronic single nucleotide polymorphisms rs689 and rs3842752, a synonymous A12A variant (rs3842744) in 1 of 759 MODY cases, and a synonymous P21P variant (rs11564720) in 1 of 759 MODY cases and 1 of 222 Caucasian controls.

DISCUSSION

We report the largest series of *INS* gene mutations identified by screening 1,044 patients diagnosed with diabetes from birth to 45 years of age. Heterozygous missense mutations were found in 37 probands of whom 35 were diagnosed during infancy (the first 12 months of life) and

33 had permanent neonatal diabetes diagnosed before 6 months of age. *INS* mutations were common in patients diagnosed with diabetes in the first 6 months of life (23% of cases tested) but far less frequent in patients diagnosed in childhood or early adulthood (two novel mutations, 0.3% of cases). We have also excluded *INS* mutations as a common cause of congenital hyperinsulinism.

The majority of patients with an *INS* gene mutation are sporadic cases that result from de novo mutations. Dominant inheritance was observed in 7 of 35 (20%) families. A similar proportion of *KCNJ11* mutations are spontaneous (21), hence PND is primarily a sporadic form of diabetes.

We identified 16 different *INS* mutations in the 35 patients with permanent diabetes diagnosed in infancy (the first 12 months of life). Ten of these mutations have previously been reported (10), including the three most common mutations, A24D, F48C, and R89C, that account for 16 of 35 (46%) of cases. Most mutations are located within the A or B chains of insulin, and nine are predicted to interfere with the formation of disulphide bridges between cysteine residues either by replacing a cysteine residue or introducing an additional cysteine residue. In vitro experimental studies like those described by Liu et al. (22) are required to explore the effect of such mutations upon insulin processing and secretion.

Novel *INS* mutations diagnosed in PND. Six novel missense mutations (H29D, L35P, G84R, C96S, S101C, and Y103C) were identified in seven families. Three of these mutations involve cysteine residues within the insulin A chain and are therefore predicted to affect the normal folding of the proinsulin molecule. S101C was identified in two families: in ISPAD180 the finding that both children are heterozygous for the mutation but neither parent is a carrier suggests that the mutation has arisen de novo in one parent who must be a germline mosaic (Fig. 2). In the second family, the mutation was not present in the unaffected father, but DNA was not available from the mother.

The novel C96S mutation occurs at the site of a previously reported mutation (C96Y) identified in a patient with PND (10) and in the Akita mouse model (11). The same amino acid substitution (Cys>Ser) is present at the adjacent residue (C95S) in the Munich mouse model (23). Studies of these mice indicate that mutant proinsulin is trapped and accumulated in the ER, leading to induction of the ER stress response, inhibition of protein synthesis, and ultimately β -cell death (11,24–26).

The other three novel mutations, H29D (residue B5), L35P (B11), and G84R, do not involve cysteine residues. The H29D mutation had arisen de novo and affects a residue containing an imidazole ring, which packs closely to the C96-C31 inter-disulfide bridge between the A and B chains (27). This mutation has been shown to disrupt the

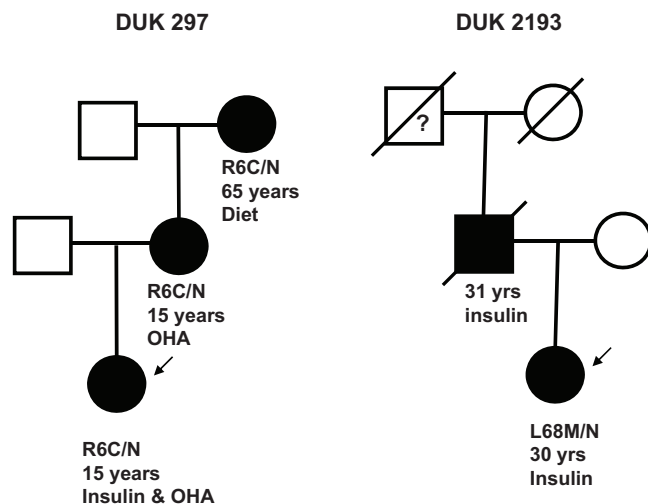


FIG. 5. Partial pedigrees for novel *INS* mutations identified in probands with MODY and young type 2 diabetes. Filled symbols represent patients with diabetes. The genotype is shown underneath each symbol. NN denotes no mutation identified. Below the genotype is the age at diagnosis of diabetes and current treatment. Arrows indicate the proband.

folding and conformation of proinsulin in vitro, decreasing the thermodynamic stability of the protein in the ER (28).

The L35P mutation affects a residue located in the B chain of the insulin molecule. The B chain contains two β turns at C31-H34 and G44-G47. These turns are highly conserved and guide the folding of the protein to form the inter-disulfide bond with the A chain (29). Residue L35 is highly conserved across all insulins and IGFs and is located adjacent to β turn 1. Due to the importance of this region in forming the C96-C31 interchain disulfide bond, we predict that a substitution of this leucine by proline would introduce prohibited steric contacts with C96, resulting in impaired disulfide bond formation.

The pathogenicity of the mutation G84R is less certain. Neither parent is affected with diabetes, but DNA samples were not available for testing. G84 is located within the C-peptide, a 31-amino acid linker between the A and B chains of proinsulin that is not highly conserved throughout evolution. The G84 residue is conserved from human to *Xenopus*, but an arginine (R) residue is present at this position in platypus. The patient with the G84R mutation is Vietnamese, and the possibility that this is a rare Asian polymorphism of no clinical significance cannot be excluded. In vitro studies have shown that the proinsulin molecule requires at least five linker amino acids (acting as the C-peptide) to produce the correct orientation of the molecule for the critical disulfide bond formation between the A and B chains (30), but it is not known which are the crucial amino acids within the C-peptide. Functional studies may help to determine the significance of this variant on insulin biosynthesis in vivo.

Novel INS mutations in MODY and young-onset type 2 diabetes. Two novel mutations were identified within the cohort of patients with MODY (R6C) and young-onset type 2 diabetes (L68M). R6C affects a residue within the signal peptide, while L68 is located within the C-peptide, which shows a lower level of evolutionary conservation. The sum of the genetic, evolutionary, and structural evidence suggests that while R6C is probably a pathogenic MODY mutation, L68M is probably a rare nonfunctional variant. Functional studies will be required to investigate these mutations further.

Clinical characteristics of patients with INS mutations. All mutation carriers identified through the infancy-onset cohort presented with symptomatic hyperglycemia or diabetic ketoacidosis and were treated with insulin. The majority (85%) of patients with an *INS* mutation were diagnosed before 6 months of age, and 96% had diabetes at 1 year, but two family members were diagnosed at 7 and 22 years of age (Fig. 3). In contrast, none of the three family members with the R6C mutation or the proband heterozygous for L68M was initially treated with insulin, and their age at diagnosis ranged from 15 to 65 years.

Our initial study showed that the age at onset of diabetes can vary within families (10), and in this larger series it has become clear that variable penetrance is common not only within families but also between families with the same mutation. For example, the four affected individuals from family ISPAD166 with the G32R mutation were diagnosed between 5 months and 22 years of age, whereas the proband from another family was diagnosed at 4 weeks of age. The age at diagnosis for A24D mutations carriers ranged from 4 weeks to 7 years. These findings suggest that other genetic or environmental factors may affect penetrance.

Both the mouse models show a more severe phenotype

in male than in female mice, with more severe diabetes symptoms in the male Akita mice (11) and lower birth weight in the Munich mice (23). While we saw no difference in the age at diagnosis of diabetes, the insulin dose, or glycemic control, and birth weight was lower for male than female *INS* mutation carriers, suggesting a more severe insulin secretory defect in utero. The reasons underlying this sex-specific phenotypic variability are not known, either in man or mouse.

No difference in birth weight was observed between patients with *INS* mutations and those with activating *KCNJ11* or *ABCC8* mutations. Although patients with *INS* gene mutations are diagnosed later (median 11 vs. 8 weeks, $P < 0.01$), the ranges overlap and hence patients diagnosed within the first 6 months of life with permanent diabetes require molecular genetic testing to confirm the genetic subtype. We suggest that newly diagnosed patients with neonatal diabetes (aged <6 months) should be referred for testing of the most common forms of transient neonatal diabetes and PND to determine the genetic etiology. This would include analysis for chromosome 6q24 abnormalities as well as *KCNJ11* and *INS* mutations, followed by *ABCC8* if these tests are negative.

Prevalence of INS mutations. *INS* mutations accounted for 23% (33 of 141) of neonatal diabetes diagnosed before 6 months of life, when patients with known etiological mutations were excluded. These patients were selected from the Exeter collection of permanent diabetes diagnosed before 6 months of age. In this series, mutations in the *KCNJ11*, *ABCC8*, and *INS* genes are the cause in 31, 10, and 12% of cases, respectively, while mutations in *GCK*, *EIF2AK3*, or *FOXP3* genes account for a further 8% of cases. Thus, mutations in known genes account for $\sim 60\%$ of all cases, indicating that additional genes for this form of diabetes remain to be identified.

Both *KCNJ11* and *INS* mutations are a rare cause of permanent diabetes diagnosed between 6 and 12 months of age, but *INS* gene mutations may be more frequent in this group (five *INS* mutation carriers identified vs. three *KCNJ11* mutations in our patient cohort).

In summary, we report the largest series of *INS* mutation carriers. These mutations are the second most common cause of PND but a rare cause of diabetes in childhood or adulthood, including MODY. We recommend that the *INS* gene should be screened for mutations in all children diagnosed with diabetes in the first year of life.

NOTE ADDED IN PROOF

Since submission and acceptance of this article, the L68M variant identified in the proband of family DUK2193 has been shown to be inherited from her unaffected mother. This result suggests that L68M is unlikely to be the cause of early-onset diabetes in this family.

APPENDIX

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