

**Heterozygous Missense Mutations in the Insulin Gene are linked to Permanent Diabetes appearing in the Neonatal Period or in Early-Infancy:
A report from the French ND Study Group**

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ABSTRACT

Objective: Permanent neonatal diabetes (PND) is defined by chronic hyperglycemia due to severe non-autoimmune insulin deficiency diagnosed in the first months of life. Several genes, including *KCNJ11* and *ABCC8* which encode the two subunits of the ATP-sensitive K⁺ (K_{ATP}) channel, can cause PND. Mutations in the Insulin (*INS*) gene have been recently described in ND families. Our study aimed to investigate the genetic anomalies and clinical heterogeneity in PND patients who are negative for a K_{ATP} channel mutation.

Research Design and Methods: We screened the *INS* gene by direct sequencing in 38 PND patients and in one child with non-autoimmune early-infancy diabetes, where no mutation in *GCK*, *KCNJ11* and *ABCC8* was identified. A detailed clinical phenotyping of the patients was carried out to specify the diabetes features in those found with an *INS* mutation.

Results: We identified 3 missense mutations in the *INS* gene in 4 probands. 2/4 mutations were inherited in a dominant manner, and the familial description evidenced a marked variability in age of diagnosis and disease progression. In our cohort, the *INS* mutations may represent ~10% of all permanent ND cases, having a later presentation of diabetes and no associated symptoms compared to cases with K_{ATP} channel mutations.

Conclusions: Heterozygous *INS* gene mutations can cause isolated permanent early-infancy diabetes, and should be assessed in neonatal as well as in childhood diabetes appearing like type 1, when autoimmune markers are absent. New pharmacogenomic strategies may be applicable as residual β-cell function is still present in some patients.

KEYWORDS. Diabetes, Genetics, Insulin biosynthesis, Insulin gene, Mutation, Neonatal diabetes

ABBREVIATIONS. ER, Endoplasmic reticulum; GCK, Glucokinase; INS, Insulin; ISPAD, International Society of Paediatric and Adolescent Diabetes; K_{ATP}, ATP-sensitive potassium; ND, neonatal diabetes; PND, permanent neonatal diabetes; SUR1, sulfonylurea receptor 1; TND, transient neonatal diabetes.

Diabetes mellitus in childhood, including the so-called “neonatal diabetes”, is unrelated to auto-immune destruction of the pancreatic β cells in the vast majority of cases (1). Neonatal diabetes (ND) is a rare (around 1:300,000 newborns) but potentially devastating metabolic disorder characterized by mild to severe hyperglycemia with low levels of circulating insulin within the first months of life (1). The presentation of the disease can be transient (TND) or permanent (PND), which differ in the duration of insulin dependence early in the first months/years of life, and in the molecular mechanisms responsible for the severe insulin secretion defect. More than half of TND cases are associated with abnormalities of an imprinted region on chromosome 6q24 (2), whereas mutations in the two subunits (Kir6.2 and SUR1) of the ATP-sensitive potassium (K_{ATP}) channel of the pancreatic β -cell have been characterized as a common cause of both TND and PND (3-5). In addition, a few cases of PND were attributed to mutations in the genes encoding the glycolytic enzyme glucokinase (*GCK*) (6) and insulin promoter factor-1 (*IPF-1*) (7), and syndromic cases including ND are caused by rare mutations in *PTF1A*, *FOXP3*, *GLIS3*, *TCF2* and *EIF2AK3* (1).

Our previous studies from the French ND case series, and others from the ISPAD cohort, have demonstrated that dominant mutations in both subunits of the K_{ATP} channels expressed in the neuroendocrine cells lead to a range in the severity of the disease. These phenotype specificities depend on the mutation in Kir6.2 or SUR1: from mild transient hyperglycemia to PND for SUR1 mutations (4,8,9), and from PND to more severe phenotypes associated with developmental delay and epilepsy usually for Kir6.2 and for some SUR1 mutations (10,11). In the patients referred to the French ND study group, more than 50 cases presenting

with very early onset diabetes are not yet defined for their molecular origin (8). This suggests that other gene defects are involved in key mechanisms regulating insulin processing and secretion, and/or survival process of insulin-producing β cell.

Very recently, Bell and colleagues, using a positional cloning approach in a pedigree including four diabetic members (age at diagnosis from 13 to 52 weeks), identified the insulin (*INS*) gene as responsible for diabetes in this family and replicated their findings in unrelated ND patients and the ISPAD cohort showing 10 heterozygous missense mutations in 16 probands (12).

In the present study, 38 PND patients from the French ND cohort and one child with non-autoimmune early-infancy diabetes, who are negative for a mutation in *GCK*, *KCNJ11* and *ABCC8* (4,8,10), were screened for the *INS* gene by direct sequencing. The H family (Table 1) was referred to us to sequence *ABCC8* as mutations in this gene may be present in patients with diabetes from the neonatal period to young adulthood (4,8). As *GCK* (*MODY2*) and *HNF1A* (*MODY3*) sequences were also found to be normal in this family, the proband-HA was included in the current study, despite diabetes appearing like non-autoimmune early-onset type 1 rather than to *bona fide* neonatal diabetes.

The three exons of *INS* were amplified in two fragments by using PCR (supplementary Table 1), and both strands were sequenced by using a standard protocol (4) and 3730xl DNA analyzer (Applied biosystems, Foster City, CA). Three missense mutations: A24D (c.71 C>A in exon 2), R89C and C96Y (c.265 C>T and c.287 G>A in exon 3) were identified in four probands diagnosed with a permanent form of diabetes. A detailed description of these patients is shown in Table 1. The same three mutations were found in PND patients from the original report (12). We also investigated whether the identified

mutations were inherited from a parent or if arose *de novo* by sequencing a DNA sample of the parents when available. In two families (H and B), the R89C mutation was inherited in a dominant manner from the mother. The sister of proband HA who presented with early-onset diabetes was also found with the R89C mutation (Table 1). The A24D and C96Y mutations were not inherited from a parent in families F and G. Such family relationships were confirmed by genotyping a panel of six microsatellite markers as previously used (4). Eleven patients with TND from the French cohort, in which chromosome 6 anomalies had been excluded (1), were also screened for the *INS* gene; but no mutation was identified in these patients.

All three mutations are located in critical regions of the preproinsulin molecule, and may affect the proteolytic processing of insulin precursors or disrupt insulin biosynthesis and induce endoplasmic reticulum (ER) stress, as proposed in the previous studies (12,13). The two amino acid changes at the signal peptide and A-chain C-peptide cleavage sites (A24D and R89C) are present in several unrelated ND patients (in three and four patients, respectively, when considering the current and previously reported cases (12)); this likely reflects the potential deleterious effect of these preproinsulin cleavage mutants on the folding and secretion of the normal insulin peptide. Importantly, the C96Y mutation found in two unrelated patients (this study and previous report (12)) was also shown to act in a dominant manner in the Akita diabetic mouse and consequently to activate the unfolded protein response (13).

The clinical features of four proband children, of one relative child (sister of proband HA) and of the two affected mothers (except for data at birth and at presentation which were not available for the two mothers) are shown in Table 1. Diabetes was diagnosed

in the children at a median age of 8.5 months (range: 25 days to 4.25 years). One child presented with mild hyperglycemia (7.2 mmol/l) whereas the four others had marked hyperglycemia (mean plasma glucose at diagnosis was 27.3, range 7.2 to 49.5 mmol/l). One case (FZ-A24D) presented with severe ketoacidosis, one (HA-R89C) with hyperglycemia and ketoaciduria and three (HL-R89C, BG-R89C, GI-C96Y) were diagnosed based on polyuria and polydipsia. The age at diagnosis in family H was consistent with familial type 1 diabetes. However, autoantibodies associated with type 1 diabetes (islet cell antibody, GAD65 and insulinoma-associated protein 2 antigen) were all negative in the two siblings, as well as in the other three younger patients. Pancreas ultrasonography was normal when performed (in 4/5 of the children cases). Two out of five of the young cases were small for gestational age (<3rd centile) including one of the offspring of a mother with diabetes mellitus (BG-R89C), suggesting a certain degree of insulin deficiency *in utero*. Initial insulin was required for those five patients and could never be stopped. At the last follow-up examination (age range: 22 months to 9.8 years), insulin dose ranged from 0.42 to 0.70 unit/kg/day (median 0.60 unit/kg/day) with a good metabolic control in each of the patient. These are relatively low insulin requirements, especially for the children and mother in family H, compared to what is required in type 1 diabetes patients in the same time frame (usually around 1 unit/kg/day). Consistent with a partially preserved β -cell secretory function, C-peptide levels in those two children were detectable and a normal response to a glucagon test was observed when they were 7.5 and 3.4 years of age, that is 3 and 1 year, respectively, after diabetes onset (Table 1). Interestingly, in patient HL, before the start of insulin therapy (at 4 years and 3 months), 2-hours post prandial glucose level was moderately high (9 mmol/l),

insulinemia was high (126 mUI/l) with a C-peptide level of 4.4 ng/ml, which proves an endogenous insulin secretion in this affected child at the age of 3 years and 5 months, an unusual situation in type 1 autoimmune diabetes.

Three patients (among whom a brother and a sister) have inherited the R89C mutation from their diabetic mother. In family H, the mother (HB) was discovered with diabetes at 3 years of age and has a good metabolic control at age 38 with a low dose of insulin (around 0.35 unit/kg/day). In contrast, the mother in family B, who was discovered at 3 months of age to have diabetes mellitus, had a poor metabolic control over the years developing severe retinopathy, neuropathy and macroangiopathy; at present (aged 35) she had to undergo amputation of both feet.

Clinically, two main differences were found by comparing in our cohort the children with an *INS* mutation and those with early-onset diabetes due to K_{ATP} channel mutations: the disease starts later with a median age of 8.5 months compared to 57 and 33 days in the cases with a mutation of *KCNJ11* or *ABCC8*, respectively (Table 2); no neuropsychological and neuromotor dysfunctions are present in children with an *INS* mutation in contrast to the children with a K_{ATP} channel mutation, where 6/17 ND-SUR1 and 4/18 ND-Kir6.2 patients in our cohort do have such developmental anomalies. From our findings, diabetes caused by an *INS* mutation was found to be isolated in contrast to what has been reported in some of the patients previously described (12). Indeed, none of the patients from our cohort had acanthosis nigricans, a finding suggestive of insulin resistance, whereas four patients in the previous study have such skin lesion.

In the current study, the three mutations identified were present in 5 children and two mothers with childhood onset diabetes, and not found in the transient form in accordance with the original report (12). In our cohort,

the *INS* mutations account for 13% of ND cases where the genetic etiology was unknown, and for ~10% of all permanent ND cases, compared to 4% for *ABCC8* and 35% for *KCNJ11* mutations (4,10 and unpublished results). A higher prevalence of PND due to *INS* mutations was reported in the ISPAD cohort (~20%), which could be explained by more strict inclusion criteria in the French cohort, particularly age of diagnosis and presentation at diagnosis for the patients who were mostly referred as newborns. The exception of family H is of great importance as children with an *INS* mutation can present clinically as type 1 diabetes patients. This is of special relevance as the age of diagnosis of type 1 diabetes has decreased over the years with many children diagnosed before or around 4 years of age (14), and highlights the need of knowing the status for autoimmune markers in those children; if negative, then a monogenic form of diabetes should be searched for. Another biological feature that may help distinguish diabetes linked to *INS* mutations from autoimmune type 1 diabetes is a higher insulin level at presentation.

Highly variable penetrance and clinical presentation of diabetes, both within and between families, is indeed documented from our study cohort for the patients carrying an *INS* mutation. Furthermore, previous works had reported familial hyperproinsulinemia with normal to mild glucose intolerance in adults caused by *INS* mutations at residues R89P/H (15,16) and H34D (17). Cellular biology experiments are needed to further understand these contrasting disease phenotypes. A clinical variability in the age at onset of diabetes in early-infancy was also reported in patients known with the Wolcott-Rallison syndrome, another disease linked to ER stress (18). This severe condition is due to mutations in the *EIF2AK3* gene that encodes the pancreatic eukaryotic initiation factor 2 α kinase or PERK. Severe defects in fetal/neonatal β cell proliferation and

differentiation were observed in the PERK-deficient mice, which results in low β cell mass, defects in proinsulin trafficking, abrogation of insulin secretion and culminate in PND (19).

A better understanding of the mechanism(s) leading to β -cell dysfunction in the patients with diabetes caused by an *INS* mutation will be crucial to define new treatments. As in the Akita mouse model, it may relate to the general concept of protein toxicity (20). The protein toxicity and overload in the ER are molecular defects already known to be involved in congenital diabetes insipidus where the disease begins after the neonatal period. Indeed this delay represents the time to destroy vasopressin secreting neurons (21). The *INS* mutations associated with permanent early-infancy diabetes extend this concept demonstrated in diabetes insipidus to diabetes mellitus. Strategies to increase insulin secretion by up-regulating the normal allele of the *INS* gene may be applicable, as long term residual β -cell function may exist in some patients with diabetes due to an *INS* mutation.

In conclusion, heterozygous *INS* gene mutations are a cause of isolated permanent early-onset diabetes, and should be searched for in neonatal as well as in childhood diabetes appearing like type 1, when autoimmune markers are absent, and when

there is a strong autosomal dominant pattern of inheritance.

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TABLE 1. Clinical characteristics of patients with permanent early-infancy diabetes who are carriers of an *INS* mutation

| Characteristic | Family F | Family H | | | Family B | | Family G |
|-------------------------------|--------------|--------------|-------------------------|----------|-------------------------|----------|-------------------------|
| | Proband-FZ | Proband-HA | Sister-HL | Mother | Proband-BG | Mother | Proband-GI |
| Sex | F | M | F | F | F | F | M |
| Ethnicity | European | European | European | European | European | European | Middle Eastern |
| Mutation | A24D | R89C | R89C | R89C | R89C | R89C | C96Y |
| At birth | | | | | | | |
| Weight (g/percentile) | 2080/<3 | 2920/25 | 3650/80 | NA | 2090/<3 | NA | 3000/10 |
| Gestation week | 39 | 38.5 | 38 | NA | 38 | NA | 41.5 |
| At presentation | | | | | | | |
| Age (m [months] or y [years]) | 0.8m | 4y 3m | 2y 4m | 4y | 8.5m | 3m NA | 4.8m |
| Presentation | Ketoacidosis | Ketoaciduria | Polyuria/ polydipsia | NA | Polyuria/ polydipsia | | Polyuria/ polydipsia |
| Glucose (mmol/l) | 38.8 | 18.4 | 7.2 | NA | 49.5 | NA | 22.5 |
| Autoantibodies | 0 | 0 | 0 | NA | 0 | NA | 0 |

| | | | | | | | |
|--|--------------|-----------|-----------|--------------|--------------|--------------|--------|
| Pancreas ultrasonography | N | N | N | NA | N | NA | NA |
| Neuropsychological assessment | N | N | N | N | N | N | N |
| C-peptide (ng/ml) | | | | | | | |
| Basal | Undetectable | 0.5 | 0.5 | Undetectable | Undetectable | Undetectable | NA |
| Under glucagon (peak level) ² | NA | 3.3 | 2.6 | Undetectable | NA | NA | NA |
| Current status | | | | | | | |
| Age (m [months] or y [years]) | 22m | 9y 10m | 6y 1m | 38y | 6y 6m | 35y | 4y 10m |
| Weight (Kg) | 12.3 | 25.5 | 19 | 51 | 23 | 54 | 17 |
| Type of insulin therapy | pump | injection | injection | injection | pump | injection | pump |
| Insulin dose (U/kg/day) | 0.60 | 0.56 | 0.42 | 0.32 | 0.70 | 0.75 | 0.70 |
| HbA _{1c} (%) ¹ | 7.3 | 6.5 | 6.7 | 7 | 7.5 | 7.1 | NA |

¹Upper limit of normal values for HbA_{1c}: 5.9% ; ²Normal response: more than 150% over baseline.
N, normal; NA, not available

TABLE 2. Comparison of clinical characteristics of patients with diabetes caused by an *INS* mutation, *KCNJ11* mutation or *ABCC8* mutation

| Characteristic | Molecular genetic etiology | | | <i>P</i> ³ |
|--|----------------------------|-------------------------------------|------------------------------------|-----------------------|
| | <i>INS</i> (n = 5) | <i>KCNJ11</i> (n = 18) ¹ | <i>ABCC8</i> (n = 17) ² | |
| Age at diagnosis (d [days] or m [months] or y [years]) | 8.5m (25d-4.25y) | 57d (1-127d) | 33d (1-125d) | <0.001 |
| Gestational age (weeks) | 38 (38-41.5) | 39 (38-41) | 40 (34-41) | NS |
| Birth weight (g) | 2920 (2080-3650) | 2710 (2110-3260) | 3040 (1660-3350) | NS |
| < 3rd centile (n) ⁴ | 2 | 4 | 5 | NS |

Results are given in median, range are in parentheses; NS, not significant.

¹ the *KCNJ11* group includes 16 cases with PND and 2 presenting with TND

² the *ABCC8* group includes 2 cases with PND and 15 presenting with TND

³ Differences between groups were calculated using Mann-Whitney and Fisher's exact tests.

⁴ n, number of patients.