Insights From Molecular Characterization of Adult Patients of Families With Multigenerational Diabetes

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Multigenerational diabetes of adulthood is a mostly overlooked entity, simplistically lumped into the large pool of type 2 diabetes. The general aim of our research in the past few years is to unravel the genetic causes of this form of diabetes. Identifying among families with multigenerational diabetes those who carry mutations in known monogenic diabetes genes is the first step to then allow us to concentrate on remaining pedigrees in which to unravel new diabetes genes. Targeted next-generation sequencing of 27 monogenic diabetes genes was carried out in 55 family probands and identified mutations verified among their relatives by Sanger sequencing. Nine variants (in eight probands) survived our filtering/prioritization strategy. After likelihood of causality assessment by established guidelines, six variants were classified as "pathogenetic/likely pathogenetic" and two as "of uncertain significance." Combining present results with our previous data on the six genes causing the most common forms of maturity-onset diabetes of the young allows us to infer that 23.6% of families with multigenerational diabetes of adulthood carry mutations in known monogenic diabetes genes. Our findings indicate that the genetic background of hyperglycemia is unrecognized in the vast majority of families with multigenerational diabetes of adulthood. These families now become the object of further research aimed at unraveling new diabetes genes.

We recently reported that 3% of hyperglycemic adults diagnosed as having type 2 diabetes are, in fact, affected by a multigenerational disease (1). Usually, these patients present with the typical cluster of metabolic abnormalities observed in type 2 diabetes. Notably in these patients, age at diagnosis, though greatly variable, is older than that in patients with maturity-onset diabetes of the young (MODY) but younger than that in patients with classic type 2 diabetes (1). In order to unravel the genetic causes of this form of diabetes, we first wanted to identify those families whose hyperglycemia is due to mutations in genes that are already known to cause monogenic diabetes, so that the remaining families may become the object of further research aimed at unraveling new diabetes genes.

We have previously reported that in 13% of such families, hyperglycemia was caused by mutations in one of the most common MODY genes (i.e., *HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, and *NEUROD1*) (1).

Here, by means of a gene panel–based next-generation sequencing approach that has been reported successful (2,3), we tested whether in some of the remaining families hyperglycemia is sustained by either mutations in uncommon MODY genes that we did not investigate in our previous effort or mutations in genes involved in other forms of monogenic diabetes.

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RESEARCH DESIGN AND METHODS

Study Design

We examined 55 probands and all available relatives from unrelated families with multigenerational diabetes (i.e., present in ≥3 consecutive generations) ascertained as previously described (1) and in which no mutations in the six most common MODY genes had been found using Sanger sequencing (1).

The study was performed according to the Declaration of Helsinki, and the protocol was approved by the local ethical committee. All subjects provided written informed consent.

Next-Generation Sequencing—Targeted Design

Targeted resequencing was carried out in the proband of each study family (n = 55). An exon-capture assay was designed to include coding regions and splice sites of 27 genes causing monogenic forms of diabetes (i.e., MODY, neonatal diabetes, and/or syndromic forms of diabetes) (Supplementary Table 1) using Illumina Design Studio (http://designstudio.illumina.com). Coordinates were obtained from the human reference sequence GRCh37, and the cumulative target size was 99.666 base pairs. The final design covered a total of 647 amplicons with an in silico estimated amplicon coverage of 99%.

Library Preparation and Sequencing

Genomic DNA was extracted from peripheral blood using the DNA Isolation Kit for Mammalian Blood (Roche).

Targeted capture and library preparation was carried out by a TruSeq Custom Amplicon Kit (Illumina, San Diego, CA) from 250 ng of double-stranded DNA according to manufacturer's instructions. Briefly, upstream and downstream oligonucleotides were hybridized to genomic DNA and unbound oligonucleotides were washed away using ELM4, SW1, and UB1 washing reagents. This was followed by an extension ligation process that connected hybridized upstream and downstream oligonucleotides by using DNA ligase. Extension-ligation products were amplified by PCR and fitted with index adaptor sequences for sample multiplexing using the TruSeq Custom Amplicon Index Kit (Illumina). The PCR product was purified from reaction components using AMPure XP beads (Beckman Coulter); each library sample underwent quantity normalization according to the TruSeq Custom Amplicon protocol and compatible indexed samples were pooled.

The pooled libraries were paired-end (2 × 151) sequenced on a micro flow cell with v3 chemistry on a MiSeq instrument (Illumina).

Read Mapping and Variant Calling

Sequences were automatically demultiplexed using the MiSeq Reporter software, running with TruSeq Amplicon standard settings, and written to FASTQ files. Sequences were then aligned against the GRGh37 (hg19) human reference assembly, yielding Binary Alignment/Map files, from which single-nucleotide polymorphisms and short indels were called for each individual sample and reported in Variant Call Format files.

Variants were filtered to include only those with ≥10× depth of coverage and mapping quality read values >30.

Variant Annotation, Filtering, Prioritization, and Classification

Functional annotation of variants was carried out with ANNOVAR (4). The Integrative Genomics Viewer version 2.2 (5) was used for visual inspection of read and variant data. All variants then underwent a mixed filtering/prioritization strategy. In detail, both synonyms and variants reported as having an allelic frequency >1/20,000 (assuming a dominant genetic model for a prevalence of MODY equal to 1.1/10,000 [6]) in the Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org), an implemented version of the Exome Aggregation Consortium database (7), were filtered out. The remaining variants (including nonsense, missense, coding insertion/deletion, and those affecting splicing) were then subjected to a functional prioritization.

Variants were retrieved when they were nonsense, frameshift, in-frame indels, and variants affecting splicing. Conversely, missense variants were retrieved only after careful bioinformatic evaluation. Accordingly, the possible functional impact of nonsynonymous amino acid substitutions was assessed in silico by thirteen prediction tools including SIFT (8), PolyPhen-2 (9), fathmm v2.3 (10), fathmm-MKL (11), MetaLR (12), MetaSVM (12), DANN (13), VEST3 (14), CADD v1.3 (15), PROVEAN v1.1 (16), MutationAssessor 1.0 (17), MutationTaster 2 (18), and LRT (19). These tools were selected because of their maintenance frequency, estimation congruency, or superior classification records (20–22). This approach represents an extension of a similar one we recently developed (23).

For each missense variant, the score values as obtained by these tools were first binarized; they became 1 when the following conditions were met and 0 otherwise: SIFT score <0.05, PolyPhen-2 HDIV >0.453, fathmm <0, fathmm-MKL >0.5, MetaLR >0.5, MetaSVM >0, DANN >0.8, VEST3 <0.05, CADD >0, PROVEAN <–2.5, MutationAssessor >1.9, LRT = D, and MutationTaster = A or D. It has to be considered that LRT and MutationTaster provide categorical classifications only. For LRT the categories and their meanings are D for "deleterious," N for "predicted neutral," and U for "unknown"; for MutationTaster they are A for "disease causing automatic" (namely, predicted as disease causing in ClinVar), D for "disease causing," N for "polymorphism," and P for "polymorphism automatic" (namely, predicted as neutral in ClinVar). Finally, a total "pathogenicity score" (with a possible range of 0–13) was obtained for each variant by summing all the single 13 binary scores as previously derived. Missense variants with a total pathogenicity score <7 (i.e., arbitrarily fixed below the median of possible range) were then filtered out. All remaining variants were then validated by Sanger sequencing and further investigated in the probands' families.

For each variant surviving the filtering/prioritization pipeline, likelihood of causality was then addressed by applying the established guidelines from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) (24,25). In this algorithm, pathogenicity is estimated by considering

the entire body of evidence, including population, bioinformatic, functional, and segregation data as well as data available from the literature and from specific diabetes-related databases (24,25). In addition, the recently freely available ClinGen Pathogenicity Calculator (26) has also been used. Accordingly, variants were classified as "pathogenic," "likely pathogenic," "of uncertain significance" (VUS), "likely benign," and "benign."

Sanger Sequencing

All variants with putatively deleterious effects as derived by the reduction pipeline were verified by Sanger sequencing and further investigated in the probands' families. All amplicons, as obtained from genomic DNA sample by PCR using gene-specific oligonucleotide primer pairs (available upon request), were subjected to direct sequencing in both forward and reverse directions on an automated AB 3130XL (Applied Biosystems, Foster City, CA) using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Results were analyzed with GeneScreen software (http://dna.leeds.ac.uk/genescreen/).

RESULTS

Clinical features of the 55 study probands are shown in Table 1.

The average read depth across the targeted gene regions in the 55 samples was 1,636 per base (± 1,460 SD) with ≥10 reads for 97.5% of bases. Average quality scores across bases of all mapped reads was 35 (Sanger/Illumina 1.9 encoding), while mean of the mapping quality scores across targeted gene regions was 49.

A mean of 186 (± 16.8) variants per sample were identified (see details in Supplementary Table 2). Nine variants (in eight probands) survived the data reduction pipeline (Table 2 and Supplementary Table 3) and were confirmed by Sanger sequencing (Supplementary Fig. 1). Each genotype variant was then tested in all family members whose DNA and clinical features were available (Supplementary Table 4). After assessing the likelihood of causality by using both the ACMG/AMP guidelines and the ClinGen Pathogenicity Calculator, seven out of the nine variants were classified as "pathogenic/likely pathogenic," while two were classified as "VUS" by both methods (Table 2).

Of the seven variants classified as pathogenic/likely pathogenic, three were novel, two have been previously reported to cause MODY, while two were only observed in gnomAD (Table 2). None of these variants was found in our in-house exome database (n = 150).

Among these seven variants, we found a missense heterozygous mutation (Val233Leu) in the common MODY gene *HNF1A* in proband P-1. This mutation, which we missed in our previous Sanger sequencing (1), segregates with diabetes in our family (Fig. 1) and has been already reported as causing MODY among Japanese (27). Notably, this normal-weight proband was diagnosed before age 25 years (Table 3), thus being definable as a MODY patient also from a classic clinical point of view (28).

Table 1—Clinical features of the study probands

	Males (%)	Age (years)	Age at diagnosis (years)	BMI (kg/m^2)	Waist circumference (cm)	HbA_{1c} (% [mmol/mol])	Antihyperglycemia treatment			Hypertension (%)	Dyslipidemia (%)	Micro-/macroalbuminuria (%)
							Diet only (%)	OADs (%)	Insulin ± OADs (%)			
FDA probands (n = 55)	51	51 ± 13	33 ± 10	30.1 ± 6.1	101 ± 14	8.8 ± 2 [72 ± 22]	5.5	32.7	61.8	51	89	47

Continuous variables are reported as mean ± SD and categorical variables are reported as percentages. OADs, oral antidiabetes drugs.

Table 2—List of variants identified by targeted next-generation sequencing that survived filtering/prioritization pipeline

Patient	Gene (RefSeq) variant	dbSNP	Bioinformatic pathogenicity score	Allele frequency (gnomAD, European non-Finnish)	Classification (according to ACMG/AMP guidelines) (24,25)	Classification (according to ClinGen Pathogenicity Calculator) (26)	Reference
P-1	*HNF1A* (NM_000545.6) c.697 G>C p.Val233Leu	n.a.	11	Absent	Pathogenic	Pathogenic	Tonooka et al., 2002 (27)
P-2	*ABCC8* (NM_000352.4) c.2473 C>T p.Arg825Trp	rs779736828	12	$8.9e^{-06}$	Likely pathogenic	Likely pathogenic	Vaxillaire et al., 2007 (29)
	KLF11 (NM_003597.4) c.51_53 ACAT>A p.Ile17del	rs758083789	n.a.	$8.9e^{-06}$	Likely pathogenic	Likely pathogenic	gnomAD
P-3	*BLK* (NM_001715.2) c.338 T>G p.Val113Gly	n.a.	9	Absent	Likely pathogenic	Likely pathogenic	Present work
P-4	*PAX4* (NM_006193.2) c.593 C>T p.Ala198Val	n.a.	9	Absent	Likely pathogenic	Likely pathogenic	Present work
P-5	*WFS1* (NM_001145853.1) c.2129 C>G p.Thr710Ser	rs200136995	8	$2.7e^{-05}$	Likely pathogenic	Likely pathogenic	gnomAD
P-6	*GLIS3* (NM_001042413.1) c.1186 C>T p.Gln396Ter	n.a.	n.a.	Absent	Likely pathogenic	Likely pathogenic	Present work
P-7	*FOXP3* (NM_014009.3) c.323 C>T p.Thr108Met	n.a.	7	$2.9e^{-05}$	VUS	VUS	De Benedetti et al., 2006 (41)
P-8	*INS* (NM_001185098.1) c.202 C>A p.Leu68Met	rs121908279	7	$4.5e^{-05}$	VUS	VUS	Edghill et al., 2008 (42)

dbSNP, Single Nucleotide Polymorphism database; n.a., not available.

As far as mutations in uncommon MODY genes are concerned, four mutations were found in three probands.

In P-2 proband, a novel digenic *KLF11/ABCC8* mutation pattern was detected. The Arg825Trp *ABCC8* missense mutation, either inherited from the mother or occurring de novo (Fig. 1), has been already reported as causative of MODY by Vaxillaire et al. (29), while the Ile17del in *KLF11*, inherited from the father's side and cosegregating with diabetes in all the affected proband's relatives whose DNA was available for sequencing (Fig. 1), is novel. Also, this slightly overweight proband was diagnosed before age 25 years and is, therefore, clinically definable as a MODY patient (28). Our finding is compatible with the notion of a digenic MODY presentation (30,31), although because of the lack of genetic information

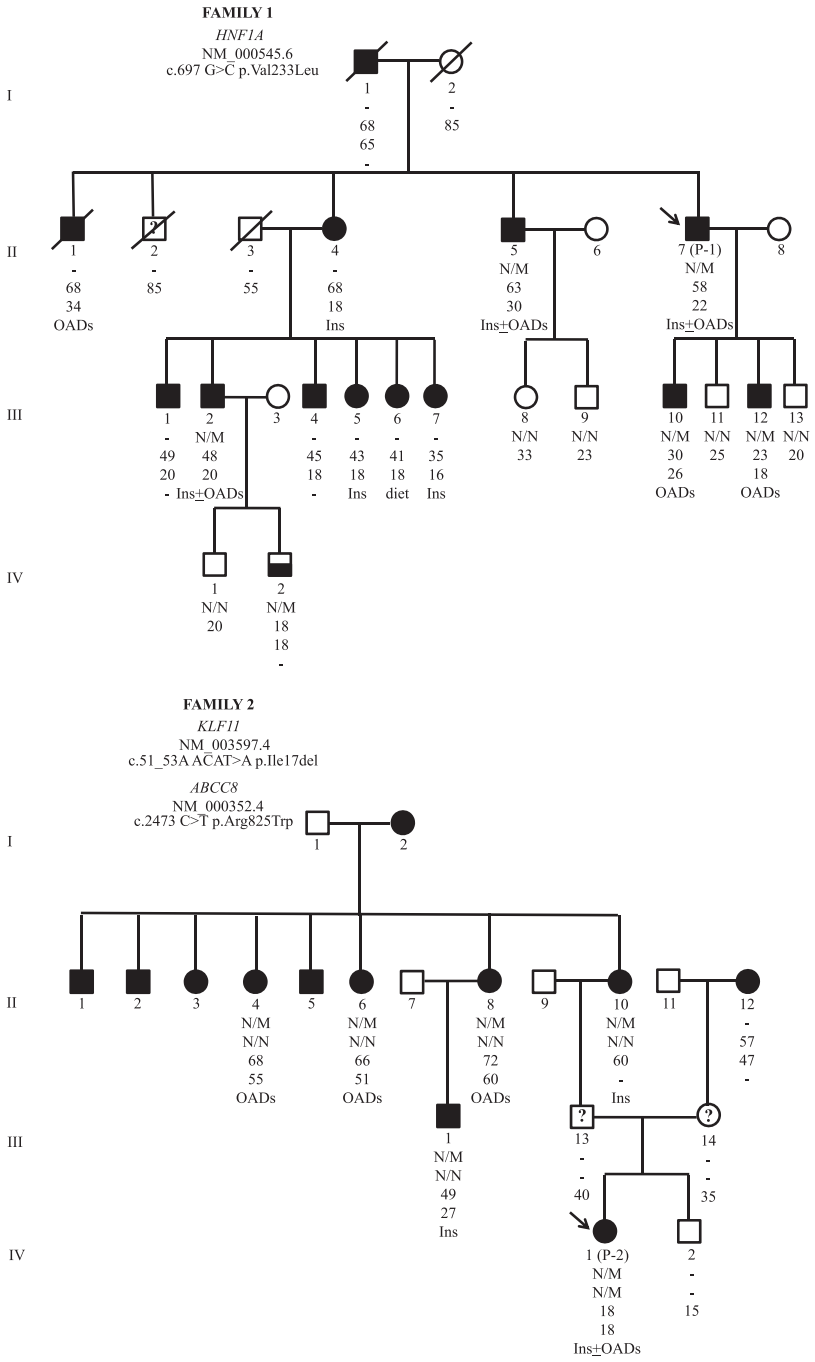

Figure 1—Partial pedigrees of the six probands' families carrying pathogenic or likely pathogenic variants. Filled symbols represent patients with diabetes. Half-filled symbols denote individuals with prediabetes (i.e., presenting impaired fasting glucose or impaired glucose tolerance or HbA_{1c} level ≥5.7%, according to 2017 ADA criteria [28]). Glycemic status and, when available, additional clinical information on individuals with unavailable genotype were reported by family probands. The genotype is shown underneath each symbol. N/M denotes mutation, while N/N denotes no mutation. Below the genotype are age in years at observation, age in years at diabetes diagnosis, and the specific antihyperglycemia treatment. Arrow indicates the study proband. Ins, insulin treatment; OADs, oral antidiabetes drugs.

in additional family members, no definite words can be said on this possibility.

P-3 proband and her affected mother carried a novel missense heterozygous mutation (Val113Gly) in *BLK* (Fig. 1), a gene whose role in MODY is debated (32,33). Unfortunately, the proband's sister did not allow for genetic testing, making it thus impossible to get deeper insights on the role of *BLK* Val113Gly in hyperglycemia in this family.

Finally, a novel heterozygous missense mutation (Ala198Val) in *PAX4* was found in proband P-4 and his affected sister (Fig. 1). Notably, the proband's maternal aunts did not allow for genetic testing. This mutation is located in the protein homeodomain that is essential for its transcription repression activity. Only few *PAX4* mutations, mainly in Asians, have been so far reported (34), with one of them residing in the same protein homeodomain as ours (34). Our present finding highlights the need to further address the role of *PAX4* as a MODY gene also among Europeans.

Interestingly, a heterozygous missense Thr710Ser mutation in *WFS1* was found in proband P-5 as well as in his affected sister and father, but not in his affected mother (Fig. 1), with no consanguinity reported between the two parents. In contrast, no mutations were observed in his affected mother. This finding is in line with recent observations reporting that heterozygous mutations in *WFS1* (the gene in which homozygous or compound heterozygous mutations are responsible for Wolfram syndrome, a recessive disorder characterized by optic atrophy, juvenile-onset diabetes, hearing loss, and other abnormalities [35]) may be

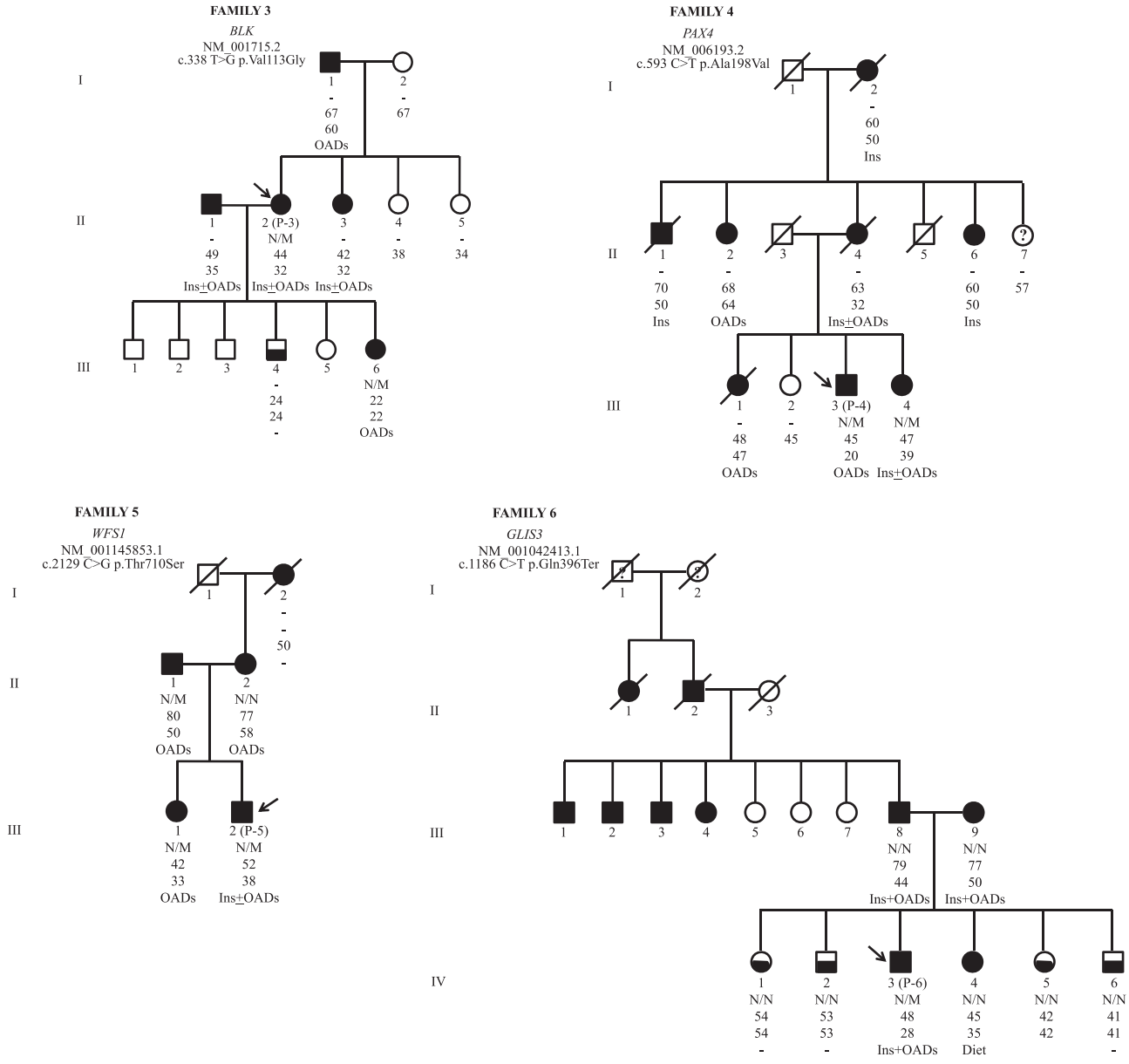

Figure 1—*Continued*.

responsible for an autosomal dominant form of nonsyndromic adult-onset familial diabetes (36,37).

Finally, a de novo *GLIS3* nonsense mutation (Gln396Ter) was found in P-6 proband (Fig. 1). No consanguinity was reported between the two parents. With the exception only of a variant reported in a heterozygous state in a MODY patient (36), *GLIS3* mutations have been exclusively reported to cause a recessive neonatal diabetes syndrome, also including congenital hypothyroidism and polycystic kidney disease (38). No such abnormalities were observed in our patient. Notably, since both of the proband's parents have diabetes and given that Gln396Ter turned out to be a de novo mutation, diabetes in this family is clearly due to mutation(s) in yet unidentified gene(s), rather than to the *GLIS3* mutation we report here, whose real role in causing hyperglycemia remains unknown.

Among the two variants classified as VUS, a missense *FOXP3* Thr108Met variant was found in P-7 proband and her mother with diabetes (Supplementary Fig. 2). Mutations in *FOXP3* cause the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (39), which also includes early-onset type 1 diabetes (40).

The Thr108Met mutation has been previously reported in a 7-year-old boy with a mild clinical IPEX phenotype not associated with diabetes (41). No clinical or laboratory evidence of autoimmune diseases has been observed in the proband or in her carrier mother with diabetes, both being negative at anti-GAD and anti-TPO antibodies assessment. Thus, the possibility that this variant has a pathogenic role (either in a dominant fashion or in compound heterozygosis with mutations in other genes) in altering glucose homeostasis cannot be excluded a priori and deserves to be addressed in further studies.

A previously reported *INS* Leu68Met missense variant, located within C-peptide but of uncertain pathogenic significance (42), was found in P-8 proband. It is of note that this variant has not been observed in gnomAD. Since no additional family members were available for genetic testing (Supplementary Fig. 2), the variant's role in hyperglycemia also remains elusive in this case.

DISCUSSION

Three percent of hyperglycemic adult patients with diabetes routinely diagnosed as having type 2 diabetes belong to families with a multigenerational form of disease (1), which is poorly characterized and whose genetic background is mostly unknown (1). This study is part of a broader attempt we are pursuing aimed at getting deeper insights into the molecular causes of this form of diabetes.

Our present and previous (1,43) findings considered together indicate that in approximately one-fourth of these families, hyperglycemia is sustained by heterozygous mutations in genes involved in monogenic forms of diabetes, mainly but not exclusively including MODY genes. Conversely, in the remaining ~75% of families the genetic cause of hyperglycemia remains to be unraveled. These latter families wait in a temporary nosographic limbo we have

Table 3—Clinical features of family probands carrying pathogenic/likely pathogenic and VUS variants

Patient	Gene (a.a. change)	Sex	Age (years)	BMI (kg/m^2)	Age at diagnosis (years)	Diabetes duration (years)	HbA_{1c} (% [mmol/mol])	Hyperglycemia treatment	Hypertension	Dyslipidemia	Micro-/macroalbuminuria
P-1	*HNF1A* (p.Val233Leu)	M	58	22.9	22	36	7.5 [58]	Insulin ± OADs	No	No	No
P-2	*ABCC8* (p.Arg825Trp) *KLF11* (p.Ile17del)	F	18	25.8	18	0	12.3 [111]	Insulin ± OADs	No	Yes	No
P-3	*BLK* (p.Val113Gly)	F	44	31.8	32	12	7.9 [63]	Insulin ± OADs	Yes	Yes	No
P-4	*PAX4* (p.Ala198Val)	M	45	30.2	20	25	8.4 [68]	OADs	Yes	Yes	Yes
P-5	*WFS1* (p.Thr710Ser)	M	52	31.9	38	14	8.4 [68]	Insulin ± OADs	Yes	Yes	Yes
P-6	*GLIS3* (p.Gln396Ter)	M	48	35.1	28	20	11.0 [97]	Insulin ± OADs	Yes	Yes	Yes
P-7	*FOXP3* (p.Thr108Met)	F	64	30.4	40	24	9.2 [77]	Insulin ± OADs	Yes	Yes	Yes
P-8	*INS* (p.Leu68Met)	F	24	46.1	20	4	6.7 [50]	OADs	No	Yes	Yes

Rows P-1 through P-6 indicate probands carrying pathogenic/likely pathogenic variants. Rows P-7 and P-8 indicate probands carrying VUS. Hypertension was diagnosed if individuals were currently receiving antihypertensive drugs or if their systolic or diastolic blood pressure was >130 or >85 mmHg, respectively. Dyslipidemia was diagnosed if patients were currently receiving antilipid agents or if they had total cholesterol ≥200 mg/dL or triglycerides ≥150 mg/dL or HDL cholesterol <40 mg/dL for males and <50 mg/dL for females. Micro-/macroalbuminuria was diagnosed if the morning urinary albumin/creatinine ratio was >2.5 or >3.5 mg/mmol in males and females, respectively, or >30 μg/mg in both sexes. a.a., amino acid; OADs, oral antidiabetes drugs.

proposed to define as familial diabetes of the adulthood (FDA) (1), an admission of ignorance that we definitively need to address. Although the definition of FDA does not suffer from limitations regarding age at disease onset and weight status comprised in the definition of MODY (28), it is entirely possible that for the few adult nonobese patients diagnosed before age 25 years it may overlap with the present definition of MODY. Several possibilities may be envisaged about the genetic background of FDA. Some patients may harbor either mutations in intronic/regulatory regions or large copy number alterations in the same genes we have here investigated but that were not covered by our sequencing method. It is also possible that some of these patients have mutations in one of the very few genes of highly uncommon monogenic diabetes that were not included in our sequencing panel (44). Finally, it is also possible that some patients carry mutations in genes whose instrumental role in regulating glucose homeostasis is hitherto unknown, as our recent discovery of *APPL1* as a new diabetes gene in one of these families clearly indicates (43).

Our present study has to be viewed as a further step toward better refining the genetic background of multigenerational forms of diabetes of adulthood, allowing the identification of families in which to try to eventually unravel new diabetes genes. Conversely, in the absence of clearly suggestive clinical and/or anamnestic features, our findings are not meant to encourage performing genetic testing for diagnostic purposes in adult individuals with multigenerational diabetes outside of research work.

In conclusion, the genetic background of hyperglycemia in the vast majority of adult individuals belonging to families with multigenerational diabetes remains unknown. Such families represent a unique research opportunity to apply next-generation sequencing approaches aimed at unraveling new diabetes genes and possibly new pathogenic pathways underlying abnormal glucose homeostasis in humans. Among these approaches are whole-exome (43) and whole-genome (44) sequencing, with the latter performed in the most informative individuals as selected among those sharing transmitted haplotypes identified by dense genome-wide association studies.

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Author Contributions. S.Pe. conceived and ran experiments, analyzed the data, and wrote the manuscript. V.T. supervised the study, analyzed the data, and wrote the manuscript. S.Pr. conceived and supervised the study, analyzed the data, and wrote the manuscript. O.L. and P.P. recruited and phenotyped patients. L.M., F.A., E.L., H.D., M.C., and E.M. conceived and ran experiments. T.B., D.C., and T.M. conducted bioinformatic analyses. M.G.S. conducted statistical analyses. All authors reviewed and edited the manuscript. S.Pr. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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