Full Title: Insights from Molecular Characterization of Adult Patients of Families with Multigenerational Diabetes Mellitus.

Running title: Monogenic diabetes-gene mutations in adult diabetic patients.

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Abstract

Multigenerational diabetes of the adulthood is a mostly overlooked entity, simplistically comprised in the large basin of type 2 diabetes. The general aim we are pursuing in last years is to unravel the genetic causes of such form of diabetes. Identifying among families with multigenerational diabetes those carrying mutations in known monogenic diabetes genes is the first step to then concentrate on remaining pedigrees where to unravel new diabetogenes.

Targeted Next Generation Sequencing of 27 monogenic diabetes-genes has been carried out in 55 family probands and identified mutations verified among their relatives by Sanger sequencing.

Nine variants (in 8 probands) survived our filtering/prioritization strategy. After likelihood of causality assessment by established guidelines, 6 variants were classified as "pathogenetic/likely pathogenetic" and 2 as "of uncertain significance".

Combining present with our previous data on the six genes causing the most common forms of maturity-onset diabetes of the young allows inferring that 23.6% families with multigenerational diabetes of the 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 the adulthood. These families now become the object of further research aimed at unraveling new diabetogenes.

Introduction

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, thought greatly variable, is older than that in patients with maturity onset diabetes of the young (MODY) but younger than that with classical type 2 diabetes (1). In order to unravel the genetic causes of this form of diabetes we firstly 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 diabetogenes.

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 successfully reported gene panel-based next generation sequencing (NGS) approach (2, 3), we tested whether in some of the remaining families hyperglycemia is sustained by either mutations in uncommon MODY genes we did not investigated in our previous effort or mutations in genes involved in other forms of monogenic diabetes.

Methods

Study design

We examined 55 probands, and all available relatives, from unrelated families with multigenerational diabetes (i.e. present in $\geq$ 3 consecutive generations) ascertained as previously described (1) and in which no mutations in the 6 most common MODY-genes have been found by 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 DNA Isolation Kit for Mammalian blood (Roche).

Targeted capture and library preparation was carried out by a Truseq Custom Amplicon kit (TSCA, Illumina, CA, USA) from 250ng 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, CA, USA). The PCR product was purified from reaction components using AMPure XP beads (Beckman-Coulter) and each library sample underwent quantity normalization according to the TSCA protocol and compatible indexed samples were pooled.

The pooled libraries were paired-end (2x151) sequenced on a micro flow cell with V3 chemistry on a MiSeq instrument (Illumina, CA, USA).

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 (BAM) files, from which

SNPs and short-Indels were called for each individual sample and reported in Variant Call Format (VCF) files.

Variants were filtered to include only those with ≥ 10X depth of coverage and mapping quality reads values exceeding 30.

Variants 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 synonymous and variants reported as having an allelic frequency >1/20,000 (assuming a dominant genetic model for a prevalence of MODY being 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 (ExAC) (7), were filtered out. The remaining variants (including nonsense, missense, coding insertion/deletion and affecting splicing) were then subjected to a functional prioritization.

Variants were retrieved when being nonsense, frameshift, in-frame indels and affecting splicing. Conversely, missense variants were retrieved only after careful bioinformatic evaluation. Accordingly, the possible functional impact of non-synonymous amino acid substitutions was assessed *in silico* by thirteen prediction tools including: SIFT (8), PolyPhen2 (9), fathmm v2.3 (10), fathmm-MKL (11), MetaLR (12), MetaSVN (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 and became 1 when the following conditions were met and 0 otherwise: SIFT score <0.05, PolyPhen2 HDIV >0.453, fathmm <0, fathmm-MKL >0.5, MetaLR >0.5, MetaSVN >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, which are D, N and U

for LRT, where D stands for "deleterious", N for "predicted neutral", U for "unknown"; A, D, N, P for MutationTaster, where A stands 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 previous 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 proband's 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 database (24, 25). In addition, also the recently free available ClinGen Pathogenicity Calculator (26) has 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 proband's families. All amplicons, as obtained from genomic DNA sample by polymerase chain reaction (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, CA, USA) using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). 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 1636 per base (SD ±1460) 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 (SD ±16.8) variants per sample were identified (see details in Supplementary Table 2). Nine variants (in 8 probands) survived data reduction pipeline (Table 2 and Supplementary Table 3) and have been confirmed by Sanger Sequencing (Supplementary Figure 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, 7 out the 9 variants were classified as "pathogenetic/likely pathogenetic", while 2 were classified as "VUS ", by both methods (Table 2).

Of the 7 variants classified as pathogenic/likely pathogenic, 3 were novel, 2 have been previously reported to cause MODY, while 2 were only observed in gnomAD (Table 2). None of these variants was found in our in-house exome database (n=150).

Among these 7 variants, we found a missense heterozygous mutation (i.e. Val233Leu) in the common MODY-gene *HNF1A* in proband P-1. This mutation, which we have missed at our previous Sanger sequencing (1), segregates with diabetes in our family (Figure1) and has been already reported as causing MODY among Japanese (27). Notably, this normal weight proband was diagnosed before age 25 years, thus being definable as a MODY patient also from a classical clinical point of view (28).

As far as mutations in uncommon MODY-genes is concerned, 4 mutations have been found in 3 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* (Figure 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 co-segregating with diabetes in all affected proband's relatives whose DNA was

available for sequencing (Figure 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, due to the lack of genetic information in additional family members, no definite words can be said on such possibility.

P-3 proband and her affected mother carried a novel missense heterozygous mutation (i.e.Val113Gly) in *BLK* (Figure1), a gene whose role on MODY is debated (32, 33). Unfortunately, the proband's sister did not allow for genetic testing, making thus impossible get deeper insights on the role of *BLK* Val113Gly on hyperglycemia in this family.

Finally, a novel heterozygous missense mutation (i.e. Ala198Val) in *PAX4* was found in proband P-4 and his affected sister (Figure 1). Notably, proband's maternal aunts did not allow for genetic testing. This mutation is located in the protein homeodomain which 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 than ours (34). Our present finding highlights the need of further addressing 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 (Figure 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 of Wolfram Syndrome -WS, a recessive disorder characterized by optic atrophy, juvenile-onset diabetes mellitus, hearing loss and other abnormalities [35]), may be responsible of an autosomal dominant form of non-syndromic adult-onset familial diabetes (36, 37).

Finally, a "de novo" *GLIS3* nonsense mutation (i.e. Gln396Ter) was found in P-6 proband (Figure 1). No consanguinity was reported between the two parents. With the only exception 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 proband's parents are diabetics 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 here report 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 diabetic mother (Supplementary Figure 2). Mutations in *FOXP3* cause the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (39), which includes also 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 and laboratory evidences of autoimmune diseases have been observed in the proband and in her carrier diabetic mother, both being negative at anti-GAD and anti-TPO antibodies assessment. Thus, if this variant has a pathogenic role (either in a dominant fashion or in compound heterozygosis with mutations in other genes) in altering glucose homeostasis is a possibility that cannot be excluded *a priori* and that deserves further studies to be addressed.

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 Figure 2), also in this case the variant role on hyperglycemia remains elusive.

Discussion

Three percent hyperglycemic adult diabetic patients 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 such form of diabetes mellitus.

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 into monogenic forms of diabetes, mainly but not exclusively including MODYs. Conversely, in the remaining ~75% families the genetic cause of hyperglycemia remains unraveled. These latter families wait in a temporary nosographic limbo we have 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 of limitations regarding age at disease onset and weight status comprised in that of MODY (28), it is entirely possible that for the few adult non-obese patients diagnosed before age 25 years it can overlap with the present definition of MODY X. 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 discovering of *APPL1* as a new diabetogene in one of these families clearly indicates (43).

Our present study has to be viewed as a further step toward a better refining of the genetic background of multigenerational forms of diabetes of the adulthood, allowing the identification of families in which trying to eventually unravel new diabetogenes. 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 mellitus outside research work.

In conclusion, the genetic background of hyperglycemia in the vast majority of adult individuals belonging to families with multigenerational diabetes mellitus remains unknown. Such families represent a unique research opportunity to apply next generation sequencing approaches aimed at unraveling new diabetogenes 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 at dense GWAS.

Article Information

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Author Contributions

S. Prudente conceived and supervised the study, analyzed the data and wrote the manuscript. V.T. supervised the study, analyzed the data and wrote the manuscript. S. Pezzilli conceived and run experiments, 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 run 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. Prudente 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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Table 1. Clinical features of the study-probands.

	Males (%)	Age (yrs)	*Age* at diagnosis (yrs)	BMI (kg/m^2)	Waist circumference (cm)	HbA1c (%) (mmol/mol)	Anti-hyperglicaemia treatment			Hypertension (%)	Dyslipidemia (%)	Micro-/macro-albuminuria (%)
							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 were reported as mean ± SD whereas categorical variables are reported as percentages.
FDA: Familial Diabetes of the Adulthood; BMI: body mass index; HbA1c: Glycated Haemoglobin; OADs: Oral Anti-diabetes Drugs.

Table 2. List of variants identified by targeted NGS that survived filtering/prioritization pipeline.

Patient	Gene (RefSeq) variant	dbSNP	Bioinformatic pathogenicity score	Allele Frequency (gnomAD-European non Finnish)	Classification (ACMG/AMP Guidelines)*	Classification (ClinGen Pathogenicity Calculator)**	Reference
P-1	*HNF1A* (NM_000545.6) c.696 G>C p.Val233Leu	n.a.	11	Absent	Pathogenic	Pathogenic	Tonooka N. *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 M.*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 F. *et al*. 2006 (41)
P-8	*INS* (NM_001185098.1) c.202 C>A p.Leu68Met	rs121908279	7	$4.5\ e^{-05}$	VUS	VUS	Edghill EL. *et al.* 2007 (42)

White rows indicate pathogenic/likely pathogenic variants. Rows highlighted in gray indicate variants of uncertain significance (VUS). NGS: Next generation Sequencing; n.a:not available; gnomAD: The Genome Aggregation Database (http://gnomad.broadinstitute.org); ACMG: American College of Medical Genetics and Genomics; AMP: Association of Molecular Pathology;
*Classification as obtained according to ACMG/AMP guidelines (24, 25).
** Classification as obtained by using the ClinGen Pathogenicity Calculator (26)

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

Patient	Gene (a.a. change)	Sex	Age (yrs)	BMI (Kg/m^2)	Age at diagnosis (yrs)	Diabetes duration (yrs)	HbA1c % (mmol/mol)	Hyperglicaemia treatment	Hypertention	Dyslipidemia	Micro-/Macro-albuminuria
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

White rows indicate probands carrying pathogenic/likely pathogenic variants. Rows highlighted in gray indicate probands carrying variants of uncertain significance (VUS).
a.a.: amino acid; BMI: body mass index; HbA1c: glycated haemoglobin; OADs: oral antidiabetes drugs.
Hypertension was diagnosed if individuals were currently receiving anti-hypertensive drugs or if their systolic or diastolic blood pressure was >130/85 mmHg.
Dyslipidemia was diagnosed if patients were currently receiving anti-lipid agents or if they had total cholesterol ≥200 mg/dl, or triglycerides ≥150 mg/dl, or HDL-cholesterol <40 mg/dl for male and <50 mg/dl for female.
Micro- and macro-albuminuria was diagnosed if morning urinary albumin creatinine ratio (ACR; mg/mmol) was >2.5/3.5 in males and female and >30 in both sexes, respectively.

Figure 1. Partial pedigrees of the 6 proband's families carrying pathogenic or likely pathogenic variants.

Filled symbols represent patients with diabetes. Half-filled symbols denote individuals with pre-diabetes (i.e. when presenting impaired fasting glucose or impaired glucose tolerance or HbA1c level $\geq$ 5.7%, according to ADA criteria 2017 [28]). Glycemic status and, when available additional clinical information, of individuals with unavailable genotype were reported by family probands.

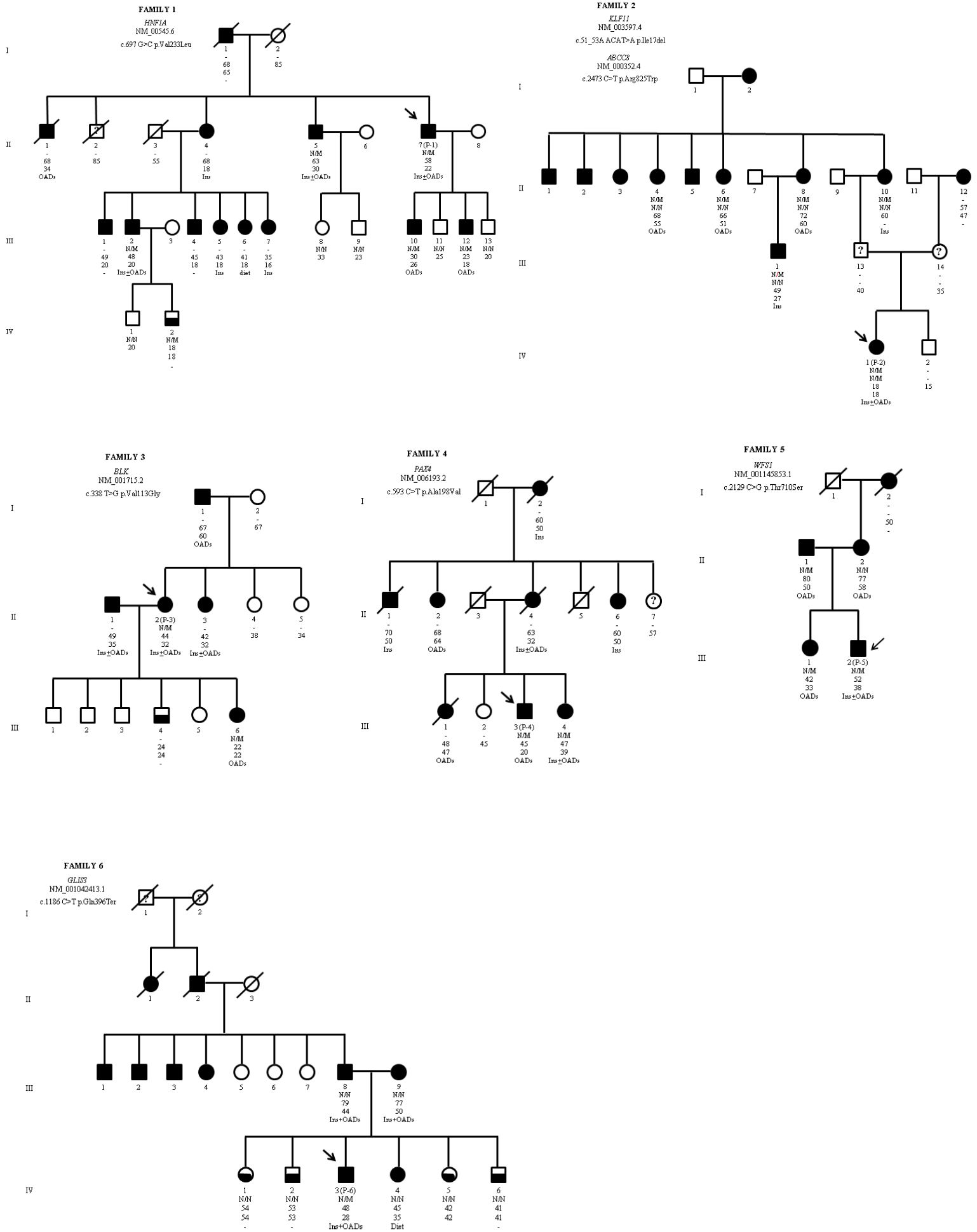
The genotype is shown underneath each symbol. N/M denotes mutation, while N/N denotes no mutation.

Below the genotype are: age at observation, age at diabetes diagnosis and the specific anti-hyperglycemia treatment.

Arrow indicates the study proband.

Ins: insulin treatment; OADs: oral antidiabetes drugs.

Figure 1.


FAMILY 1
HNF1A
NM_00545.6
c.697 G>C p.Val233Leu
FAMILY 2
KLF11
NM_003597.4
c.51_53A ACAT>A p.Ile17del
ABCC8
NM_000352.4
c.2473 C>T p.Arg825Trp
FAMILY 3
BLK
NM_001715.2
c.338 T>G p.Val113Gly
FAMILY 4
PAX4
NM_006193.2
c.593 C>T p.Ala198Val
FAMILY 5
WFS1
NM_001145853.1
c.2129 C>G p.Thr710Ser
FAMILY 6
GLIS3
NM_001042413.1
c.1186 C>T p.Gln396Ter

SUPPLEMENTARY MATERIALS

Insights from Molecular Characterization of Adult Patients of Families with Multigenerational Diabetes Mellitus

Serena Pezzilli, Ornella Ludovico, Tommaso Biagini, Luana Mercuri, Federica Alberico, Eleonora Lauricella, Hamza Dallali, Daniele Capocefalo, Massimo Carella, Elide Miccinilli, Pamela Piscitelli, Maria Giovanna Scarale, Tommaso Mazza, Vincenzo Trischitta, Sabrina Prudente.

SUPPLEMENTARY TABLES

Supplementary Table S1. List of the 27 monogenic diabetes-genes included in the sequencing panel.

GENE	Ref Seq	Diabetes type	OMIM
ABCC8	NM_000352.4	MODY/ND	600509
APPL1	NM_012096.2	MODY	604299
BLK	NM_001715.2	MODY	191305
CEL	NM_001807.4	MODY	114840
EIF2AK3	NM_004836.6	SD	604032
FOXP3	NM_014009.3	SD	300292
GATA4	NM_001308093.1	ND	600576
GATA6	NM_005257.5	ND	601656
GCK	NM_000162.3	MODY/ND	138079
GLIS3	NM_001042413.1	SD	610192
HNF1A	NM_000545.6	MODY	142410
HNF1B	NM_000458.3	MODY/ND	189907
HNF4A	NM_000457.4	MODY	600281
IER3IP1	NM_016097.4	SD	609382
INS	NM_001185098.1	MODY/ND	176730
KCNJ11	NM_000525.3	MODY/ND	600937
KLF11	NM_003597.4	MODY	603301
NEUROD1	NM_002500.4	MODY/ND	601724
NEUROG3	NM_020999.3	ND	604882
PAX4	NM_006193.2	MODY	167413
PDX1	NM_000209.3	MODY/ND	600733
PTF1A	NM_178161.2	ND	607194
RFX6	NM_173560.3	SD	612659
SLC19A2	NM_006996.2	SD	603941
SLC2A2	NM_000340.1	SD	138160
WFS1	NM_001145853.1	SD/ADD	606201
ZFP57	NM_001109809.2	ND	612192

OMIM: Online Mendelian Inheritance in Men (https://www.omim.org); MODY: Maturity Onset Diabetes of the Young; ND: neonatal diabetes. SD: syndromic diabetes; ADD: autosomal dominant diabetes.

Supplementary Table S2. Variant filtering and prioritization in 55 study-samples

Identified variants	Mean	SD
Total variants	186	16.8
High confidence variant calls	159.5	14.1
After exclusion of synonymous and intronic variants	20.5	2.9
After exclusion of variants with MAF >0.00005 (gnomAD- European Non Finnish)	0.42	0.3
Predicted damaging and confirmed by Sanger Sequencing	0.18	0.5

SD: standard deviation; gnomAD: The Genome Aggregation Database (http://gnomad.broadinstitute.org)

Supplementary Table S3. Individual prediction scores for each missense variant presenting a total score ≥7 when subjected to 13 bioinformatic tools.

PATIENT	GENE	REF SEQ	NUCLEOTIDE CHANGE	AMINOACID CHANGE	PATHOGENICITY PREDICTION SCORES													
					SIFT	POLYPHEN2 HDIV	LRT	MUTATION TASTER	MUTATION ASSESSOR	FATHMM	FATHMM-MKL	PROVEAN	MetaSVM	VEST3	CADD	DANN	MetaLR	TOTAL SCORE
P-1	*HNF1A*	NM_000545.6	Exon 3 c.697 G>C	p.Val233Leu	1	1	1	1	1	1	1	0	1	0	1	1	1	11
P-2	*ABCC8*	NM_000352.4	Exon 20 c.2473 C>T	p.Arg825Trp	1	1	1	1	1	1	1	1	1	0	1	1	1	12
P-3	*BLK*	NM_001715.2	Exon 5 c.338 T>G	p.Val113Gly	1	1	0	1	1	0	1	1	1	0	1	1	0	9
P-4	*PAX4*	NM_006193.2	Exon 5 c.593 C>T	p.Ala198Val	1	1	0	1	0	1	1	0	1	0	1	1	1	9
P-5	*WFS1*	NM_001145853.1	Exon 8 c.2129 C>G	p.Thr710Ser	0	0	1	1	0	1	1	0	1	0	1	1	1	8
P-7	*FOXP3*	NM_014009.3	Exon 4 c.323 C>T	p.Thr108Met	1	1	0	0	0	1	0	0	1	0	1	1	1	7
P-8	*INS*	NM_001185098.1	Exon 2 c.202 C>A	p.Leu68Met	0	1	0	1	1	1	0	0	0	0	1	1	1	7

Supplementary Table S4. Clinical and genetic characteristics of examined family members with available genotypic information.

Family	Family member	Mutation carrier	Gender	Age (yrs)	Age at diagnosis (yrs)	BMI (Kg/m^2)	Glycemic status	FPG	PG 2h-OGTT	HbA1c % (mmol/mol)	Hyperglycemia treatment	Hypertension	Dyslipidemia	Micro-/Macro-albuminuria
1	II-1 **(P-1)**	yes	M	58	22	22.9	DM	na	na	7.5 (58)	Ins+OADs	no	no	no
	III-8	no	F	33	na	22.5	NG	85	68	na	na	na	na	na
	III-9	no	M	23	na	19.3	NG	85	98	na	na	na	na	na
	III-10	yes	M	30	26	27.1	DM	na	na	6.1 (43)	OADs	no	no	no
	III-11	no	M	25	na	21.9	NG	78	72	na	na	na	na	na
	III-12	yes	M	23	18	24.9	DM	na	na	6.0 (42)	OADs	no	no	no
	III-13	no	M	20	na	22.4	NG	81	62	na	na	na	na	na
	IV-1	no	M	20	na	33.7	NG	86	78	na	na	na	na	na
	IV-2	yes	M	18	18	28.4	PD	112	126	na	na	no	no	no
2	IV-1 **(P-2)**	yes	F	18	18	25.8	DM	na	na	12.3 (111)	Ins+OADs	no	yes	no
	II-4	yes	F	68	55	22.9	DM	na	na	8.1 (65)	OADs	yes	yes	yes
	II-6	yes	F	66	51	30.0	DM	na	na	7.8 (62)	OADs	yes	yes	yes
	II-8	yes	F	72	60	22.8	DM	na	na	7.0 (53)	OADs	yes	yes	yes
	II-10	yes	F	60	u	u	DM	na	na	u	Ins	u	u	u
	III-1	yes	M	49	27	27.1	DM	na	na	11.3 (100)	Ins	no	yes	yes
3	II-2 **(P-3)**	yes	F	44	32	31.8	DM	na	na	7.9 (63)	Ins+OADs	yes	yes	no
	III-6	yes	F	22	22	24.8	DM	na	na	5.8 (40)	OADs	no	yes	yes
4	III-3**(P-4)**	yes	M	45	20	30.2	DM	na	na	8.4 (68)	OADs	yes	yes	yes
	III-4	yes	F	47	39	49.8	DM	na	na	5.6 (38)	Ins+OADs	yes	yes	yes
5	III-2 **(P-5)**	yes	M	52	38	31.9	DM	na	na	8.4 (68)	Ins+OADs	yes	yes	yes
	II-1	yes	M	80	50	30.4	DM	na	na	10.2 (88)	OADs	yes	no	no
	II-2	no	F	77	58	24.6	DM	na	na	9.9 (85)	OADs	yes	yes	no
	III-1	yes	F	42	33	30.0	DM	na	na	11.2 (99)	OADs	no	yes	no

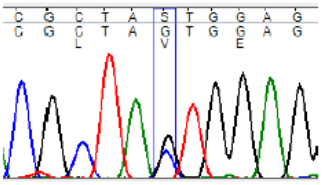
Family	Family member	Mutation carrier	Gender	Age (yrs)	Age at diagnosis (yrs)	BMI (Kg/m^2)	Glycemic status	FPG	PG 2h-OGTT	HbA1c (%) mmol/mol	Hyperglycemia treatment	Hypertension	Dyslipidemia	Micro-/Macro-albuminuria
6	IV-3 **(P-6)**	yes	M	48	28	35.5	DM	na	na	11.0 (97)	Ins+OADs	yes	yes	yes
	III-8	no	M	79	44	34.3	DM	na	na	8.4 (68)	Ins+OADs	yes	yes	yes
	III-9	no	F	77	50	37.1	DM	na	na	7.9 (63)	Ins+OADs	yes	yes	yes
	IV-2	no	M	53	53	28.0	PD	na	na	5.8 (40)	na	no	yes	yes
	IV-4	no	F	45	35	35.9	DM	na	na	7.0 (53)	diet	no	yes	no
	IV-5	no	F	42	42	43.8	PD	na	na	6.6 (49)	na	no	yes	yes
	IV-6	no	M	41	41	34.3	PD	na	na	6.0 (42)	na	yes	yes	yes
7	IV-3 **(P-7)**	yes	F	64	40	30,4	DM	na	na	9.2 (77)	Ins+OADs	yes	yes	yes
	IV-1	yes	F	41	26	21,5	DM	na	na	5.6 (38)	OADs	no	no	yes
8	III-1 **(P-8)**	yes	F	24	20	46,1	DM	na	na	6.7 (50)	OADs	no	yes	yes

BMI: body mass index; NG: normoglycemic; DM: diabetes mellitus; PD: pre-diabetes (as defined, according to ADA 2017 criteria, when presenting impaired fasting glucose or impaired glucose tolerance or HbA1c level $\geq$ 5.7%); OGTT: oral glucose tolerance test; FPG: fasting plasma glucose; PG: plasma glucose; HbA1c: glycated haemoglobin; Ins: insulin; OAD: oral antidiabetes drugs; nt: not assessed; u: unavailable.
Hypertension was diagnosed if individuals were currently receiving anti-hypertensive drugs or if their systolic or diastolic blood pressure was $\geq$130/85 mmHg.
Dyslipidemia was diagnosed if individuals were currently receiving anti-lipid agents or if they had total cholesterol $\geq$200 mg/dl, or triglycerides $\geq$150 mg/dl, or HDL-cholesterol <40 mg/dl for male and <50 mg/dl for female.
Micro- and macro-albuminuria was diagnosed if morning urinary albumin creatinine ratio (ACR; mg/mmol) was >2.5/3.5 in males and female and >30 in both sexes, respectively.

SUPPLEMENTARY FIGURES

Supplementary Figure S1. Sanger Sequencing confirmation of the 9 variants, which survived to filtering/prioritization pipeline.

P-1
HNF1A
NM_000545.6



c.697 G>C p.Val233Leu

P-2
KLF11
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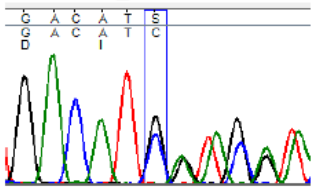

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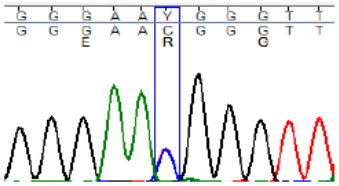

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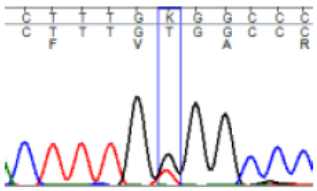

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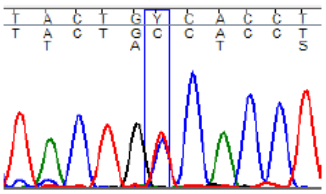
P-4
PAX4
NM_006193.2



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P-5
WFS1
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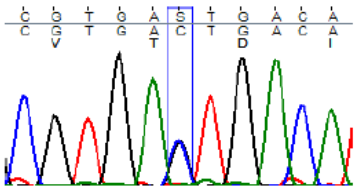

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P-6
GLIS3
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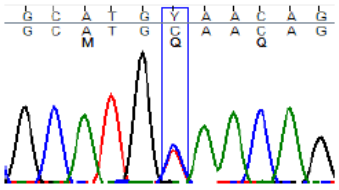

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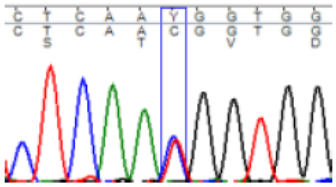

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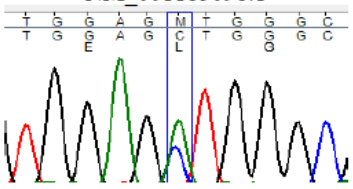
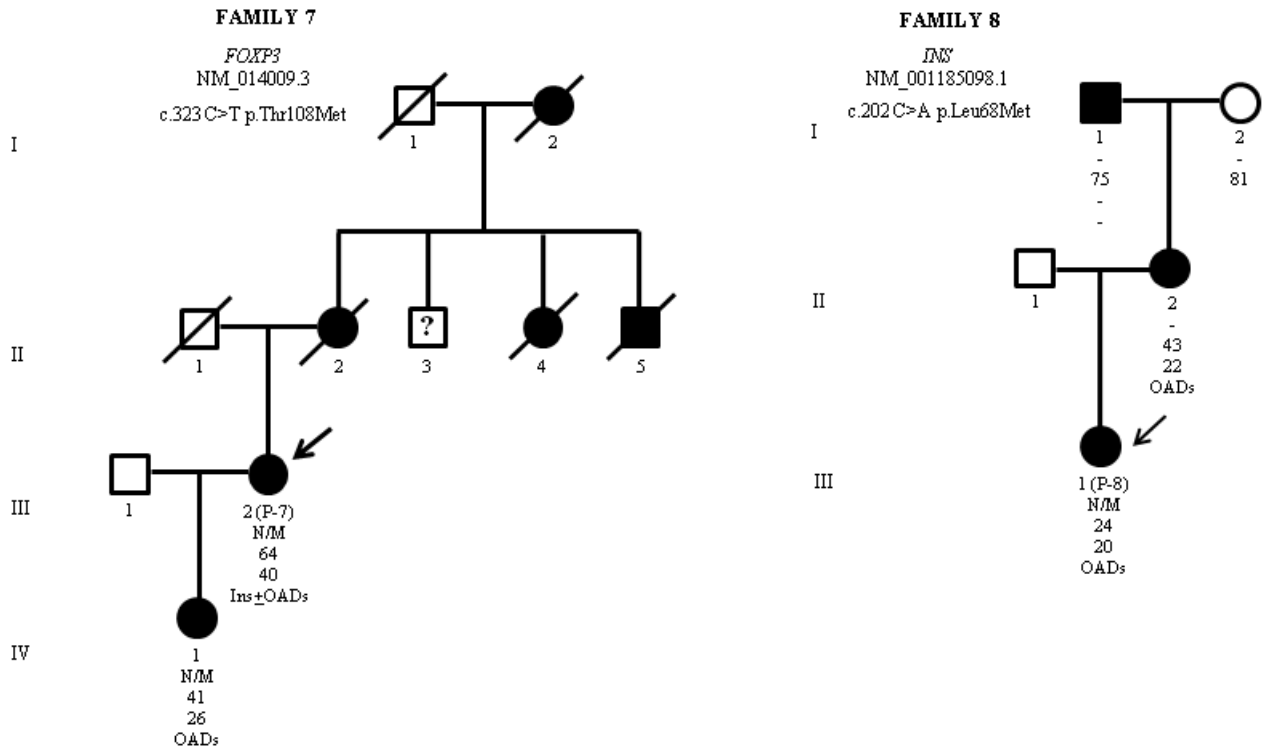


c.202 C>A p.Leu68Met

Supplementary Figure S2. Partial pedigrees of the 2 proband's families carrying VUS.



Filled symbols represent patients with diabetes. The genotype is shown underneath each symbol. N/M denotes mutation, while N/N denotes no mutation.
Glycemic status of individuals with unavailable genotype were reported by the family probands.
Below the genotype are: age at observation, age at diabetes diagnoses and the specific anti-hyperglycemia treatment. Arrow indicates the study proband.
VUS: variant of uncertain significance; Ins: insulin treatment; OADs: oral antidiabetes drugs.