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Functional Investigations of *HNF1A* Identify Rare Variants as Risk Factors for Type 2 Diabetes in the General Population

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Variants in *HNF1A* encoding hepatocyte nuclear factor 1α (HNF-1A) are associated with maturity-onset diabetes of the young form 3 (MODY 3) and type 2 diabetes. We investigated whether functional classification of *HNF1A* rare coding variants can inform models of diabetes risk prediction in the general population by analyzing the effect of 27 *HNF1A* variants identified in well-phenotyped populations (n = 4,115). Bioinformatics tools classified 11 variants as likely pathogenic and showed no association with diabetes risk (combined minor allele frequency [MAF] 0.22%; odds ratio [OR] 2.02; 95% CI 0.73–5.60; P = 0.18). However, a different set of 11 variants that reduced HNF-1A transcriptional activity to <60% of normal (wild-type) activity was strongly associated with diabetes in the general population (combined MAF 0.22%; OR 5.04; 95% CI 1.99–12.80; P = 0.0007). Our functional investigations indicate that 0.44% of the population carry *HNF1A* variants that result in a substantially increased risk for developing diabetes. These results suggest that functional characterization of variants within MODY genes may overcome the limitations of bioinformatics tools for the purposes of presymptomatic diabetes risk prediction in the general population.

Maturity-onset diabetes of the young (MODY) is a dominantly inherited subtype of diabetes, estimated to account for 1–2% of all diabetes cases and is caused by mutations across 10 or more genes (1). MODY arises most commonly from mutations in *HNF1A* (2) and *GCK* (3,4), and less commonly from mutations in *HNF4A* (5), *HNF1B* (6), and *INS* (7). Genetic studies in multiethnic cohorts have shown that variants in MODY genes may also predispose carriers to the risk of diabetes later in life (8–10). In the hepatocyte nuclear factor 1α (*HNF1A*) gene, population-specific variants with very low minor allele frequency (MAF) have demonstrated an increased risk for type 2 diabetes in selected populations. The *HNF1A* E508K rare variant (MAF 0.45% in Mexicans and almost absent in other populations) is associated with type 2 diabetes in the Mexican population (odds ratio [OR], 5.48; $P = 4.4 \times 10^{-7}$) (11), and the G319S variant is associated with early-onset type 2 diabetes in the Oji-Cree population (OR 4.0 in homozygous carriers and OR 1.97 in heterozygous carriers) (12). Meta-analyses have shown the common variants I27L and A98V slightly increase the type 2 diabetes risk (I27L: OR 1.09; $P = 8.1 \times 10^{-15}$; MAF 33%; A98V: OR 1.22; $P = 5.1 \times 10^{-10}$; MAF

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2.7%) (13). Although concurrent large association studies conclude that common variants in *HNF1A* (MAF >5%) do not associate with type 2 diabetes (14,15), certain combinations of variants (I27L and A98V) have, in vivo, shown a modest but significant association with impairment in glucose-stimulated insulin secretion. I27L alone has been associated with an increased type 2 diabetes risk (OR 1.5; $P = 0.002$) in elderly (age >60) and overweight (BMI >25 kg/m^2) patients (OR 2.3; $P = 0.002$) (16).

The spectrum of the in vitro functional consequence of *HNF1A* variants differs largely. Analyses of *HNF1A* mutations that cosegregate with familial early-onset diabetes (MODY) have demonstrated that they most often cause diabetes as a result of HNF-1A haploinsufficiency (loss of function), by none or severely impaired binding and transactivation of HNF-1A target genes (<~30% compared with wild-type), and/or by reducing HNF-1A protein stability (17–19). Similar investigations of the type 2 risk variants G319S and E508K have shown a milder effect on HNF-1A function compared with MODY variants by reducing the HNF-1A transactivation potential to <40–60% (11,20), whereas DNA binding properties have remained intact. The functional consequence of common variants, however, are mild when assessed alone (~70% transactivation by L27 and ~60% by V98) compared with combined (~50% by L27 and V98) variants (16). The DNA binding properties and protein levels of these common variants have remained intact.

A diagnosis of MODY can alter treatment and is important for prognostic evaluation (21), and identification of individuals at risk for diabetes later in life can target lifestyle interventions (22). The increasing availability of next-generation sequencing offers an opportunity to identify individuals who carry variants that may cause MODY or elevate the diabetes risk.

The spectrum of rare coding variants in seven of the most common MODY genes was recently investigated in well-phenotyped cohorts of the general population (23). This study concluded that 0.5–1.5% of randomly selected individuals carry rare variants that are interpreted as causal for MODY in the Human Gene Mutation Database (HGMD) or fulfill bioinformatics criteria for pathogenicity but that most of these carriers were euglycemic through middle age. We hypothesized that among these rare variants, only a subset might predispose to type 2 diabetes and that they could be better distinguished from benign variants and MODY variants using functional investigations compared with the bioinformatics variant prediction tools commonly used in biomedical research.

To test this hypothesis, we focused on rare coding variants identified in the *HNF1A* gene, assessing HNF-1A function. HNF-1A is a transcription factor that regulates the expression of several liver- and pancreas-specific genes involved in glucose transport and glucose/amino acid/lipid metabolism (24), and mutations in *HNF1A* are associated with the most common form of MODY in most populations (MODY3) (25), resulting in reduced β-cell insulin secretion and an early disease onset (before 25–35 years).

RESEARCH DESIGN AND METHODS

Study Design

We sought to investigate whether the functional classification of *HNF1A* rare variants can inform models of diabetes risk prediction in the general population. By studying all protein-coding *HNF1A* rare variants identified by exome sequencing in the three well-phenotyped population cohorts (described below), using *1*) bioinformatics tools used to aid medical diagnostics or *2*) functional assays assessing transactivation, DNA binding ability, and nuclear localization, we could estimate the effect of individual variants and diabetes risk. We reasoned that risk variants could be validated based on statistics from a public knowledge base for type 2 diabetes genetics (http://www.type2diabetesgenetics.org).

Variant Selection and Phenotypic Description of the Study Populations

We studied protein-coding rare variants in *HNF1A* identified by exon sequencing and reported in a recent study (23). The variants were identified in 4,115 random individuals from the Framingham Heart Study (FHS) Offspring cohort (n = 1,653), the Jackson Heart Study (JHS) cohort (n = 1,691), and type 2 diabetes case and control patients ascertained from the extremes of genetic risk (extreme type 2 diabetes [T2D] cohort, n = 771).

The FHS is a three-generation prospective family study of individuals of European ancestry designed to identify factors that contribute to cardiovascular disease, and the Offspring cohort consists of 5,124 adult children and spouses (enrolled in 1971) of the original participants (26). The JHS is a large community-based observational study of participants who are of African American origin. The study participants, who were recruited from urban and rural areas of the Jackson, Mississippi, metropolitan statistical area, consist of 6,500 men and women and 400 families (27). The extreme T2D cohort was originally selected from 27,500 individuals in three prospective cohorts: the Malmö Preventive Project (Sweden) (28), the Scania Diabetes Registry (Sweden), and the Botnia Study (Finland), selecting lean and young case patients with type 2 diabetes and old and obese control patients. Individuals with age of diabetes diagnosis younger than 35 years were excluded in an attempt to avoid consideration of patients with type 1 diabetes or MODY. In general, the individuals in the three cohorts differ in sex, age, and BMI. JHS individuals had a higher mean BMI (and represented by more women) than individuals in the FHS cohort, whereas the mean age was higher in FHS. Mean fasting plasma glucose values were approximately similar across the cohort case and control patients but were highest in JHS case patients.

Bioinformatics Classification

We used five different in silico prediction tools to classify the variants bioinformatically: sorting tolerant from intolerant (SIFT) (29), PolyPhen-2 (HumVar model), Combined Annotation Dependent Depletion (CADD) (30), MutationTaster (31), and Align Grantham Variation and

Grantham Deviation (GVGD) (32,33). For CADD we used a cutoff value of 15 (>15, pathogenic). The variants were assigned two classes: likely pathogenic or unlikely pathogenic. A variant was classified as likely pathogenic if it was scored as pathogenic in at least four of the five variant interpretation software tools used. A variant was classified as unlikely pathogenic if it was scored as pathogenic in less than four of the five variant interpretation software tools used.

Functional Assays

Plasmids

We used human pancreatic *HNF1A* cDNA (National Center for Biotechnology Information Entrez Gene BC104910.1) in the pcDNA3.1/HisC vector (Thermo Fisher) for the functional studies (19), and all *HNF1A* variants were made using the QuikChange XL Site-directed Mutagenesis Kit (Agilent Technologies). We verified the sequence of all constructs by Sanger sequencing. Constructs encoding the *HNF1A* mutants p.P112L, p.R263C, p.Q446*, and p.P447L, previously reported to cause MODY3 (18,19), were used as controls in the functional assays. One plasmid preparation was prepared for each of the 27 rare variants and compared with multiple wild-type ($n > 3$) and MODY control variant plasmid preparations ($n > 2$). Reporter gene constructs used in the transactivation assay were pGL3-RA, containing the rat albumin promoter and HNF-1A recognition sequence (nucleotide −170 to +5) next to the *Firefly* luciferase gene (pGL3-Basic vector backbone; Promega), and the control reporter vector pRL-SV40 (Promega), encoding the *Renilla* luciferase gene.

Transactivation Analysis

HeLa cells, grown as described previously (19), were cotransfected with reporter plasmid pGL3-RA, internal control pRL-SV40, and wild-type *HNF1A* or *HNF1A* variant plasmids. Empty vector pcDNA3.1/HisC was used as a control for the basal activity of promoter. Cells were transfected with Lipofectamine 2000 (Thermo Fisher) according to the manufacturer's protocol. Transcriptional activity on the rat albumin promoter was measured 24 h posttransfection with the Dual-Luciferase Assay System (Promega) in a Chameleon luminometer (Hidex). *Firefly* luciferase activity was normalized by correction for transfection efficiency of *Renilla* luciferase (internal control). p.P112L and p.P447L were included as MODY control *HNF1A* variants for severely reduced transactivation activity.

Nuclear and Cytoplasmic Localization by Immunofluorescence

Nuclear and cytoplasmic localization of wild-type and HNF-1A variant proteins were assessed by an indirect immunofluorescence assay, as described previously (11). HeLa cells were transfected for 24 h with wild-type or *HNF1A* variant plasmids using the protocol described by jetPRIME (Polyplus). HNF-1A was detected using primary antibody anti-Xpress and secondary antibody Alexa Fluor 488 (Thermo Fisher). Cell nuclei were counterstained with DAPI (Thermo Fisher). Finally, cells were analyzed on a TCS SP2 confocal microscope (Leica) with a ×63 objective. Images were analyzed with the Leica Application Suite (LAS). The MODY variant p.Q466* was included as a control for impaired localization (cytosolic retention) (34). A minimum of 200 cells was analyzed for nuclear versus nuclear and cytosolic staining.

DNA Binding Analysis

We used an in vitro coupled transcription and translation system (TNT Coupled Reticulocyte Lysate System; Promega) for expression of HNF-1A proteins essentially as described before (34). An electrophoretic mobility shift assay was performed to investigate the ability of equal amounts of in vitro synthesized HNF-1A proteins to bind to a [γ-^{32}P]-radiolabeled rat albumin oligonucleotide containing an HNF-1A binding site. For this, bound products were analyzed by 6% DNA retardation gel electrophoresis (Thermo Fisher), followed by autoradiography (LAS-1000 Plus; Fujifilm Medical System). DNA binding was quantified using the intensity of HNF-1A protein-oligonucleotide complexes (from two independent expression reactions and three separate gel analyses) using Image Gauge 3.12 software (Fujifilm Medical Systems). MODY variants p.P112L and p.R263C were included as controls for severely reduced and no DNA binding ability, respectively.

Protein Expression Analysis

Wild-type and HNF-1A variant protein expression levels were measured using cell lysates from transfected HeLa cells lysed in passive lysis buffer (Promega) and analyzed by SDS-PAGE and immunoblotting with HNF-1A antibody (Cell Signaling). We quantified protein levels by densitometric analysis and normalized to actin antibody (Santa Cruz Biotechnology).

Statistical Analyses

Experiments were performed on at least three independent occasions with three parallels, unless otherwise specified, and all data are expressed as means ± SD. Statistical analyses were performed using the Student *t* test, and a *P* value of <0.05 was considered significant.

We used counts from the Supplementary Table 3 in Flannick et al. (23) for all statistical analyses to discern whether groups of variants were associated with diabetes in the three study cohorts. Variants were grouped based on classifiers (bioinformatics vs. functional evaluation). Counts of carriers were summarized separately for individuals with diabetes and control subjects for each class in each cohort and meta-analyzed using a fixed-effect model performed by the method of Mantel and Haenszel, as implemented with the Metan version 9 module in Stata/IC 13.0 software (StataCorp LP), with each study cohort treated as a separate group. All results are presented as ORs with 95% CIs with nominal *P* values from the meta-analysis.

RESULTS

In this study, we evaluated 27 nonsynonymous *HNF1A* variants with MAF <1% found in individuals from three population-based cohorts described in the RESEARCH

Table 1—Functional and bioinformatic evaluation of rare nonsynonymous *HNF1A* variants from the study cohorts (23)

Nucleotide change	Amino acid change	Functional evaluation (this study)			Previously reported				Bioinformatic evaluation/population frequencies							Variant classification		Number of variant carriers					
																		FHS cohort		JHS cohort		T2D cohort	
		Transcriptional activity (%)	DNA binding (%)	Nuclear localization (%)	Phenotype/ family segregation	Functional effect	HGMD	Ref.	SIFT	PolyPhen-2 (HumVar)	Align GVGD	MutationTaster	CADD	ExAC	1000G	Bioinformatic evaluation (this study)	According to clinical diagnostic practice	DM (n = 218) (n/MAF)	No DM (n = 1,435) (n/MAF)	DM (n = 346) (n/MAF)	No DM (n = 1,345) (n/MAF)	DM (n = 362) (n/MAF)	No DM (n = 409) (n/MAF)
																		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
c.335C>T	P112L	14	19	—	MODY/yes	TA <30%, DNA-binding 30–40% (19, 34); TA ~50% (35)	MODY	(19, 34, 35, 47)	Del	Prob	C65	Disease causing	34	—	—	Likely pathogenic (5/5)	Class 5	—	—	—	—	—	—
c.1340C>T	P447L	18	—	—	MODY/yes	TA ≤20%, low DNA-binding (18, 48)	MODY	(2, 18, 37, 48, 49)	Del	Prob	C0	Disease causing	34	—	—	Likely pathogenic (4/5)	Class 5	—	—	—	—	—	—
c.787C>T	R263C	—	5	—	MODY/T2D/yes	TA <30%, no DNA-binding (19, 36)	MODY	(19, 36, 50)	Del	Prob	C0	Disease causing	35	9.13×10^{-6}	—	Likely pathogenic (4/5)	Class 5	—	—	—	—	—	—
c.1396C>T	Q466*	—	—	15	MODY/NA	TA <30%, NL ~3% (19, 34)	MODY	(19, 34)	—	—	—	—	40	—	—	NA	Class 5	—	—	—	—	—	—
c.965A>G	Y322C	30	—	63	Uncertain MODY/NA	NA	MODY	(51)	Del	Prob	C0	Disease causing	24,7	0.000132	Yes	Likely pathogenic (4/5)	Class 3	—	—	3 (0.43)	3 (0.11)	—	—
c.818_820del	E275del	30	44	68	MODY/NA	NA	MODY	(52)	—	—	—	—	—	—	—	NA	Class 3	1 (0.23)	0	—	—	—	—
c.392G>A	R131Q	35	58	59	MODY/yes	TA ~50% (18, 53)	MODY	(2, 18, 53, 54)	Del	Prob	C35	Disease causing	34	8.25×10^{-6}	—	Likely pathogenic (5/5)	Class 3	—	—	—	—	1 (0.14)	0
c.1522G>A	E508K	40	—	59	Uncertain MODY/T2D/no	TA ~40%; NL ~60% (11)	MODY	(11, 51, 55)	Del	Poss	C0	Disease causing	32	0.0004403	—	Likely pathogenic (4/5)	Class 2	1 (0.23)	0	—	—	—	—
c.1405C>T	H469Y	41	—	80	MODY/T1D/no	NA	—	(25, 52, 56)	Del	Prob	C0	Disease causing	23,5	0.0001613	—	Likely pathogenic (4/5)	Class 2	0	2 (0.07)	0	1 (0.037)	—	—
c.1544C>A	T515K	46	—	74	Uncertain MODY/NA	NA	MODY	(51)	Del	Poss	C0	Disease causing	35	6.634×10^{-5}	—	Likely pathogenic (4/5)	Class 3	—	—	1 (0.15)	0	—	—
c.1541A>G	H514R	51	—	57	T2D/no	NA	T2D	(57, 58)	Tol	Prob	C0	Disease causing	22,5	0.0002903	—	Unlikely pathogenic (3/5)	Class 3	1 (0.23)	0	—	—	—	—
c.1513C>A	H505N	54	—	64	Uncertain MODY/NA	NA	MODY	(51)	Tol	Prob	C0	Disease causing	26,1	0.000108	Yes	Unlikely pathogenic (3/5)	Class 3	0	1 (0.035)	—	—	—	—
c. 298C>A	Q100K	57	71	86	NA/NA	NA	—	—	Tol	Ben	C0	Disease causing	20,9	1.162×10^{-6}	—	Unlikely pathogenic (2/5)	Class 3	—	—	1 (0.15)	0	—	—
c.307G>A	V103M	59	65	78	MODY/NA	NA	—	(59, 60)	Tol	Prob	C0	Disease causing	25,6	3.61×10^{-5}	—	Unlikely pathogenic (3/5)	Class 2	1 (0.23)	0	—	—	—	—
c.1696C>A	H566N	59	—	73	NA/NA	NA	—	—	Tol	Ben	C0	Disease causing	23,1	—	—	Unlikely pathogenic (2/5)	Class 3	—	—	0	1 (0.037)	—	—
c.142G>A	E48K	61	—	75	Uncertain MODY/T1D/ conflicting results	NA	MODY	(61, 62)	Tol	Ben	C0	Disease causing	18,32	0.0001564	—	Unlikely pathogenic (2/5)	Class 3	0	1 (0.035)	—	—	—	—
c.1360A>G	S454G	64	—	78	NA/NA	NA	—	—	Tol	Ben	C0	Disease causing	10,98	—	—	Unlikely pathogenic (1/5)	Class 3	—	—	—	—	1 (0.14)	0
c.1469T>C	M490T	63	—	73	NA/NA	NA	—	—	Del	Prob	C0	Disease causing	25,8	9.36×10^{-6}	—	Likely pathogenic (4/5)	Class 3	—	—	—	—	0	1 (0.12)
c.1748G>A	R583Q	63	—	78	Late-onset NIDDM/T2D/no	NA	MODY	(63–65)	Tol	Ben	C0	Disease causing	23,6	0.0005041	Yes	Unlikely pathogenic (2/5)	Class 3	0	4 (0.14)	—	—	—	—
c.92G>A	G31D	65	—	81	Uncertain MODY/NA	NA	MODY	(61, 66–69)	Del	Ben	C0	Disease causing	22,8	0.0007105	Yes	Unlikely pathogenic (3/5)	Class 2	0	5 (0.17)	1 (0.15)	0	0	1 (0.12)
c.854C>T	T285M	64	—	87	NA/NA	NA	—	—	Del	Prob	C0	Disease causing	24	2.889×10^{-5}	—	Likely pathogenic (4/5)	Class 3	0	1 (0.035)	—	—	—	—
c.185A>G	N62S	65	—	70	Uncertain MODY/NA	NA	MODY	(64)	Tol	Poss	C0	Disease causing	22,1	0.0001206	—	Unlikely pathogenic (3/5)	Class 3	0	1 (0.035)	0	1 (0.037)	—	—
c.1532A>G	Q511R	66	—	73	NA/NA	NA	—	—	Del	Prob	C0	Disease causing	28	1.66×10^{-5}	—	Likely pathogenic (4/5)	Class 3	0	1 (0.035)	—	—	—	—
c.1729C>G	H577D	69	—	74	T1D/uncertain MODY/no	NA	—	(70, 71)	Del	Ben	C0	Disease causing	24,2	0.0001424	Yes	Unlikely pathogenic (3/5)	Class 2	0	1 (0.035)	—	—	—	—
c.533C>T	T178I	67	—	79	NA/NA	NA	—	—	Del	Ben	C0	Disease causing	18,39	—	—	Unlikely pathogenic (3/5)	Class 3	0	1 (0.035)	—	—	—	—
c.827C>G	A276G	69	—	70	Uncertain MODY/NA	NA	MODY	(51, 72)	Del	Poss	C0	Disease causing	28,7	—	—	Likely pathogenic (4/5)	Class 3	0	1 (0.035)	—	—	—	—
c.1165T>G	L389V	68	—	79	Uncertain MODY/NA	NA	MODY	(51, 73, 74)	Tol	Poss	C0	Disease causing	12,49	0.0005422	Yes	Unlikely pathogenic (2/5)	Class 2	—	—	4 (0.58)	14 (0.52)	—	—
c.290C>T	A97V	71	—	85	NA/NA	NA	—	—	Del	Poss	C65	Disease causing	25,2	6.689×10^{-5}	—	Likely pathogenic (5/5)	Class 3	—	—	0	1 (0.037)	—	—

Continued on p. 339

Table 1—Continued

Nucleotide change	Amino acid change	Functional evaluation (this study)			Previously reported				Bioinformatic evaluation/population frequencies							Variant classification		Number of variant carriers					
																		FHS cohort		JHS cohort		T2D cohort	
		Transcriptional activity (%)	DNA binding (%)	Nuclear localization (%)	Phenotype/ family segregation	Functional effect	HGMD	Ref.	SIFT	PolyPhen-2 (HumVar)	Align GVGD	MutationTaster	CADD	ExAC	1000G	Bioinformatic evaluation (this study)	According to clinical diagnostic practice	DM (n = 218) (n/MAF)	No DM (n = 1,435) (n/MAF)	DM (n = 346) (n/MAF)	No DM (n = 1,345) (n/MAF)	DM (n = 362) (n/MAF)	No DM (n = 409) (n/MAF)
																		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
c.824A>C	E275A	78	—	81	NA/NA	NA	—	—	Del	Prob	C0	Disease causing	26,5	3.661×10^{-5}	Yes	Likely pathogenic (4/5)	Class 3	0	1 (0.035)	—	—	—	—
c.341G>A	R114H	83	—	81	Uncertain MODY/NA	NA	MODY	(74, 75)	Tol	Ben	C0	Polymorphism	22,9	3.318×10^{-5}	—	Unlikely pathogenic (1/5)	Class 3	—	—	1 (0.15)	0	0	1 (0.12)
c.586A>G	T196A	101	—	85	Uncertain MODY/NA	NA	MODY	(51, 76)	Tol	Ben	C0	Disease causing	14,81	0.0003306	—	Unlikely pathogenic (1/5)	Class 2	—	—	—	—	0	1 (0.12)
c.293C>T	A98V	62	—	84	Risk T2D	Reduced effect in combination with L27	Serum C-peptide and insulin response	(13, 16, 77)	Del	Ben	C0	Polymorphism	23,4	0.03672	Yes	Unlikely pathogenic (2/5)	Class 1	9 (2.06)	53 (1.85)	4 (0.58)	16 (0.59)	32 (4.4)	21 (2.6)
c.1460G>A	S487N	83	—	83	Cardiovascular disease, increased risk/earlier age of diagnosis	Low	Cardiovascular disease, increased risk, association with	(16, 74, 78, 79)	Del	Ben	C0	Polymorphism	15,32	0.3465	Yes	Unlikely pathogenic (2/5)	Class 1	148 (33.9)	890 (31)	90 (13)	336 (12.5)	227 (31.4)	249 (30.4)
c.79A>C	L27I*	86	—	86	Risk T2D	I27L: small effect	Insulin resistance, association with	(13, 62, 74)	Tol	Ben	C0	Polymorphism	25,6	0.3533	Yes	Unlikely pathogenic (1/5)	Class 1	165 (37.8)	936 (32.6)	95 (13.7)	318 (11.8)	259 (35.7)	277 (33.9)

The variants are ranged based on their functional effect, and MODY control variants and common variants are separated from the rare *HNF1A* variants from the study cohorts by a solid line. We used five in silico tools for the bioinformatics classification: SIFT, PolyPhen-2 (HumVar), Align GVGD, MutationTaster, and CADD. SIFT predictions: Del, deleterious; Tol, tolerated. PolyPhen-2 (HumVar) predictions: Prob, probably damaging, Poss, possibly damaging; Ben, benign. Align GVGD: the prediction classes form a spectrum (C0, C35, C65), with C65 most likely to interfere with function and C0 least likely. MutationTaster: Disease causing or polymorphism. For CADD we used a cutoff threshold of 15 (>15 pathogenic). For a variant to be classified as likely pathogenic, it was scored as pathogenic in at least four of the five variant interpretation software tools used. For a variant to be classified as unlikely pathogenic, it was scored as pathogenic in less than four of the five variant interpretation software tools used. DM, diabetes mellitus; *n*, number of carriers; NA, not annotated; 1000G, 1000 Genomes.

DESIGN AND METHODS (23). Of these, 18 variants have been reported before in the literature, and 9 variants have not been reported previously (Table 1). We found no association with type 2 diabetes for the whole group of 27 rare nonsynonymous *HNF1A* alleles (n = 62 carriers) (MAF 0.75%; OR 1.52; 95% CI 0.86–2.69; P = 0.15) (Table 2).

Bioinformatics Classification

The variants were assigned to two classes, as described in RESEARCH DESIGN AND METHODS, using five in silico prediction tools (SIFT, PolyPhen2, CADD, MutationTaster, and Align GVGD) (Table 1). Following such a method, 11 of the 27 low-frequency nonsynonymous variants were classified as likely pathogenic (Table 1) and showed no association with diabetes risk (MAF 0.22%, OR 2.02; 95% CI 0.73–5.60; P = 0.18) (Table 2).

Functional Evaluation

We performed a separate evaluation of variant consequence on in vitro protein function. Because HNF-1A is a transcription factor, we performed two separate functional tests focusing on transactivation and nuclear localization of all 27 rare variants. For comparison, we also analyzed three of the common *HNF1A* variants (MAF >1%) identified in the cohorts (p.L27I, p.A98V, and p.S487N) and four previously characterized MODY3-causing variants (p.P112L, p.R263C, p.P447L, and p.Q466*) (18,19,34). The positions of these variants within the HNF-1A protein sequence are shown in Fig. 1.

First, we investigated the effect of individual variants on HNF-1A transcriptional activity in transiently transfected HeLa cells using a luciferase reporter construct, whose expression is regulated by HNF-1A binding to a rat albumin promoter (Fig. 2). As a control for severely reduced transcriptional activity, we used two well-characterized MODY3-causing variants (p.P112L and p.P447L) (18,19). The individual effect of the 27 rare variants on normal HNF-1A transcriptional activity varied, ranging from no effect/nearly normal activity to substantial effect (<60% activity compared with wild-type) (Fig. 2), but could be clearly distinguished from the severe effects demonstrated by the MODY3-causing controls (<20% activity).

Second, we monitored the nuclear versus cytoplasmic localization of the HNF-1A variant proteins using transiently transfected HeLa cells (Fig. 3). This assay was designed to detect impaired nuclear protein transport, a loss-of-function mechanism described for certain MODY variants (19). A previously reported MODY3-causing variant, p.Q466*, known to be retained in the cytoplasm, was included for comparison (34). Most of the 27 HNF-1A variant proteins demonstrated efficient nuclear translocation similar to wild-type HNF-1A, with a strong immunofluorescent signal restricted to the cell nucleus in most of the cells counted (Fig. 3). A few variants showed a reduced number of cells with nuclear accumulation alone (<60%) but not to the extent of the p.Q466* MODY control variant included, demonstrating a strongly reduced number of cells with nuclear staining alone (<20%) and increased cytoplasmic accumulation. Functional data of all variants are summarized in Table 1.

Diabetes Risk Prediction

We next tested whether the two functional assays could improve classification of variants that increase the risk of diabetes. Variants were first subdivided according to a range of different thresholds (percentage transcriptional activity or percentage nuclear localization compared with wild-type) (Table 3). Association with diabetes was then tested for each transactivation (TA) model. As summarized in Table 3, a model including variants having a modest effect on TA (>70% activity) or nuclear localization (>80% of all cells analyzed) did not perform differently from a model that includes all rare variants. In contrast, a model only including variants with a strong effect on normal HNF-1A function (i.e., <60% TA activity, or <70% in nuclear localization) compared with wild-type showed a strong and significant association with diabetes risk (OR 5.04; 95% CI 1.99–12.80; P = 0.0007; and OR 4.44; 95% CI 1.50–13.12; P = 0.007, respectively). Combining these two models did not increase the association compared with classification by TA only. Moreover, the effect sizes remained similar for the range of stricter classes (<40% and <50% activity compared

Table 2—Association analysis between bioinformatics classification of *HNF1A* rare variants and type 2 diabetes in study cohorts

	FHS cohort		JHS cohort		Extreme T2D cohort			
Bioinformatic classification	DM (n = 218) n (%)	No DM (n = 1,435) n (%)	DM (n = 346) n (%)	No DM (n = 1,345) n (%)	DM (n = 362) n (%)	No DM (n = 409) n (%)	Meta-analysis OR (95% CI)*	P value
All rare variants	4 (1.8)	20 (1.4)	11 (3.2)	21 (1.6)	2 (0.6)	4 (1.0)	1.52 (0.86–2.69)	0.15
Likely pathogenic	1 (0.5)	6 (0.4)	4 (1.2)	5 (0.4)	1 (0.3)	1 (0.2)	2.02 (0.73–5.60)	0.18
Likely benign	3 (1.4)	14 (1.0)	7 (2.0)	16 (1.2)	1 (0.3)	3 (0.7)	1.34 (0.67–2.69)	0.40

We used five in silico tools for the bioinformatics classification: SIFT, PolyPhen-2 (HumVar), Align GVGD, MutationTaster, and CADD (Table 1). For a variant to be classified as likely pathogenic, it was scored as pathogenic in at least four of the five variant interpretation software tools used. For a variant to be classified as unlikely pathogenic, it was scored as pathogenic in less than four of the five variant interpretation software tools used. DM, diabetes mellitus; n, number of carriers. *ORs were estimated by formal fixed-effect meta-analysis performed by the method of Mantel and Haenszel.

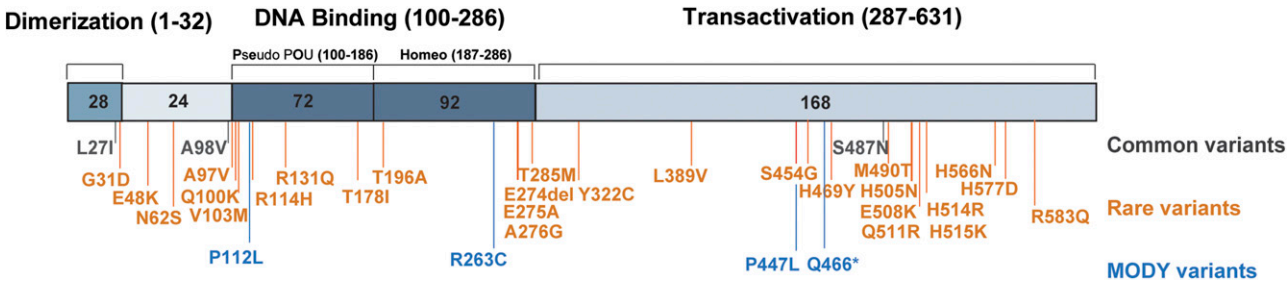

Figure 1—Position of *HNF1A* variants in the HNF-1A protein sequence identified in the study cohorts. Schematic illustration shows rare nonsynonymous *HNF1A* variants (orange), common variants (gray), and MODY3-associated variants (blue) identified in the cohorts studied (7). The total number of rare mutations reported in MODY families to date (25) is shown as the number for each functional domain.

with wild-type), albeit with decreasing levels of significance as fewer alleles met the stricter criteria.

Thus, our data suggest that a reduction to <60% of normal TA function is the best discriminator between diabetes risk and neutral *HNF1A* variants. By using this classification, we were able to classify as functionally impaired 11 variants (Y322C, E275del, R131Q, E508K, H469Y, T515K, H514R, Q100K, H505N, V103M, and H566N), with a cumulative carrier frequency of 0.44% (i.e., allele frequency of 0.22%) in the studied populations (Tables 1 and 3), and the remaining rare variants showed no evidence of association (OR 0.77; 95% CI 0.35–1.72; $P = 0.53$) (Table 3). Notably, only 5 of the 11 functionally impaired variants had a consistently high pathogenicity score in the bioinformatics prediction tools (Table 1), illustrating the challenges with inferring biological function from in silico classifications alone.

Replication

As an independent replication of the observed associations, we searched the publically available Type 2 Diabetes Genetics Database (http://www.type2diabetesgenetics.org) for all rare *HNF1A* variants investigated in our assays. In that database, we identified 16 of the 27 studied variants (Supplementary Table 1). The class of functionally impaired alleles that were present in this database ($n = 7$ variants) was significantly associated with type 2 diabetes (OR 3.01; 95% CI 1.87–4.83; $P = 2 \times 10^{-6}$), but no association was seen for the nonimpaired class ($P = 0.70$) (Table 4). The replication signal was dominated by the Mexican p.E508K variant, but the remaining variants also showed a consistent

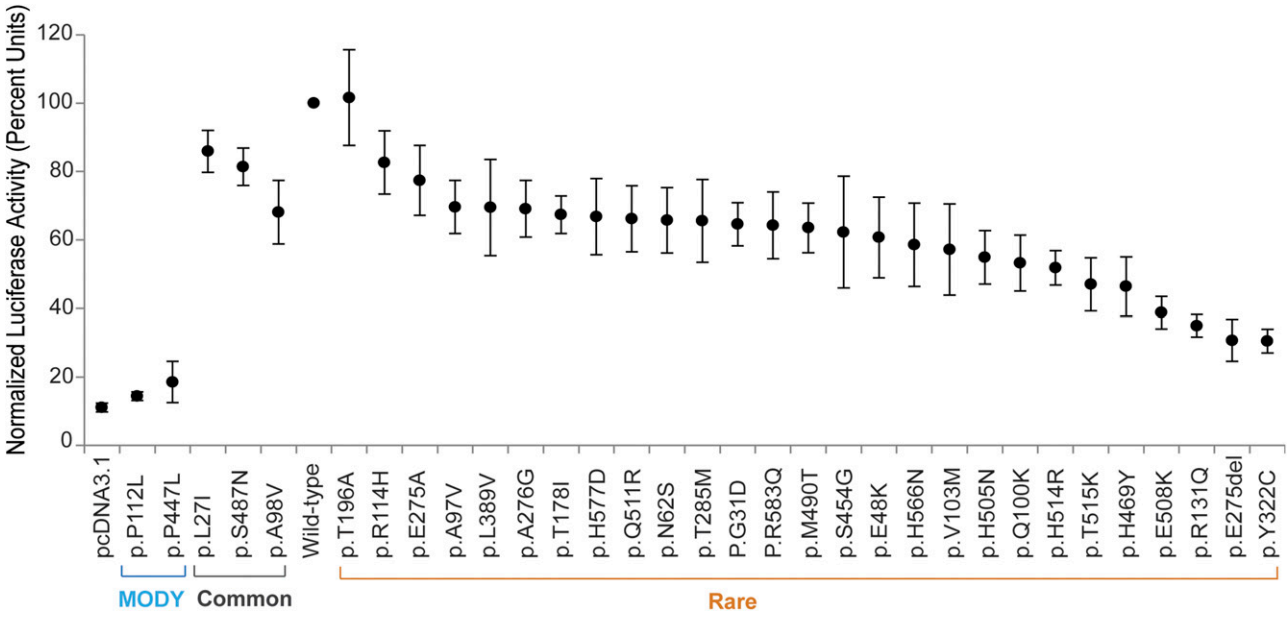

Figure 2—Assessment of transcriptional activity of HNF-1A protein variants using a luciferase reporter assay. HeLa cells were transiently transfected with wild-type or variant *HNF1A* plasmids together with reporter plasmids pGL3-RA and pRL-SV40. Luciferase measurements are given in percentage activity compared with wild-type. Each point represents the mean (the range bars indicate the 95% CIs) of nine readings. Three parallel readings were conducted on each of 3 experimental days. Two MODY variants and three common variants were included in the study.

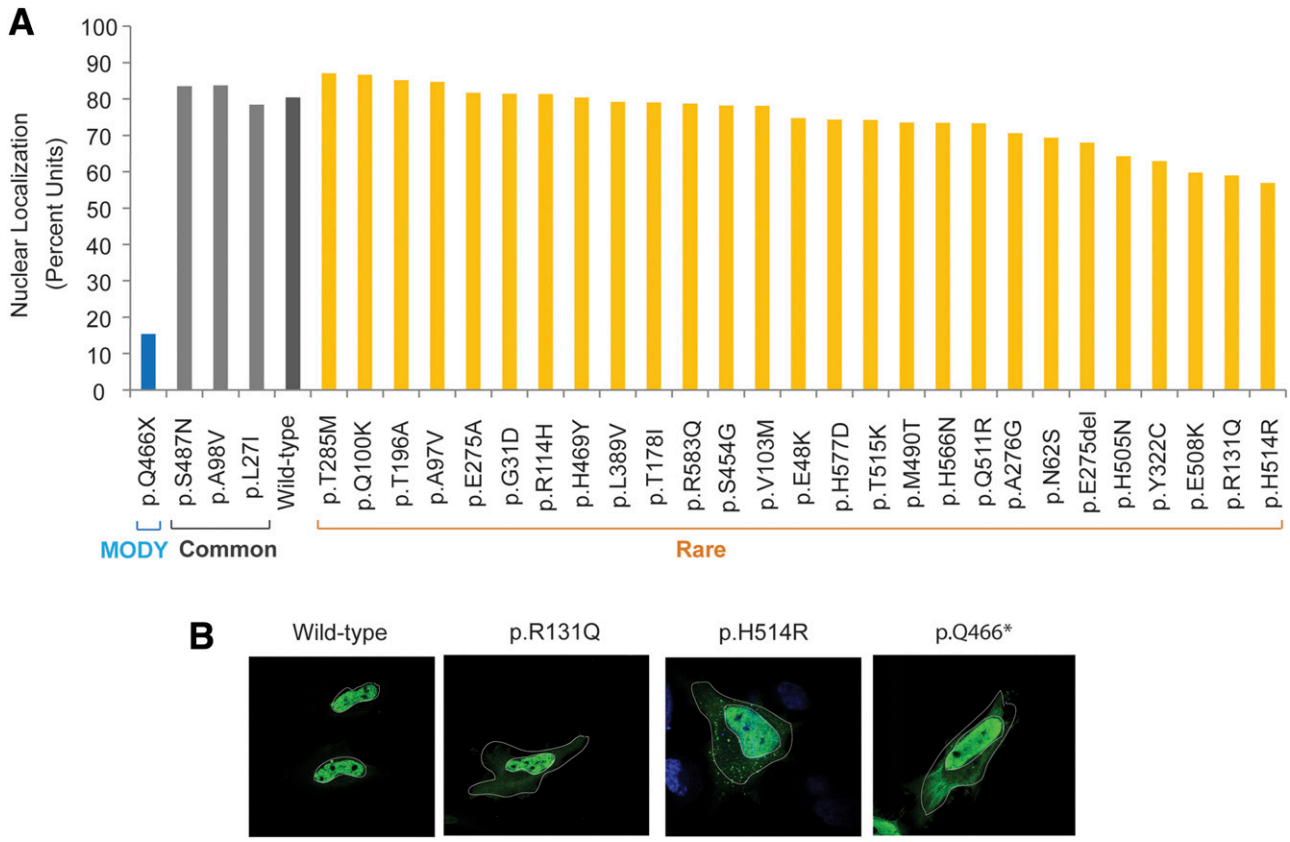

Figure 3—Analysis of nuclear localization of HNF-1A protein variants in HeLa cells. Cells were transiently transfected for 24 h and Xpress-epitope–tagged HNF-1A protein variants detected by immunofluorescence. *A*: Subcellular localization in a minimum of 200 cells was assessed for each *HNF1A* variant. The percentage of cells with nuclear accumulation alone is presented. *B*: Representative images of cells of the two most impaired nuclear localization variants. One MODY3 variant (p.Q466*) with abnormal subcellular localization was included as a control. HNF-1A was detected using tag-specific antibody and Alexa Fluor 488 (green). DNA staining (DAPI) is shown in blue. In more detail, the cytoplasmic signals of the cells expressing p.R131Q were more uniform, whereas the cells expressing p.H514R revealed a pattern resembling aggregated particles. For the purpose of clarity, the nuclei and cell membrane have been marked with a white line.

direction of effect (OR 1.63; 95% CI 0.85–3.10; P = 0.14). These numbers support the chosen thresholds as predictive of diabetes risk, although much larger samples of variant carriers will be necessary to fully calibrate the estimated risk from functionally impaired *HNF1A* variants.

HNF-1A Loss-of-Function Mechanism

To better understand the molecular mechanism for the loss of function mediated by the predicted risk variants (according to our transactivation assay), we extended our functional investigations for the 11 variants that demonstrated the strongest association with diabetes risk in the cohorts (activity <60% compared with wild-type). Because 4 of the 11 variants are located in the DNA-binding domain of HNF-1A, we used an electrophoretic mobility shift assay to test whether impaired DNA binding could be the explanation for the reduced TA (Supplementary Fig. 1). Two MODY3 variants (p.P112L and p.R263C) previously shown to severely reduce DNA binding were included for comparison. Using equal amounts of wild-type versus variant proteins, we observed significantly reduced DNA binding ($P < 0.05$) for one variant (p.E275del) (Supplementary Fig. 1*A* and *B*), explaining its inability to fully transactivate. The level of binding was, however, not as weak (44%) as the MODY-causing control variants included for severely reduced DNA binding (<20%).

We also questioned whether the low TA capacity of the 11 variants could be caused by reduced expression of HNF-1A. Indeed, we observed a significantly lower level of expressed protein ($P < 0.05$) for 4 of the 11 variants investigated (p.Y322C, p.E508K, p.H514R, and p.T515K) (Supplementary Fig. 2).

DISCUSSION

We have investigated the potential pathogenic effect of 27 rare *HNF1A* variants previously identified in the FHS and JHS population-based cohorts, and Swedish and Finnish type 2 diabetes case and control subjects ascertained from the extremes of genetic risk (23). Using traditional bioinformatics tools to classify these variants into likely/unlikely pathogenicity resulted in only a nonsignificant trend between their predicted pathogenicity and type 2 diabetes. Using functional assays, however, we revealed a model in which transcriptional activity of <60% was robustly

Table 3—Functional classification of *HNF1A* variants and their association with type 2 diabetes in the study cohorts (23)

	FHS cohort		JHS cohort		Extreme T2D cohort			
	DM (n = 218)	No DM (n = 1,435)	DM (n = 346)	No DM (n = 1,345)	DM (n = 362)	No DM (n = 409)	Meta-analysis	
Classification model	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	OR (95% CI)*	P value
TA <40%	2 (0.9)	0 (0)	3 (0.9)	3 (0.2)	1 (0.3)	0 (0)	5.91 (1.69–20.68)	0.005
TA <50%	2 (0.9)	2 (0.1)	3 (0.9)	4 (0.3)	1 (0,3)	0 (0)	3.77 (1.22–11.70)	0.02
TA <60%	4 (1.8)	3 (0.2)	5 (1.4)	5 (0.4)	1 (0.4)	0 (0)	5.04 (1.99–12.80)	0.0007
TA <70%	4 (1.8)	19 (1.3)	10 (2.9)	20 (1.5)	2 (0.6)	2 (0.5)	1.67 (0.92–3.04)	0.09
TA <80%	4 (1.8)	20 (1.4)	10 (2.9)	21 (1.6)	2 (0.6)	2 (0.5)	1.60 (0.88–2.90)	0.12
NL <60%	2 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.3)	0 (0)	9.85 (1.09–89.12)	0.04
NL <70%	3 (1.4)	2 (0.1)	3 (0.9)	4 (0.3)	1 (0.3)	0 (0)	4.44 (1.50–13.12)	0.007
NL <80%	4 (1.8)	11 (0.8)	8 (2.3)	19 (1.4)	2 (0.6)	1 (0.2)	1.90 (0.99–3.64)	0.05
TA <60% or NL <60%	4 (1.8)	20 (1.4)	10 (2.9)	21 (1.6)	2 (0.6)	2 (0.5)	**5.04 (1.99–12.80)**	**0.0007**
TA <60% or NL <70%	4 (1.8)	4 (0.3)	5 (1.4)	6 (0.4)	1 (0.3)	0 (0)	4.19 (1.73–10.12)	0.001
TA >60% and NL >70%	0 (0)	17 (1.2)	6 (1.7)	16 (1.1)	1 (0.3)	4 (1.0)	0.77 (0.35–1.72)	0.53

Selected cutoffs shown in boldface type. DM, diabetes mellitus; n, number of carriers; NL, nuclear localization; TA, transcriptional assay.
*ORs were estimated by formal fixed-effect meta-analysis performed by the method of Mantel and Haenszel.

associated with type 2 diabetes. We found a similar albeit weaker effect in an independent publicly available data set (www.type2diabetesgenetics.org) ($P = 2.0 \times 10^{-6}$), with some residual effect left after excluding the well-known p.E508K Mexican risk variant ($P = 0.14$). Studies of rare variants are intrinsically challenging, because power decreases rapidly with decreasing allele frequencies and effect estimates will have large uncertainties. The current sample is powered to detect an OR of >2.0 given variants present in 1% of the study population and an OR >2.9 for variants with a combined 0.44% carrier frequency such as found for the 60% TA threshold (at nominal significance) (Supplementary Figs. 3 and 4). Thus, much larger replication samples are needed if the true effects of these rare variants are in the lower end of our risk estimate. Likewise, the small numbers of individuals carrying impaired variants make it challenging to disentangle possible difference among the three cohorts.

Although our functional assay demonstrated reduced HNF-1A function for 11 of the 27 rare variants, we emphasize that the degree of functional impairment (TA <60%) is much less pronounced than what is seen for true *HNF1A* MODY variants that show dominant disease transmission in families (Table 1). These variants show an almost complete lack of HNF-1A function with transcriptional activity and/or DNA binding <20% compared with the normal protein (18,19,34–37).

The penetrance of MODY variants is estimated at ~63% by age 25, increases to 93.6% by age 50, and to 98.7% by age 75 (38). Although the clinical expression of MODY varies between families and even within families (39,40), most carriers will develop diabetes during their life-time compared with the two- to fivefold increased risk estimated for variants characterized in the current study. The effects are similar to the recently reported p.E508K variant associated with a fivefold increased type 2 diabetes risk in the Mexican and Latino population (11). The variant appears in ~2.1% of individuals with type 2 diabetes and in 0.35% in control subjects in these populations but are apparently absent in other populations. The E508K allele has also demonstrated incomplete penetrance, suggesting that it is a moderate diabetes allele and different from the true *HNF1A* MODY variants that demonstrate high disease penetrance (38). Reduced penetrance of another *HNF1A* variant (G319S) and variable clinical presentation of type 2 diabetes has also been shown (12). The G319S variant, which is private for the Oji-Cree population and common among individuals with diabetes (40%), influences the age of onset of type 2 diabetes, BMI, and plasma glucose levels through a gene-dosage effect. Homozygous

Table 4—Enrichment of functionally impaired *HNF1A* alleles in the type 2 diabetes genetics database*

	Case subjects (n = 8,379)	Control subjects (n = 8,478)		
	n (%)	n (%)	OR (95% CI)†	P value
Impaired	68 (0.81)	23 (0.27)	3.01 (1.87–4.83)	2.0×10^{-6}
Nonimpaired	35 (0.42)	39 (0.46)	0.91 (0.58–1.44)	0.70

*Web site address: http://www.type2diabetesgenetics.org. †ORs and corresponding P values were calculated using the χ^2 test.

individuals for the S319 allele were diagnosed at an earlier age and had lower BMI and higher plasma glucose levels than the heterozygous (G319/S319) carriers (12).

Our findings may have clinical implications. Our data indicate that as much as 0.44% of the general population may carry *HNF1A* variants, with considerable effect on diabetes risk. Such information may lead to increased awareness of the relevance of *HNF1A* screening in diabetes laboratories in patients with a (non-MODY) late-onset type 2–like diabetes.

Moreover, compared with traditional bioinformatics tools, functional studies of *HNF1A* gene variants seem needed to better translate genomic signals and may be a faithful indicator of the true effect of these variants as risk factors for type 2 diabetes (this principle may be applicable also for other genes and change the way of how to investigate the effect of gene variants in complex diseases). Only 5 of the 11 variants identified as functionally impaired in our study were consistently scored as damaging by commonly used variant assessment software. Likewise, HGMD labeled many apparently neutral variants as MODY, which is in accordance with other studies showing that HGMD has to be used with caution (41). These limitations are not fully overcome by the manual five-class classification system used by many diagnostics laboratories, (42) including ours. This system is only applicable to Mendelian disease. Thus, most of the variants fall into the class 3 (variant of unknown significance) or class 2 (likely benign) categories (Table 1), as exemplified with the E508K variant that is associated with a fivefold increased risk of type 2 diabetes but does not show strong familial segregation and low penetrance compared with MODY criteria.

Although the magnitude of reduced HNF-1A function is larger for *HNF1A* variants causing MODY3 (38,43,44) compared with the reduced function observed for the rare *HNF1A* variants investigated in this report, a natural question is whether sulfonylurea treatment might be beneficial for subjects carrying *HNF1A* variants with $<60\%$ TA activity or $<70\%$ in the nuclear localization assay (11). To our knowledge, no studies have systematically explored whether the Oji-Cree or the Mexican Americans carrying *HNF1A* variants with some degree of functional impairment are more responsive to sulfonylurea compared with type 2 diabetes without the variants. Still, this might be interesting to explore in future clinical studies.

A limitation of our study is that the 27 *HNF1A* variants investigated were identified in relatively small cohorts and may not be representative across other samples and populations. However, our study was performed in two population-based cohorts of different genetic and environmental backgrounds (here Europeans and African Americans) (23), thus providing some evidence to support that this is not a restricted phenomenon in a particular cohort or ancestry group. Hence extending the study to surveys of an even wider range of human populations by investigating larger cohorts of multiethnic backgrounds will be important. By this, the relevance of personal genomic sequencing the *HNF1A* gene for disease risk prediction can be better evaluated (45).

The current study was restricted to functional investigations of variants in the most commonly affected MODY gene, *HNF1A*, where common variation with small effects on type 2 diabetes risk have also been found at least in some populations (11,12,16). Our findings illustrate that functional assays can pinpoint rare *HNF1A* variants of high effect on type 2 diabetes risk, thus suggesting that in-depth functional assays could be a fruitful approach to also detect additional low-frequency variants in other reported MODY genes (23), as was found for *PPARG*, a gene implicated in lipodystrophy and insulin resistance (46).

In conclusion, we have shown here that functional verification of the biological effect of rare *HNF1A* variants identified by high throughput sequencing of large populations is needed to distinguish impaired from neutral variants and to correctly estimate the risk for developing MODY and type 2 diabetes. Functional assays can play an important role in rigorous evaluation of variants' causality. Until now, predictive genetic testing of variants causing type 2 diabetes has not been relevant because of small effects in few populations. To fully determine the true power of such risk variants, follow-up studies of functional analysis of gene variants from larger cohorts should be performed. Our study highlights the necessity of increased awareness regarding the relevance of MODY genes, and *HNF1A* in particular, in the contribution of type 2 diabetes risk.

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