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Mutations in *HNF1A* Result in Marked Alterations of Plasma Glycan Profile

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A recent genome-wide association study identified hepatocyte nuclear factor 1- α (*HNF1A*) as a key regulator of fucosylation. We hypothesized that loss-of-function *HNF1A* mutations causal for maturity-onset diabetes of the young (MODY) would display altered fucosylation of *N*-linked glycans on plasma proteins and that glycan biomarkers could improve the efficiency of a diagnosis of *HNF1A*-MODY. In a pilot comparison of 33 subjects with *HNF1A*-MODY and 41 subjects with type 2 diabetes, 15 of 29 glycan measurements differed between the two groups. The DG9-glycan index, which is the ratio of fucosylated to nonfucosylated triantennary glycans, provided optimum discrimination in the pilot study and was examined further among additional subjects with *HNF1A*-MODY ($n = 188$), glucokinase (*GCK*)-MODY ($n = 118$), hepatocyte nuclear factor 4- α (*HNF4A*)-MODY ($n = 40$), type 1 diabetes ($n = 98$), type 2 diabetes ($n = 167$), and nondiabetic controls ($n = 98$). The DG9-glycan index was

markedly lower in *HNF1A*-MODY than in controls or other diabetes subtypes, offered good discrimination between *HNF1A*-MODY and both type 1 and type 2 diabetes (C statistic ≥ 0.90), and enabled us to detect three previously undetected *HNF1A* mutations in patients with diabetes. In conclusion, glycan profiles are altered substantially in *HNF1A*-MODY, and the DG9-glycan index has potential clinical value as a diagnostic biomarker of *HNF1A* dysfunction. *Diabetes* 62:1329–1337, 2013

Genome-wide association studies are providing novel insights into the genetic architecture and biological basis of many diseases, but immediate translation into clinical practice has been limited. We recently performed a genome-wide association study of the human plasma *N*-glycome and found evidence of association involving common variants near the hepatocyte nuclear factor 1- α (*HNF1A*) gene; follow-up functional experiments established *HNF1A* as a master regulator of plasma protein fucosylation (1). Fucosylation, a specific type of glycosylation, comprises the addition of fucose residues to glycans. Here we evaluate the hypothesis that mutations causing a more severe deficit in *HNF1A* function (resulting in the monogenic subtype of diabetes known as *HNF1A* maturity-onset diabetes of the young [MODY; *HNF1A*-MODY]) are associated with marked alterations of plasma glycome composition, and we assess the value of glycan profiles as a diagnostic biomarker for *HNF1A*-MODY.

Most human proteins are posttranslationally modified by the addition of complex oligosaccharide structures (glycans) (2). Despite the impact on protein structure and function, the clinical consequences of changes in the human glycome remain largely unexplored, primarily because reliable analytical techniques have been developed only recently (3). In recent studies, *HNF1A* was shown to promote both the *de novo* and salvage pathways for the synthesis of guanosine diphosphate–fucose (1) and to regulate fucosyltransferase VI (1,4). *HNF1A* thereby controls the outer-arm (antennary) fucosylation of proteins with *N*-linked glycans through effects on both the supply of activated precursors and the incorporation of fucose (1,4).

Mutations disrupting *HNF1A* are responsible for the most common subtype of monogenic diabetes, *HNF1A*-MODY (5). Like other forms of MODY, *HNF1A*-MODY is characterized

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by autosomal dominant inheritance and pancreatic β -cell dysfunction. This typically leads to diabetes diagnosed in the second to fourth decade of life in the absence of β -cell autoimmunity and insulin resistance (5,6). In clinical practice, diagnostic differentiation between *HNF1A*-MODY and other causes of early-onset diabetes (including type 1 and type 2 diabetes and other forms of MODY) is complicated by the overlap of phenotypic features. In most countries only a minority of cases of *HNF1A*-MODY are referred for definitive molecular testing (i.e., *HNF1A* sequencing), and many patients with *HNF1A*-MODY are misdiagnosed with type 1 or type 2 diabetes (7). Failures or delays in accurate molecular diagnosis can have clinical repercussions because, unlike other forms of diabetes, the optimal treatment for *HNF1A*-MODY is low-dose sulfonylureas (8). Patients with undiagnosed *HNF1A*-MODY may spend many years receiving inappropriate treatment (including exogenous insulin) and experiencing suboptimal glycemic control (9).

We aimed to test the hypothesis that inactivating mutations in *HNF1A*, such as those found in *HNF1A*-MODY, are associated with decreased antennary fucosylation of circulating proteins and to evaluate the clinical translational potential of measuring glycan profiles in diabetes.

RESEARCH DESIGN AND METHODS

Subjects for initial and validation studies. Full details of the subjects are available in Table 1 and the Supplementary Appendix. Subjects carrying mutations in *HNF1A* ($n = 221$), glucokinase (*GCK*; $n = 118$), and hepatocyte nuclear factor 4- α (*HNF4A*; $n = 40$) were recruited from five European centers. Subjects with MODY had an established heterozygous loss-of-function mutation confirmed by sequencing in a certified diagnostic center. All MODY mutations were considered pathogenic if they met one or more of the following criteria: mentioned in previously published reports, presence of a truncating mutation, cosegregation of the mutation with a MODY phenotype within the family, and absence of the variant in normal chromosomes. In addition, we recruited 208 subjects with clinically labeled type 2 diabetes who were diagnosed at an age younger than 45 years, 98 subjects with clinically labeled type 1 diabetes, and 98 subjects who acted as nondiabetic controls. Most samples were collected when the subject was in a fasting state, although fasting status does not influence glycan levels (10).

Glycan release, labeling, and analysis. All samples were stored at -80°C before analysis. Glycan release, labeling, and analysis using hydrophilic interaction high-performance liquid chromatography and sialidase digestion was performed as previously reported (11,12). Chromatograms from fluorescently labeled glycans were separated into 16 glycan groups (GP series) and 13 desialylated glycan groups (DG series), composing a total of 29 peaks (Supplementary Table 1). The amounts of glycans present in each peak were expressed as the percentage of the total plasma glycome. Glycan analysis was performed in two centers: the National Institute for Bioprocessing Research and Training (Dublin, Ireland) and the Glycobiology laboratory of Genos Ltd. (Zagreb, Croatia). Both laboratories used the same columns and separation conditions and previously have demonstrated reproducibility of analytic results within and between laboratories (1,10).

Study design and analysis. All glycan traits were compared using Mann-Whitney *U* tests in an initial study of 33 subjects with *HNF1A*-MODY and 41 subjects with type 2 diabetes. Based on these results (Supplementary Table 1), the glycan ratio of DG9 to [DG8+DG9], hereafter referred to as the DG9-glycan index, was chosen for further follow-up.

The validation study was performed using 188 subjects with *HNF1A*-MODY, 118 subjects with *GCK*-MODY, 40 subjects with *HNF4A*-MODY, 167 subjects with type 2 diabetes, and 98 subjects with type 1 diabetes cases, plus 98 nondiabetic controls. There was no overlap between the initial and validation studies. We first sought evidence for important covariates through analysis of parameters including age, sex, BMI, HDL, triglycerides, sample origin, processing laboratory, and sample type (plasma vs. serum). An adjusted model incorporated significant covariates (age, BMI, sample origin, processing laboratory) as well as those already known to affect specific glycan traits (sex). An additional analysis was performed without covariates. The use of the DG9-glycan index as a discriminator of diabetes subtypes was analyzed using receiver operator characteristic (ROC) curves from which the C statistic was obtained.

TABLE 1
Clinical characteristics of subjects included in the initial and validation studies

Characteristic	Initial study			Validation study				
	<i>HNF1A</i> -MODY ($n = 33$)	Type 2 diabetes ($n = 41$)	<i>HNF1A</i> -MODY ($n = 188$)	<i>GCK</i> -MODY ($n = 118$)	<i>HNF4A</i> -MODY ($n = 40$)	Type 1 diabetes ($n = 98$)	Type 2 diabetes ($n = 167$)	Nondiabetic controls ($n = 98$)
Sex (% male)	33	46	45	43	43	51	60	51
BMI (kg/m^2)	25.4 (22.3–28.1)	34.1 (29.7–39.5)	23.3 (21.5–26.1)	22.9 (19.4–26.0)	24.6 (22.7–27.3)	26.5 (23.9–29.5)	31.0 (27.8–35.6)	27.1 (22.7–31.4)
Age at diabetes diagnosis (years)*	21.2 (15.6–29.0)	32.0 (27.0–36.0)	18.0 (15.0–27.0)	26.0 (12.0–37.0)	29.5 (20.0–39.0)	25.5 (14.0–32.0)	40.0 (37.0–43.0)	N/A
Duration of diabetes (years)*	12.0 (9.3–30.0)	6.8 (1.0–12.3)	16.0 (4.0–28.0)	3.0 (0.0–10.0)	17.7 (6.0–34.0)	14.1 (9.7–20.0)	12.0 (6.0–22.0)	N/A
Age at sampling (years)	38.9 (30.0–56.1)	40.2 (34.7–48.2)	40.0 (24.5–50.2)	32.0 (17.0–51.0)	52.0 (31.8–58.2)	42.3 (32.6–50.1)	51.7 (46.2–60.9)	53.7 (49.3–59.1)
HbA1c (%)	7.4 \pm 1.8	9.0 \pm 2.1	7.1 \pm 1.2	6.5 \pm 0.7	7.5 \pm 1.7	8.8 \pm 0.8	7.8 \pm 1.4	5.5 \pm 0.3
FPG (mmol/L) [†]	6.6 \pm 1.8	9.7 \pm 3.6	7.6 \pm 2.7	7.1 \pm 1.0	7.6 \pm 2.6	9.7 \pm 4.4	8.4 \pm 2.5	5.7 \pm 0.5
Total cholesterol (mmol/L)	4.5 \pm 0.8	4.7 \pm 1.0	4.9 \pm 1.4	4.5 \pm 0.9	4.9 \pm 1.2	4.8 \pm 1.3	4.7 \pm 1.3	5.3 \pm 1.0
HDL-cholesterol (mmol/L)	1.6 \pm 0.4	1.1 \pm 0.3	1.5 \pm 0.5	1.3 \pm 0.4	1.5 \pm 0.4	1.5 \pm 0.4	1.2 \pm 0.4	1.6 \pm 0.5
Triglycerides (mmol/L)	N/A	2.0 (1.2–2.6)	1.0 (0.7–1.5)	0.8 (0.6–1.3)	0.8 (0.7–1.2)	1.0 (0.8–1.4)	1.8 (1.3–2.4)	1.1 (0.8–1.5)

Normally distributed variables reported as mean \pm SD; all others reported as median (25th–75th centiles). FPG, fasting plasma glucose; N/A, not available. *Subjects with diabetes only (i.e., excluded carriers of a nondiabetic mutation). [†]Unavailable for subjects from Edinburgh.

Performance of the DG9-glycan index as a clinical tool was evaluated by calculating sensitivity and specificity and other measures for the detection of *HNF1A*-MODY at various thresholds. In particular, we estimated posttest diagnostic probabilities based on data from an etiological investigation of young adults with diabetes from the U.K. that indicated pretest probabilities of 4% for *HNF1A*-MODY in young-onset type 2 diabetes and 1% in type 1 diabetes (13).

The effect of the type of *HNF1A* mutation, the HNF1A isoform, and the mutated functional domain of HNF1A on DG9-glycan index levels was assessed. *HNF1A* mutations were classified as either protein-changing mutations (missense mutations resulting in a change of amino acid) or truncating mutations (which generate a premature stop codon). In addition, protein-changing mutations were grouped as exons 1–6 [affecting isoforms *HNF1A* (A), (B) and (C)], exon 7 [isoforms *HNF1A* (A) and (B)] and exons 8–10 [isoform *HNF1A* (A) only] (14,15). Protein-changing mutations also were grouped according to the affected functional domain: dimerization, DNA binding, or transactivation (14,15).

We also assessed evidence for pathogenicity of the *HNF1A* missense mutations included in the validation study and examined whether the DG9-glycan index correlated with other indicators of pathogenicity. These included cosegregation within families, functional characterization of mutant proteins, and *in silico* prediction of the effect of the amino acid substitution on protein function. ***HNF1A* sequencing.** In the subsequent case-finding study, we evaluated the value of the DG9-glycan index as a screening test for identifying *HNF1A*-MODY in a set of individuals with young-onset diabetes (diagnosed up to age 45 years) who had not previously been suspected of having an *HNF1A* mutation. We tested subjects with a DG9-glycan index <0.16 from the initial or validation studies with clinical labels of type 1 ($n = 7$) and type 2 diabetes ($n = 41$), as well as subjects with diabetes of any type diagnosed up to age 45 years from general population cohorts from Croatia ($n = 6$) and Scotland ($n = 3$) in whom glycan profiles had been measured previously (1). The 10 exons of *HNF1A* were amplified by PCR and bidirectional sequencing performed using M13 primers and a Big Dye Terminator Cyclor Sequencing kit v1.1 (Applied Biosystems, Warrington, U.K.). Reactions were analyzed on an ABI 3730 capillary sequencer (Applied Biosystems), and results were compared with the reference sequence (NM_000545.3) using Mutation Surveyor v3.97 (SoftGenetics, Cambridge, U.K.). Mutation testing was undertaken in family members when available to establish cosegregation. *In silico* analysis of missense mutations was performed using the software program Condel (CONsensus DELeterious score of missense single nucleotide polymorphisms) (16). Condel produces a weighted average of scores from three computational tools [SIFT, Polyphen2, and Mutation-Assessor (17–19)] and classifies missense single nucleotide polymorphisms as probably “deleterious” (i.e., pathogenic) or probably “neutral” (i.e., benign).

All analyses were performed using SPSS version 17.0. The study was performed in accordance with the latest version of the Declaration of Helsinki.

RESULTS

HNF1A-MODY and measures of antennary fucosylation.

In the initial study, we found marked differences in the plasma glycome profiles between 33 subjects with *HNF1A*-MODY

and 41 subjects with early-onset type 2 diabetes. Fifteen of 29 glycan measures differed significantly between the two groups ($P < 0.05$) (Supplementary Table 1). Patterns were consistent with the known effects of HNF1A on fucosylation (1), in that subjects with loss-of-function mutations in *HNF1A* were characterized by an increase in the proportion of glycans without antennary fucose.

Validation study. For the validation study, we focused on DG9 and DG8 as measures of triantennary glycans with and without antennary fucose, respectively (Fig. 1). Therefore, the DG9-glycan index [DG9-to-(DG8+DG9) ratio] summarizes the proportion of triantennary glycans that are fucosylated. As well as consistency with the existing data on HNF1A effects on fucosylation (1) and strong evidence from the initial study (Supplementary Table 1), triantennary glycans are not affected by the removal of fibrinogen during coagulation (G. Lauc, unpublished observations), allowing our validation studies to include both serum and plasma samples.

The distributions of DG9-glycan index measures for the 709 individuals in the validation study differed significantly between the subject groups (Fig. 2; Supplementary Table 2). Median (interquartile range) DG9-glycan index levels were substantially lower in subjects with *HNF1A*-MODY [0.09 (0.06–0.13)] than in those with young-onset type 2 diabetes [0.25 (0.18–0.33); $P = 1 \times 10^{-39}$ vs. *HNF1A*-MODY], type 1 diabetes [0.28 (0.20–0.34); $P = 1 \times 10^{-34}$ vs. *HNF1A*-MODY], or *GCK*-MODY [0.25 (0.18–0.31); $P = 5 \times 10^{-32}$ vs. *HNF1A*-MODY]. DG9-glycan index levels in subjects with *HNF1A*-MODY were also lower when compared with controls [0.24 (0.19–0.29); $P = 1 \times 10^{-32}$ vs. *HNF1A*-MODY] and against all other diabetic patients combined [0.25 (0.18–0.31); $P = 5 \times 10^{-55}$ vs. *HNF1A*-MODY]. Consistent with evidence that *HNF4A* also regulates fucosylation (1), cases of *HNF4A*-MODY showed DG9-glycan index levels between those in *HNF1A*-MODY and other forms of diabetes [0.18 (0.09–0.24); $P = 2 \times 10^{-7}$ vs. *HNF1A*-MODY].

Adjustment for significant covariates had no appreciable impact on the magnitude or significance of differences in DG9-glycan index values between groups (Supplementary Table 2).

Receiver operating characteristic (ROC) curve analyses. To test whether glycan profiling had potential as a clinically

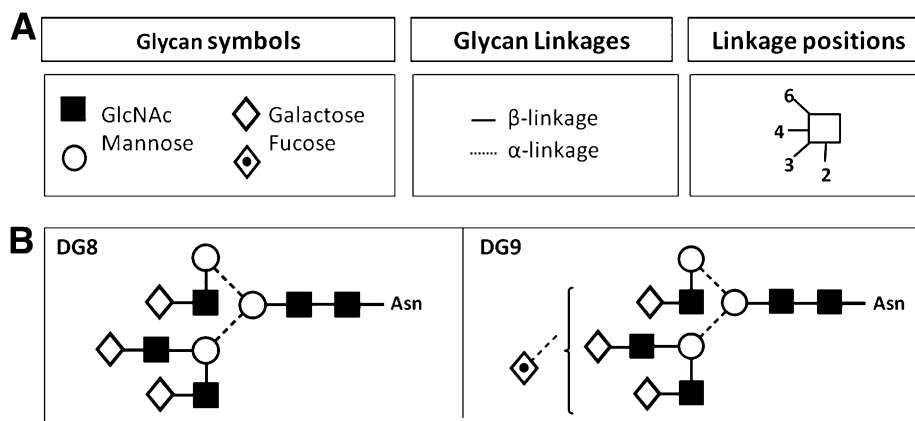


FIG. 1. A: Structural symbols for *N*-glycans, their linkages, and the abbreviations used. The Oxford nomenclature has been used to represent *N*-linked glycan composition and structure (20). **B:** Representations of the structures of the major glycans in DG8 and DG9 high-performance liquid chromatography peaks. Both are triantennary glycans. The only difference is the presence of a terminal fucose residue (◊) attached to one of the antennae of the glycans in DG9.

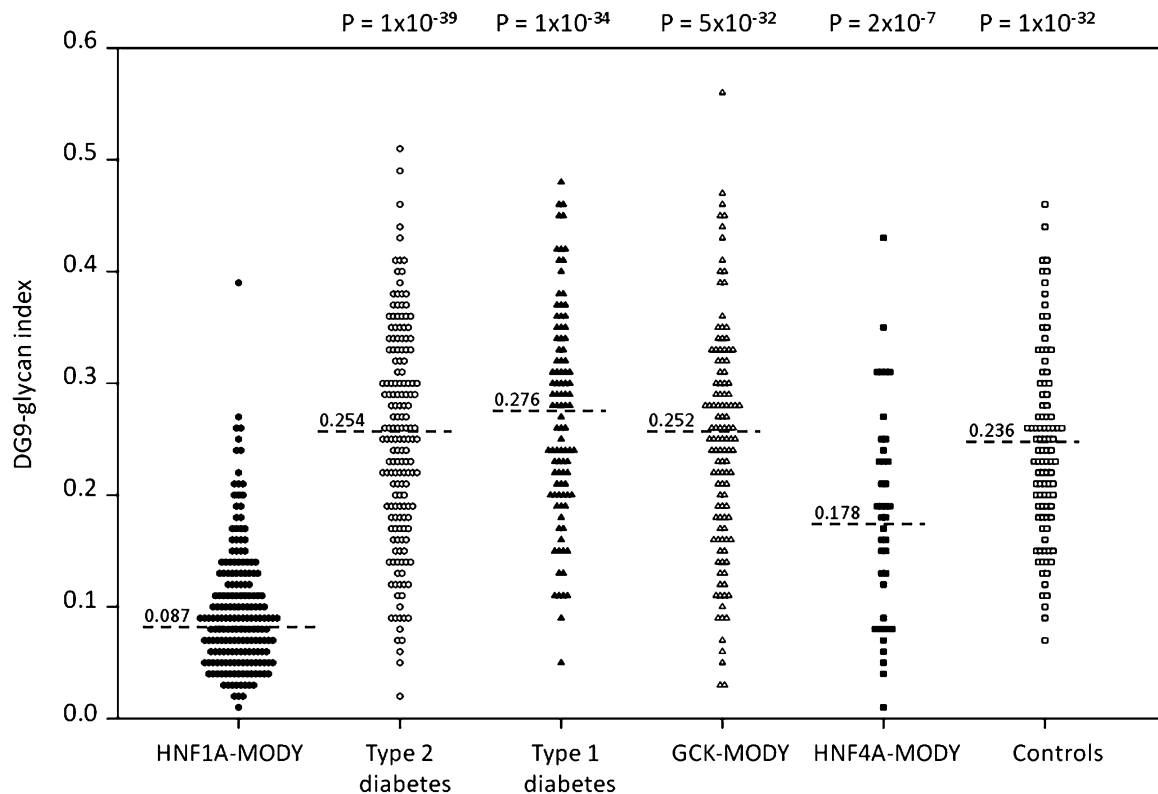


FIG. 2. Dot histograms illustrating the DG9-glycan index in different diabetes subtypes and nondiabetic control subjects. Subjects are represented by the following symbols: ● = *HNF1A*-MODY; ○ = type 2 diabetes; ▲ = type 1 diabetes; △ = *GCK*-MODY (*GCK*-MODY); ■ = *HNF4A*-MODY (*HNF4A*-MODY); □ = nondiabetic controls. *P* values are calculated by Mann-Whitney *U* tests in comparison with subjects with *HNF1A*-MODY. The median value of the DG9-glycan index for each diabetes subtype is highlighted adjacent to a black dashed line.

valid screening test, C statistic measures of discriminative accuracy were derived from ROC curve analyses (Fig. 3; Supplementary Table 3). The C statistic was 0.94 for *HNF1A*-MODY against type 1 diabetes and 0.91 for *HNF1A*-MODY against early-onset type 2 diabetes. Similar discrimination was observed for the comparison of *HNF1A*-MODY and *GCK*-MODY (C statistic 0.90), but the DG9-glycan index performed less well in differentiating *HNF4A*-MODY and *HNF1A*-MODY (C statistic 0.76). These measures were not affected by sample type (serum vs. plasma; Supplementary Table 3).

We have reported previously that high-sensitivity C-reactive protein (hs-CRP) is a sensitive and specific biomarker for *HNF1A*-MODY (21,22). In the validation dataset, the ability of the DG9-glycan index to discriminate between *HNF1A*-MODY and type 2 diabetes was comparable with hs-CRP (C statistic 0.91 and 0.94, respectively). However, the DG9-glycan index provided superior discrimination of *HNF1A*-MODY from type 1 diabetes compared with hs-CRP (C statistic 0.94 vs. 0.83 for hs-CRP).

Other glycan measures. As described earlier, our primary validation analyses focused on the DG9-glycan index. However, the availability of full glycome profiles for the validation samples allowed us to explore the relative performance of other measures highlighted in the initial study. Other glycan ratios, such as DG7 to (DG5+DG6), representing the proportion of biantennary fucosylated glycans, offered good discrimination between diabetes subtypes (Supplementary Table 3). The DG7-to-(DG5+DG6) ratio provided near perfect discrimination between *HNF1A*-MODY and type 2 diabetes (C statistic >0.99) in plasma

samples, but it performed less well in analyses of serum samples (C statistic 0.78).

Correlates of the DG9-glycan index within the *HNF1A*-MODY group. Eighteen subjects within the *HNF1A*-MODY group did not have diabetes when glycans were sampled. DG9-glycan index levels were not different in the patients with *HNF1A*-MODY with and without diabetes ($P = 0.45$). This further confirms that these changes in antennary fucosylation are specific to loss-of-function *HNF1A* mutations and are unrelated to dysglycemia. DG9-glycan index levels were not correlated with the age at diabetes diagnosis ($P = 0.38$).

Analysis by mutation type showed median DG9-glycan index was lower in subjects with *HNF1A*-MODY with protein-changing mutations than those with truncating mutations (0.08 vs. 0.10; $P = 0.003$) (Table 2). This difference in DG9-glycan index levels did not remain significant when analysis was restricted to one individual per family (the proband), a maneuver that renders the observations independent at the expense of a reduced sample size ($P = 0.18$). Analysis by functional domain indicated lower median DG9-glycan index in subjects with *HNF1A*-MODY with missense mutations affecting the dimerization/DNA-binding domains compared with those with missense mutations in the transactivation domain (0.08 vs. 0.13; $P = 0.04$). The latter relationship was consistent whether we considered all subjects with *HNF1A*-MODY or only the probands ($P = 0.04$ and 0.002, respectively). The *HNF1A* isoform affected had no effect on DG9-glycan index levels ($P = 0.30$).

We also examined whether the DG9-glycan index could be used as a marker of *HNF1A* function to provide additional

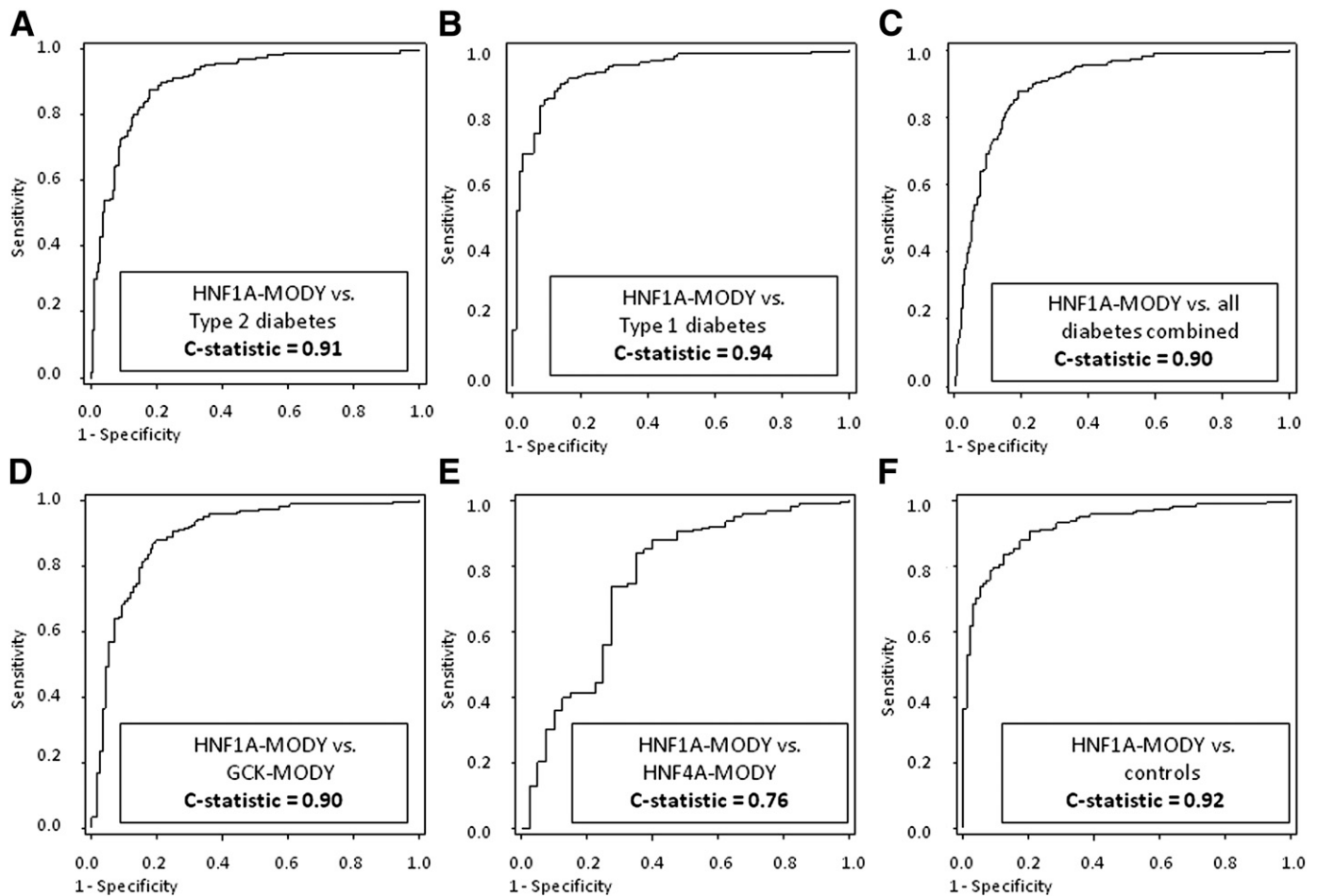


FIG. 3. ROC curves illustrating the performance of the DG9-glycan index to discriminate *HNF1A*-MODY and type 2 diabetes (A); *HNF1A*-MODY and type 1 diabetes (B); *HNF1A*-MODY and other diabetes subtypes combined (C); *HNF1A*-MODY and *GCK*-induced MODY (*GCK*-MODY) (D); *HNF1A*-MODY and *HNF4A*-MODY (E); and *HNF1A*-MODY and nondiabetic control subjects (F).

evidence regarding pathogenicity. All missense *HNF1A* mutations in this study were classified as probably “deleterious” (i.e., pathogenic) or probably “neutral” (i.e., benign) using the software program Condel. Four missense *HNF1A* mutations (in five subjects) had Condel scores within the

benign range: c.92G>A p.Gly31Asp, c.142G>A p.Glu48Lys, c.871C>A p.Pro291Thr, and c.1816G>A p.Gly606Ser. Family data for these mutations were generally less supportive of pathogenicity because of incomplete cosegregation, later age at diagnosis of diabetes, or unavailability of family

TABLE 2

DG9-glycan index levels in subjects with *HNF1A*-MODY according to type and position of *HNF1A* mutation

	Subjects with <i>HNF1A</i> -MODY (n)	DG9-glycan index*	P	Unrelated subjects with <i>HNF1A</i> -MODY (n)	DG9-glycan index*	P
Truncating mutations	83	0.10 (0.07–0.21)	0.003	39	0.10 (0.07–0.14)	0.180
Protein-changing mutations	105	0.08 (0.05–0.21)		44	0.08 (0.05–0.14)	
Classified by isoform affected						
Exons 1–6 [isoforms <i>HNF1A</i> (A), (B) and (C)]	93	0.08 (0.05–0.21)		42	0.08 (0.05–0.11)	
Exon 7 [isoforms <i>HNF1A</i> (A) and (B)]	11	0.09 (0.05–0.18)	0.300	1	0.13	N/A
Exons 8–10 [isoform <i>HNF1A</i> (A) only]	1	0.21		1	0.21	
Classified by affected functional domain						
Dimerization/DNA-binding domains [†]	62	0.08 (0.05–0.17)	0.043	26	0.08 (0.05–0.11)	0.002
Transactivation domain [‡]	17	0.13 (0.08–0.25)		4	0.21 (0.17–0.24)	

P values calculated using Mann-Whitney U and Kruskal-Wallis tests. The Mann-Whitney U test was used to test truncating versus protein-changing mutations and dimerization/DNA-binding versus transactivation domains, while the Kruskal-Wallis test was used to compare the isoforms. *Data expressed as median (25th–75th centiles). [†]Dimerization domain (amino acids 1–32) and DNA-binding domain (amino acids 91–281). [‡]Transactivation domain (amino acids 282–631).

members for testing. The median (interquartile range) DG9-glycan index levels in these five subjects were significantly higher compared with the remainder of the group with *HNF1A* missense mutations [0.16 (0.11–0.21) vs. 0.08 (0.05–0.11); $P = 0.01$].

Clinical potential of the DG9-glycan index. To examine the performance of the DG9-glycan index as a diagnostic screen in clinical practice, we based analyses on U.K. data showing that subjects with unrecognized *HNF1A*-MODY account for approximately 4% of young-onset type 2 diabetes (diagnosed ≤ 45 years) and 1% of type 1 diabetes (13). On the basis of the validation study, we estimate that a diagnostic threshold for the DG9-glycan index of 0.16 confers 88% sensitivity and 81% specificity for the discrimination of *HNF1A*-MODY from young-onset type 2 diabetes and 88% sensitivity and 88% specificity for equivalent comparisons with type 1 diabetes. In contrast, an age younger than 25 years at diagnosis of diabetes, which is the most widely used diagnostic feature for MODY, has lower sensitivity (64%) but higher specificity (99%) for the discrimination of *HNF1A*-MODY from young-onset type 2 diabetes. We calculate that a patient with diabetes who is diagnosed before or at age 45 years, who has an existing clinical label of type 2 diabetes, and who is found to have a DG9-glycan index ≤ 0.16 has a posttest probability of harboring an underlying *HNF1A* mutation of 16%, whereas the same patient with a DG9-glycan index > 0.16 has a 1% posttest probability of having unrecognized *HNF1A*-MODY.

***HNF1A*-MODY case-finding in diabetic subjects.** The results of the validation study indicated that a diagnostic threshold of 0.16 for the DG9-glycan index provided optimum discrimination from both type 1 and type 2 diabetes. *HNF1A* sequencing was performed in 57 subjects with young-onset diabetes and who were clinically labeled as having type 1 or type 2 diabetes, all of whom had a DG9-glycan index ≤ 0.16 . These subjects were either from the initial or validation studies or recruited from general population cohorts in Croatia and Scotland. Three of these 57 individuals were found to have *HNF1A* mutations.

The first proband (Supplementary Fig. 1A) was heterozygous for the missense mutation c.608G>A p.Arg203His in exon 3, which previously has been shown to be causal for MODY (23). The phenotype is consistent with *HNF1A*-MODY with a two-generation history of young-adult onset diabetes, and, although treated with insulin and labeled with type 1 diabetes since diagnosis at age 31, this patient was found to have residual endogenous insulin secretion 17 years after diagnosis (C-peptide, 0.27 nmol/L). Two sisters with insulin-treated diabetes also carry the Arg203His mutation. One sister has stopped basal-bolus insulin successfully after the diagnosis of MODY and her diabetes is well controlled with gliclazide; the proband is considering a similar change in therapy. In the second proband (Supplementary Fig. 1B), a novel missense variant c.139G>C, p.Gly47Arg was identified in exon 1. This subject, now aged 53 years, was diagnosed with presumed type 2 diabetes at age 37. Her mother, diagnosed with type 2 diabetes at age 70, also carries the mutation. A normoglycemic son, currently 29 years old, does not carry the mutation. In the third proband (Supplementary Fig. 1C), a novel missense mutation c.751G>A, p.Ala251Thr was found in exon 4. This proband was diagnosed with diabetes at age 43 and is being managed with sulfonylurea (and metformin) therapy 25 years later. Sensitivity to sulfonylureas and maintenance of diabetes control for many

years while taking these agents is typical of subjects with *HNF1A*-MODY. The proband's mother and a sister had diabetes in old age.

To assess pathogenicity for the two novel variants (Gly47Arg and Ala251Thr), we first established that both variants were absent from 400 normal chromosomes and the October 2011 release of the consensus calls for the 1000 Genomes Project (24). The Ala251Thr variant is not reported in the National Heart, Lung, and Blood Institute's Exome Sequencing Project ($N = 6503$; accessed June 2012 via the Exome Variant Server) (25), whereas the Gly47Arg variant is reported in a single European American individual. The phenotypic characteristics of this subject are not declared: because the Exome Sequencing Project includes cases of metabolic and cardiovascular disease, this finding does not exclude a pathogenic role for this variant. Second, we assessed whether the mutated residues were conserved across species. Both Gly47 and Ala251 are highly conserved: Ala251 is conserved in eight of the nine orthologs tested, including frog, chicken, and six mammalian orthologs, whereas Gly47 is conserved in seven of the nine orthologs tested, including *Xenopus*, chicken, and five mammalian orthologs. *In silico* prediction software was more ambiguous: Condel predicted that the variant Gly47Arg is "neutral" and that Ala251Thr is "deleterious." Overall, we regard the clinical and bioinformatic data for these two variants as supportive, but not conclusive, evidence in favor of pathogenicity. The low DG9-glycan index levels associated with Gly47Arg and Ala251Thr (0.12 and 0.15, respectively) provide additional support for pathogenicity. Definitive evidence of a causal role would require more extensive clinical (including the detection of these mutations in additional MODY families) and functional studies.

DISCUSSION

The study confirms the hypothesis that the glycan profile of plasma proteins is altered substantially in those with *HNF1A* mutations. We also demonstrated that these differences could be exploited as biomarkers in diabetes diagnostics and showed that the DG9-glycan index can discriminate *HNF1A*-MODY from both type 1 and type 2 diabetes.

Recent efforts to improve diagnostic performance by identifying biochemical markers specific for MODY subtypes have met with varying success (21,26–32). We recently demonstrated that individuals with *HNF1A*-MODY have low levels of C-reactive protein and that hs-CRP assays can discriminate well between *HNF1A*-MODY and both type 2 diabetes and *HNF4A*-MODY (21,22,33). However, hs-CRP does not provide good discrimination between *HNF1A*-MODY and type 1 diabetes. Furthermore, hs-CRP is an acute inflammatory marker, and diagnostic discrimination can be disturbed by intercurrent infection. Potential advantages of the DG9-glycan index in this context include stability over time (10) and differentiation of *HNF1A*-MODY from both common types of diabetes. Although there is some indication that glycan profiles are affected by acute inflammation (34), all four subjects with *HNF1A*-MODY and elevated levels of hs-CRP (> 10 mg/L) in the current study had DG9-glycan indices below 0.16. This suggests that the DG9-glycan index is less prone to spurious elevation from intercurrent infection than hs-CRP, although this will require confirmation in larger numbers.

The ability to discriminate between *HNF1A*-MODY and type 1 diabetes in subjects with recently diagnosed diabetes is particularly important because diagnostic misclassification can lead to the unwarranted decision to recommend lifelong therapy with exogenous insulin. Detectable C-peptide can indicate *HNF1A*-MODY rather than type 1 diabetes of long duration, but it is not helpful close to a diagnosis of diabetes because a substantial proportion of type 1 diabetes patients retain some production of endogenous insulin (35). In this study we did not explicitly examine type 1 diabetes during the honeymoon period; however, glycan profiles are stable within an individual over time, which suggests these measures will continue to provide useful discriminative power from type 1 diabetes close to diagnosis (36). In principle, therefore, the addition of the DG9-glycan index to existing biomarkers such as hs-CRP (21), 1,5-anhydroglucitol (29), pancreatic autoantibodies (37), and C-peptide (13,35) should improve the capacity for clinical discrimination of all major diabetes subtypes.

With respect to clinical utility, our study showed that it was possible to identify subjects with *HNF1A* mutations using the DG9-glycan index. Given the high sensitivity and specificity of the DG9-glycan index, the proportion of cases found was lower than might have been expected (3 of 57; 5%). There are several possible explanations. The first is that the estimate of discriminatory power based on the C statistics calculated during the validation study is inflated, perhaps because of overfitting (38). An alternative explanation is that discovery of novel cases of *HNF1A*-MODY might have been compromised in our study samples by the extent of existing clinical investigation (e.g., approximately 30% of the Oxford samples had undergone *HNF1A* sequencing). Another intriguing possibility is that a small proportion of individuals in large, population-based cohorts have low DG9-glycan index levels due to low-frequency alleles in genes other than *HNF1A*, which play an important role in protein fucosylation, such as those encoding the fucosyltransferases *FUT6* and other genes (1). Further validation of the DG9-glycan index in unselected groups of subjects with young-onset diabetes will be required to assess performance in a more typical clinical scenario in which extensive prior screening for monogenic disease has not been undertaken.

The use of the DG9-glycan index in clinical practice currently is restricted by the cost and limited availability of accurate glycan profiling. Clinical translation is, therefore, dependent on the implementation of a focused assay for specific glycan moieties rather than the global chromatographic profiling used in this study. The DG9-glycan index seems to be the most promising candidate from this study, although the validation study suggests that other measures [such as the DG7-to-(DG5+DG6) ratio] might have superior performance when plasma (rather than serum) samples are available—a finding that requires further confirmation.

There is some evidence for a genotype-phenotype relationship of glycan levels within subjects with *HNF1A*-MODY, including that DG9-glycan index levels are lower in those with protein-changing rather than truncating *HNF1A* mutations. We reported a similar relationship in our studies of hs-CRP; in this case, restricting the analysis to independent probands did not abolish the significance (22). These observations would be consistent with a dominant-negative effect, which has been reported for some *HNF1A* mutations (39,40). Missense mutations disrupting the DNA-binding and dimerization domains have significantly lower

DG9-glycan index levels than those affecting the trans-activation domain. This indicates a more severe functional outcome of *HNF1A* mutations that disturb DNA binding. This finding is consistent with previous reports of decreased age at diabetes diagnosis in subjects with *HNF1A*-MODY who have missense mutations within the DNA-binding/dimerization domains compared with those in the trans-activation domain (14,15).

This study primarily aimed to define the role of glycans as a potential adjunct in the diagnosis of *HNF1A*-MODY and, in particular, the capacity of this measure to improve the targeting of (relatively expensive and therefore restricted) diagnostic sequencing. However, anticipated reductions in the cost of diagnostic sequencing are likely to encourage more liberal access to *HNF1A* sequencing, including many individuals with a relatively low risk of MODY. In this setting, the problem shifts toward correctly interpreting the clinical significance of the many novel *HNF1A* variants that will be uncovered, and we provide preliminary evidence that biomarkers of *HNF1A*, such as the DG9-glycan index, may prove useful in this context.

In summary, the use of the DG9-glycan index both as a biomarker for *HNF1A*-MODY and as a promising biochemical measure of *HNF1A* function represents a compelling example of the potential for rapid clinical translation of a genetic discovery originating from a genome-wide association analysis.

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