# Biomarker discovery in diabetes mellitus and lipid metabolism: multi-platform glyco(proteo)mic approaches

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# CHAPTER 3

# 3. Development of an exoglycosidase plate-based assay for detecting α1-3,4 fucosylation biomarker in individuals with HNF1A-MODY

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Reprinted and adapted: Daniel Demus, Paulina A Urbanowicz, Richard A Gardner, Haiyang Wu, Agata Juszczak, Tamara Štambuk, Edita Pape Medvidović, Katharine R Owen, Olga Gornik, Nathalie Juge, Daniel I R Spencer, Development of an exoglycosidase plate-based assay for detecting α1-3,4 fucosylation biomarker in individuals with HNF1A-MODY, Glycobiology, Volume 32, Issue 3, March 2022, Pages 230-238, https://doi.org/10.1093/glycob/cwab107

## ABSTRACT

Maturity-onset diabetes of the young due to hepatocyte nuclear factor-1 alpha variants (HNF1A-MODY) causes monogenic diabetes. Individuals carrying damaging variants in *HNF1A* show decreased levels of α1-3,4 fucosylation, as demonstrated on antennary fucosylation of blood plasma *N*-glycans. The excellent diagnostic performance of this glycan biomarker in blood plasma *N*-glycans of individuals with HNF1A-MODY has been demonstrated using liquid chromatography methods. Here, we have developed a high-throughput exoglycosidase plate-based assay to measure α1-3,4 fucosylation levels in blood plasma samples. The assay has been optimised and its validity tested using 1000 clinical samples from a cohort of individuals with young-adult onset diabetes including cases with HNF1A-MODY. The α1-3,4 fucosylation levels in blood plasma showed a good differentiating power in identifying cases with damaging *HNF1A* variants, as demonstrated by ROC curve analysis with AUC values of 0.87 and 0.95. This study supports future development of a simple diagnostic test to measure this glycan biomarker for application in a clinical setting.

# INTRODUCTION

Glycosylation is a co-/post-translational modification of proteins which is important for protein structure, stability and function, and influences most biological processes including cell signalling. There are two types of glycosylation: *N*-glycosylation where glycans are attached to a protein via a glycosidic bond to asparagine and *O*-glycosylation where the glycosidic bond links glycans to either serine or threonine[101]. Advances in analytical technologies have helped to further understand the role of glycosylation in human health and disease. Alterations in glycosylation patterns occur in certain pathological conditions such as cancers[36], autoimmune diseases and in response to lifestyle changes[37, 102]. Glycosylation features such as sialylation, fucosylation and galactosylation have been explored in different diseases as potential biomarkers[36] for early diagnosis[103] or for monitoring the effectiveness of treatments[68, 104]. The measurement of glycosylation features may also be applied to estimate and track the biological age of healthy individuals[105].

Maturity onset diabetes of the young due to damaging alleles in hepatocyte nuclear factor-1 alpha (HNF1A-MODY) is a rare type of diabetes caused by an autosomal dominant mutation in the single gene, *HNF1A*, which is involved in regulating β-cell development and insulin secretion[74]. HNF1A-MODY is characterised by defects in insulin secretion and onset of hyperglycemia in the 2$^{nd}$-4$^{th}$ decade of life[81]. A correct diagnosis is a key factor for optimal disease management, as early stages of HNF1A-MODY can be effectively controlled with orally administered sulfonylureas[106]. Diagnosis of HNF1A-MODY is challenging as it often depends on the awareness of physicians and the availability of relatively expensive genetic testing for confirming the presence of pathogenic variants in the *HNF1A* gene. Moreover, HNF1A-MODY shares clinical features with other types of diabetes which is estimated to lead to misdiagnosis in around 80% of cases in the UK[79]. A MODY probability calculator has been developed to help physicians detect those at higher risk of MODY[78]. Moreover, due to the autosomal dominant inheritance pattern of HNF1A-MODY, a correct diagnosis helps identify other family members affected by this disorder[81].

Certain variants in the *HNF1A* gene result in altered glycosylation patterns associated with HNF1A-MODY. Genome-wide association studies (GWAS) show that damaging loss-of-function variants in the *HNF1A* gene lead to downregulation of fucosyltransferases that perform the α1-3 and α1-4 fucosylation of glycans[82]. Further studies proposed that decreased α1-3 and α1-4 fucosylation of *N*-glycans (antennary fucosylation) from blood plasma proteins can be used as a differentiating biomarker for HNF1A-MODY[53]. The use of antennary fucosylation as a biomarker of HNF1A-MODY was previously demonstrated[51, 53] and we recently showed the excellent inter-laboratory performance of the *N*-glycan biomarker using liquid chromatography (LC) methods[52]. Nevertheless, the applicability of LC-based methods in clinical practice is limited due to their low robustness and equipment-related costs, therefore development of a simpler analytical approach to test for this well-studied biomarker is warranted.

Here, we have developed an exoglycosidase plate-based assay which enables high-throughput measurements of levels of fucose residues that are attached via α1-3 and α1-4 linkages to glycoconjugates present in blood plasma. The assay has been optimised and its performance validated on 1000 clinical blood plasma samples from individuals with young-adult onset diabetes, including groups of individuals with different variants in the *HNF1A* gene. The differentiating power of α1-3,4 fucosylation levels is comparatively discussed with the previously published results testing antennary fucosylated *N*-glycans as a biomarker for HNF1A-MODY using LC methods.

# MATERIALS AND METHODS

## Material

The blood samples were obtained from the HNF1A-MODY cohort[51]. Briefly, participants of this study, were recruited from two European centres via the Young Diabetes in Oxford (YDX) study in the UK (n=499) and the Croatian National Diabetes Registry (CroDiab) in Croatia (n=501). The study inclusion criteria were the age of 18 years or older and diabetes diagnosis before the age of 45 years. Biochemical inclusion criteria were: fasting C-peptide ≥ 0.2 nmol/L, which indicates endogenous insulin production and negative GAD antibodies (GADA) to exclude type 1 diabetes patients. Informed consent was obtained from all participants. Sequencing of *HNF1A* and systematic and functional assessment of rare *HNF1A* alleles, performed as part of the previous study[51], allowed to divide the participants into four groups with (likely) damaging *HNF1A* variants , (likely) benign *HNF1A* variants, a group of cases with variants of unknown significance (VUS) and a group without variants in the *HNF1A* gene.



## α1-3,4 fucosylation level measurements

Patients' blood plasma samples and pooled blood plasma standard (FRNCP0125, VisuCon) were applied in the exoglycosidase plate-based assay. The pooled plasma standard was applied in the assay optimization experiments and used as a process control in the patients cohort study. Blood plasma samples were thawed, vortexed and centrifuged briefly at 600 rpm for 30 s prior to the exoglycosidase plate-based assay. Duplicate 10 µL aliquots of each sample were transferred manually into a 96-well PCR plate (4ti-0960, 4titude) and diluted 5-fold in 50 mM citrate buffer, pH 6. The plate was then sealed with a pierce foil seal (4ti-0531, 4titude), incubated at 100°C for 10 min to enhance denaturation of proteins, then cooled down at 4°C and centrifuged briefly at 600 rpm for 30 s.

The pierce foil seal was removed before the plate was placed on a Hamilton STARlet liquid handling robot where the next steps were performed using a semi-automated program. The blood plasma samples were treated with an exoglycosidase for fucose release or left untreated (no exoglycosidase). Briefly, 11 µL α1-3,4 specific fucosidase

(E1_10125)[85] was added at 3 µM final enzyme concentration in 250 mM citrate buffer, pH 6. The dilution buffer (11 µL) was added to the untreated samples. The plate was then removed from the robot, sealed again, mixed on a plate shaker for 1 min and centrifuged at 800 rpm for 30 s. Following incubation at 37°C overnight (16 ± 1 h), the plate was cooled down at 4°C and briefly centrifuged at 600 rpm for 30 s, the seal removed and the plate was placed back in the robot where 54 µL of ultrapure water was added to each well. The plate was sealed, the samples were mixed by vortexing for 1 min and then centrifuged at 1400 rpm for 40 min.

The seal was then removed and the plate placed into the robot which transferred 54.4 µL of supernatant into a 384-well microplate (4ti-0234, 4titude). An L-fucose assay kit containing reaction buffer, NADP+, fucose dehydrogenase and fucose standard (K-FUCOSE, Megazyme), diaphorase (D5540, Sigma) and resazurin (199303, Sigma Aldrich, UK) were used in the following steps. A standard curve was prepared in 4 replicates in the 384-well microplate with L-fucose concentration ranging from 0, 0.86, 1.69, 2.52, 3.35, 4.17 and 5.00 ng/µL. A reagent mix (containing 10.9 µL of reaction buffer, pH 9.5, 5.4 µL of 1 mM resazurin solution and 2.7 µL of NADP+) and an enzymatic reagent mix (containing 1.1 µL of fucose dehydrogenase and 5.4 µL of 10 U/mL diaphorase solution) were added to each sample. The plate was then removed from the robot, sealed and incubated for 3 h in the dark at 21°C. The plate was then centrifuged at 600 rpm for 30 s and placed in a plate reader (Enspire 2300, Perkin Elmer Enspire, USA) for fluorescence measurements at 24°C with an excitation wavelength (λex) = 571 nm and an emission wavelength (λem) = 586 nm. Three consecutive measurements were taken with excitation illumination from above the plate at a height of 10 mm. Fluorescence signals from the three measurements were averaged.

## Data processing and statistical analysis

Data processing, visualization and statistical analysis were performed using Microsoft Excel and R platform version 1.1.463. Receiver Operating Characteristic (ROC) curve analysis was applied to estimate diagnostic performance, testing three *HNF1A* variant groups: (likely) damaging, (likely) benign and diabetes cases without *HNF1A* mutation.

ROC curves, area under the curve (AUC), optimal sensitivity, specificity and cutoff values were generated using "cutpointr" R package. Data outliers were identified with the 1.5xIQR rule and detected per mutation group[89]. Statistical significance of differences in α1-3,4 fucosylation levels between mutation groups was determined using the Mann-Whitney U test for pairwise comparison and Kruskal-Wallis test for global comparison ($p < 0.05$). The Spearman's correlation method was used to test α1-3,4 fucosylation levels against a panel of inflammatory and metabolic markers available within the cohort. Body mass index (BMI) and C-reactive protein (CRP) level values were available in the clinical data for 866 and 916 patients, respectively. The Spearman's correlation method was used to evaluate α1-3,4 fucosylation levels of glycoconjugates in blood plasma and antennary fucosylation levels of plasma *N*-glycans measured as indexes as a part of the previous HNF1A-MODY study[52]. A generalised linear model adjusted for either age or sex and high-sensitivity CRP (hsCRP) levels as confounding variables was used to investigate associations between α1-3,4 fucosylation levels (dependent variable) and sex or age.

Chromeleon version 7.2 (Thermo Fisher) was used to export fluorescence traces as an open text format. UHPLC data processing, quantitation and graphical overlays of chromatograms were performed using HappyTools version 0.0.2 build 1800521a[88]. Microsoft Excel was used to export fluorescence readout generated by the micro-plate reader EnSpire (Perkin Elmer).

For intra-plate variation, α1-3,4 fucosylation levels (pg/µL) were averaged for 6 replicates of human blood plasma standards (VisuCon), then standard deviation (SD) and coefficient of variation (CV) values were calculated. The averaged α1-3,4 fucosylation levels per each assay plate were used to estimate inter-plate variation, as described by SD and CV values.

# RESULTS

## Development of the plate assay for measurements of α1-3,4 fucosylation levels in plasma samples

The exoglycosidase plate-based assay developed in this work is based on the use of a novel α1-3,4 specific fucosidase (E1_10125), which is characterised by the ability to remove fucose residues from glycoconjugates present in blood plasma[52, 85]. Importantly, E1_10125 is capable of removing antennary fucose residues from *N*-glycans terminated with sialic acids, which is an advantage over commercially available α1-3,4 specific fucosidases. The released L-fucose residues are then subjected to an enzymatic redox reaction to produce a fluorescence signal which is directly proportional to the amount of released L-fucose residues. The released L-fucose monosaccharides are oxidised to L-fucono-1,5-lactone by a L-fucose dehydrogenase which in turn reduces NADP+ to NADPH. The NADPH is then oxidised by a diaphorase which leads to the reduction of resazurin and the formation of the fluorescent product resorufin in molar proportions stoichiometric to the released L-fucose monosaccharides (**Figure 1**).

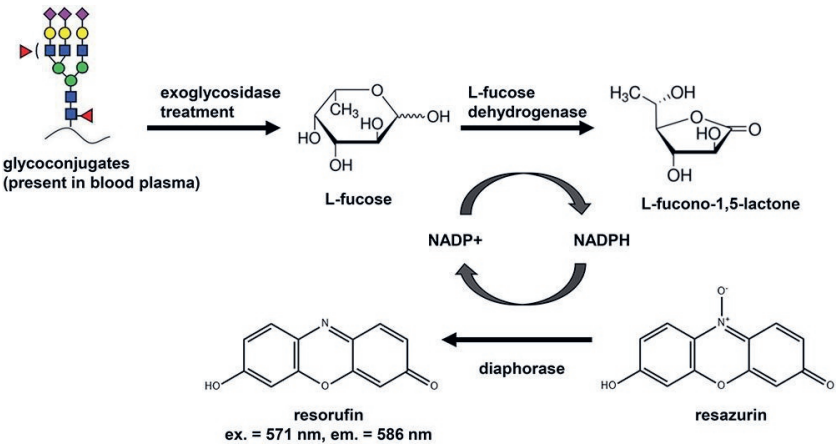



**Figure 1. Schematic of the enzymatic redox reaction resulting in the formation of fluorescent product resorufin that forms the detection mechanism of the exoglycosidase plate-based assay.** Resorufin measured at an excitation (ex.) wavelength of 571 nm and an emission (em.) wavelength of 586 nm. [NAPD+: nicotinamide adenine dinucleotide phosphate (oxidised); NADPH: nicotinamide adenine dinucleotide phosphate (reduced)]. This figure is available in black and white in print and in colour at *Glycobiology* online.

The fluorescence signal reaches maximum levels following 3 hours incubation and provides the best signal to background ratios. Each plasma sample was either treated with the exoglycosidase or untreated and processed in the same manner throughout the different steps of the plate-based assay. The fluorescence signal of the untreated sample was subtracted from the fluorescence signal of the exoglycosidase treated sample to exclude fluorescence background and possible interferences coming from the sample matrix. The amount of fucose in the plasma samples was then calculated based on the fucose standard curve. No fluorescence signal was generated by the E1_10125 alone, which might have influenced fucosylation level readouts and the consistency of measurements (data not shown). In this type of assay based on fluorescence measurements, readouts of low concentrations of monosaccharides might be limited by the fluorescence background signal. To be accurate, the fluorescence signal of the sample (following background subtraction) should always be greater than 0 (**Supplementary Table S1**).

**Table S1. Detection of very low fucose levels in blood plasma samples might be limited by the sample background fluorescence signal.** The table presents a summary of an experiment using 3 sets of plasma samples spiked with fucose standard of known concentration (in a range from 1200.00 to 0.04 pg/µL). The fucose-spiked denatured plasma samples were not exoglycosidase treated and were processed by the plate-based assay: the samples were centrifuged, the supernatants were subjected to redox reactions and the fluorescence signal measurements. Data are expressed as the mean and standard deviation (SD) of fluorescence signal together with coefficient of variation (CV) and signal to background (S/B) ratio for each concentration. S/B values below 0 (marked bold) indicate concentrations of fucose in plasma samples that were below limit of detection of the assay.

| Fucose concentration in spiked plasma samples (pg/µL) | Average signal - S (U), n = 3 | SD | CV | Background signal - B (U) | Average background signal – $B_A$ (U) | $S/B_A$ |
|---|---|---|---|---|---|---|
| 1200.00 | 104152 | 1309 | 1% | 34374 | 34833 | 2.99 |
| 600.00 | 71160 | 1596 | 2% | 32480 | | 2.04 |
| 300.00 | 51872 | 856 | 2% | 35263 | | 1.49 |
| 150.00 | 40864 | 1074 | 3% | 34817 | | 1.17 |
| 75.00 | 38249 | 1019 | 3% | 34653 | | 1.10 |
| 37.50 | 35735 | 1746 | 5% | 34190 | | 1.03 |
| 18.75 | 34320 | 1697 | 5% | 32224 | | **0.99** |
| 9.38 | 32343 | 44 | 0% | 32193 | | **0.93** |
| 4.69 | 34245 | 1360 | 4% | 39285 | | **0.98** |
| 2.34 | 33753 | 1639 | 5% | 35227 | | **0.97** |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.17 | 35390 | 179 | 1% | 37565 | | 1.02 |
| 0.59 | 34515 | 1292 | 4% | 35536 | | **0.99** |
| 0.29 | 33617 | 1605 | 5% | 34514 | | **0.97** |
| 0.15 | 34145 | 899 | 3% | 35346 | | **0.98** |
| 0.07 | 33578 | 1613 | 5% | 32183 | | **0.96** |
| 0.04 | 34112 | 227 | 1% | 32312 | | **0.98** |

To determine the optimal concentration of E1_10125 during the development of the assay, 2, 4, 8 and 16 µM concentrations of the enzyme were tested on 10 µL pooled blood plasma samples. The results showed similar levels of released fucose residues irrespective of the enzyme concentration (**Supplementary Figure S1A**). Since protein concentration may vary between patient plasma samples[107], we ensured that the final concentration of E1_10125 provides maximum and consistent release of fucose regardless of protein content in plasma samples, as demonstrated on a range of pooled plasma volumes (6, 8, 10, 12 and 14 µL) (**Supplementary Figure S1B**). Based on these results, the optimal final concentration was determined to be 3 µM. Of note, in the development stage, a broader range of plasma volumes (2 – 18 µL) was tested. However, plasma volumes ≥ 16 µL led to the formation of a larger protein pellet after centrifugation, which disrupted aspiration and transfer of fucose-containing supernatants on the robotic platform, resulting in the need for manual intervention. This issue was not observed during a validation and the sample cohort analysis when using 10 µL of patients' plasma samples, which indicates that a range of 6 – 14 µL mimics the protein content variation sufficiently. Furthermore, we demonstrated that E1_10125 fucosidase at 3 µM showed high efficiency (~ 75%) in removing antennary fucose residues from intact glycoproteins present in blood plasma samples under assay conditions. This was directly compared to the efficiency of E1_10125 when applied to released *N*-glycans and showed that E1_10125 does not require released glycan substrates (**Supplementary Figure S2**).

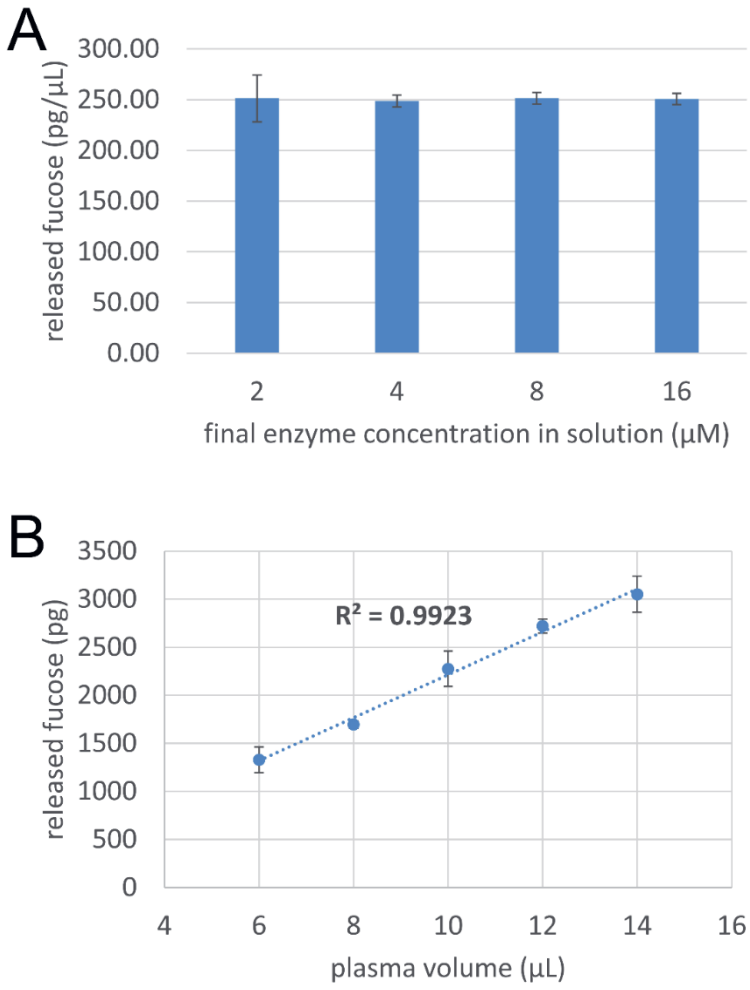
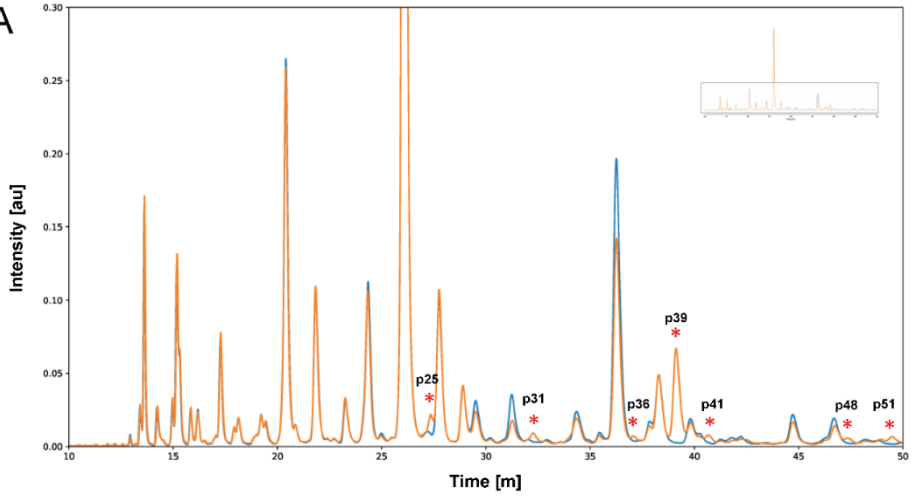
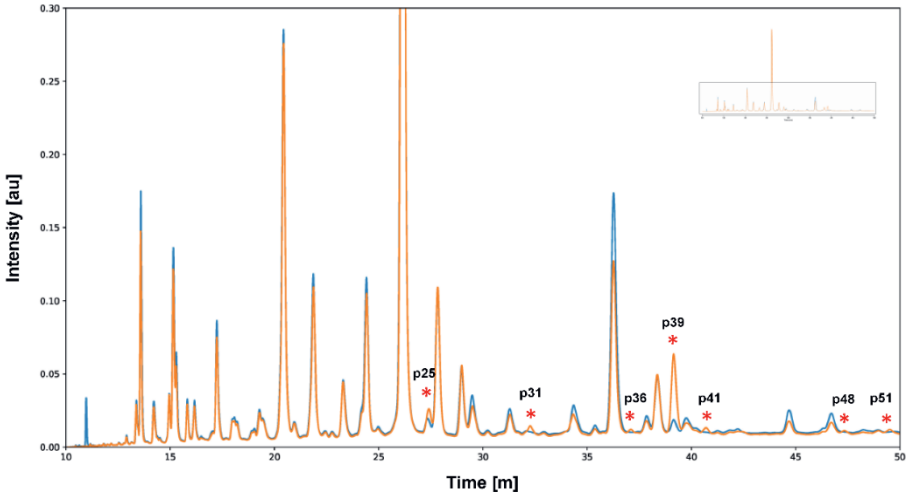



**Figure S1. Performance of E1_10125 fucosidase applied at different concentrations to 10 µL plasma standard samples**, n = 3 (A) and E1_10125 fucosidase applied at the final concentration of 3 µM to a range of plasma volumes, n = 3 each (B). E1_10125 provides maximum fucose release from blood plasma conjugates at the range of 2 – 16 µM final concentrations (A). 3 µM final concentration was proven to provide a good linearity ($R^2$ = 0.9923) for the released fucose amounts within the tested plasma volumes that mimicked the protein content variation in 10 µL of clinical blood plasma samples.

A




B




C

| | | p25 | p31 | p36 | p39 | p41 | p48 | p51 | sum |
|---|---|---|---|---|---|---|---|---|---|
| **Fucosidase treated plasma** | set 1 | 0.00393 | 0.00128 | 0.00006 | 0.00293 | 0.00027 | 0.00042 | 0.00043 | 0.0093 |
| | set 2 | 0.00409 | 0.00123 | 0.00005 | 0.00273 | 0.00029 | 0.00031 | 0.00035 | 0.0090 |
| | **Average** | **0.00401** | **0.00125** | **0.00005** | **0.00283** | **0.00028** | **0.00036** | **0.00039** | **0.0092** |
| | **STD** | **0.00012** | **0.00004** | **0.00001** | **0.00014** | **0.00001** | **0.00008** | **0.00006** | **0.0002** |
| **Fucosidase untreated plasma** | set 1 | 0.00628 | 0.00336 | 0.00084 | 0.02336 | 0.00192 | 0.00157 | 0.00250 | 0.0398 |
| | set 2 | 0.00676 | 0.00296 | 0.00075 | 0.01983 | 0.00189 | 0.00089 | 0.00134 | 0.0344 |
| | **Average** | **0.00652** | **0.00316** | **0.00080** | **0.02160** | **0.00190** | **0.00123** | **0.00192** | **0.0371** |
| | **STD** | **0.00034** | **0.00029** | **0.00006** | **0.00249** | **0.00002** | **0.00048** | **0.00082** | **0.0038** |

**Figure S2. The efficiency of E1_10125 fucosidase in removing antennary fucose residues from released *N*-glycans and intact glycoproteins present in blood plasma samples.** Fluorescence UHPLC chromatograms of released procainamide labeled plasma *N*-glycans derived from 8 µL pooled plasma standard (A) and plasma pellet obtained from the plate assay (B), and a table showing relative abundance of α1-3,4 fucosylated *N*-glycan peaks in full plasma *N*-glycome obtained from the plasma pellets (C). E1_10125 fucosidase digested samples (blue) were overlaid with enzymatically untreated sample chromatograms (orange). Chromatograms were normalised to the most abundant peak. α1-3,4 fucosylated glycan structure peaks are marked with asterisks (*). *N*-glycan release, exoglycosidase digestions, chromatographic separation of *N*-glycan structures and relative peak area extraction were performed as described previously[52, 84], and the content of antennary fucosylated *N*-glycans calculated for both sets of samples, each set n =2. Briefly, two sets (n = 2) of blood plasma pellets from 10 µL plasma standard (VisuCon) from a set of exoglycosidase treated and untreated samples for the plate-based assay were saved for *N*-glycan release to assess a degree of fucose release performed on denatured intact glycoproteins. Additionally, 8 µL pooled plasma standard (n = 2 sets) was used for *N*-glycan release and digestion by E1_10125 as positive control. Both sets were then compared for the degree of fucose release from intact glycoproteins vs. released *N*-glycans. The results showed that E1_10125 allows full (100%) α1-3,4 linked fucose release from released *N*-glycans whereas E1_10125 at the final concentration of 3 µM allows approximately 75% fucose release from *N*-glycans of denatured intact glycoproteins, which was calculated based on relative abundance of α1-3,4 fucosylated *N*-glycan peaks in fucosidase treated (0.0092 ± 0.0002) and untreated (0.0371 ± 0.0038) plasma samples (C).

## Performance of the assay in analysing α1-3,4 fucosylation levels as a biomarker for HNF1A-MODY

Having optimised the exoglycosidase plate-based assay, 1000 blood plasma samples from the HNF1A-MODY cohort were analysed to assess α1-3,4 fucosylation levels and evaluate their diagnostic performance for the identification of cases with diabetes carrying damaging variants in the *HNF1A* gene.

Based on systematic and functional assessment of rare *HNF1A* alleles, which had been performed previously[51], the 947 participants were grouped into four *HNF1A* variant types: (likely) damaging (n = 18), (likely) benign (n = 8), variants of unknown significance (VUS, n = 5) and no *HNF1A* rare variants (n = 916). Clinical characteristics of study participants are summarised in **Table 1**.

**Table 1. Clinical characteristics of study participants**

| | (Likely) damaging allele | (Likely) benign<br><br>n = 8 cases | Variants of unknown significance (VUS) | No rare *HNF1A* allele variant |
|---|---|---|---|---|
| Sex, male (n) | 5 | 4 | 2 | 529 |
| Age at recruitment (years) | 40 ± 17 | 48 ± 11 | 54 ± 14 | 47 ± 11 |
| Age at diagnosis (years) | 26 ± 9 | 36 ± 6 | 37 ± 10 | 35 ± 7 |
| Diabetes duration (years) | 13 ± 12 | 9 ± 7 | 17 ± 11 | 12 ± 10 |
| BMI (kg/m$^2$) | 26 ± 5 | 35 ± 5 | 28 ± 6 | 31 ± 7 |
| hsCRP (mg/L) | 0.8 ± 1.3 | 5.2 ± 5.7 | 4.0 ± 6.3 | 6.2 ± 29.7 |
| HbA1c (%) | 7.7 ± 1.7 | 8.8 ± 2.1 | 6.7 ± 1.3 | 7.9 ± 2.7 |
| C-peptide (nmol/L) | 0.42 ± 0.21 | 0.81 ± 0.62 | 0.60 ± 0.57 | 5.11 ± 71.47 |
| Total cholesterol (mmol/L) | 4.75 ± 1.09 | 5.07 ± 0.88 | 4.74 ± 1.21 | 4.69 ± 1.23 |
| HDL (mmol/L) | 1.4 ± 0.3 | 1.1 ± 0.2 | 1.3 ± 0.4 | 1.3 ± 3.4 |
| Triglycerides (mmol/L) | 1.2 ± 0.5 | 2.0 ± 1.0 | 1.1 ± 0.3 | 2.0 ± 1.7 |

Mean values are presented for each group (± standard deviation)

From the 1000 samples tested, 947 showed a background to fluorescence signal > 0 and blood plasma α1-3,4 fucosylation levels from these samples were subsequently subjected to statistical analyses.

Box plot analysis was used to evaluate differences in α1-3,4 fucosylation levels between the four *HNF1A* variant groups. The analysis showed significant differences ($p \leq 0.05$) in α1-3,4 fucosylation levels between groups of (likely) damaging vs. (likely) benign and (likely) damaging vs. cases without rare *HNF1A* variants (**Figure 2**). No significant difference in α1-3,4 fucosylation levels was observed between (likely) benign and a group of cases without rare *HNF1A* variant groups. Data outliers were identified within each group, 1 case within (likely) damaging group and 24 cases within cases without *HNF1A* mutation and removed prior to the further analyses[89]. The average fucose content in blood plasma for each defined group was as follows: 79.26 ± 35.63 pg/µL for (likely) damaging, 241.01 ± 125.41 pg/µL for (likely) benign, 185.71 ± 111.15 pg/µL for VUS and 193.60 ± 99.58 pg/µL for cases without rare *HNF1A* variants.

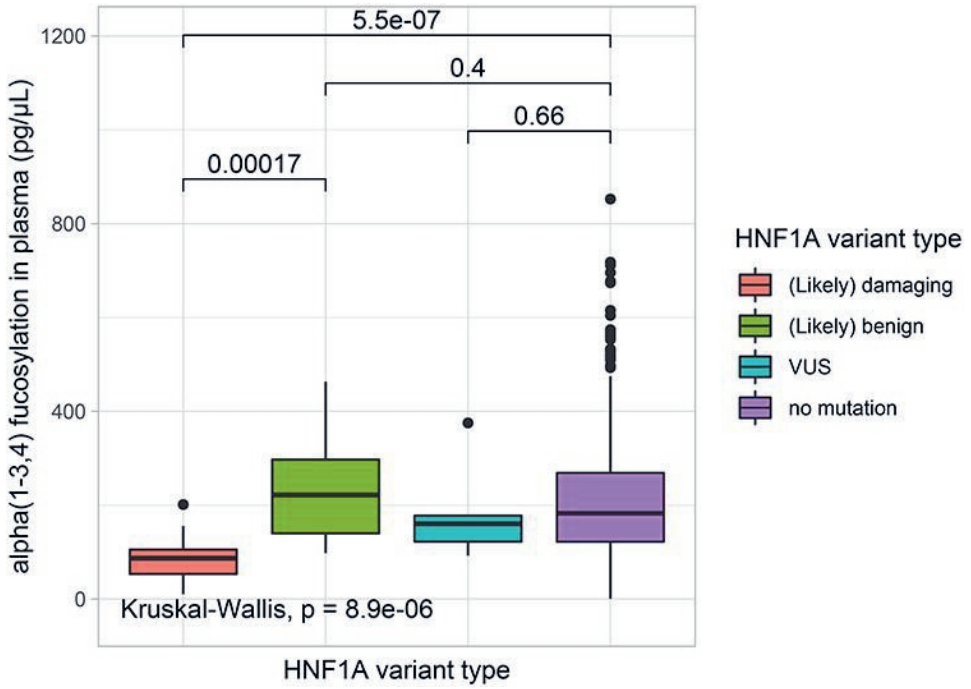



**Figure 2. Box plots presenting differences in α1-3,4 fucosylation levels in blood plasma (pg/µL) measured using the exoglycosidase plate-based assay for groups of patients with different *HNF1A* variant groups.** Each box represents 25th–75th percentile, the median is marked by a vertical line, whiskers indicate values that are within 1.5 × IQR of the hinge. Outliers are displayed as black filled circles. The lines and numbers above the box plots indicate the *P*-value when comparing two categories using The Wilcoxon–Mann–Whitney test. The analysis with $P \leq 0.05$ is considered statistically significant.

Next, receiver operating characteristics (ROC) curves were used to estimate the differentiating power of α1-3,4 fucosylation levels to identify patients with HNF1A-MODY carrying damaging variants in the *HNF1A* gene. The ROC analysis showed that α1-3,4 fucosylation levels provide good differentiating power between a group of (likely) damaging cases and the group of individuals without rare *HNF1A* variant , as determined by the measurement of the area under the curve (AUC) that was found to be 0.87 with 94% sensitivity, 71 % specificity at the cut-off of 129.43 pg/µL (**Figure 3A**). Additionally, the diagnostic performance of α1-3,4 fucosylation levels was tested for (likely) damaging vs. (likely) benign variant groups giving an AUC of 0.95 with 94% sensitivity, 75% specificity at the cutoff of 137.59 pg/µL (**Figure 3B**). By applying α1-3,4

fucosylation levels as a biomarker for HNF1A-MODY, two cases from the VUS group would have been classified as carrying damaging mutations.

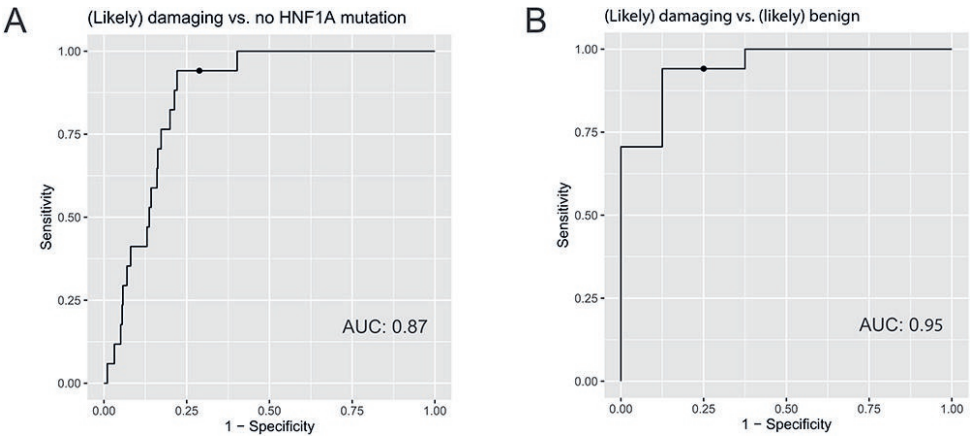



**Figure 3. ROC curves illustrating the performance of α1-3,4 fucosylation levels in differentiating cases with (likely) damaging vs. no *HNF1A* variants (A) and (likely) damaging vs. (likely) benign variants in the *HNF1A* gene (B).** The AUC values are displayed for each ROC curve. The optimal cut-off points are displayed as a dot on the precision recall curve for each ROC curve.

## Intra- and inter-assay variations for measurements of α1-3,4 fucosylation levels in blood plasma samples using the exoglycosidase plate-based assay

In order to evaluate the effectiveness and robustness of the exoglycosidase plate-based assay, 72 plasma standard samples, were distributed over 12 assay plates measured on different days. The results showed good repeatability and precision for measurements of α1-3,4 fucosylation levels in blood plasma samples with CVs of 9% for average intra-plate variation and 10% for inter-plate variation (**Table 2**).

## Associations between α1-3,4 fucosylation levels, age, sex and clinical markers

We investigated associations between α1-3,4 fucosylation levels and either sex or age within individuals with diabetes without rare variants in the *HNF1A* gene. A significant association between α1-3,4 fucosylation levels and sex (regression coefficient $\beta = 0.25$, $\rho = 0.0005$) but not age was observed. Average α1-3,4 fucosylation levels were found to be 180.9 ± 97.8 pg/µL in females (n = 362) and 204.2 ± 100.2 pg/µL in males (n = 510).

**Table 2. Intra- and inter-assay variations for measurements of α1-3,4 fucosylation levels in blood plasma samples by the exoglycosidase plate-based assay.** Intra- and inter-assay variations are described by coefficient of variation (CV) values that were calculated based on the amount of released fucose for human blood plasma standards (n = 6) distributed over 12 assay plates.

| Intra-plate variation | | | |
|---|---|---|---|
| Plate | Average fucose level in plasma (pg/µL), n = 6 | SD | CV |
| 1 | 235.65 | 13.11 | 6% |
| 2 | 213.89 | 55.97 | 26% |
| 3 | 224.09 | 30.18 | 13% |
| 4 | 200.53 | 10.62 | 5% |
| 5 | 250.55 | 6.62 | 3% |
| 6 | 219.50 | 17.17 | 8% |
| 7 | 242.71 | 10.95 | 5% |
| 8 | 213.80 | 27.16 | 13% |
| 9 | 256.58 | 7.85 | 3% |
| 10 | 230.28 | 8.42 | 4% |
| 11 | 175.92 | 19.79 | 11% |
| 12 | 220.35 | 22.09 | 10% |
| Average intra-plate | | | 9% |
| Inter-plate variation | 223.65 | 22.14 | 10% |

Previously, we reported significant correlations between CRP levels and antennary fucosylation levels, which were measured using antennary fucosylated *N*-glycan traits by an LC-based method[52]. Here, we tested α1-3,4 fucosylation levels against a panel of inflammatory and metabolic markers (**Table 1**). We found a weak correlation between α1-3,4 fucosylation and CRP levels (correlation coefficient $r = 0.29$, $p < 2.2 \times 10^{-16}$) within the unselected sample cohort of young-adult onset diabetes (**Supplementary Figure S3A**). Another weak correlation was found for α1-3,4 fucosylation levels and body mass index (BMI) with a correlation coefficient r = 0.17, p < 1.2 x 10-6 (**Supplementary Figure S3B**) in the same cohort.

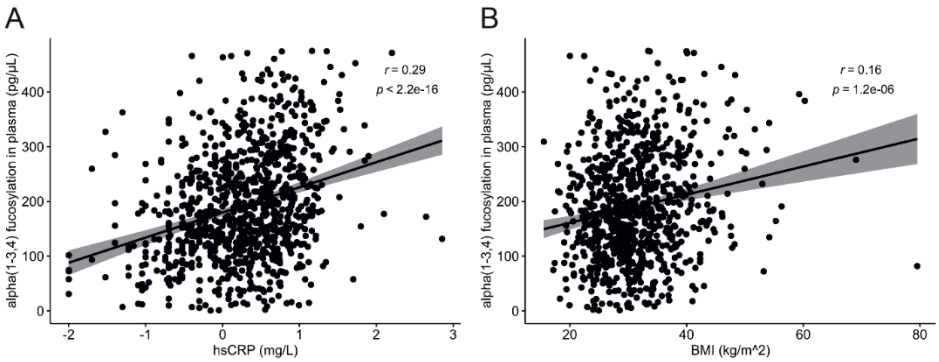



**Figure S3. Correlations between CRP and α1-3,4 fucosylation levels (A), and BMI and α1-3,4 fucosylation levels (B) observed within the sample cohort**. hsCRP concentrations were logarithmically transformed.

We next compared the fucosylation indexes previously measured using an LC-based method[52], which reflect levels of antennary fucosylation in the full plasma *N*-glycome, with the α1-3,4 fucosylation levels in blood plasma samples measured by the exoglycosidase plate-based assay. The analysis was carried out for 31 cases that had been diagnosed with mutations in the *HNF1A* gene, which included groups of (likely) damaging, (likely) benign variants and VUS. The Spearman's correlation analysis showed a strong correlation between the fucosylation indexes and α1-3,4 fucosylation levels in blood plasma samples with a correlation coefficient r = 0.85 (**Figure 4A**). When considering all previously analysed 320 diabetes cases with and without mutations in the *HNF1A* gene, a significant but weaker correlation with the correlation coefficient r = 0.68 was obtained (**Figure 4B**)

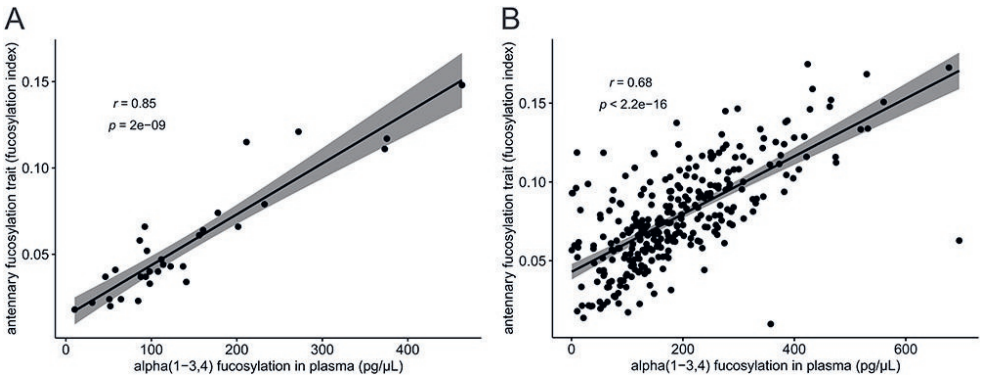



**Figure 4. Correlations between *N*-glycan antennary fucosylation levels measured as indexes by LC method in the previous study (Demus et al. 2021[52]) and α1-3,4 fucosylation levels measured by the exoglycosidase plate-based assay for 31 HNF1A-MODY positive cases (A), and 320 diabetes cases including 31 HNF1A-MODY positive and 289 negative cases (B).**

# DISCUSSION

Diagnosis of HNF1A-MODY is challenging as current clinical criteria to guide genetic screening for MODY have been proven insensitive[108] and increasingly non-specific[109]. Overlapping of clinical features of MODY with other types of diabetes, poor access and the cost of genetic testing often leads to misdiagnosis of MODY patients and incorrect disease management[109]. Since the GWAS study from 2010 revealed that damaging variants in the *HNF1A* gene are associated with decreased levels of antennary fucosylated *N*-glycans[82], this potential glycan biomarker for HNF1A-MODY has been studied using a combination of fluorescently labelled glycans and LC separation techniques. Its diagnostic accuracy has been tested on large cohorts of cases with diabetes carrying different variants in the *HNF1A* gene. Individuals with damaging alleles in the *HNF1A* gene are known to have malfunctioning molecular mechanisms in which HNF1α factor is involved. The HNF1α is a master regulator of several genes, among others, genes controlling β-cell function and growth[74] and genes encoding fucosyltrasferases[82]. Therefore, individuals that carry damaging variants in the *HNF1A* gene represent decreased levels of α1-3,4 fucosylation. The excellent differentiating performance of blood plasma antennary fucosylated *N*-glycans as a biomarkers for HNF1A-MODY with AUCs of over 0.90 has been reported[51-53]. By applying those glycan biomarkers, it was possible to identify rare cases with damaging variants in the *HNF1A* gene. However, this analytical method has not been translated into a widely available diagnostic tool.

As a response to the current demand for a simple and effective diagnostic tool for the detection of this well-studied *N*-glycan biomarker for HNF1A-MODY, we have developed the exoglycosidase plate-based assay with fluorescence output to measure α1-3,4 fucosylation levels in blood plasma samples. This assay employs a novel α1-3,4 specific fucosidase (E_10125), which is capable of releasing fucose residues from glycoconjugates present in blood plasma samples[85]. This fucosidase works efficiently on *N*-glycan structures terminated with sialic acids and shows high efficiency (~ 75%) in removing *N*-glycan antennary fucose residues from intact glycoproteins present in

plasma samples. This advantage has been exploited in this study. The remaining portion (~ 25%) of undigested fucose from *N*-glycans, when applying E1_10125 fucosidase to denatured plasma samples, might be due to the inaccessibility of fucose residue substrates to the enzyme in this case where the glycans remain attached to the proteins. Nevertheless, the plate-based assay targets α1-3,4-linked fucose from all glycoconjugates (*N*- and *O*-glycosylated proteins and glycolipids) present in plasma samples and, as demonstrated by the consistency and excellent linearity of released fucose levels, the undigested portion of *N*-glycan fucose residues does not interfere with the results.



The validity of the assay was tested on 1000 blood plasma samples from a diabetes cohort, which included diagnosed cases with and without rare variants in the *HNF1A* gene. The ROC analysis showed that α1-3,4 fucosylation levels assessed from blood plasma samples by the exoglycosidase plate-based assay provide very good discriminatory power to identify cases with damaging variants in the *HNF1A* gene, as tested against a group of cases with benign (AUC = 0.95) and no rare variants (AUC = 0.87) in the *HNF1A* gene. The results for cases with damaging vs. no rare variants in *HNF1A* are similar to the AUCs of 0.90 for two single glycans structures reported using a LC-based method from the same sample cohort[51]. We previously determined an antennary fucosylation trait, which reflects overall changes in antennary fucosylation in blood plasma *N*-glycome[52]. Here, *N*-glycan antennary fucosylation levels measured as indexes by applying this derived antennary fucosylation trait were correlated with α1-3,4 fucosylation levels of blood plasma glycoconjugates measured by the exoglycosidase plate-based assay for 31 HNF1A-MODY cases and all 320 cases with young-adult onset non-autoimmune diabetes, separately. The respective Spearman's correlation coefficients of 0.85 and 0.68 obtained in the correlation analysis are considered relatively low and might suggest varying levels of α1-3,4-linked fucose residues released from *O*-glycans and glycolipids present in blood plasma. Higher values for the correlation coefficient would have been expected in the case of proportionate amounts of α1-3,4-linked fucose derived from *N*-glycans and other

glycoconjugtates in plasma samples. Nevertheless, the significance of the correlation analysis together with results obtained for the intra- and inter-plate variations from the current cohort study demonstrate the excellent repeatability of measurements and the validity of the exoglycosidase plate-based assay.

The performance of a biomarker based on α1-3,4 fucosylation might be influenced by inflammatory events that affect glycosylation profiles[52, 98]. A large proportion (~ 50%) of *N*-glycans containing α1-3,4 fucosylation derives from acute phase proteins expressed during inflammation[94] and, here, we have confirmed that α1-3,4 fucosylation levels weakly correlate with CRP levels (correlation coefficient r = 0.29) that are indicative of inflammation as well as a non-alcoholic fatty liver disease related to participants BMI[99]. For the later, overexpression of fucosyltransferase FUT6 has been reported, which might lead to altered expression of other fucosyltrasferases due to their interconnection with the HNF1α transcription factor and the availability of guanosine diphosphate-fucose (GDP-fucose) donor substrate[82, 110]. Furthermore, the weak correlation between α1-3,4 fucosylation levels and BMI (correlation coefficient r = 0.17), which has also been observed within the current sample cohort, might be explained by the obesity-linked pro-inflammatory state, and secretion of inflammatory mediators as well as CRP[111]. In addition, we found that the α1-3,4 fucosylation levels were associated with sex, but not age, with higher α1-3,4 fucosylation levels in male individuals, consistent with previous findings reporting that fucosylation is gender dependent[112]. Together this data indicates that different cut-off values for α1-3,4 fucosylation levels will need to be applied to men and women in a real diagnostic setting.

CRP, which is already widely used in clinical testing, has been previously evaluated as a biomarker for HNF1A-MODY and provided a good diagnostic performance with an AUC of 0.83 (88% sensitivity and 69% specificity at the cutoff 0.81 mg/L), although not as performant as antennary fucosylated *N*-glycan and α1-3,4 fucosylation biomarkers[51]. In addition, the application of CRP as a specific biomarker for HNF1A-MODY is limited

as CRP levels rise rapidly during inflammation[113] which may confound the results. The influence of inflammation on the performance of the glycan biomarker for HNF1A-MODY is possible yet still less significant than in the case of CRP. Altogether, the good performance of α1-3,4 fucosylation as a biomarker for HNF1A-MODY implies that employing easily accessible technology, such as the described exoglycosidase plate-based assay, could serve as a screening step to identify high risk cases and select individuals for the *HNF1A* sequencing which is the current diagnostic gold standard test. This is likely to be cost-saving by narrowing the number of cases requiring expensive genetic testing.



The sequences of *FUT3*, *FUT5*, and *FUT6* are highly polymorphic and the presence of single-nucleotide polymorphisms (SNPs) in genes encoding fucosyltransferases might affect α1-3,4 fucosylation levels measured by the plate-based assay[114]. Genetic variations can lead to inactivation of fucosyltrasferases, for example, inactive FUT3 in individuals with Lewis negative blood type[115]. Information on the presence of SNPs in genes encoding fucosyltransferases was not available in the clinical data of the sample cohort used in this study. Therefore, we are not able to estimate the significance of occurrence of these SNPs on α1-3,4 fucosylation levels measured by the plate-based assay. Exclusion of individuals with loss of function SNPs in fucosyltransferase genes might lead to further improvement of the AUC and different cut-off values for α1-3,4 fucosylation biomarker. We consider this aspect as a limitation of the current study with a potential for future research and investigation.

There are several advantages to the exoglycosidase plate-based assay over other methods used for glycosylation analysis. First, the exoglycosidase plate-based assay allows timely and cost-effective high-throughput screening of large numbers of plasma samples. Currently, the LC-based approach requires extensive sample preparations and a long chromatographic separation of fluorescently-tagged glycans, often exceeding 1 hour per sample. In comparison, our exoglycosidase plate-based assay provides semi-automated sample preparation using the robotic platform followed by readouts of

fluorescence signals that allow the assessment of absolute α1-3,4 fucosylation levels from 96 plasma samples within 24 hours. Furthermore, the assay uses a simple technology based on fluorescence readouts and generates results that are easy to process and interpret, with no need for high-end instrumentation or expertise. This type of exoglycosidase assay has a potential to become an alternative to immuno-/lectin-based biochemical assays, the use of which might be limited by the availability and binding affinity of the detection antibodies and lectins[116]. It is expected that advances in glycosidase discovery will enhance a broader application of the exoglycosidase plate-based assay and enable the measurement of absolute levels of other monosaccharides in various types of samples, including released glycans, intact proteins, biopharmaceuticals and clinical samples[85]. In conclusion, the exoglycosidase plate-based assay developed in this work enables robust and high-throughput screening of absolute α1-3,4 fucosylation levels in blood plasma samples. The cohort study confirmed the excellent performance of α1-3,4 fucosylation levels as a clinical biomarker for HNF1A-MODY, which allows identification and classification of cases with diabetes carrying damaging variants in the *HNF1A* gene. The results of this work should facilitate the translation of this glycan biomarker into clinical practice and the development of a clinically relevant, widely available diagnostic test.

## Acknowledgments

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 722095. The authors gratefully acknowledge the support of the Biotechnology and Biological Sciences Research Council (BBSRC) with contribution from the Innovate UK Biocatalyst grant Glycoenzymes for Bioindustries (BB/M029042/) and the BBSRC Institute Strategic Programme Grant Gut Microbes and Health BB/R012490/1 and its constituent project BBS/E/F/000PR10356 (Theme 3, Modulation of the gut microbes to promote health throughout life). The research was supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. AJ was a Diabetes UK George Alberti fellow during the research.

Authors would like to thank Archana Mili Shubhakar (Ludger) for the technical expertise in the assay automation. Matthew Doherty (Ludger) for help in creating figures. Osmond Rebello (former Ludger) for working simultaneously on the development of glycosidase plated-based assays.

The CroDiab study was approved by Ethics Committee of Clinical Hospital Merkur and University of Zagreb, Faculty of Pharmacy and Biochemistry, Croatia. The study was conducted in accordance with the Declaration of Helsinki. The YDX study was approved by the Oxfordshire Local Research Ethics Committee, and all subjects gave written informed consent.