Altered high-density lipoprotein composition is associated with risk for complications in type 2 diabetes mellitus in South Asian descendants: a cross-sectional, case-control study on lipoprotein subclass profiling

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ORIGINAL ARTICLE

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Altered high-density lipoprotein composition is associated with risk for complications in type 2 diabetes mellitus in South Asian descendants: A cross-sectional, case-control study on lipoprotein subclass profiling

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Abstract

Background: Composition of high-density lipoproteins (HDL) is emerging as an important determinant in the development of microvascular complications in type 2 diabetes mellitus (T2DM). Dutch South Asian (DSA) individuals with T2DM display an increased risk of microvascular complications compared with Dutch white Caucasian (DwC) individuals with T2DM. In this study, we aimed to investigate whether changes in HDL composition associate with increased microvascular risk in this ethnic group and lead to new lipoprotein biomarkers.

Materials and Methods: Using ^{1}H nuclear magnetic resonance spectroscopy and Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA) software, plasma lipoprotein changes were determined in 51 healthy individuals (30 DwC, 21 DSA) and 92 individuals with T2DM (45 DwC, 47 DSA) in a cross-sectional, case-control study. Differential HDL subfractions were investigated using multinomial logistic regression analyses, adjusting for possible confounders including BMI and diabetes duration.

Results: We identified HDL compositional differences between healthy and diabetic individuals in both ethnic groups. Specifically, levels of apolipoprotein A2 and HDL-4 subfractions were lower in DSA compared with DwC with T2DM. Apolipoprotein A2 and HDL-4 subfractions also negatively correlated with waist circumference, waist-to-hip ratio, haemoglobin A1c, glucose levels and disease duration in DSA with T2DM, and associated with increased incidence of microvascular complications.

Conclusion: While HDL composition differed between controls and T2DM in both ethnic groups, the lower levels of lipid content in the smallest HDL subclass (HDL-4) in DSA with T2DM appeared to be more clinically relevant, with higher odds of having diabetes-related pan-microvascular complications such as

retinopathy and neuropathy. These typical differences in HDL could be used as ethnicity-specific T2DM biomarkers.

KEYWORDS

Dutch South Asians, high-density lipoprotein composition, microvascular complications, NMR, type 2 diabetes mellitus

1 | INTRODUCTION

The worldwide rising prevalence of type 2 diabetes mellitus (T2DM) is one of the major challenges to public health in the 21st century.[1] Being overweight and obese are the most prominent risk factors for T2DM. However, South Asians (SA) have a significantly higher risk of developing T2DM compared with white Caucasians (wC) even with a body mass index (BMI) of about 23, which strongly suggests that different pathophysiological pathways in this particular ethnic group may lead to T2DM progression.[2] In addition, compared with other ethnic groups, SAs tend to develop T2DM 5-10 years earlier,[2] and SA patients with T2DM are more prone to develop microvascular complications such as diabetic retinopathy, with a faster progression and greater disease severity compared with western European patients with T2DM.[3–5]

Previous studies showed that as the reduction of, in particular, low-density lipoproteins (LDL) by statins were associated with insulin resistance and increased risk of hyperglycaemic complications in T2DM, high-density lipoproteins (HDL) may be a better biomarker in diabetes progression.[6–8] HDL is composed of various lipids and proteins including apolipoproteins, enzymes and lipid transfer factors, and exhibit large heterogeneity in size, density and composition. The various HDL fractions have different functionalities with respect to mediating cholesterol efflux, anti-oxidation, anti-inflammation and anti-thrombotic processes.[9,10] Low plasma HDL cholesterol (HDL-C) levels, for instance, were found associated with an increased risk of T2DM and cardiovascular disease,[11–13] while also the importance of HDL functionality to cardiovascular disease incidence and prognosis of heart failure was observed,[14–16] one of the main complications among patients with T2DM. Emerging evidence further indicated that HDL particle quality has a causative impact on diabetes.[17–20]

Most of these studies were performed in Western populations; however, very little is known about HDL functional changes in individuals with T2DM from other ethnic groups. Given that SA represent a unique high-risk population with different pathophysiological pathways to T2DM progression, dysfunction of HDL might be an important aspect overlooked in this population. Therefore, we hypothesized that specific differences in HDL composition in Dutch SA (DSA) with T2DM relate to their increased vulnerability to diabetes-related microvascular complications. In the present study, we aimed to investigate whether differences in plasma HDL composition in DSA with T2DM in relation to Dutch wC (DwC) with T2DM and their respective non-diabetic controls are associated with diabetes-related microvascular complications to provide possible new biomarkers. For this, we used ^{1}H nuclear magnetic resonance (NMR) spectroscopy and the validated Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA) software[21–26] to measure plasma HDL subclass and lipid concentrations for HDL composition determination in DSA individuals with and without T2DM, compared with DwC without or with T2DM.

2 | MATERIALS AND METHODS

2.1 | Study population

For the present cross-sectional study, baseline samples were used from the MAGNetic resonance Assessment of VICTOza efficacy in the Regression of cardiovascular dysfunction in type 2 diAbetes mellitus (MAGNA VICTORIA) study from two previous randomized controlled trials (RCT; ClinicalTrials.gov NCT01761318[27] and NCT02660047,[28] respectively), together with age- and gender-matched healthy controls from both ethnic groups.[29] In the first trial, 50 DwC with T2DM were recruited while 51 DSA with T2DM were recruited in the second trial. Patients with type 1 diabetes mellitus were excluded in both trials. One DwC patient with T2DM and four DSA patients with T2DM withdrew from the RCTs. Healthy controls were recruited by advertisement in Leiden University Medical Centre (Leiden, The Netherlands) and local newspapers, as mentioned elsewhere.[29] In addition, 51 healthy non-diabetic control participants (30 DwC-C and 21 DSA-C) were prospectively enrolled with a similar age and sex distribution to the corresponding patients with T2DM. Ethnicity was based on the self-identified and self-reported biological parents' and ancestors' origins. Participants with complete informed consent were included. The study conformed to the revised Declaration of Helsinki and ethical approval was obtained from the Institutional Review Board (Leiden University Medical Center, Leiden, The Netherlands).

For the present study, we excluded participants of whom plasma samples were not available.

2.2 | Sample preparation for ^{1}H nuclear magnetic resonance spectroscopy

Sample preparation was performed consistently with the requirements of the Bruker B.I.LISA lipoprotein analysis protocol. The EDTA plasma samples were thawed at room temperature and immediately homogenized by inverting the tubes 10 times. Next, 200 µl of plasma

was manually transferred to a Ritter 96 deep-well plate. A Gilson 215 liquid handler robot was used to mix 120 µl of plasma with 120 µl, 75 mM disodium phosphate buffer in H_2O/D_2O (80/20), pH 7.4, containing 6.15 mM NaN_3 and 4.64 mM sodium 3-[trimethylsilyl] d4-propionate (Cambridge Isotope Laboratories). Using a modified second Gilson 215 liquid handler, 190 µl of each sample was transferred into 3 mm Bruker SampleJet NMR tubes. Subsequently, the tubes were sealed by POM ball insertion and transferred to the SampleJet autosampler where they were kept at 6°C while queued for acquisition.

2.3 | Nuclear magnetic resonance spectroscopy measurement and processing

All proton NMR (^{1}H-NMR) experiments were acquired on a 600-MHz Bruker Avance Neo spectrometer (Bruker Corporation) equipped with a 5-mm triple resonance inverse (TCI) cryogenic probe head with a Z-gradient system and automatic tuning and matching.

The NMR spectra were acquired following the Bruker B.I.Methods protocol. Before the measurements, a standard 3 mm sample of 99.8% methanol-d4 (Bruker) was used for temperature calibration.[30] A standard 3 mm QuantRefC sample (Bruker) was measured as the quantification reference and for quality control. All experiments were recorded at 310 K. The duration of the π/2 pulses was automatically calibrated for each sample using a homonuclear-gated nutation experiment on the locked and shimmed samples after automatic tuning and matching of the probe head.[31] For water suppression, presaturation of the water resonance with an effective field of γB1 = 25 Hz was applied during the relaxation delay and the mixing time of the NOESY1D experiment.[32]

The NOESY1D experiment was recorded using the first increment of a NOESY pulse sequence with a relaxation delay of 4 s and a mixing time of 10 ms.[33] After applying four dummy scans, 32 scans of 98 304 points covering a sweep width of 17 857 Hz were recorded.

The lipoprotein values were extracted from the NOESY1D plasma spectra by submitting the data to the commercial Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA) platform.[21–26] This approach extracts information about lipoproteins and lipoprotein subfractions in plasma. In the current study, we focused on the HDL particles; dissected into four subclasses (sorted according to increasing density and decreasing size; i.e. HDL-1, HDL-2, HDL-3 and HDL-4), component concentration and composition. Lipid content including total cholesterol, free cholesterol, phospholipids and triglycerides within total HDL and HDL subclasses represents the composition of HDL, called HDL main fractions and subfractions. The calculated esterified cholesterol was not included in the present study.

2.4 | Isolation of primary human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords obtained at the Leiden University Medical Centre after written informed consent was obtained and the umbilical cord was collected and processed anonymously. The umbilical cord was rinsed with phosphate-buffered saline (PBS) to remove any remaining blood. To detach the endothelial cells from the umbilical cord, trypsin/EDTA (BE17-161E; Lonza) was used. After 20 min of incubation at 37°C, the trypsin was inactivated with 20% fetal bovine serum in PBS, and the entire solution in the umbilical cord was collected. The umbilical vein was flushed with PBS one more time to ensure that all detached cells were collected before centrifugation at 300 g for 7 min. Cells were then cultured in 1% gelatin pre-coated flasks in endothelial growth medium (EGM2 medium, C-22011 supplemented with SupplementMix; Promocell) with 1% antibiotics (penicillin/streptomycin, 15 070 063; Gibco). We cultured HUVECs from two different donors (one boy and one girl). We pooled them together and froze them in several vials for future experiments once they were confluent.

2.5 | High-density lipoprotein isolation

Leftover plasma samples from ^{1}H NMR measurements were used to isolate HDL based on the protocol from a previous study by Mulder et al.[34] First, a 1:2 mixture of 36% polyethylene glycol (PEG 6000, 442 714 K; VWR International) in 10 mM HEPES (pH 8, H3375; Sigma-Aldrich) and plasma was prepared. Following that, samples were incubated for 30 min on ice to precipitate apolipoprotein B-containing lipoproteins, and centrifuged for 30 min at 2200 *g*. The HDL-containing supernatants were collected, kept at 4°C and used the following day for HDL functionality tests.

2.6 | High-density lipoprotein anti-thrombotic capacity

Primary HUVECs were used to test anti-thrombotic properties of HDL. In the 96-well plate, HUVECs (passage 3) were seeded at a density of 4×10^5 cells/well. The following day, HUVECs were pre-incubated for 30 min with 2% apolipoprotein B-depleted plasma or an equal volume of precipitation reagent in HEPES as a control. Tumour necrosis factor α (H8916; Sigma-Aldrich) was then added at a concentration of 10 ng/ml. After another 5 h of incubation, the cell surface was washed once with HBSS, no calcium and no magnesium (14170120; Gibco). Each well received 50 µl of normal pooled plasma before being placed in the fluorometer for a 10-min incubation at 37°C. The formation of thrombin was started by mixing 10 µl of the fluorogenic substrate with calcium (TS50.00 FluCa-kit; Thrombinoscope BV). The final reaction volume was 60 µl. Thrombin formation was measured every 20 s for 60 min and calibrated using Thrombinoscope software (Data S1, Figure S4a). To assess HDL anti-thrombotic capacity, endogenous thrombin potential (ETP) was measured. To limit potential variation because of different plate conditions, HDL anti-thrombotic capacity measurements were

performed at the same time using the same batch of pooled HUVECs and reagents. For each individual, measurements were taken in three technical replicates. To avoid batch effects, each plate contains samples from four groups.

2.7 | Statistical analyses

Differences in baseline characteristics were tested between four groups, namely DwC healthy controls (DwC-C), DwC-T2DM, DSA healthy controls (DSA-C) and DSA-T2DM. Categorical variables were expressed as n (%) and differences between groups were tested with the chi-squared test or Fisher's exact test. Normally distributed continuous variables were expressed as mean (SD), and differences were evaluated by post hoc tests of unpaired one-way ANOVA test; skewed continuous variables were presented as median (25-75th percentile) and differences were assessed using the Kruskal-Wallis test.

Based on ^{1}H NMR spectroscopy, a total of 112 lipoprotein main fractions and subfractions were extracted. To confirm the reliability of the NMR measurements, correlation analyses were performed between clinical routine measurements and corresponding parameters from NMR. We first extracted concentrations of all lipoprotein subclasses and performed a Pearson's correlation between clinical routine measurements [total triglycerides, total cholesterol, LDL-cholesterol (LDL-C), and HDL-C] and corresponding parameters from NMR.

Four groups (DSA-T2DM, DSA-C, DwC-T2DM and DwC-C) were considered as four outcomes, and concentrations of HDL main fractions and subfractions were scaled (z-normalization, i.e. with mean = 0 and SD = 1) to identify lipoproteins with different concentrations and multinomial logistic regression analysis (MLR) was used. We set a specific reference for each comparison: between DSA-T2DM and DSA-C, DSA-C was the reference; between DwC-T2DM and DwC-C, DwC-C was the reference; between DSA-T2DM and DwC-T2DM, DwC-T2DM was the reference; between DSA-C and DwC-C, DwC-C was the reference. The analyses were adjusted for several potential confounding factors. First, age (continuous variable), sex (dichotomous variable) and current smoking status (dichotomous variable) were adjusted for Model 1. Second, Model 1 was further adjusted for BMI (continuous variable; Model 2). Third, when comparing individuals with T2DM from two ethnic groups, we set the disease duration of healthy individuals as zero and adjusted the diabetes duration (continuous variable; Model 3). We considered a $p < .05$ as significant. The results were expressed as regression coefficient (β), and odds ratios with a 95% confidence interval to evaluate the differences between concentrations of HDL main fractions and subfractions and different groups. We followed the STROBE guidelines to report our findings.

Next, based on HDL main fractions and subfractions, supervised dimension reduction analysis called sparse partial least squares discriminant analysis (sPLS-DA) was performed by using the 'mixOmics' package in R.[35] sPLS-DA enables the selection of the most predictive or discriminative features in the data to classify the samples. Thus, by using this analysis, we aimed to rank and validate the features according to their contribution between DSA-T2DM and DSA-C, DwC-T2DM and DwC-C, DSA-T2DM, and DwC-T2DM, and DSA-C and DwC-C. We then compared the rank of features determined by sPLS-DA and those generated from multinomial logistic regression by using Spearman's correlation to validate further the findings. The contribution of loading features was calculated based on two components for each sPLS-DA model.

Pearson's correlation analyses were performed to reveal associations between differential HDL subfractions and laboratory markers including fasting levels of gamma-glutamyl transferase, haemoglobin A1c (HbA1c), and glucose and anthropometrics markers including visceral adipose tissue (VAT), waist circumference and waist-to-hip ratio.

We then investigated the correlation between disease duration and differential HDL subfractions, and further examined the associations between HDL subfractions and pan-microvascular-related complications, which were defined as composite diabetes-related complications including diabetic nephropathy, diabetic neuropathy and diabetic retinopathy. A T2DM participant with one or more of these complications was considered as having a pan-microvascular-related complication. This analysis was performed separately for DSA and DwC populations, and the Mann-Whitney-Wilcoxon test was used to assess the statistical differences between the cases (e.g. with pan-microvascular-related complications) and controls (e.g. without pan-microvascular-related complications) for each ethnic group. Statistical analyses were performed in R (version 4.1.0) and GraphPad Prism version 8 (GraphPad Inc.).

3 | RESULTS

3.1 | Clinical characteristics

After the consecutive exclusion of one type 1 diabetes mellitus individual from the MAGNA VICTORIA study, five individuals who withdrew from the RCT and three individuals with missing plasma samples, 92 individuals with T2DM were included. None of the healthy controls were excluded (Figure 1). In total, 143 individuals were included in the present study: 47 DSA-T2DM subjects (19 men/28 women), 21 DSA-C (nine men/15 women), 45 DwC-T2DM subjects (25 men/20 women) and 30 DwC-C (16 men/14 women) (Table 1).

Compared with DSA-C, systolic blood pressure, body surface area, BMI, waist circumference, waist-to-hip ratio, fasting glucose, HbA1c, triglyceride levels and VAT were higher in the DSA-T2DM group, while total cholesterol, HDL-C and LDL-C were lower (Table 1). In the DwC-T2DM group, body surface area, BMI, waist circumference, waist-to-hip ratio, total cholesterol, LDL-C, total body fat and subcutaneous adipose tissue were higher compared with DwC-C, together with a higher proportion of current smokers. Comparing individuals with T2DM between the ethnic groups, DSA subjects had a longer diabetes duration, a higher incidence of vascular-related

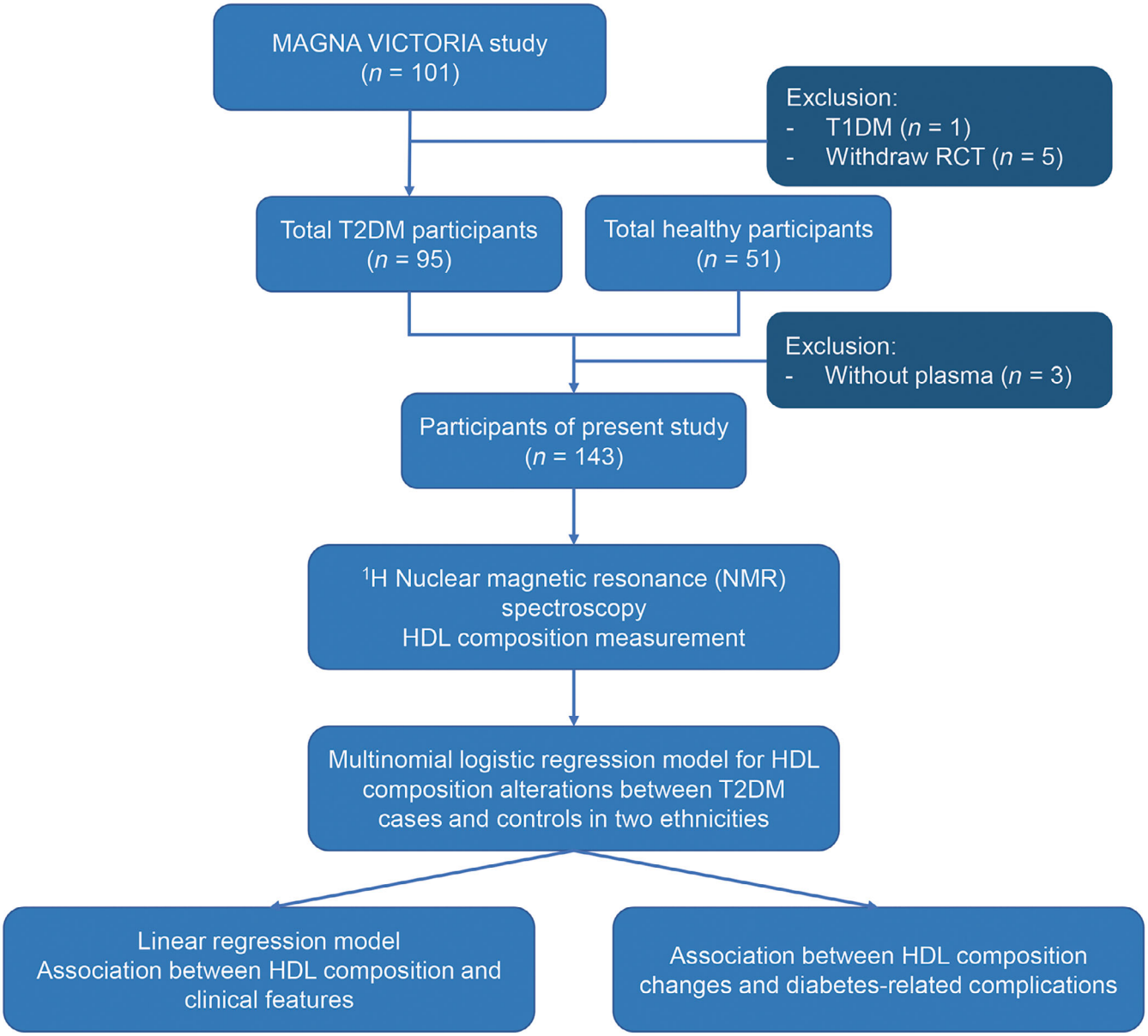

FIGURE 1 Flow chart of the study. HDL, high-density lipoprotein; T1DM, type 1 diabetes; T2DM, type 2 diabetes mellitus.

complications (e.g. retinopathy and macrovascular problems) and a higher albumin/creatine ratio compared with DwC subjects.

3.2 | Comparison between nuclear magnetic resonance-based results versus clinical chemistry approach

The NMR-based lipoprotein subclass measurements are shown in Figure 2A. To verify the quality of the NMR measurements we compared total triglycerides, total cholesterol, HDL-C and LDL-C with the clinical chemistry measurements revealing a high correlation in the total cohort ($R = 0.81\text{-}0.99$, $p < 0.00001$) as well as any single group (Data S1, Figure S1).

3.3 | Different high-density lipoprotein compositions between healthy and diabetic individuals in two ethnic groups

To identify differences in HDL composition between the various groups, 32 HDL main fractions and subfractions were tested (Data S1, Table S1) using multinomial logistic regression analyses. Comparing healthy and diabetic individuals in DSAs, we found that 14 HDL subfractions were different (five higher and nine lower) in DSA-T2DM compared with DSA-C (Figure 2B and Data S1, Table S2, Model 1); 21 HDL subfractions were different (four higher and 17 lower) in DwC-T2DM compared with DwC-C (Figure 2C and Data S1, Table S3, Model 1). After the adjustment of BMI (Model 2), only four HDL subfractions remained significant [apolipoprotein (Apo)A2

TABLE 1 Clinical characteristics of study participants

	Dutch South Asian			Dutch white Caucasian			Dutch South Asian T2DM versus Dutch white Caucasian T2DM
	Control (n = 21)	T2DM (n = 47)	*p*-value[a]	Control (n = 30)	T2DM (n = 45)	*p*-value[a]	*p*-value[b]
Demographics							
Age, years	48.3 ± 8.1	54.9 ± 10.1	.0367	57.9 ± 7.9	59.0 ± 6.5	NS	NS
Women, n (%)	15 (71.4)	28 (59.6)	NS	14 (46.7)	20 (44.4)	NS	NS
Current smoker, n (%)	3 (14.3)	7 (14.9)	NS	1 (3.3)	9 (20.0)	.0437	NS
Medical history diabetes							
Duration diabetes mellitus, years	-	17.9 ± 10	-	-	10.3 ± 6.0	-	<.0001
Nephropathy, n (%)	-	10 (21.3)	-	-	11 (23.4)	-	NS
Neuropathy, n (%)	-	14 (29.8)	-	-	15 (31.9)	-	NS
Retinopathy, n (%)	-	24 (51.1)	-	-	5 (10.6)	-	<.0001
Macrovascular, n (%)	-	13 (27.7)	-	-	2 (4.3)	-	.0037
Medication use, n (%)							
Metformin	-	45 (95.7)	-	-	45 (100)	-	NS
Sulphonylurea derivatives	-	8 (17.0)	-	-	13 (28.9)	-	NS
Insulin	-	36 (76.6)	-	-	29 (64.4)	-	NS
Antihypertensive medication	-	34 (72.3)	-	-	34 (75.6)	-	NS
ACE-inhibitors	-	13 (27.7)	-	-	17 (37.8)	-	NS
Statins	-	36 (76.6)	-	-	36 (80.0)	-	NS
Blood pressure							
Systolic blood pressure (mmHg)	123.5 ± 13.7	144.6 ± 21.5	.0003	126.2 ± 12.1	141.3 ± 15.0	.0007	NS
Diastolic blood pressure (mmHg)	80.2 ± 11.8	85.3 ± 10.0	NS	80 ± 77-83	86.9 ± 8.8	.0158	NS
Anthropometrics							
BSA, m^2	1.7 ± 0.2	1.9 ± 0.2	<.0001	1.9 ± 0.2	2.2 ± 0.2	<.0001	<.0001
BMI, kg/m^2	23.5 ± 3.0	29.5 ± 4.0	<.0001	24.3 ± 3.3	32.3 ± 3.9	<.0001	.0018
Waist circumference, cm	82.0 ± 7.4	101.0 ± 9.5	<.0001	86.6 ± 9.1	110.4 ± 8.9	<.0001	<.0001
Waist-to-hip ratio	0.9 ± 0.1	1 ± 0.1	<.0001	0.9 ± 0.1	1 ± 0.1	<.0001	.0039
Laboratory markers							
Fasting glucose, mmol/L	5.0 ± 0.3	8.1 ± 3.0	<.0001	5.2 ± 0.5	7.8 ± 2.1	−<.0001	NS
HbA1c, mmol/mol	35.5 ± 2.4	67.8 ± 11.3	<.0001	35.5 ± 2.7	64.9 ± 10.7	<.0001	NS
GGT, IU/L	-	28 (18-37.5)	-	-	32 (21-45)	-	NS
Total cholesterol, mmol/L	5.4 ± 0.8	4.2 ± 0.9	<.0001	5.7 ± 1.1	4.8 ± 1.0	.0018	.0226
HDL-cholesterol, mmol/L	1.6 ± 0.3	1.2 ± 0.3	.0028	1.9 ± 0.5	1.3 ± 0.3	<.0001	NS
LDL-cholesterol, mmol/L	3.4 ± 0.7	2.1 ± 0.8	<.0001	3.3 ± 1.0	2.6 ± 0.8	.0017	.0416

(Continues)

TABLE 1 (Continued)

	Dutch South Asian			Dutch white Caucasian			Dutch South Asian T2DM versus Dutch white Caucasian T2DM
	Control (n = 21)	T2DM (n = 47)	*p*-value[a]	Control (n = 30)	T2DM (n = 45)	*p*-value[a]	*p*-value[b]
Triglycerides, mmol/L	0.9 ± 0.3	1.8 ± 1.4	.0031	0.9 (0.7-1.2)	2.1 ± 1.3	<.0001	NS
Serum creatinine, μmol/ml	68.0 (60.0-79.0)	67.0 (59.0-83.5)	NS	73.0 (68.0-85.0)	68.0 (57.0-80.0)	NS	NS
Urinary markers							
Albumin/creatine ratio (mg/mmol)	-	2.7 (0.55-8.45)	-	-	0.7 (0-2.5)	-	.0037
Micro-albuminuria, n (%)[c]	-	15 (31.9)	-	-	7 (15.6)	-	-
Macro-albuminuria, n (%)[d]	-	7 (14.9)	-	-	1 (2.2)	-	-
Radiology based markers							
Total body fat, %	32.4 ± 7.1	37.1 ± 9.1	NS	32.5 ± 7.1	37.2 ± 9.3	<.0001	NS
VAT, cm^2	73.2 ± 29.8	166.4 ± 55.8	<.0001	74.7 ± 34.1	205.6 ± 75.6	<.0001	NS
SAT, cm^2	233.2 (195.9-258.8)	300.1 (228.3-371.4)	.0561	189.7 (148.8-238.6)	335.7 (262.4-419.5)	<.0001	NS

Note: Data are presented as mean ± SD, median (25-75 percentile), or percentage.
Note: Most of these data have been published before.[27–29]
Abbreviations: ACE, angiotensin-converting enzyme; BMI, body mass index; BSA, body surface area; GGT, gamma-glutamyl transferase; HbA1c, haemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.
[a]Post-hoc tests of unpaired one-way ANOVA, Kruskal-Wallis test or chi-squared test, $p < .05$.
[b]Post-hoc tests of unpaired one-way ANOVA or Kruskal-Wallis test, chi-squared test or Fisher's exact test, $p < .05$.
[c]Albumin-creatinine ratio between 3.0 and 30 mg/mmol.
[d]Albumin-creatinine ratio >30 mg/mmol.

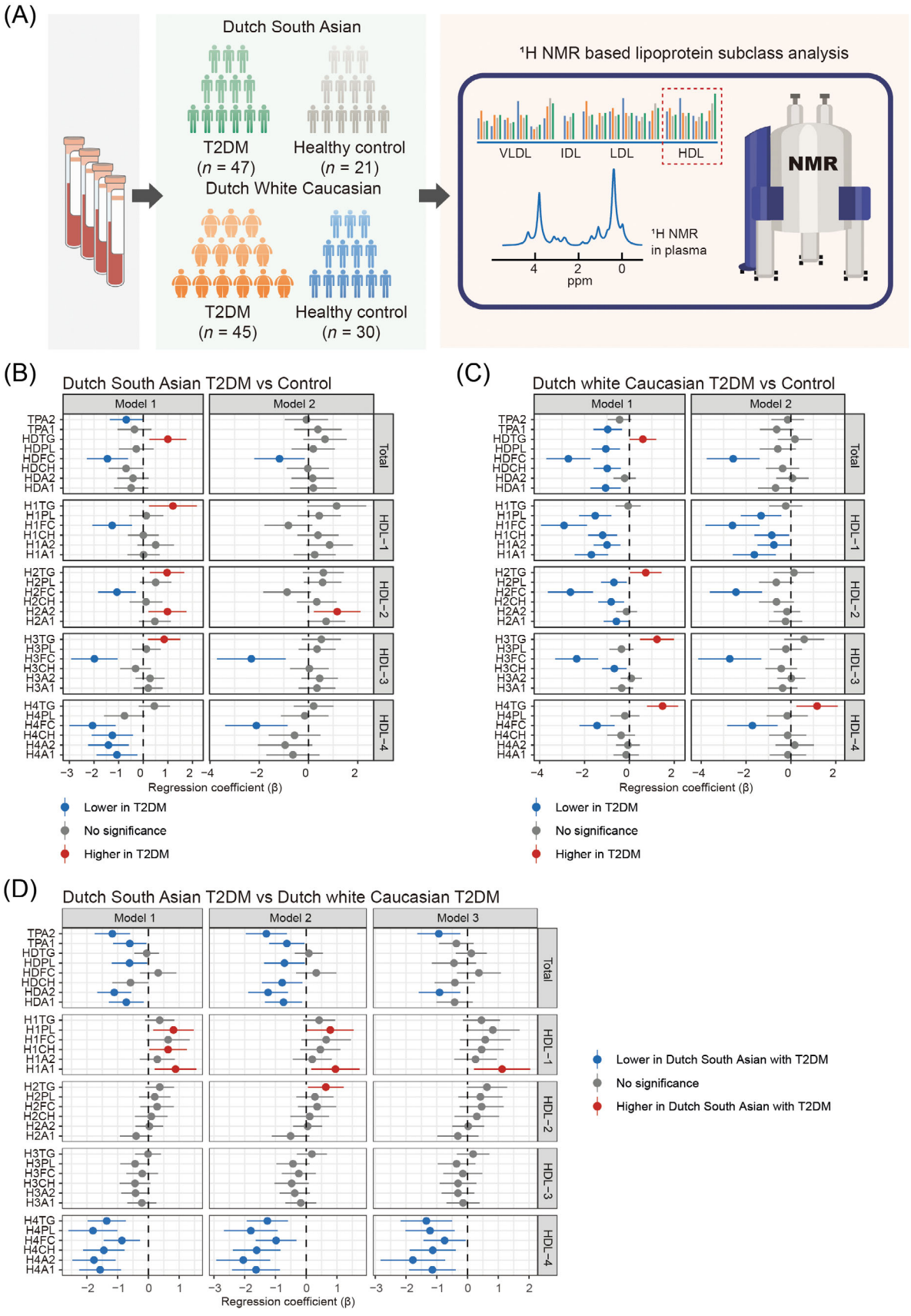

FIGURE 2 Legend on next page.

content in HDL-2 was higher and free cholesterol content in total HDL, HDL-3 and HDL-4 were lower] in DSA-T2DM (Figure 2B and Data S1, Table S2, Model 2); 10 HDL subfractions persisted (triglyceride content in HDL-4 was higher and HDL-1 subfractions except H1TG and free cholesterol content in HDL were lower) in DwC-T2DM (Figure 2C and Data S1, Table S3, Model 2). As multinomial logistic regression analysis was used to evaluate the association between HDL composition and the odds of having T2DM, the common trend in both ethnic groups showed that higher free cholesterol content in most HDL subclasses was associated with lower odds of having T2DM (Data S1, Tables S2 and S3). In particular, higher ApoA2 content in HDL-2 was associated with higher odds of having T2DM in DSA. In DwC, higher HDL-1 subfractions were associated with lower odds of having T2DM, and higher triglyceride content in the smallest and dense HDL (i.e. HDL-4) was associated with higher odds of having T2DM (Data S1, Tables S2 and S3).

Furthermore, sPLS-DA, a supervised machine learning method combining variable selection and classification in a one-step procedure was performed by using HDL-related features. A clear separation between T2DM and healthy controls was observed in both ethnicities, and the top five ranked features in DSA-T2DM were free cholesterol content in total HDL and subclasses (HDL-2, HDL-3 and HDL-4) and ApoA2 in HDL-4 (HDFC, H2FC, H3FC, H4FC and H4A2). Those in DwC-T2DM were free cholesterol content in total HDL and subclasses (HDL-1, HDL-2 and HDL-4) and ApoA1 in HDL1 (HDFC, H1FC, H2FC, H3FC and H1A1), (Data S1, Figure S2c-d). The ranks generated from MLR and sPLS-DA tightly correlated in both ethnic groups (Data S1, Figure S2e-f). Our findings revealed ethnic differences in HDL composition between healthy and diabetic individuals that were not detectable by routine lipid or laboratory assessments.

3.4 | High-density lipoprotein composition in plasma revealed ethnicity specificity in individuals with and without type 2 diabetes in two ethnic groups

When we compared T2DM subjects between DSA and DwC, notable differences were observed in HDL-4 subclass composition. Total ApoA1 and ApoA2, and phospholipid content in total HDL were lower in DSA-T2DM when compared with DwC-T2DM; whereas large HDL subclass composition such as ApoA1, cholesterol and phospholipid content in HDL-1 (H1A1, H1CH and H1PL) were higher in DSA-T2DM compared with DwC-T2DM (Data S1, Table S4 and Figure 2D, Model 1). After further adjustment for BMI and diabetes duration, the regression coefficient of differential HDL subfractions, albeit attenuated, remained different (Data S1, Table S4 and Figure 2D, Model 2 and Model 3), particularly the HDL-4 subclass composition and ApoA2 presence. Moreover, a significant difference in T2DM was observed between the two ethnic groups when using sPLS-DA (Figure 3A). Ranking HDL-related feature measurements by discriminating capability, we found that HDL-4 subclass composition and ApoA2 presence had the greatest contribution (Figure 3B). Notably, the rank generated from MLR and sPLS-DA highly correlated ($\rho = 0.97$, $p < 0.00001$, Figure 3C), which further validated our previous findings.

Considering the higher prevalence of T2DM in DSA, we also compared HDL composition among healthy individuals of both ethnicities and found that the majority of the HDL compounds were lower in DSAs, with only the triglyceride content in the HDL subclasses showing comparable distributions (Data S1, Table S5 and Figure S3a). Meanwhile, similar but less profound findings generated from sPLS-DA were found in healthy individuals between the two ethnic groups (Data S1, Figure S2b-d). These results suggested that ApoA2 and the HDL-4 subclass composition were different in T2DM and had ethnic specificity.

3.5 | High-density lipoprotein anti-thrombotic capacity reduction in type 2 diabetes in both ethnicities

Anti-thrombotic capacity was measured in 142 subjects to evaluate HDL functionality (47 DSA-T2DM, 21 DSA-C, 45 DwC-T2DM and 29 DwC-C). Based on the thrombin generation curve, diabetic plasma generated more thrombin than non-diabetic plasma samples in both ethnic groups (Data S1, Figure S4b,d). Furthermore, the endogenous thrombin potential was significantly higher in patients with T2DM versus healthy controls in both ethnicities (Data S1, Figure S4c,e), revealing that HDL functionality in anti-thrombin formation was impaired in both DSA and DwC with T2DM.

3.6 | Associations between differential high-density lipoprotein subfractions and clinical outcomes

We further investigated the associations between differential HDL subfractions (ApoA2 and HDL-4 subclass composition) and clinical

FIGURE 2 The ethnicity-specific distinction of HDL composition between DSA and DwCs based on ^{1}H NMR. (A) Scheme of ^{1}H NMR in the present study. (B) Forest plot of differential lipoprotein subfractions between DSA-C and DSA-T2DM. (C) Forest plot of differential lipoprotein subfractions between DwC-C and DwC-T2DM. (D) Forest plot of differential lipoprotein subfractions between DSA-T2DM and DwC-T2DM. The regression coefficient and 95% confidence interval were depicted by a horizontal line with a dot. A non-significant association was represented by the colour grey, a positive association was represented by the colour red, and a substantial negative association was represented by the colour blue. Model 1: adjusted age, gender and current smoking status; Model 2: Model 1 + BMI; Model 3: Model 2 + diabetes duration. A1, apolipoprotein A1; A2, apolipoprotein A2; CH, cholesterol; DSA, Dutch South Asians; DwC, Dutch white Caucasians; FC, free cholesterol; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL low-density lipoprotein; PL, phospholipid; T2DM; type 2 diabetes mellitus; TG, triglyceride; VLDL, very low-density lipoprotein. Abbreviations of lipoprotein main fractions and subfractions are shown in Table S1.

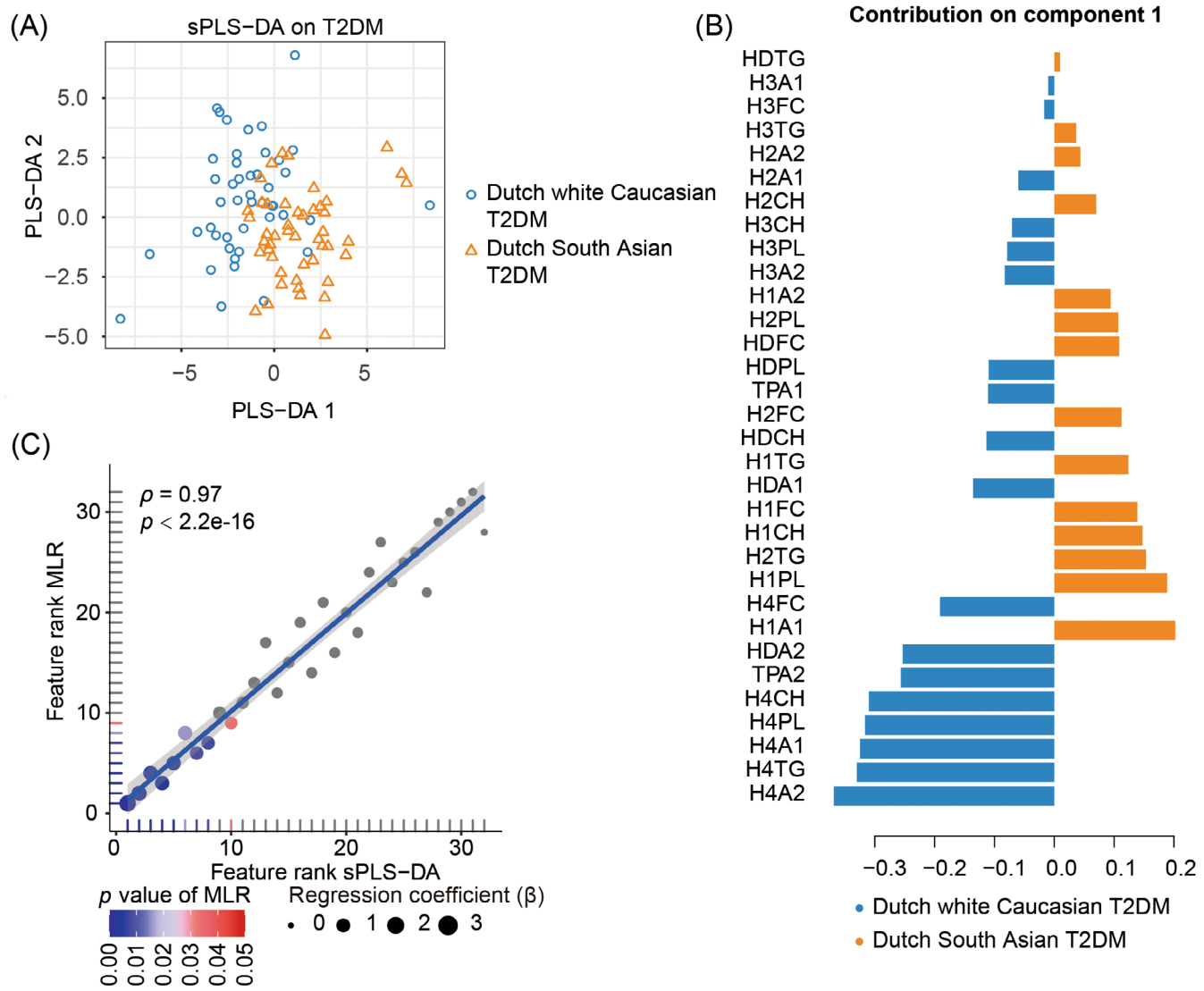

FIGURE 3 sPLS-DA differentiates DSA-T2DM from DwC-T2DM. (A) sPLS-DA performed on high-density lipoprotein main fractions and subfractions separates DSA-T2DM from DwC-T2DM. (B) Loading values of features with the contribution to differentiating DSA-T2DM from DwC-T2DM in the sPLS-DA. (C) Spearman's correlation between the rank generated from MLR and sPLS-DA. Dot colour represents the *p*-value of MLR, and dot size represents the absolute value of regression coefficient. DSA, Dutch South Asians; DwC, Dutch white Caucasians; MLR, multinomial logistic regression (analysis); sPLS-DA, sparse partial least squares discriminant analysis; T2DM; type 2 diabetes mellitus. Abbreviations of lipoprotein main fractions and subfractions are shown in Table S1.

outcomes (laboratory and anthropometric markers). In DSA-T2DM, except H4TG, the other lipid content in HDL-4 subclass negatively correlated with waist-to-hip ratio and waist circumference; ApoA1 and ApoA2, cholesterol and phospholipid content in HDL-4 (H4A1, H4A2, H4CH and H4PL) revealed a negative correlation with VAT; H4CH, H4FC and H4PL negatively correlated with HbA1c and H4FC and H4PL negatively correlated with fasting glucose. Interestingly, H4A1 and H4A2 positively correlated with gamma-glutamyl transferase (Data S1, Figure S5a).

In DwC-T2DM, TPA2 and HDA2 negatively correlated with waist-to-hip ratio; HDA2 and H4A2 correlated negatively with waist circumference while H4FC showed a negative correlation with fasting glucose levels (Data S1, Figure S5b). These results suggested that changes in HDL composition were clinically relevant, particularly in DSA-T2DM, which could partly reflect long-term dysregulated blood-glucose control.

3.7 | Associations between differential high-density lipoprotein subfractions and pan-microvascular-related complications

As a longer disease duration may lead to a higher incidence of complications (Figure 4A,B), we examined the correlation between HDL subfractions and diabetes duration in cases of both ethnicities. Notably, we discovered that ApoA2 and all HDL-4 subfractions except H4TG

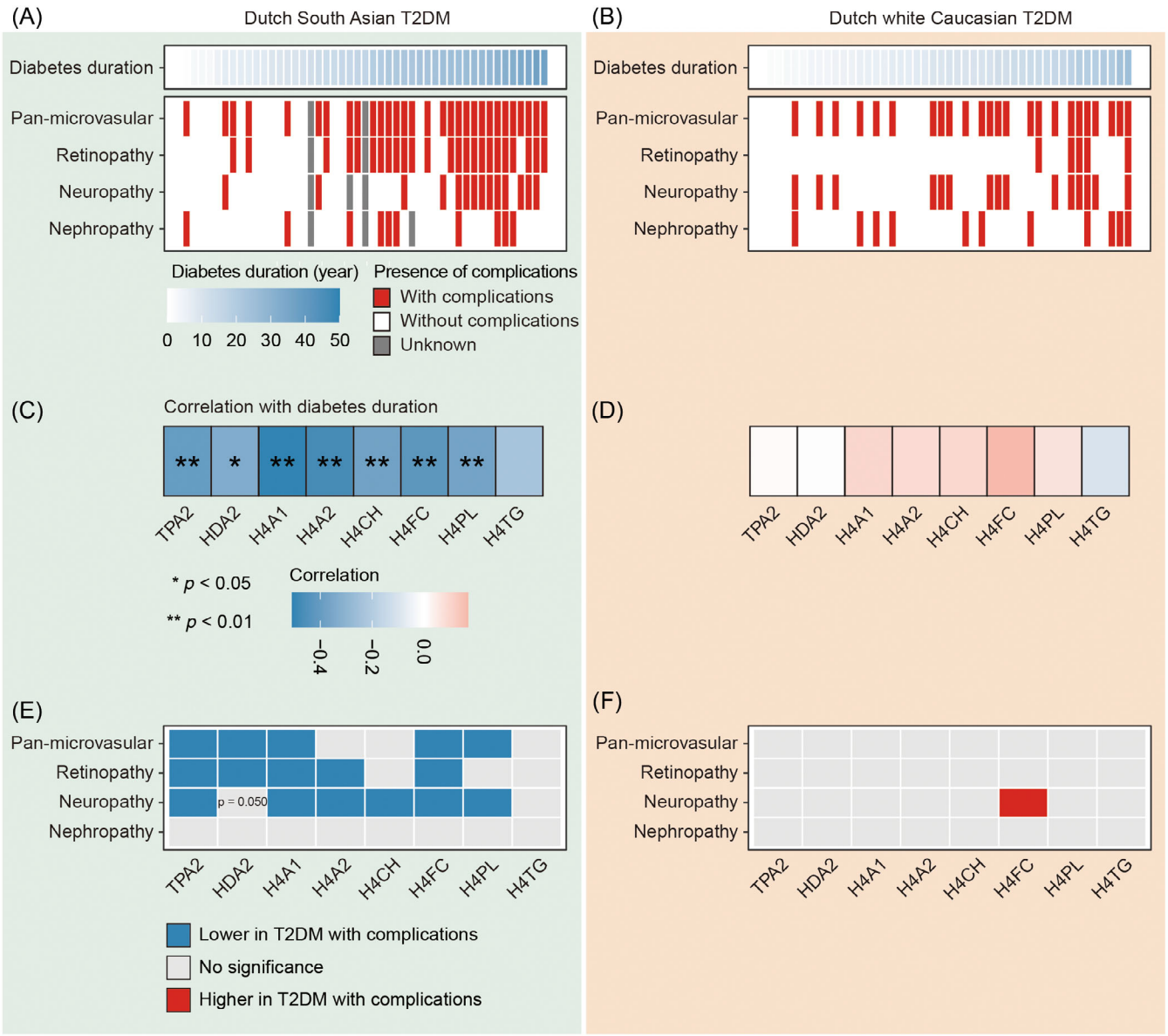

FIGURE 4 Associations between differential HDL subfractions and diabetes-related microvascular complications. (A) Association between disease duration and presence of diabetes-related microvascular complications in DSA-T2DM. (B) Association between disease duration and presence of diabetes-related microvascular complications in DwC-T2DM. (C) Pearson's correlation between diabetes duration and differential HDL subfractions in DSA-T2DM. (D) Correlation between diabetes duration and differential HDL subfractions in DwC-T2DM. Pearson's correlation analysis was performed. The colour of scale bar represented Pearson's correlation R. *p < .05; **p < .01. (E) Summary heatmap showing the associations between differential HDL subfractions and diabetes-related microvascular complications in DSA-T2DM. (F) Summary heatmap showing the associations between differential HDL subfractions and diabetes-related microvascular complications in DSA-T2DM. DSA, Dutch South Asians; DwC, Dutch white Caucasians; HDL, high-density lipoprotein; T2DM; type 2 diabetes mellitus. Abbreviations of lipoprotein main fractions and subfractions are shown in Table S1.

negatively associated with diabetes duration in DSA-T2DM while we did not find these associations in DwC-T2DM (Figure 4C,D).

Given glycaemic control is associated with multiple diabetes-related complications, particularly microvascular problems; we then investigated the associations between differential HDL subfractions (ApoA2 and HDL-4 subclass composition) and microvascular complications. Interestingly, there was no association between HDL subfractions and pan-microvascular-related complications in wC subjects with T2DM; while, in DSA-T2DM, we observed that total ApoA2, HDA2, H4A1, H4FC and H4PL were lower in patients with pan-microvascular-related complications. When we compared single complications, TPA2, HDA2, H4A1, H4A2 and H4FC were significantly lower in DSA-T2DM with retinopathy, while H4FC was higher in DwC-T2DM with retinopathy (Figure 4B). In DSA-T2DM, TPA2, H4A1, H4A2, H4CH, H4FC and H4PL were significantly lower in diabetic neuropathy while none of these HDL subfractions were associated with diabetic neuropathy in

DwC-T2DM (Figure 4E,F). None of the differential HDL subfractions changed between groups with and without diabetic nephropathy. These data suggest that changes in HDL composition, particularly TPA2 and HDL-4 subclass composition (except H4TG in DSA), were associated with diabetes-related microvascular complications such as neuropathy and retinopathy.

4 | DISCUSSION

In the present study, we observed HDL compositional differences between healthy and diabetic individuals in both ethnic groups. Specifically, we discovered that lower ApoA2 presence and HDL-4 subclass compositional changes in DSA individuals with T2DM were associated with a higher incidence of diabetes-related pan-microvascular complications such as retinopathy and neuropathy compared with DwC individuals with T2DM. These results indicated that differences in HDL composition might be used as an ethnicity-specific biomarker for DSA with T2DM and may provide a mechanism underlying the increased risk of microvascular complications in DSA.

Previous studies investigating HDL particle subspecies did not account for the HDL composition, particularly the lipid content, and the majority of these studies were conducted on white ethnic groups.[36,37] Only a single study showed HDL-2 and HDL-3 were associated with insulin resistance and beta cell function in SA at risk of T2DM.[38] In our study, we included both DSA and DwC populations and generated HDL main fractions and subfractions, including 32 features, providing a higher resolution of HDL lipoprotein fraction than HDL-C alone. sPLS-DA based on HDL composition could not only distinguish healthy controls from T2DM in both ethnic groups but also show the distinction between T2DM in these two ethnic groups. This suggested that this technique may be clinically meaningful beyond the measurement of HDL-C, particularly in different ethnicities.

We observed ethnicity-specific associations between HDL composition and T2DM and found a loss of large HDL subclass and higher triglycerides in the smallest HDL subclass in DwC with T2DM, consistent with the finding of the PREVEND study that HDL size is inversely associated with T2DM risk.[36] Interestingly, in DSA with T2DM, we observed an opposite trend that the lipid content in the smallest HDL subclass was specifically lower and triglycerides were higher in the largest HDL subclass when adjusting for age, gender and current smoking status. After BMI adjustment, only free cholesterol content in HDL persisted, suggesting that BMI plays a vital role in affecting HDL composition in DSA-T2DM and highlighting the importance of ethnic-specific guidelines for BMI. Furthermore, a randomized trail proved that a structured weight management programme incorporating a total diet replacement weight loss phase was acceptable to individuals of SA ethnicity and could achieve T2DM remissions similarly to other populations, indicating the favourable aspects of dietary weight management in this high-risk population.[39]

In our study, with individuals with T2DM of both ethnicities receiving similar medical care, we still discovered that total ApoA1 and ApoA2, as well as HDL-4 subclass composition, were significantly lower in DSA with T2DM than DwC with T2DM. In particular, small HDL particles played a role in cellular cholesterol efflux, and had anti-oxidative, antithrombotic, anti-inflammatory and antiapoptotic capacities.[40,41] These observations support our findings in DSA and we hypothesize that in DSA with T2DM, impaired HDL function might be explained by the loss of functional small HDL subfractions; while in DwC with T2DM, increased triglyceride content in small HDL subclass might lead to HDL dysfunction. Our in-house HDL functional assay revealed that individuals with T2DM in both ethnic groups had significantly reduced anti-thrombotic capacity because of impaired HDL function, which is also consistent with earlier findings showing that individuals with T2DM had impaired HDL function in terms of its capacity to suppress tumour necrosis factor-induced vascular cell adhesion molecule-1 expression in endothelial cells in vitro.[19] A previous study by Bakker et al. reported that in obese DSA, without T2DM, only the ability of HDL to prevent LDL oxidation was reduced in overweight DSA when compared with obese DwC (without T2DM).[25] Following these findings, we discovered that obese DSA with T2DM had a specific change in HDL composition, implying that T2DM could be an additional ethnicity-specific risk factor for cardiovascular disease.

HDL-4 subfractions in DSA with T2DM had the most clinical relevance, but not in DwC with T2DM. There were notable associations between HDL-4 subfractions with both waist circumference and waist-to-hip ratio, which were reported in the HELIUS study as critical predictors for T2DM regardless of ethnic background.[42] In addition, lipid content of HDL-4 was discovered to have associations with glycaemic control parameters, which were risk factors for diabetes progression.[43–45] In the current study, we also observed that HDL-4 subfractions negatively correlated with diabetes duration in DSA, and exhibited remarkable differences between patients with and without pan-microvascular complications, particularly neuropathy and retinopathy. In contrast, there was no link between HDL compositional differences and diabetes-related complications in DwC subjects with T2DM. This could indicate that changes in HDL composition occur because of hyperglycaemia and disease duration rather than as a driving factor associated with diabetes-related complications. HDL-related pharmacological strategies that have been tested involving HDL-mimetic peptides, showed remarkable effects on reducing inflammation, preventing oxidation and promoting cholesterol efflux.[46–48] We found that HDL-4 subfractions were associated with disease duration in DSA with T2DM, suggesting the necessity of future studies to test the HDL-mimetic peptide effects on T2DM in individuals of SA descent.

The strength of our study is that we measured the HDL composition in detail in two ethnic groups of diabetic and non-diabetic individuals. Our findings further indicated impaired HDL function in T2DM, linking it with diabetes-related complications. Meanwhile, we revealed ethnic differences in HDL composition. Of note, there are still several limitations to our study. First, our study is a cross-sectional study, which precludes statements on causality. Second, sample sizes are relatively small, which might limit generalization potential and preclude stratification analyses. Third, DSA with T2DM have a longer disease

duration than DwC with T2DM in our study, which may affect incidence of complications. However, DSA-T2DM showed much faster disease progression than DwC-T2DM, which is a typical ethnic hallmark; future studies with a larger sample size and multiple ethnicities are needed to verify our observations. Fourth, statin use could also affect HDL concentration, composition and function,[49,50] suggesting that differences in HDL composition between healthy controls and T2DM may not be solely because of diabetes status. Fifth, because of limited plasma availability, we were only able to perform an in-house HDL anti-thrombotic capacity assay, without performing cholesterol efflux capacity, anti-oxidation capacity or anti-inflammatory capacity measurements. Besides, we could not perform experiments to evaluate the anti-thrombotic capacity between large/buoyant HDL and small dense HDL. Sixth, the biological response of HUVECs is variable; therefore, our in-house assay is not comparable with clinical chemistry determinations.

5 | CONCLUSIONS

In conclusion, Dutch patients with T2DM of both wC and SA descent exhibited altered HDL composition when compared with healthy individuals, but revealed a distinct phenotype. In DSA subjects, lower ApoA2 and HDL-4 were associated with a higher incidence of diabetes-related complications such as retinopathy and neuropathy, suggesting that they could be used as ethnicity-specific biomarkers for patients with T2DM.

AUTHOR CONTRIBUTIONS

LY, MAG and BMvdB contributed to the study concept, design and analysis; LY analysed and interpreted data, and critically revised the manuscript; RL-G interpreted data and revised the manuscript; AV performed NMR measurements; HJvE and MBB collected data for the MAGNA VICTORIA studies, IMJ supervised the MAGNA VICTORIA studies, with HJL serving as study director; PCNR and MAG interpreted NMR data; and LY, TJR and BMvdB drafted the manuscript. The final manuscript was read, commented on and approved by all the authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

All data and methods supporting the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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