Thromboinflammation in high-risk human populations

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CHAPTER 4

Diacylglycerol abnormalities in diabetic nephropathy in Dutch South Asians and Dutch white Caucasians with type 2 diabetes mellitus: lipidomic phenotyping of plasma in a cross-sectional study

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Abstract

Objective: Type 2 diabetes mellitus (T2DM) confers a higher risk for complications in South Asian individuals as compared to other ethnic groups. It has been hypothesized that altered lipid metabolism may at least partly mediate this risk. Here we investigate lipidomic changes between Dutch South Asians (DSA) and Dutch white Caucasians (DwC) with and without T2DM and their association with clinical features.

Methods: Plasma samples were measured using the targeted quantitative LC/MS-based Shotgun Lipidomics Assistant (SLA) platform in a cross-sectional study, including 51 healthy participants (21 DSA, 30 DwC) and 92 participants with T2DM (47 DSA, 45 DwC), resulting in a comprehensive mapping of the circulating lipidome. Unbiased weighted correlation network analysis was used to identify clinically relevant lipid modules (lipid clusters associated with disease) and key mediatory lipids.

Results: In both the DSA and DwC populations, differences in lipidomic profiles (lipid classes and lipid species) between T2DM patients and healthy controls were found. DSA-T2DM lipid changes correlated to clinical features, particularly diacylglycerols (DGs), and associated with glycemic control and renal function. Furthermore, when compared to DwC controls, DSA controls already had a diabetes-prone lipid profile in their circulation at baseline.

Conclusions: This study demonstrates ethnic disparities in the circulating lipidomic profiles of T2DM patients and healthy controls. Our results revealed an ethnic distinction of lipid modules in relation to clinical outcomes. Additionally, we have identified specific diacylglycerols, particularly DG 18:1_18:2, as potential biomarkers for glycemic control and renal function in DSA-T2DM.

Keywords: Lipidomics, Dutch South Asian, Dutch white Caucasian, type 2 diabetes mellitus, diabetic nephropathy

Introduction

One of the major challenges to public health in the twenty-first century is the worldwide rise in type 2 diabetes mellitus (T2DM) prevalence. T2DM is characterized by insulin resistance and insufficient compensatory insulin secretion, the mechanism of which varies by ethnicity [1]. South Asians (SAs), as one of the high-risk populations, have a higher T2DM incidence than other ethnic groups [2]. As a result the South Asian (SA) population with T2DM tend to develop the disease at an earlier age, around 5-10 years ahead, and often with a lower body mass index (BMI), compared to white Caucasians (wC), thus revealing a distinct disease phenotype [2]. SAs possess a distinct body composition characterized by a higher prevalence of abdominal obesity and a larger proportion of visceral fat [3]. This unique phenotype contributes to the production and secretion of specific inflammatory cytokines, which can result in an elevated chronic low-grade inflammatory state and increasing the risk of developing T2DM among this population [4, 5]. Furthermore, SA patients with T2DM were found to be more prone to develop microvascular complications such as diabetic nephropathy (DN), as well as progressing to end-stage renal disease at a faster rate than Caucasian European patients with T2DM [6, 7].

While emerging lipidomic approaches generally revealed specific molecular lipid changes leading to T2DM (6-8), most of these studies were only performed in single ethnicity lacking differential information on T2DM development between different ethnic groups. Of note, a number of epidemiological studies highlighted the role of dyslipidemia in relation to the incidence of T2DM [8, 9], hinting that development of dyslipidemia may be a sign of future T2DM. Additionally, several studies found that dyslipidemia was associated with an increased risk of diabetes-related microvascular complications such as nephropathy, neuropathy, and retinopathy [10-12]. Given that dyslipidemia patterns differ by race/ethnicity [13] and may influence disease outcome [14], it suggests that lipid metabolism may play a vital role in ethnic differences in risk and progression of T2DM. In the present study, we measured lipidomic phenotypes in Dutch South Asian (DSA) and Dutch white Caucasian (DwC) participants, with or without T2DM, using the differential mobility mass

spectrometry (DMS/MS)-based Shotgun Lipidomics Assistant (SLA) platform [15]. Based on this platform, we sought to investigate differences in lipid class and lipid species correlating with disease risk and progression between these two ethnic groups.

Material and methods

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] [16] and [NCT02660047] [17], respectively), together with age and gender matched healthy controls from both ethnic groups [18]. The details of both trials can be found elsewhere [16, 17]. Both trials had the following inclusion criteria: BMI ≥ 23, age between 18 and 74 years, and HbA1c levels between 6.5% and 11.0% (≥ 47.5 and ≤ 96.4 mmol/mol). Patients were allowed to take specific glucose-lowering medication (metformin, sulfonylurea derivatives, or insulin) at a stable dosage for at least 3 months prior to participating in the study. They could also use antihypertensives and statins. Exclusion criteria included the use of glucose-lowering medication other than those specified, renal disease, congestive heart failure (NYHA class III-IV), uncontrolled hypertension (systolic blood pressure > 180 mm Hg and/or diastolic blood pressure > 110 mm Hg), or recent acute coronary or cerebrovascular events within 30 days before study enrolment. We excluded samples with missing plasma, diagnosed with T1DM, and individuals who withdraw from the randomized clinical trial. In total, 47 DSA with T2DM (DSA-T2DM, age 54.9 [SD: 10.1] years, 59.6% women, BMI: 29.5 [4.0] kg/m^2), 21 DSA healthy individuals (DSA-C, age 48.3 [SD: 8.1] years, 71.4% women, BMI: 23.5 [3.0] kg/m^2), 45 DwC with T2DM (DwC-T2DM, age 59.0 [SD: 6.5] years, 44.4% women, BMI: 32.3 [3.9] kg/m^2), and 30 DwC healthy individuals (DwC-C, age 57.9 [SD: 7.9] years, 46.7% women,

BMI: 24.3 [3.3] kg/m^2) were included. Ethnicity was based on the self-identified and self-reported biological parents' and ancestors' origins. Participants with complete informed consent were included. The study was conducted in accordance with the revised Helsinki Declaration, and the Institutional Review Board granted ethical approval (Leiden University Medical Center, Leiden, the Netherlands).

Lipidomics profiling using the SLA platform

Plasma samples were prepared according to Ghorasaini *et al* [19], and analyzed on the SLA platform (Figure 1). The SLA consists of a SCIEX QTRAP 5500 mass spectrometer with a SelexION differential mobility spectroscopy (DMS) interface and a Nexera X2 ultrahigh-performance liquid chromatography system that is controlled by the SLA software. Detailed protocols on its operation can be found elsewhere [15, 19].

Statistical analyses

For the SLA data pre-processing we first calculated the missing values per lipid class for all individuals per group (DSA-T2DM, DSA-C, DwC-T2DM, and DwC-C). Per lipid class, specific lipids with more than 30% missing values in each group were excluded. Missing values were imputed with half of the minimum concentration per lipid class (Figure S1 and Table S2).

Next, to determine the relative abundance of each lipid class, we performed a calculation by normalizing the concentration of each lipid class. This involved summing up the concentrations of all lipid species within each class and dividing it by the total concentration across all lipid classes. By applying this normalization process, a more accurate understanding of how each lipid class contributes to the overall lipid composition is obtained and allows to clearly assess the proportional representation of different lipid classes. Subsequently, principle component analysis (PCA) and hierarchical cluster analysis

(HCA) were performed in all participants in both ethnicities based on relative lipid class abundance. Differences in relative lipid class abundance between healthy controls and T2DM, as well as between healthy individuals and T2DM from two ethnic groups were examined.

Multinomial logistic regression analysis (MLR) was used to differentiate the various specific lipids. The four groups were considered as outcomes (DSA-T2DM, DSA-C, DwC-T2DM, and DwC-C). The lipid concentrations were scaled (z-score normalization). When comparing DSA-T2DM to DSA-C, we used DSA-C as reference, and when comparing DWC-T2DM to DwC-C, we used DwC-C as reference. Age (continuous variable), sex (dichotomous variable), and current smoking status (dichotomous variable) were adjusted for the complete model. Multiple testing corrections were used, with a false discovery rate (FDR) of 0.05 considered significant. To assess the relationship between lipid concentrations and T2DM, the results were expressed as a regression coefficient (β) with a 95% confidence interval (CI).

For weighted correlation network analysis, the "WGCNA" R package was used to investigate the role of lipid species in association with observed clinical features [20]. Using this algorithm, a proper soft threshold was first chosen and lipids with similar concentration patterns could be grouped into multiple modules, each of which was linked to a concomitant clinical feature. These modules were tagged with colour codes. This makes it possible to identify some clinically relevant lipids within potential lipid modules in relation to respective clinical features. Diabetes-related complications-associated lipid modules were considered key modules in this study. Key mediatory lipids that correspond to the clinical parameters were derived from key lipid modules and the differentiated lipids (between the various groups, Figure S4). Pearson's correlation analysis was used to determine the relationship between those key mediatory lipids and clinical parameters related to dyslipidaemia, kidney function, and glycemic control.

To validate our observations, we used a published external dataset of Chinese IgA nephropathy patients to investigate the relation between commonly changed lipids and

renal function [21]. To this end, we first examined the changes in these lipids between healthy controls and IgA nephropathy patients. Next, the relationship between these lipids and kidney function parameters was determined using Pearson's correlation analysis.

For each ethnic group, the Wilcoxon signed-rank test was used to assess the statistical differences between cases (i.e., those with diabetes-related complications) and controls (i.e., those without diabetes-related complications). R (version 4.1.0) and GraphPad Prism version 8 (Graphpad Inc., La Jolla, CA, USA) were used for statistical analysis.

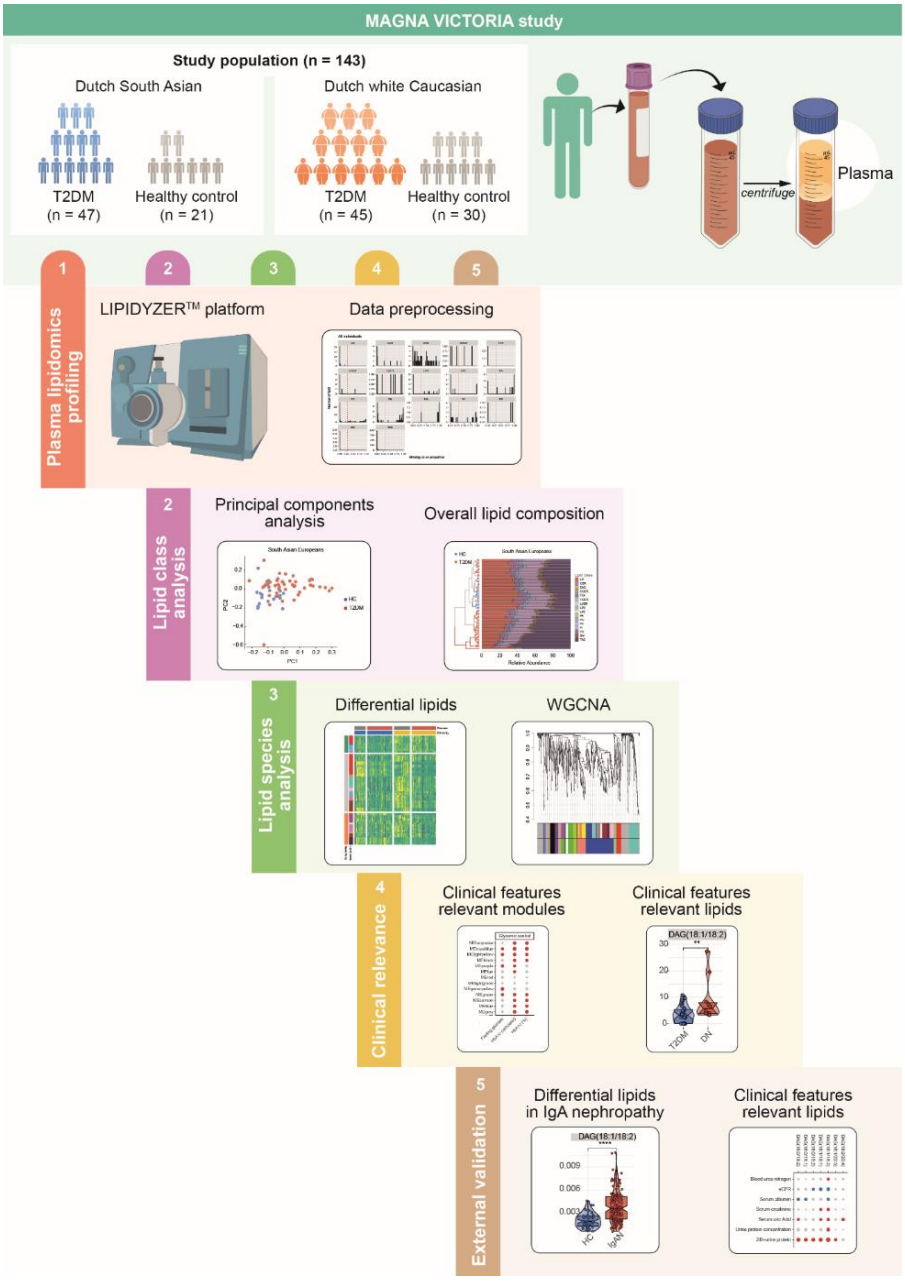

Fig. 1. Study workflow and design.

Abbreviations: *T2DM* type 2 diabetes mellitus; *WGCNA* Weighted Correlation Network Analysis

Results

Pre-processing of plasma lipidome profiles of individuals with T2DM vs. healthy participants

Targeted lipidomic analysis quantified lipids from 17 different lipid classes (Figure 1). After exclusion of lipids with 30% missing values, we distinguished 689, 686, 679, 699, and 668 lipids in DSA-T2DM, DSA-C, DwC-T2DM, and DwC-C, respectively (Figure S1), of which 654 common lipid species across lipid classes (CE, cholesteryl ester; CER [Cer d18:1/FA], ceramide; DG, diacylglyceride; DCER [Cer d18:0/FA], dihydroceramide; FA, fatty acid; HexCER, hydroxyceramide; LacCER, lactosylceramide; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TG, triglyceride) were chosen for further analysis (Figure S1 and Table S2).

Healthy individuals of Dutch South Asian ethnicity reveal a pre-diabetes lipid class profile

We first applied PCA analysis to identify clusters of subjects based upon similarities in their relative abundance of lipid classes in both ethnic groups, regardless of their prespecified group. Although the distinction was not perfect, we observed that relative lipid class abundance had better power to distinguish patients with T2DM from healthy controls in DwC than in DSA (Figure S2A, B). Hierarchical cluster analysis revealed that in DSA, all subjects were clustered into two main subclusters, one with patients with T2DM only and the other with patients with T2DM and healthy individuals; whereas in DwC, most of the healthy subjects were clustered together and separated from patients with T2DM (Figure 2). Between DSA-T2DM and DSA-C, 10 lipid classes (9 lower and 1 higher), and between DwC-T2DM and DwC-C, 11 lipid classes (10 lower and 1 higher) were changed significantly (Figure S2C, D). When DSA-T2DM and DwC-T2DM were compared, the DG lipid class was found to be more abundant, with the greatest relative difference, in DSA with T2DM than

in DwC with T2DM (Figure S2E). Similarly, we also found that the abundance of DG lipid class was higher in DSA than DwC among healthy individuals (Figure S2F). These findings indicated that based on lipid class abundance, DSA-C already had a phenotype more closely related to DSA-T2DM; there were marginal differences in lipid class abundance between T2DM in the two ethnic groups.

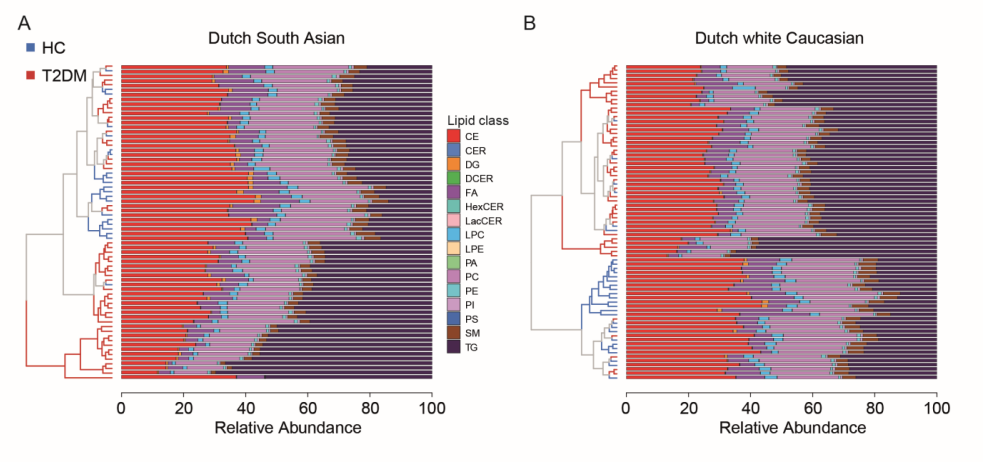

Fig. 2. Lipid class abundance between patients with T2DM and healthy controls. (A) Stack plot with the hierarchical cluster in Dutch South Asian. (B) Stack plot with the hierarchical cluster in Dutch white Caucasian.

Abbreviations: *CE* cholesteryl ester; *CER* ceramide; *DCER* dihydroceramide; *DG* diacylglyceride; *FA* fatty acid; *HC* healthy control; *HexCER* hydroxyceramide; *LacCER* lactosylceramide; *LPC* lysophosphatidylcholine; *LPE* lysophosphatidylethanolamine; *PA* phosphatidic acid; *PC* phosphatidylcholine; *PE* phosphatidylethanolamine; *PI* phosphatidylinositol; *PS* phosphatidylserine; *SM* sphingomyelin; *T2DM* type 2 diabetes mellitus; *TG* triglyceride.

Comparison of differential lipids between patients with T2DM and healthy controls in two ethnicities

After multinomial logistic regression analyses and multiple testing corrections, we found 436 differential lipids (396 higher and 40 lower) in DSA-T2DM compared to DSA-C (Figure 3A and Table S3); 519 differential lipids (471 higher and 48 lower) in DwC-T2DM compared to DwC-C (Figure 3A and Table S4). To further investigate the significance of each lipid change between two ethnicities, we compared the regression coefficients (T2DM vs healthy controls) and discovered that lipids from the DGs and TGs classes in DSA showed higher regression coefficients than those in DwC, while the CEs in DwC showed lower regression coefficients; the remaining lipids behaved similarly (Figure 3A).

We found 9 lipids that were specifically lower in DSA-T2DM (mostly from the CEs and LPCs) and 17 lipids that were specifically lower in DwC-T2DM (including lipids from the FAs, PCs, SMs, and TGs) (Figure 3B, D, and Table S5). Furthermore, 13 lipids were specifically higher in DSA-T2DM (primarily from the DGs, PEs, and TGs), while 88 lipids were specifically higher in DwC-T2DM (primarily from the CEs, DGs, PCs, PEs, and TGs) (Figure 3C, E and Table S5). These findings indicate that different lipid metabolism phenotypes were found in both ethnicities, and differential lipids, particularly DGs and TGs, contributed more to the risk of T2DM in DSA.

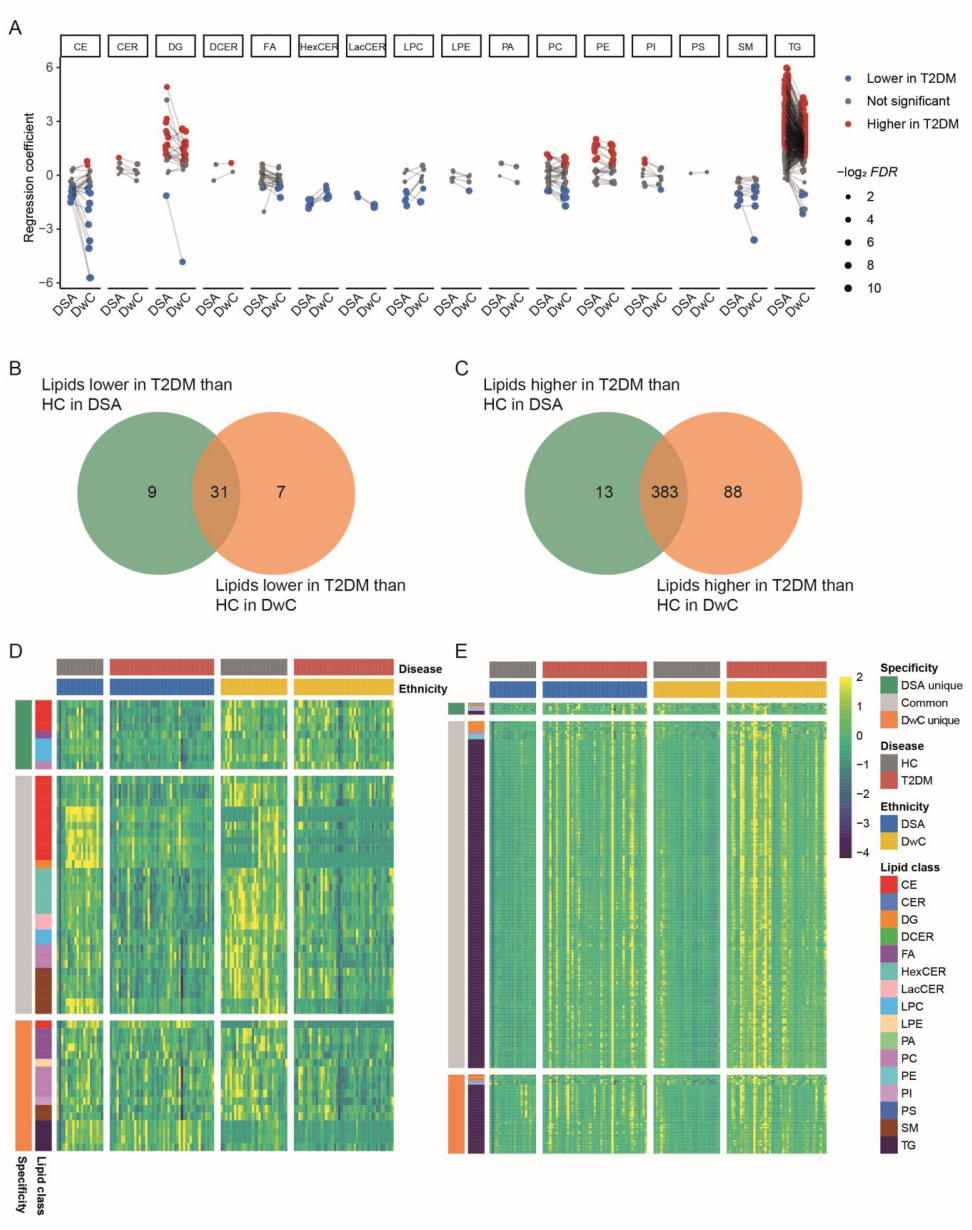

Fig. 3. Comparison of differential lipids between patients with T2DM and healthy controls in two ethnicities. (A) Differential lipids per lipid class between patients with T2DM and healthy controls, as well as a comparison across two ethnicities. The color grey represents

lipids with no significance (*FDR* > 0.05), the color blue represents lipids lower in T2DM, and the color red represents lipids higher in T2DM. The dot size represents -$\log_2$*FDR*. Venn diagram of (B) lipids lower in T2DM and (C) higher in T2DM than healthy controls (HC) in DSA and DwC. Heatmap of lipids that are commonly/uncommonly (D) lower and (E) higher in DSA and DwC with T2DM.

Abbreviations: *CE* cholesteryl ester; *CER* ceramide; *DCER* dihydroceramide; *DG* diacylglyceride; *DwC* Dutch white Caucasian; *DSA* Dutch South Asian; *FA* fatty acid; *FDR* false discovery rate; *HexCER* hydroxyceramide; *LacCER* lactosylceramide; *LPC* lysophosphatidylcholine; *LPE* lysophosphatidylethanolamine; *PA* phosphatidic acid; *PC* phosphatidylcholine; *PE* phosphatidylethanolamine; *PI* phosphatidylinositol; *PS* phosphatidylserine; *SM* sphingomyelin; *TG* triglyceride.

Ethnic distinction in associations of lipid correlation network modules with clinical features

To identify highly connected lipid modules and the relevance between baseline clinical traits and each lipid module, we performed a weighted correlation network analysis (WGCNA, Figure S3). Except for the grey module, which corresponded to the set of lipids that were not clustered in any module, the other lipids in both ethnicities were clustered into 12 modules.

Total TG concentration measured on the SLA platform matched the clinical routine measurements. In both ethnic groups, we did observe lipid modules consisting of TG species that had a positive correlation with total TG concentration (Figure 4). Surprisingly, there were noticeable differences between the two ethnic groups. In DSA-T2DM, the lipid modules were positively correlated with glycemic control parameters, and negatively correlated with HDL-cholesterol (Figure 4A). While in DwC-T2DM, the lipid modules showed positive correlations with anthropometric parameters, total cholesterol, and LDL-cholesterol and negative correlations with blood pressure and kidney function (Figure 4B). We also found several modules associated with diabetes-related complications. In DSA-T2DM, the ‘royal blue’ module, as only module, was correlated with diabetic nephropathy

(DN); whereas in DwC-T2DM, the 'light green', 'black', and 'grey60' modules were associated with diabetic retinopathy (DR) and DN, respectively (Figure 4).

By combining lipids in diabetes-related-complications modules with differential lipids, we identified 7 lipids from the DG class (two lipids showed ethnicity-specific difference) in DSA and 5 lipids from the CEs, TGs, and DG classes in DwC (Figure S4), which we considered as key mediatory lipids. Our findings revealed ethnic differences in the associations between lipid modules and clinical features, particularly in DSA-T2DM, where lipid modules were correlated with high TGs, low HDL-cholesterol, and poor glycemic control.

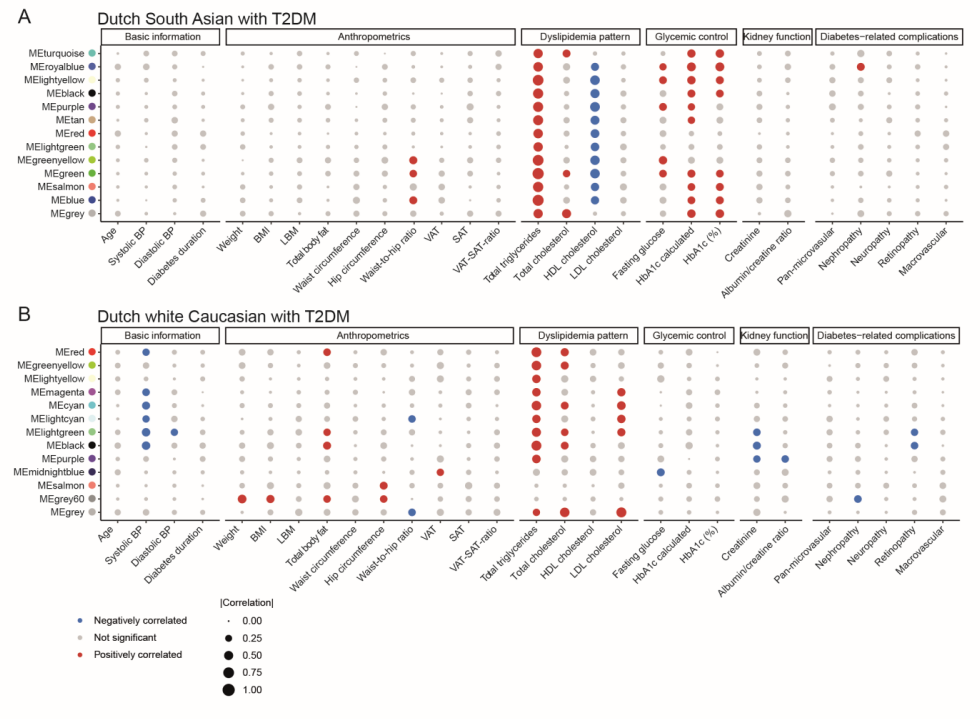

Fig. 4. Association of lipid correlation network modules with clinical features in (A) Dutch South Asians with T2DM and (B) Dutch white Caucasians with T2DM. The color grey denotes a lipid cluster with no significant associations with clinical features, the color blue denotes a lipid cluster with a negative association with clinical features, and the color red

denotes a lipid cluster with a positive association with clinical features. The correlation coefficients are represented by the size of the dots (Spearman's rank correlation test).

Abbreviations: *BP* blood pressure; *BMI* body mass index; *HbA1c* hemoglobin A1c; *HDL* high-density lipoprotein; *LBM* lean body mass; *LDL* low-density lipoprotein; *SAT* subcutaneous adipose tissue; *VAT* visceral adipose tissue.

Clinical relevance screening for key mediatory lipids from two ethnicities

The key mediatory lipids of DSA were first investigated in relation to clinical features such as dyslipidaemia, kidney function, and glycemic control parameters in both ethnicities. These lipids correlated positively with total TGs, total cholesterol, albumin/creatine ratio, and HbA1c in DSA-T2DM, but negatively with HDL-cholesterol and LDL-cholesterol (Figure 5A). Since these lipids were derived from a DN-related module, we next compared the lipid concentrations in patients with and without DN. All the lipids, except DG 18:2_20:4, were higher in DN than T2DM in DSA (Figure 5B). However, we found only a few correlations between these lipids and LDL-cholesterol in DwC-T2DM (Figure 5C). In DwC, between T2DM and DN, those lipids exhibited the opposite behaviour (Figure 5D).

Key mediatory lipids derived from modules in DwC were then examined. Only limited correlations with clinical parameters could be observed in both ethnic groups (Figure S4A, C). None of them showed associations with DR or DN in either ethnicity (Figure S4B, D). These findings suggested that DGs were more strongly associated with DSA-T2DM than DwC-T2DM and with DN and kidney function.

We finally investigated key mediatory lipids of DSA in an external Chinese cohort of patients with IgA nephropathy and found that they were all higher in patients with IgA nephropathy than in healthy controls, with DG 18:1_18:2 showing the strongest correlation with renal function parameters (Figure S5).

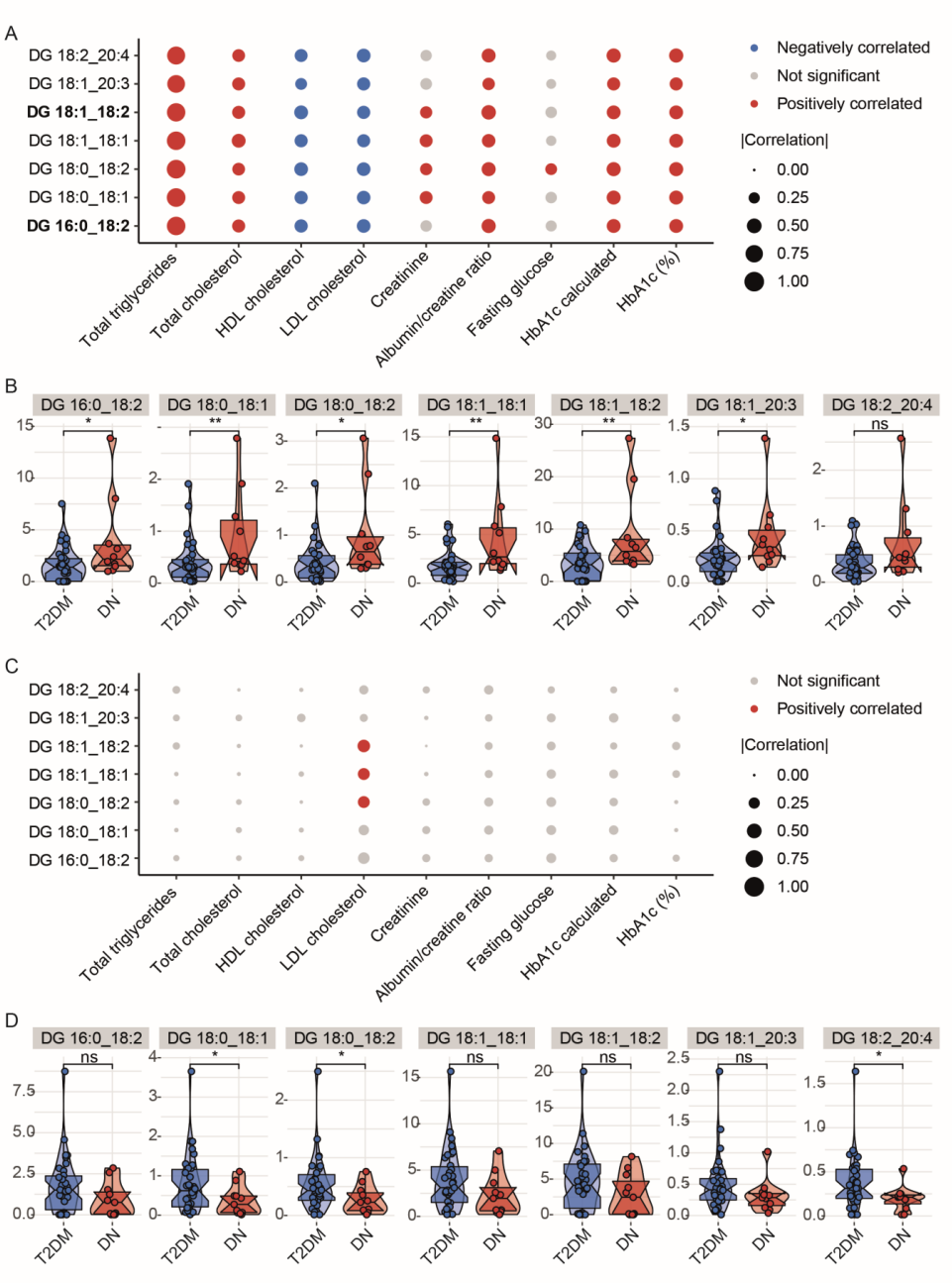

Fig. 5. Correlations between key mediatory lipids in diabetic nephropathy-associated module of Dutch South Asians and lipoproteins, kidney function, and glycemic control. (A)

Bubble plot depicting the correlations of lipids with lipoproteins, kidney function, and glycemic control in Dutch South Asians with T2DM. (B) Violin plots of lipids between T2DM with and without diabetic nephropathy in Dutch South Asians. (C) Bubble plot depicting the correlations of lipids with lipoproteins, kidney function, and glycemic control in Dutch white Caucasians with T2DM. (D) Violin plots of lipids between T2DM with and without DN in Dutch white Caucasians. Lipids in bold indicated that they were specifically different in Dutch South Asians. The color grey indicates no significant correlations with clinical features; the color blue indicates a negative correlation with clinical features, and the color red indicates a positive correlation with clinical features. The size of the dots represents the correlation coefficients (Pearson's correlation). The Wilcoxon signed-rank test was performed; *p<0.05, **p<0.01.

Abbreviations: *DG* diacylglyceride; *DN* diabetic nephropathy; *HbA1c* hemoglobin A1c; *HDL* high-density lipoprotein; *LDL* low-density lipoprotein; *T2DM* type 2 diabetes mellitus.

Discussion

In the current lipidomic phenotyping study, we discovered differences in lipid classes and lipid species between patients with T2DM and healthy individuals in both the Dutch South Asian (DSA) and Dutch white Caucasian (DwC) populations. Specifically, lipid changes in individuals with T2DM of DSA were found to be more strongly associated with clinical parameters than DwC, with diacylglycerols (DGs) showing strong associations to diabetic nephropathy and renal function. Furthermore, we observed that healthy DSA individuals already had a diabetes-prone lipid distribution. These findings imply that impaired DG metabolism in DSA could be a potential hallmark and that lipidomic phenotyping could provide detailed insights into lipid metabolic complexity and interindividual variations among T2DM patients of various ethnic groups.

Previous studies suggested that SAs may have a lower ability to secrete insulin, lower muscle mass and a higher ectopic fat deposition which contributed to the higher T2DM prevalence [3, 22]. In the current study, it is worth noting that healthy DSA individuals already revealed a diabetic lipid distribution, which partly could predict the higher risk in developing T2DM in this population. Additionally, our study revealed remarkable differences in lipidomic profiles between both ethnic groups, lending credence to previously established

associations between T2DM and dysregulated lipoprotein composition using ^{1}H NMR lipoprotein profiling [23]. Our results demonstrate distinct differences in the lipidome between patients with T2DM and healthy controls, mainly related to CE, DG, PE, SM, and TG metabolism. This was consistent with previous findings observed in the case-cohort study nested within the PREDIMED trial [24] and the longitudinal METSIM study [25]. However, conflicting results were reported in two studies based on Chinese populations [26, 27]; for instance, compared to healthy controls, FFA, SM and LPC lipid species were higher in Chinese patients with T2DM, whereas we found opposite results in Dutch patients with T2DM, further highlighting the variability in lipidomics profile between ethnicities.

The comprehensive analysis for lipidomics profiling performed in the current study allows for testing clinically relevance. As a hallmark of T2DM, insulin resistance affects regulation of lipid and lipoprotein metabolism [28, 29]. In line with previous studies, we found that lipid modules in DSA-T2DM positively correlated with TG, total cholesterol and negatively correlated with HDL-C; whereas lipid modules in DwC-T2DM correlated with total TG, cholesterol, and LDL-C, suggesting an ethnic preference in correlation with dyslipidemia patterns. Insulin resistance also impairs glucose metabolism and TG metabolism [30-32]. Interestingly, we discovered ethnicity differences in lipid modules (mainly consisting of TGs and DGs) demonstrating a correlation with both short- and long-term glycemic control exclusively in DSA-T2DM, rather than in the DwC-T2DM population. Also, our observation that certain lipid modules correlated with DN in both ethnic groups was in line with the reported dyslipidemia as a hallmark of chronic kidney disease (CKD) [33]. Moreover, a previous study revealed that patients with CKD had abnormalities in glycerolipid metabolism such as monoradylglycerolipids (MG), DGs, and TGs [34], which is also consistent with our findings in DSA-T2DM. However, we did not observe these associations in DwC-T2DM; one possible hypothesis might be a shorter duration in diabetes, which resulted in the more excessive changes in lipid metabolism.

By combining lipid abundance and lipid species analysis, we have identified a specific lipid class, DG, which contributes to the increased risk in development and progression of T2DM

among SAs. DG is derived from lipoprotein lipase (LPL)-mediated hydrolysis of TGs, and our observations reveal low DG abundance alongside high TG levels in T2DM in both ethnicities. However, DG lipid class abundance was much higher in DSA than DwC in both healthy and diabetic individuals, hinting to possible lipolysis dysregulation in SAs. Insulin resistance, a crucial factor in T2DM development, has been found to be higher in SAs than in wCs [35, 36]. LPL has been associated with insulin resistance [37, 38], and this could potentially explain the higher proportion of DG observed in our study, as higher insulin resistance in SAs impairs the ability of insulin to suppress lipolysis, leading to an increased release of fatty acids that are subsequently converted to DGs. Previous research has reported the impact of DGs on hepatic insulin resistance. Increased levels of DGs are commonly observed in animal models of lipid-induced hepatic insulin resistance [39, 40]. Furthermore, several human studies have demonstrated significant associations between total hepatic DG content or specific DG species and insulin resistance markers, such as homeostasis model assessment-estimated insulin resistance (HOMA-IR) [41-44]. These associations were found to be stronger than those observed with variables like body mass index, ceramide content, and markers of endoplasmic reticulum stress [41]. Interestingly, DGs and their targets protein kinase C (PKC) and protein kinase D (PKD) have been shown to regulate multiple critical cellular responses [45], which might be a plausible mechanism for inhibition of insulin signalling leading to hepatic insulin resistance. Once DG accumulates, it could lead to hyperactivation of PKC/PKD and play an important role in development of diabetic nephropathy [46, 47]. Our observation that DG metabolism in the circulation was disturbed, with a higher correlation to clinical outcomes, may argue that a dysregulated DG-PKC/PKD signalling network could disrupt the redox balance and lead to more oxidative stress [47], and in part could explain why DSA-T2DM patients are more vulnerable to diabetic nephropathy progression.

The strength of our study is that we measured detailed lipidomic profiles in two ethnic groups of diabetic and healthy individuals. Our findings confirmed lipidomic perturbations in patients with T2DM in both ethnic groups; meanwhile, we revealed an ethnic distinction of lipid modules in relation to clinical outcomes (e.g., glycemic control). Notably, there are

still several limitations to our study. First, our study is a cross-sectional study; therefore, we cannot address issues of causality in the association of T2DM. Second, the relatively small sample size limits the power of generalization and precludes stratification analyses. Third, as waist-to-hip ratio was found to be the most reliable predictor of T2DM in the HELIUS study, regardless of ethnicity [48], lack of a WHR matching design might be a shortcoming in our study. Fourth, oxidized lipids induced by oxidative stress play a critical role in the development and progression of T2DM [49, 50], however, we were not able to detect high-throughput oxidized lipids by using this platform. Fifth, for renal function validation we only used an external cohort of patients with IgA nephropathy instead of diabetic nephropathy, and in a singular ethnic group. Therefore, further longitudinal studies with multiple ethnic groups and larger sample sizes are needed to verify our findings.

Conclusions

In conclusion, Dutch patients with T2DM of both white Caucasian and South Asian descent exhibited altered circulating lipidomes when compared to healthy individuals of the same ethnicity. In DSA the lipid changes of especially DGs, were clinically more relevant than in DwC. These DGs, particularly DG 18:1_18:2, were associated with glycemic control and renal function in DSA patients with T2DM and Chinese patients with IgA nephropathy (validation cohort). These observations suggest that they could be used as ethnicity-specific biomarkers for diabetic nephropathy patients. In addition, lipidomics phenotyping provides detailed insight into lipid metabolic complexity and interindividual variations among patients with T2DM from various ethnic groups.

Abbreviations

ACE: Angiotensin-converting enzyme; BMI: body mass index; BP: blood pressure; CE: cholesteryl ester; CER: ceramide; CI: confidence interval; CKD: chronic kidney disease; DCER: dihydroceramide; DG: diacylglyceride; DMS: differential mobility mass spectrometry; DN: diabetic nephropathy; DR: diabetic retinopathy; DSA: Dutch South Asian; DwC: Dutch white Caucasian; eGFR: estimated glomerular filtration rate; FA: fatty acid; FDR: false discovery rate; HbA1c hemoglobin A1c; HC: healthy control; HCA: hierarchical cluster analysis; HDL: high-density lipoprotein; HDL-C: high-density lipoprotein-cholesterol; HexCER: hydroxyceramide; IgAN: IgA nephropathy; LacCER: lactosylceramide; LBM: lean body mass; LC/MS: liquid chromatography/mass spectrometry; LDL: low-density lipoprotein; LDL-C: low-density lipoprotein-cholesterol; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; LPL: lipoprotein lipase; MG: monoradylglycerolipid; MLR: multinomial logistic regression analysis ns: not significant; PA: phosphatidic acid; PC: phosphatidylcholine; PCA: principle component analysis; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PKC: protein kinase C; PS: phosphatidylserine; SAT: subcutaneous adipose tissue; SD: standard deviation; SLA: shotgun lipidomics assistant; SM: sphingomyelin; T2DM: type 2 diabetes mellitus; TG: triglyceride; VAT: visceral adipose tissue; WGCNA: weighted correlation network analysis.

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Authors' contributions

LY, MG, and BMvdB contributed to the study concept, design and analysis; LY analyzed and interpreted data, and critically revised the manuscript; AV and NB carried out the SLA

experiments and first data analysis; HvE and MB collected data for the MAGNA VICTORIA study, IMJ supervised the study, with HJL as study director; PCNR interpreted lipidomics data; LY, TJR, and BMvdB drafted the manuscript. All authors read, commented on, and approved the final manuscript.

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Availability of data and materials

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

Competing interests

The authors declare that they have no relevant financial interests or personal relationships.

Ethics approval and consent to participate

Participants were drawn from two prior randomized controlled trials (ClinicalTrials. gov NCT01761318 and NCT02660047). The study protocol was approved by the Institutional Review Board (Leiden University Medical Center, Leiden, The Netherlands), and all participants gave written informed consent.

Consent for publication

The manuscript was approved by all authors for publication.

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Supporting information

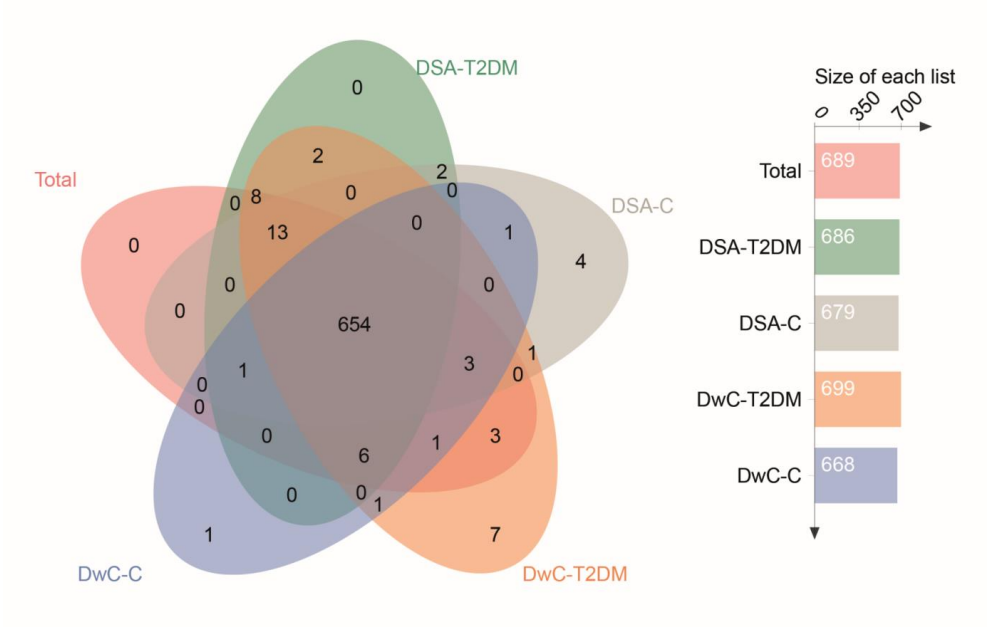

Fig. S1. Lipid species selected for analysis. Missing value percentage quantification per lipid class in total individuals, Dutch South Asians with T2DM (DSA-T2DM), Healthy Dutch South Asians (DSA-C), Dutch white Caucasians with T2DM (DwC-T2DM), and healthy Dutch white Caucasians (DwC-C). Details of missing values are shown in Additional file 2: Table S2. Common lipids with a missing value percentage of less than 30%.

Abbreviations: *DSA* Dutch South Asian; *DwC* Dutch white Caucasian; *T2DM* type 2 diabetes mellitus; *TG* triglyceride.

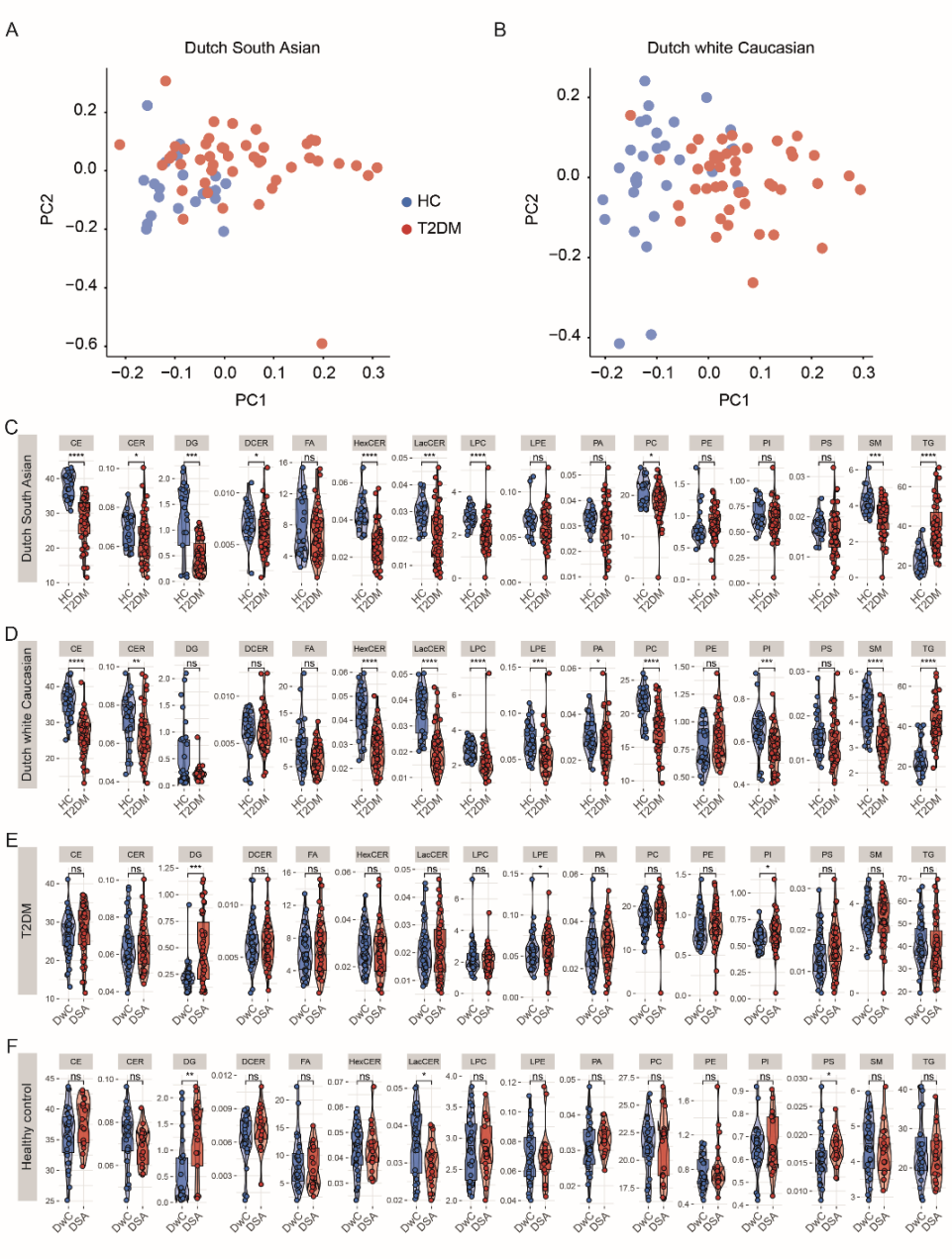

Fig. S2. Lipid abundance comparison between individuals from two ethnic groups. Principle component analysis using lipid class abundance in (A) Dutch South Asians and (B) Dutch white Caucasians. (C) Violin plot of lipid class abundance between patients with

T2DM and healthy controls in Dutch South Asians. (D) Violin plot of lipid class abundance between patients with T2DM and healthy controls in Dutch white Caucasians. (E) Violin plot of lipid class abundance between Dutch South Asians with T2DM and Dutch white Caucasians with T2DM. (F) Violin plot of lipid class abundance in healthy individuals between Dutch South Asians and Dutch white Caucasians. Wilcoxon signed-rank test was performed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Abbreviations: *CE* cholesteryl ester; *CER* ceramide; *DCER* dihydroceramide; *DG* diacylglyceride; *DSA* Dutch South Asian; *DwC* Dutch white Caucasian; *FA* fatty acid; *HC* healthy control; *HexCER* hydroxyceramide; *LacCER* lactosylceramide; *LPC* lysophosphatidylcholine; *LPE* lysophosphatidylethanolamine; *PA* phosphatidic acid; *PC* phosphatidylcholine; *PE* phosphatidylethanolamine; *PI* phosphatidylinositol; *PS* phosphatidylserine; *SM* sphingomyelin; *T2DM* type 2 diabetes mellitus; *TG* triglyceride.

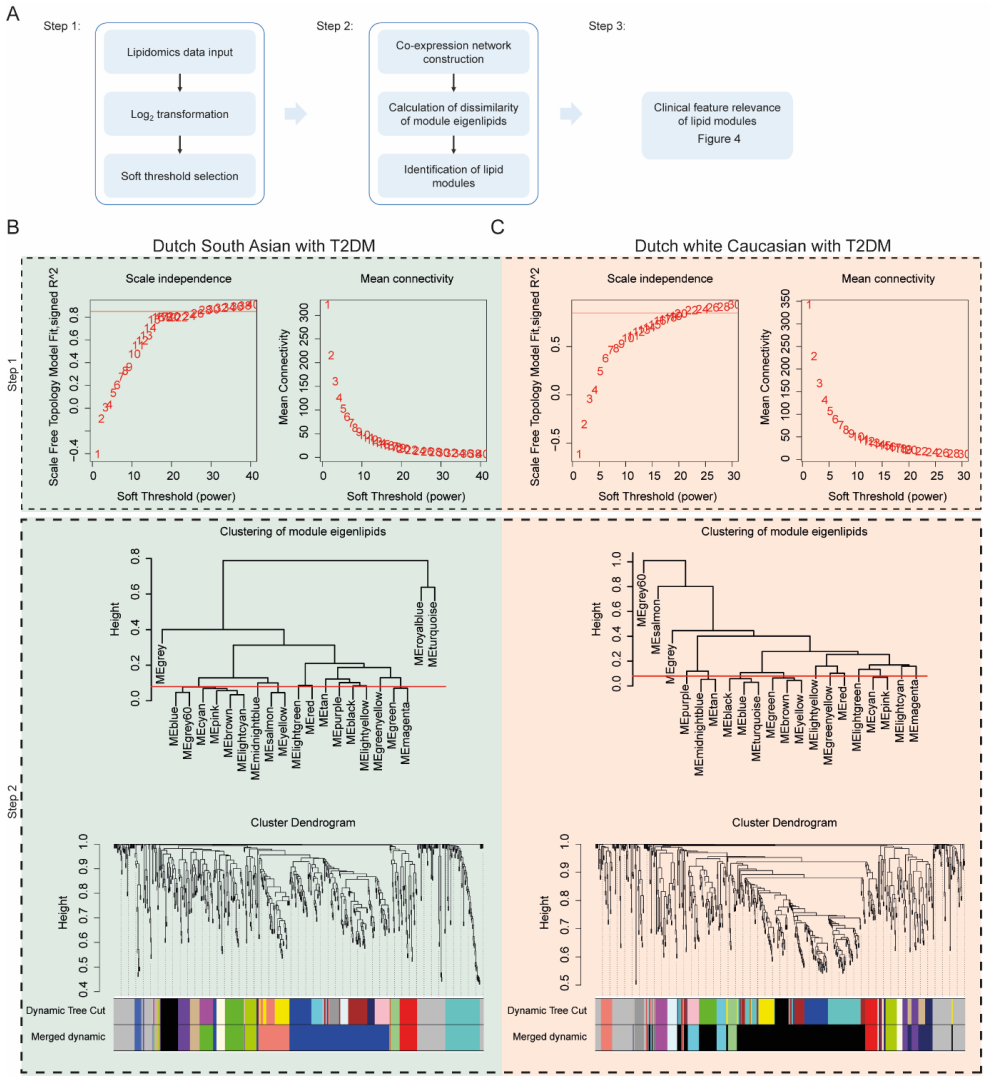

Fig. S3. Step-by-step WGCNA analysis. (A) Workflow of WGCNA analysis using the lipidomics profiles. Step 1 and Step 2 of WGCNA analysis in (B) Dutch South Asian and (C) Dutch white Caucasian.

Abbreviations: *ME* module eigenlipids

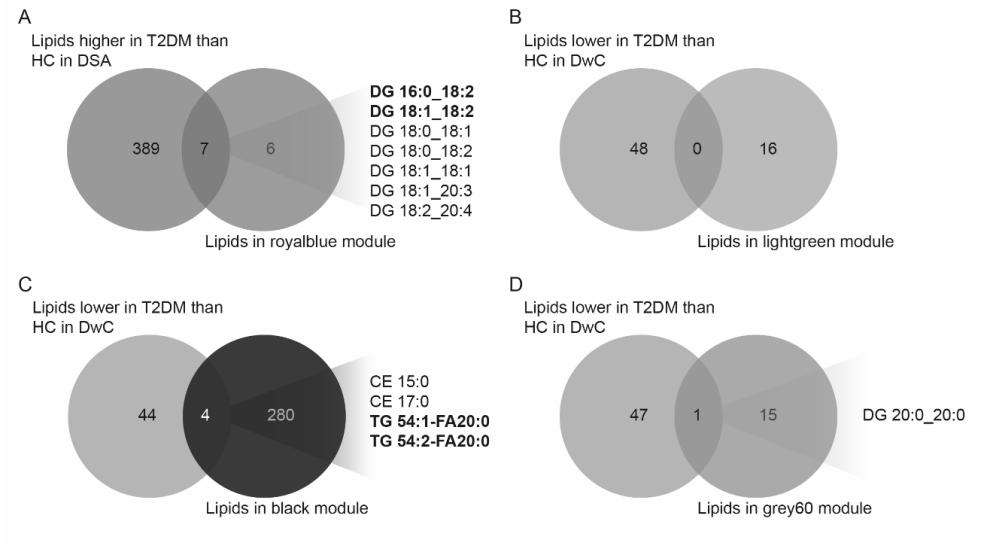

Fig. S4. Identification of key mediatory lipids. (A) Key mediatory lipid species (from diabetic nephropathy [DN] and glycemic control associated module, 'royal blue' module) in Dutch South Asians. (B) Key mediatory lipid species (from DR-associated module, 'light green' module) in Dutch white Caucasians. (C) Key mediatory lipid species (from diabetic retinopathy [DR]-associated module, 'black' module) in Dutch white Caucasians. (D) Key mediatory lipid species (diabetic nephropathy [DN]-associated module, 'grey60' module) in DwC. Bold lipids indicated that they were specifically different in DSA/DwC.

Abbreviations: *CE* cholesteryl ester; *DG* diacylglyceride; *DN* diabetic nephropathy; *DR* diabetic retinopathy; *DSA* Dutch South Asian; *DwC* Dutch white Caucasian; *HC* healthy control; *T2DM* type 2 diabetes mellitus; *TG* triglyceride.

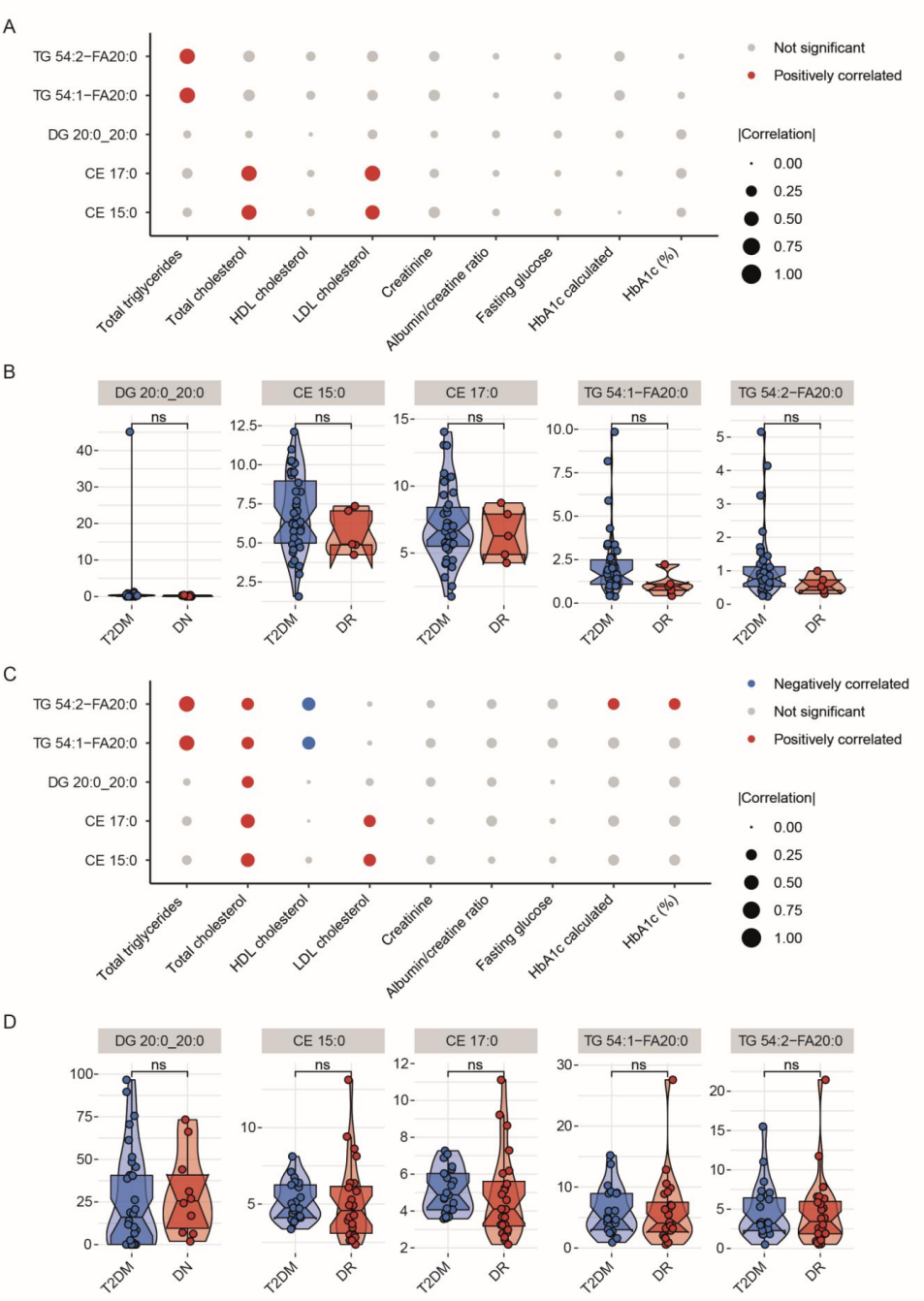
A
TG 54:2−FA20:0
TG 54:1−FA20:0
DG 20:0_20:0
CE 17:0
CE 15:0
Total triglycerides
Total cholesterol
HDL cholesterol
LDL cholesterol
Creatinine
Albumin/creatine ratio
Fasting glucose
HbA1c calculated
HbA1c (%)
Not significant
Positively correlated
|Correlation|
0.00
0.25
0.50
0.75
1.00
B
DG 20:0_20:0
CE 15:0
CE 17:0
TG 54:1−FA20:0
TG 54:2−FA20:0
ns
T2DM
DN
DR
C
Negatively correlated
Not significant
Positively correlated
|Correlation|
D

Fig. S5. Correlations between key mediatory lipids in Dutch white Caucasians and dyslipidemia, kidney function, and glycemic control. (A) A bubble plot depicting the correlations of lipids with lipoproteins, kidney function, and glycemic control in Dutch South Asians with T2DM. (B) Violin plot of lipids between T2DM with and without DN/DR in Dutch South Asians. (C) A bubble plot depicting the correlations of lipids with lipoproteins, kidney function, and glycemic control in Dutch South Asians with T2DM. (D) Violin plot of lipids between T2DM with and without DN/DR in Dutch South Asians. The color grey indicates no significant correlations with clinical features, the color blue indicates a negative correlation with clinical features, and the color red indicates a positive correlation with clinical features. The size of the dots represents the correlation coefficients (Pearson's correlation). The Wilcoxon signed-rank test was performed.

Abbreviations: *CE* cholesteryl ester; *DG* diacylglyceride; *DN* diabetic nephropathy; *DR* diabetic retinopathy; *HbA1c* hemoglobin A1c; *HDL* high-density lipoprotein; *LDL* low-density lipoprotein; *ns* not significant; *T2DM* type 2 diabetes mellitus; *TG* triglyceride.

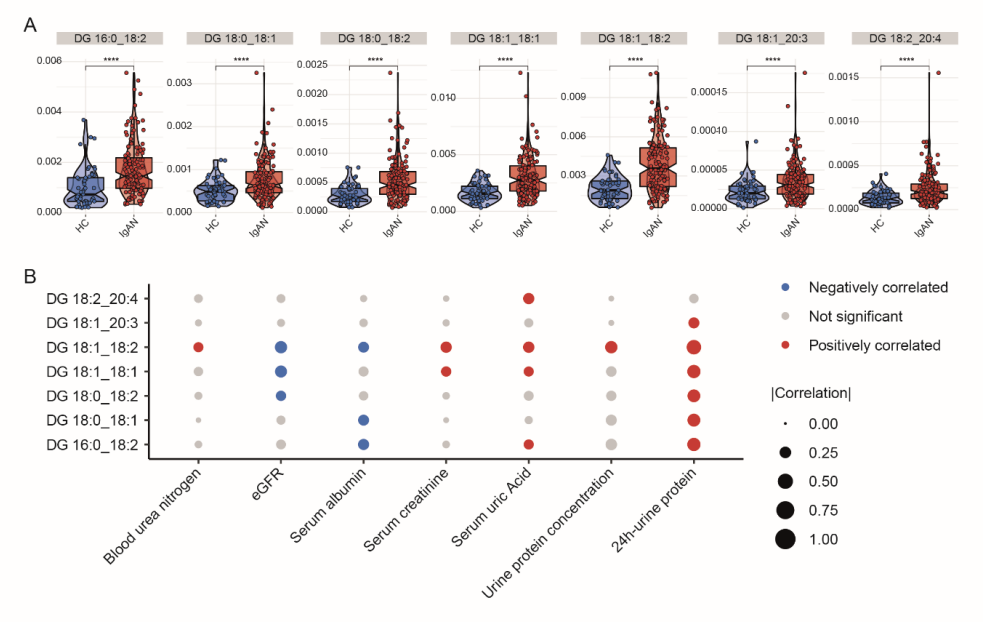

Fig. S6. The role of key mediatory lipids in DN-associated module of DSA in IgA nephropathy and their relationships with kidney function. (A) Violin plot of lipid differences between healthy controls and IgA nephropathy. (B) A bubble plot illustrating the relationships between the key mediatory lipids of Dutch South Asians and kidney function parameters in IgA nephropathy. The color grey denotes no significant correlations with clinical features, the color blue denotes a negative correlation with clinical features, and the color red denotes a positive correlation with clinical features. The correlation coefficients (Pearson's correlation) are represented by the size of the dots. The Wilcoxon signed-rank test was performed; ****p<0.0001.

Abbreviations: *CE* cholesteryl ester; *DG* diacylglyceride; *eGFR* estimated glomerular filtration rate; *HC* healthy control; *IgAN* IgA nephropathy.

Table S1. Clinical characteristics of study participants

	Dutch South Asian			Dutch white Caucasian			DSA-T2DM vs DwC-T2DM
	Control (n=21)	T2DM (n=47)	p-value[1]	Control (n=30)	T2DM (n=45)	p-value[1]	p-value[2]
Demographics							
Age (years)	48.3 (8.1)	54.9 (10.1)	0.0367	57.9 (7.9)	59.0 (6.5)	>0.9999	0.1606
Women (no, %)	15 (71.4%)	28 (59.6%)	0.3489	14 (46.7%)	20 (44.4%)	0.8498	0.1464
Current smoker (no, %)	3 (14.3%)	7 (14.9%)	1.0000	1 (3.3%)	9 (20.0%)	0.0437	0.7108
Medical history diabetes							
Duration diabetes mellitus (years)	-	17.9 (10)	-	-	10.3 (6.0)	-	<0.0001
Pan-microvascular (n, %)		30 (63.8%)	-		24 (51.1%)	-	0.3067
Nephropathy (n, %)	-	10 (21.3%)	-	-	11 (23.4%)	-	0.7174
Neuropathy (n, %)	-	14 (29.8%)	-	-	15 (31.9%)	-	0.7144
Retinopathy (n, %)	-	24 (51.1%)	-	-	5 (10.6%)	-	<0.0001
Macrovascular (n, %)	-	13 (27.7%)	-	-	2 (4.3%)	-	0.0037
Medication use			-			-	
Metformin (n, %)	-	45 (95.7%)	-	-	45 (100%)	-	0.4947
Sulfonylurea derivatives (n, %)	-	8 (17.0%)	-	-	13 (28.9%)	-	0.2682
Insulin (n, %)	-	36 (76.6%)	-	-	29 (64.4%)	-	0.2935
Anti-hypertensive medication (n, %)	-	34 (72.3%)	-	-	34 (75.6%)	-	0.7255
ACE-inhibitors (n, %)	-	13 (27.7%)	-	-	17 (37.8%)	-	0.4165
Statins (n, %)	-	36 (76.6%)	-	-	36 (80.0%)	-	0.8864
Blood pressure							
Systolic blood pressure (mmHg)	123.5 (13.7)	144.6 (21.5)	0.0003	126.2 (12.1)	141.3 (15.0)	0.0007	>0.9999
Diastolic blood pressure (mmHg)	80.2 (11.8)	85.3 (10.0)	0.1746	80 (77-83)	86.9 (8.8)	0.0158	0.8458
Anthropometrics							
Weight	63.6 (9.9)	79.7 (11.7)	<0.0001	73.3 (10.5)	96.4 (13.6)	<0.0001	<0.0001
BMI (kg/m^2)	23.5 (3.0)	29.5 (4.0)	<0.0001	24.3 (3.3)	32.3 (3.9)	<0.0001	0.0018
Total body fat (%)	32.4 (7.1)	37.1 (9.1)	0.1576	32.5 (7.1)	37.2 (9.3)	<0.0001	0.9998
Waist circumference, cm	82.0 (7.4)	101.0 (9.5)	<0.0001	86.6 (9.1)	110.4 (8.9)	<0.0001	<0.0001
Hip circumference, cm	95.2 (7.3)	104.1 (8.0)	<0.0001	98.2 (6.1)	107.6 (7.5)	<0.0001	0.1264
Waist-to-hip ratio	0.9 (0.1)	1 (0.1)	<0.0001	0.9 (0.1)	1 (0.1)	<0.0001	0.0039
VAT, cm^2	73.2 (29.8)	166.4 (55.8)	<0.0001	74.7 (34.1)	205.6 (75.6)	<0.0001	0.4342
SAT, cm^2	233.2 (195.9-258.8)	300.1 (228.3-371.4)	0.0561	189.7 (148.8-238.6)	335.7 (262.4-419.5)	<0.0001	>0.9999
VAT-SAT ratio	0.3 (0.2-0.4)	0.5 (0.4-0.7)	0.0017	0.4 (0.3-0.6)	0.6 (0.4-0.9)	0.0064	>0.9999
Glycemic control							
Fasting glucose (mmol/L)	5.0 (0.3)	8.1 (3.0)	<0.0001	5.2 (0.5)	7.8 (2.1)	<0.0001	>0.9999
HbA1c (mmol/mol)	35.5 (2.4)	67.8 (11.3)	<0.0001	35.5 (2.7)	64.9 (10.7)	<0.0001	>0.9999
Lipid panels							
Total cholesterol (mmol/L)	5.4 (0.8)	4.2 (0.9)	<0.0001	5.7 (1.1)	4.8 (1.0)	0.0018	0.0226
HDL-cholesterol (mmol/L)	1.6 (0.3)	1.2 (0.3)	0.0028	1.9 (0.5)	1.3 (0.3)	<0.0001	0.9008
LDL-cholesterol (mmol/L)	3.4 (0.7)	2.1 (0.8)	<0.0001	3.3 (1.0)	2.6 (0.8)	0.0017	0.0416
Total Triglycerides (mmol/L)	0.9 (0.3)	1.8 (1.4)	0.0031	0.9 (0.7-1.2)	2.1 (1.3)	<0.0001	0.2758
Kidney function panels							
Serum creatinine (µmol/mL)	68.0 (60.0-79.0)	67.0 (59.0-83.5)	0.4096	73.0 (68.0-85.0)	68.0 (57.0-80.0)	0.9582	0.9823
Albumin/creatine ratio (mg/mmol)	-	2.7 (0.55-8.45)	-	-	0.7 (0-2.5)	-	0.0037
Micro-albuminuria (n, %)[a]	-	15 (31.9%)	-	-	7 (15.6%)	-	-
Macro-albuminuria (n, %)[b]	-	7 (14.9%)	-	-	1 (2.2%)	-	-

Data are presented as mean (SD), median (25-75 percentile), or percentage.
Abbreviations: *ACE* Angiotensin-converting enzyme*, BMI* body mass index*, HbA1c* Hemoglobin A1c, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *SAT* subcutaneous adipose tissue, *VAT* visceral adipose tissue.
[1]Post hoc tests of unpaired One-way ANOVA, Kruskal-Wallis test, or Chi-square test, $p<0.05$
[2]Post hoc tests of unpaired One-way ANOVA or Kruskal-Wallis test, Chi-square test, or Fisher's exact test, $p<0.05$
[a]Albumin-creatinine ratio between 3.0 – 30 mg/mmol. [b]Albumin-creatinine ratio > 30 mg/mmol
Most of these data have been published before [14, 15].