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Alterations in the metabolism of phospholipids, bile acids and branched-chain amino acids predicts development of type 2 diabetes in black South African women: a prospective cohort study☆

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ABSTRACT

Background: South Africa (SA) has the highest global projected increase in diabetes risk. Factors typically associated with insulin resistance and type 2 diabetes risk in Caucasians are not significant correlates in black African populations. Therefore, we aimed to identify circulating metabolite patterns that predict type 2 diabetes development in this high-risk, yet understudied SA population.

Methods: We conducted a prospective cohort study in black SA women with normal glucose tolerance (NGT). Participants were followed for 13 years and developed (i) type 2 diabetes ($n = 20$, NGT-T2D), (ii) impaired glucose tolerance (IGT) ($n = 27$, NGT-IGT), or (iii) remained NGT ($n = 28$, NGT-NGT). Mass-spectrometry based metabolomics and multivariate analyses were used to elucidate metabolite patterns at baseline and at follow-up that were associated with type 2 diabetes development.

Results: Metabolites of phospholipid, bile acid and branched-chain amino acid (BCAA) metabolism, differed significantly between the NGT-T2D and NGT-NGT groups. At baseline: the NGT-T2D group had i) a higher lysophosphatidylcholine:lysophosphatidylethanolamine ratio containing linoleic acid (LPC(C18:2):LPE(C18:2)), ii) lower proliferation-related bile acids (ursodeoxycholic- and chenodeoxycholic acid), iii) higher levels of leucine and its catabolic intermediates (ketoleucine and C5-carnitine), compared to the NGT-NGT group. At follow-up: the NGT-T2D group had i) lower LPC(C18:2) levels, ii) higher apoptosis-related bile acids (deoxycholic- and glycodeoxycholic acid), and iii) higher levels of all BCAAs and their catabolic intermediates.

Conclusions: Changes in lysophospholipid metabolism and the bile acid pool occur during the development of type 2 diabetes in black South African women. Further, impaired leucine catabolism precedes valine and isoleucine catabolism in the development of type 2 diabetes. These metabolite patterns can be useful to identify and monitor type 2 diabetes risk >10 years prior to disease onset and provide insight into the pathophysiology of type 2 diabetes in this high risk, but under-studied population.

1. Introduction

The global burden of type 2 diabetes continues to rise in conjunction with obesity, related to a sedentary lifestyle and energy-dense diets [1]. Within sub-Saharan Africa, South Africa (SA) has the highest global projected increase in diabetes risk for the next 25 years [2]. A recent population-based study in urban-dwelling black South Africans found that the age-standardized prevalence of type 2 diabetes was 13%. Among those individuals, 40% were aged 65–74 years, and women were at higher risk than men [3].

Type 2 diabetes is a progressive disease characterized by increased insulin resistance (IR), impaired β-cell function, and eventually β-cell failure [1]. Prior to disease onset, hyperinsulinemia, as a result of increased insulin secretion and reduced hepatic clearance, develops to compensate for IR and to maintain euglycemia [4]. This compensation may last for decades. Chronic factors, including glucolipotoxicity, inflammation, endoplasmic reticulum stress, and oxidative stress may jointly contribute to exhaust this compensatory mechanism, but the mechanisms are unclear [5]. Several studies have shown that type 2 diabetes remission is possible either via lifestyle changes or bariatric surgery, but the success rates are highly dependent on disease duration

Abbreviations: SA, South Africa; NGT, Normal glucose tolerance; IGT, Impaired glucose tolerance; IR, Insulin resistance; BCAA, Branched-chain amino acid; LPC, Lysophosphatidylcholine; LPE, Lysophosphatidylethanolamine.

☆ Conflict of Interest: The authors declare no conflict of interest.

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[6,7]. Thus the need to detect early signs/markers of IR and type 2 diabetes is important to prevent long-term health complications, notably cardiovascular diseases, with related health costs [8].

Importantly, studies in SA and the USA have shown that factors typically associated with IR and type 2 diabetes risk in Caucasian populations, including circulating triglycerides, HDL-cholesterol, ectopic fat accumulation, and adipose tissue inflammation, are not significant correlates in black African populations [9–11]. These findings reinforce the fact that obesity-related disorders involve a complex interaction between genetic background, environmental and lifestyle factors. To understand this complex interaction, it is of major interest to explore the biochemical balance downstream of the genome, where metabolomics and the comprehensive analyses of metabolites provide a direct link between biochemical pathways and phenotypic changes. Previous studies have shown that elevated branched chain amino acids (BCAA) and metabolites related to their catabolism, including branched-chain keto acids (BCKA) or C3- and C5-carnitines, may predict, and potentially mediate, IR and type 2 diabetes development [12,13]. Notably, these findings were demonstrated in different Caucasian populations [14,15]. Further, low levels of glycine and lysophosphatidylcholine (LPC, C18:2) was shown to predict the risk for developing impaired glucose tolerance (IGT) in the KORA- and EPIC-cohorts from Caucasians [16].

Despite the high prevalence of type 2 diabetes in urban black SA women, metabolic pathways putatively involved in its pathophysiology have been scarcely studied in this population. Our hypothesis was that specific metabolite patterns or pathways can predict the development of type 2 diabetes in black SA women >10 years prior to diagnosis.

2. Methods

2.1. Study Design and Population

The study participants included black SA women who were the caregivers of the Birth to Twenty Plus cohort (BT20+) [17]. Briefly, between 2002 and 2003 (baseline), 2174 caregivers of the BT20+ cohort were invited to participate in the study. Of those invited, 1251 accepted and underwent blood sampling and general anthropometric measurements. From these, 476 individuals had complete blood analyte data and stored serum samples at baseline (Fig. S1). Approximately 13 years later (follow-up), between 2015 and 2016, contactable participants were asked to participate in the current follow-up study if they fulfilled the inclusion criteria: (1) an available stored serum sample; (2) normal fasting glucose at baseline; (3) a negative HIV test at follow-up; and (4) <65 years of age. Participants were excluded if they had a chronic or infectious disease, or were pregnant or lactating. At follow-up, all recruited women ($n = 144$) underwent clinical measurements. Those without known type 2 diabetes underwent an oral glucose tolerance test (OGTT). WHO diabetes diagnostic criteria were used to diagnose IGT and type 2 diabetes [18]. We included all women that had NGT at baseline (based on the 2002–2003 data) and had developed (at follow-up) either (i) type 2 diabetes ($n = 20$, NGT-T2D group) or (ii) IGT ($n = 27$, NGT-IGT group). These were matched with a third group that remained NGT ($n = 28$, NGT-NGT group). Further, we calculated a principal component model and used a statistical experimental design to match for multiple variables in order to minimize group differences in baseline measures of fasting glucose, age, BMI, and waist circumference, and also transitions in BMI and waist circumference from baseline to the 13-year follow-up [19]. A flow chart that schematically illustrates the study design and exclusion criteria is presented in Fig. S1. All participants provided written informed consent. The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (MO10556 and M150530), in accordance with the Declaration of Helsinki. Procedures and associated risks were explained to the participants who then signed a written consent prior to participation. Sample collection was carried out at the South African Medical Research Council (MRC)/University of the Witwatersrand (WITS) Developmental Pathways for Health Research Unit at the Chris Hani Baragwanath Hospital in Soweto Johannesburg. Metabolomics analyses were conducted on serum samples from all 75 women, sampled at baseline and follow-up.

2.2. Clinical Measurements

At baseline and follow-up, basic anthropometric measurements, including weight, height, waist circumference, hip circumference, and BMI, were collected after an overnight fast. At follow-up, participants underwent a 75-g OGTT after an overnight fast to determine glucose tolerance based on WHO criteria [18]. Serum glucose, total cholesterol, triglycerides, and HDL cholesterol were analyzed on the Randox RX Daytona Chemistry Analyzer (Randox Laboratories Ltd., UK). Serum insulin and C-peptide assays were analyzed on the Immulite® 1000 Immunoassay System (Siemens Chemiluminescent Technology, Germany). HbA1c levels were measured on whole blood using the D-10™ Hemoglobin Analyzer (Bio-Rad Laboratories, Inc. USA). Serum samples were stored at −80 °C until metabolomics analyses.

2.3. Sample Preparation and Metabolomics Analyses

In view of known methodological biases, all samples were prepared and analyzed in a specific order [20] keeping samples from the same subject in close connection, but with a randomized internal order and batches were balanced according to the diagnostic groups. Quality control samples (QC, pooled from all samples) were included in each batch to assist with the identification of metabolites, to monitor instrument stability, exclude background features, and minimize methodological biases that could interfere with interpretations of results.

Sample preparation was performed as described previously [21]. The multi-platform metabolomics analyses included gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF/MS) and liquid chromatography coupled with time-of-flight mass spectrometry (LC-TOF/MS, operating in positive and negative ion modes).

Detailed sample preparation, the use of internal standards, analysis protocols, and subsequent data processing methods are provided in the supplementary material.

2.4. Statistical Analysis

First, we inspected the dataset using principal component analysis (PCA) to detect groupings, outliers, and trends. To minimize the influence of confounders related to storage, we did not compare the metabolite patterns between the two samplings, i.e., baseline and follow-up. Instead, we calculated separate OPLS-discriminant analysis models (OPLS-DA) at baseline and follow-up to compare the NGT-NGT and the NGT-T2D groups. This allowed us to elucidate similarities and differences in metabolite profiles before and after disease development, respectively.

Next, the metabolite profiles of the NGT-IGT group were predicted into existing OPLS-models to enable comparisons of metabolite profile among all diagnostic groups.

The obtained models were validated, based on analysis of variance of the cross-validated OPLS scores (CV-ANOVA) to test for significance [22]. Metabolites were considered significant when fulfilling the statistical significance criteria using post-hoc linear regression on loadings calculated from the validated OPLS-models [23]. A non-parametric Wilcoxon-Mann-Whitney rank sum test and Spearman rank correlation on a 95% confidence level was used to explore relationship between putative metabolites, diagnosis and clinical endpoints. The above analyses were performed with SIMCA 14.0.0 software (Umetrics, Umeå, Sweden) and MATLAB R2016a (The MathWorks, Natick, MA, USA).

3. Results

3.1. Subject Characteristics

All clinical characteristics and anthropometric data are presented in Table 1. The groups did not differ in age, weight, BMI, or hip circumference at baseline or follow-up. Waist circumference, WHR and fasting glucose concentrations were significantly higher in the NGT-T2D group than the other two groups at baseline. Within-group changes in anthropometrical variables over the 13-year follow-up period were significant. Within the NGT-IGT and NGT-NGT groups, waist circumference (+14%, $P = 0.0004$ and +11%, $P = 0.02$) and WHR (+7%, $P = 0.001$ and +7%, $P = 0.004$) increased, respectively. Only the WHR (+6.5%, $P = 0.04$) significantly increased in the NGT-T2D group over the 13-year follow-up period.

At follow-up, the NGT-T2D group had significantly higher HbA1c and fasting glucose, insulin, HOMA-IR, and triglycerides concentrations, compared to the other two groups. The NGT-IGT group had significantly higher fasting glucose, C-peptide, and 120-min glucose compared to the NGT-NGT group at follow-up (Table 1).

3.2. Metabolomics Analysis

The combination of GC-TOF/MS and LC-TOF/MS, provided a comprehensive coverage of serum metabolites with different chemical properties. After removing the noisy peaks, we detected 1076 putative metabolites. Among these, 252 were identified and classified into amino acids, carbohydrates, fatty acids, lipids, acylcarnitines, organic acids etc. A list of the identified and classified metabolites is shown in supplementary Table S1. Additional details regarding data pre-processing, data inspection with PCA, and efforts to identify unknowns are shown in the supplementary material.

3.3. Metabolite Profiles That Describe the Development and Risk of Type 2 Diabetes in Black SA Women

With OPLS modeling, we constructed two separate models, one for baseline samples and one for follow-up samples. These models highlight the metabolite profile that significantly discriminates between the NGT-T2D and NGT-NGT groups, calculated from all 1076 putative metabolites. At baseline, when all women were NGT, the metabolite profile comprised 28 annotated and 81 non-annotated metabolites could significantly discriminate between the NGT-T2D and NGT-NGT groups (CV-ANOVA, $P < 0.05$, Fig. 1A). At follow-up, the metabolite profile was amplified, including 70 annotated and 66 non-annotated metabolites that differed significantly between the NGT-T2D and NGT-NGT groups (OPLS-DA, CV-ANOVA, $P < 0.001$, Fig. 1B). Only annotated metabolites are shown in figures. Information on the non-annotated compounds are shown in supplementary Table S2.

Several phospholipid metabolites differed at baseline between the NGT-T2D and the NGT-NGT group. Specifically, we detected a higher lysophosphatidylcholine:lysophosphatidylethanolamine (LPC(18:2):LPE (C18:2)) ratio, and lower levels of glycerophospholipid precursors (i.e., glycerol-2-phoshpate and glycerol-3-phosphate), conjugated C16:0 and C18:0-fatty acids (namely 2-hydroxypalmitic acid (C16-OH), palmitic acid methyl ester (C16:0-ME), and octadecanoic acid methyl ester (C18:0-ME)), and lower levels of the omega-6 fatty acid, eicosadienoic acid (C20:2n6).

At baseline, we could also detect lower circulating tricarboxylic acid (TCA) cycle intermediates (i.e., citric acid and isocitric acid) in the NGT-T2D group along with lower alpha-aminobutyric acid, beta-alanine, a platelet aggregation factor (PAF, C16:2), and LPE(C18:2), compared to the NGT-NGT group. At baseline, the NGT-T2D group had lower levels of two bile acid species, chenodeoxycholic acid (a primary bile acid)

Table 1
Characteristics of participants in the three diagnostic groups, at baseline and at 13-year follow-up. All participants exhibited normal glucose tolerance (NGT) at baseline.

Participant groups	NGT-NGT ($n = 28$)	NGT-IGT ($n = 27$)	NGT-T2D ($n = 20$)
Baseline (2002–2003)			
Age (years)	43 (39–48)	44 (38–49)	45 (41–46)
Fasting glucose (mmol/L)	4.8 (4.4–5.1)*	4.6 (4.4–5.0)#	5.4 (5.0–5.9)*,#
Body composition			
Weight (kg)	76.3 (62.1–88.0)	78.8 (68.0–85.0)	81.1 (73.3–106.0)
BMI (kg/m^2)	31.6 (26.1–35.4)	31.8 (27.0–34.0)	33.9 (29.8–39.1)
Waist circumference (cm)	87.0 (77.4–96.5)*	88.2 (81.0–96.0)#	96.5 (90.8–110.0)*,#
Hip circumference (cm)	113.0 (105.0–123.0)	112.0 (104.5–122.0)	117.0 (108.0–125.4)
WHR	0.76 (0.71–0.80)*,§	0.80 (0.76–0.82)#,§	0.86 (0.80–0.88)*,#
13-year follow-up			
Age (years)	56 (52–61)	58 (51–62)	58 (54–59)
Body composition			
Weight (kg)	81.4 (67.2–89.8)	83.1 (72.9–101.1)	82.9 (72.5–99.2)
BMI (kg/m^2)	33.4 (27.7–37.3)	33.9 (28.9–37.9)	32.4 (30.0–38.9)
Waist circumference (cm)	97.8 (85.4–106.5)	100.0 (93.5–110.4)	102.6 (95.4–117.7)
Hip circumference (cm)	117.4 (111.1–127.1)	121.0 (110.8–128.0)	117.5 (107.0–130.8)
WHR	0.82 (0.76–0.80)*,§	0.85 (0.80–0.90)§	0.90 (0.84–0.90)*
Glucose tolerance, insulin resistance			
Fasting glucose (mmol/L)	4.7 (4.1–5.1)*,§	5.2 (4.4–5.6)#,§	7.5 (6.3–9.3)*,#
120 min glucose (mmol/L)	5.7 (4.8–7.0)*,§	8.9 (8.3–9.5)#,§	11.8 (11.5–15.4)*,#
Fasting insulin (mIU/L)	9.7 (3.7–15.8)*	11.4 (5.6–18.3)#	18.4 (11.5–22.6)*,#
Fasting C-peptide (ng/mL)	1.8 (1.1–2.5)*,§	2.5 (1.6–2.8)§	2.0 (1.7–3.9)*
HbA1c (mmol/mol)	36.6 (33.3–38.8)*	36.6 (32.2–39.8)#	51.9 (42.1–67.2)*,#
HbA1c (%)	5.5 (5.2–5.7)*	5.5 (5.1–5.8)#	6.9 (6.0–8.3)*,#
HOMA-IR	1.8 (0.8–3.7)*	2.8 (1.0–3.7)#	6.1 (3.3–10.4)*,#
Serum lipid profile			
HDL cholesterol (mmol/L)	1.3 (1.1–1.5)*	1.2 (1.1–1.4)	1.1 (1.0–1.2)*
Total cholesterol (mmol/L)	4.6 (4.1–5.6)	4.7 (4.0–5.1)	4.7 (4.1–5.1)
Triglycerides (mmol/L)	0.8 (0.7–1.1)*	1.0 (0.8–1.2)#	1.3 (1.0–1.2)*,#

Data presented as median (25th–75th percentiles). All comparisons were based on non-parametric Wilcoxon-Mann-Whitney rank sum tests.
* $P < 0.05$ for NGT-T2D vs. NGT-NGT.
# $P < 0.05$ for NGT-T2D vs. NGT-IGT.
§ $P < 0.05$ for NGT-IGT vs. NGT-NGT.

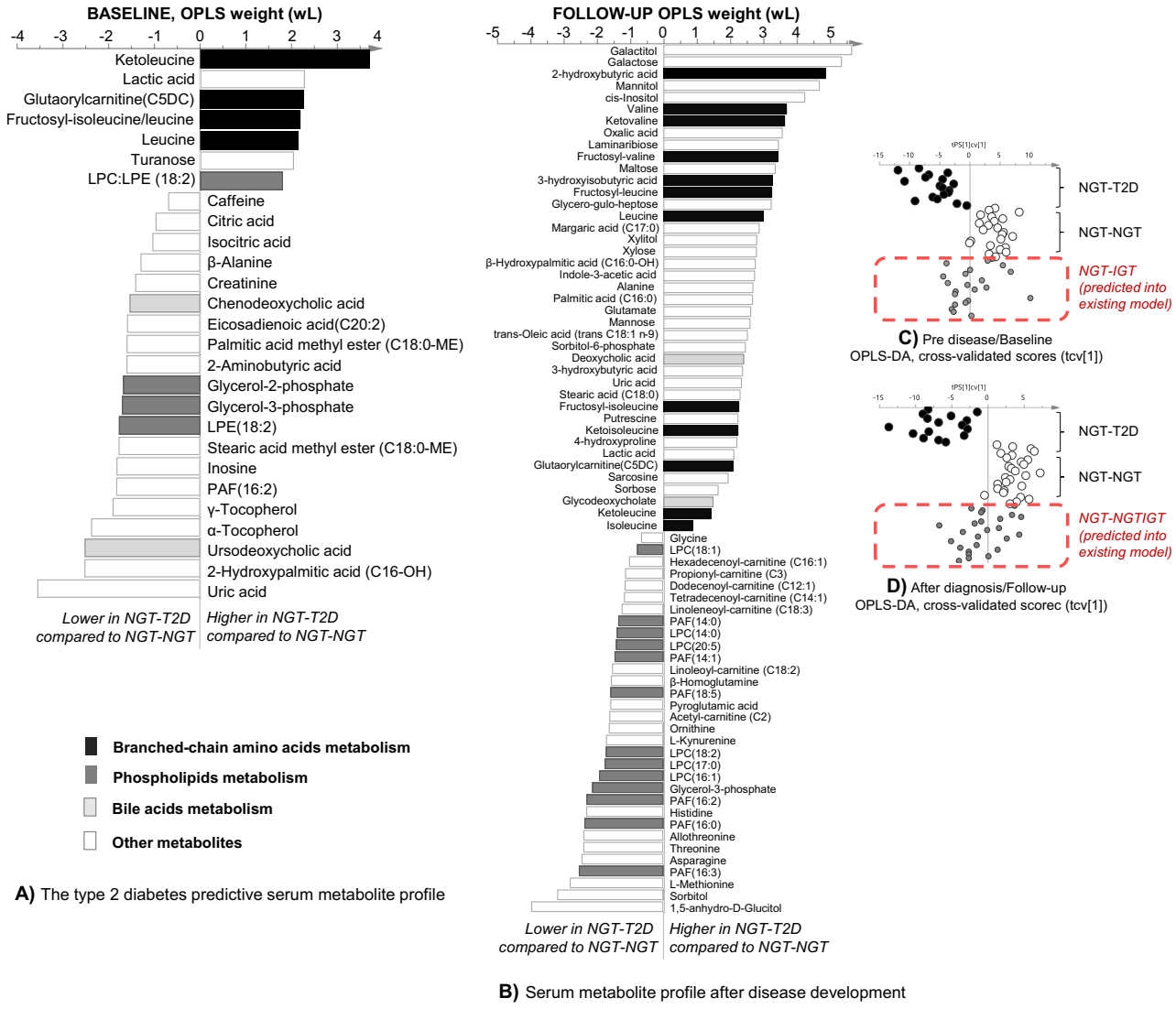

Fig. 1. Two separate multivariate models (OPLS-DA) that describe circulating type 2 diabetes metabolite profiles in black South African women, before and after disease diagnosis. (A) At baseline: the metabolite profile (OPLS model weights, w [1]) that could discriminate between normal glucose tolerance (NGT) that later developed into type 2 diabetes (NGT-T2D) and NGT that remained unchanged (NGT-NGT) during follow-up. (B) At 13-year follow-up: the metabolite profile (OPLS weights, w [1]) that could discriminate between NGT-T2D and NGT-NGT. (C) Baseline: Cross-validated (CV) OPLS-DA scores (tcv [1]) that describe subject variability prior to disease diagnosis. (D) At 13-year follow-up: Subject variability at follow-up (CV OPLS-DA scores, tcv [1]) shows the difference between NGT-T2D and NGT-NGT groups. Data for women classified as impaired glucose tolerant (IGT) were predicted into existing models to enable comparison of the complete metabolite profile. Only metabolites that were significantly altered are shown, according to the latent significant criteria on a 95% confidence level. Shading indicates metabolites related to branched-chain amino acids (black), phospholipids (dark gray), bile acid (light gray) metabolism, or unclassified metabolites (white). All identified putative metabolites are listed in Table S1.

and ursodeoxycholic acid (a secondary bile acid), compared to the NGT-NGT group.

Compared to the NGT-NGT group, both baseline and follow-up metabolite profiles of the NGT-T2D group had significantly higher circulating levels of leucine, ketoleucine (the BCKA of leucine), and a C5-carnitine, fructosyl-leucine/isoleucine (a glycated-BCAA; it was not possible to separate glycated-leucine from isoleucine). In addition, we detected consistently higher lactic acid and lower glycerol-3-posphate levels in the NGT-T2D group, compared to the NGT-NGT group, at both baseline and follow-up.

The NGT-IGT group had a metabolite profile that was in-between that of the NGT-T2D and NGT-NGT groups at both baseline and follow-up (Fig. 1C and D). This is also shown in the raw data for the relative concentrations of BCAAs and their catabolic intermediates (Fig. 2). Similar to the NGT-T2D group, leucine catabolic metabolites (ketoleucine, $P = 0.03$) were higher in the NGT-IGT group compared to the NGT-NGT group at baseline (Fig. 2A), with slightly higher valine catabolic intermediate levels in the NGT-IGT group compared to the NGT-NGT group at follow-up (Fig. 2B).

At follow-up, the NGT-T2D group had higher circulating fatty acids and lower levels of specific LPCs, containing fatty acids C14:0, C16:1, C17:0, C18:1, C18:2, and C20:5, compared to the NGT-NGT group. The bile acid pool size and composition were also altered as type 2 diabetes developed. The NGT-T2D group had higher levels of secondary and conjugated bile acids (i.e., deoxycholic acid and glycodeoxycholate) at follow-up compared to the NGT-NGT group. At follow-up the NGT-T2D group had higher levels of all BCAAs, including their related BCKAs and metabolites closely related to their catabolism (e.g., ketovaline, 3-hydroxyisobutyrate and a C5-carnitine), compared to the NGT-NGT group (Figs. 1B and 2B).

Several long-chain acylcarnitines, such as C16:1, C18:2, C18:3, and C14:1, were lower in the NGT-T2D group than in the NGT-NGT group at follow-up. As expected, several circulating carbohydrates (e.g., inositol, galactitol, galactose, laminaribiose, maltose, mannitol, and mannose)

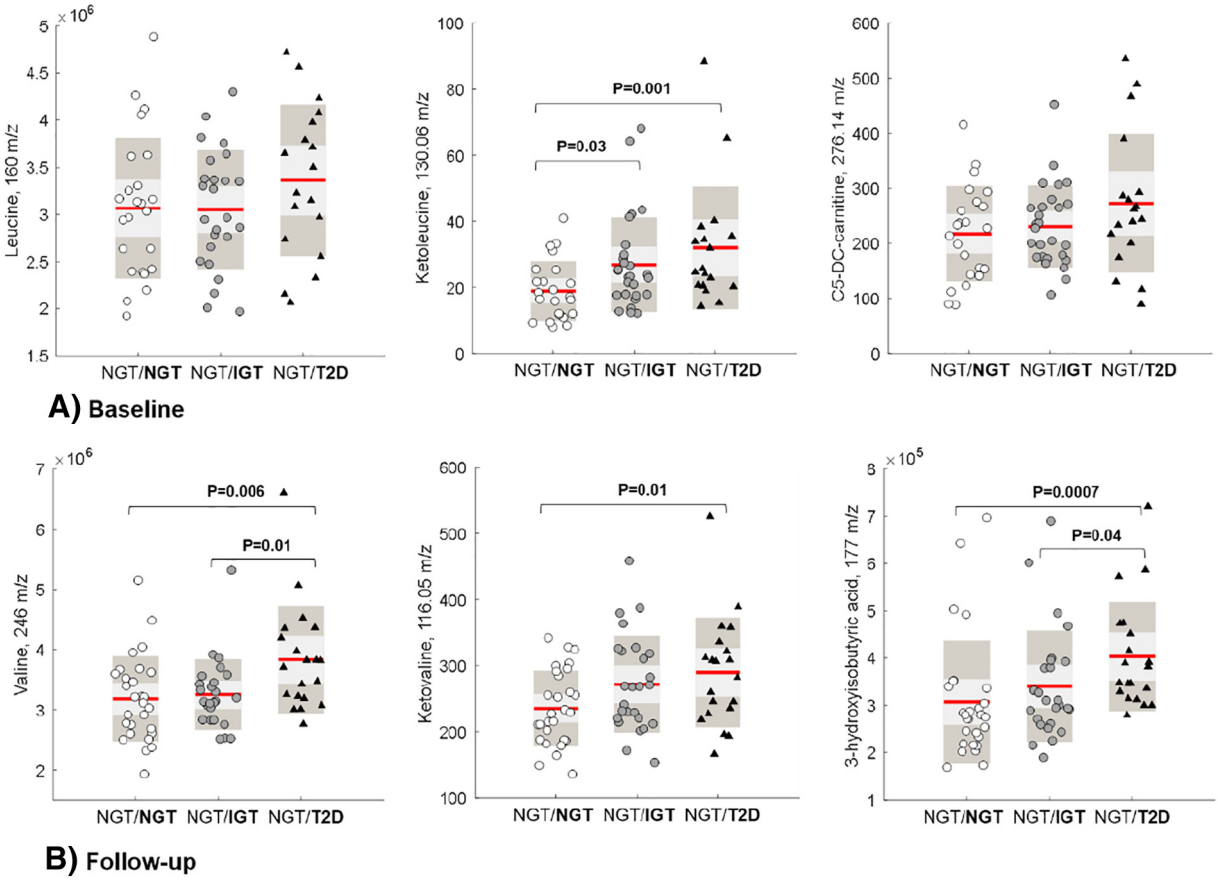

Fig. 2. Branched-chain amino acid catabolism before (baseline) and after type 2 diabetes diagnosis (13-year follow-up). Relative concentrations of serum branched-chain amino acids (BCAAs: leucine, isoleucine, and valine) and their catabolic intermediates (ketoleucine, ketovaline, C5-carnitine, and 3-hydroxyisobutyric acid) are shown for NGT-T2D compared to NGT-NGT and NGT-IGT groups, at (A) baseline: prior to type 2 diabetes diagnosis and (B) 13-year follow-up: after diagnosis of type 2 diabetes or other conditions, as indicated. (A and B) The NGT-IGT group shows a BCAA pattern in-between the NGT-T2D and NGT-NGT profiles. The unique mass channel for each metabolite, used for quantification, is indicated on the y-axis. Red lines indicate the mean values, and data points are layered over the 95% confidence interval (white box) and the standard deviation (gray boxes). *P*-values were calculated by Wilcoxon-Mann-Whitney (WMW) rank sum test to compare the concentration differences of these highlighted metabolites between diagnosis groups.

were higher in the NGT-T2D group than in the NGT-NGT group at follow-up (Fig. 1B).

A heatmap that displays baseline metabolite correlations to clinical endpoints at follow-up are shown in supplementary Fig. S2. In line with the presented metabolite profiles, we found that the baseline LPC(C18:2):LPE(C18:2) ratio, their precursors (glycerol-2 and 3-phosphate) along with leucine and its catabolic intermediates were positively correlated with HOMA-IR, insulin, Hb1Ac and C-peptide at follow-up. The heatmap also highlights a negative correlation between chenodeoxycholic acid (a primary bile acid) and ursodeoxycholic acid (a secondary bile acid) and HOMA-IR, insulin, Hb1Ac and C-peptide at follow-up. To address the potential confounding effect of anti-diabetic medications, we excluded all women that were on anti-diabetic medications at follow-up from the multivariate model ($n = 7$). This resulted in a similar albeit less pronounced metabolite pattern suggesting minor confounding of anti-diabetic medications (Supplementary Fig. S3).

4. Discussion

Our multi-platform mass spectrometry-based metabolomics analysis revealed a distinct circulating metabolite profile that predicted the development of type 2 diabetes in black SA women and presented novel and valuable information about an under-studied population with a high prevalence of type 2 diabetes. Black SA women that developed type 2 diabetes over a 13-year follow-up period had higher baseline LPC(C18:2):LPE(C18:2) ratio, a different bile acid metabolite profile, and higher levels of leucine along with its catabolic intermediates (i.e., ketoleucine and C5-carnitine), compared to women that remained NGT over the same period.

Interestingly, the metabolites linked to the same pathways were altered between diagnostic groups at both baseline and follow-up with some similarities between the NGT-IGT and NGT-T2D groups.

Metabolites related to phospholipid metabolism formed a part of the metabolic profile that predicted the development of type 2 diabetes in this population. Prior to diagnosis, the NGT-T2D group had lower LPE (C18:2), a higher LPC(C18:2):LPE(C18:2) ratio, and lower phospholipid precursors (glycerol-2- and 3-phosphates), compared to the NGT-NGT group. In contrast, manifest type 2 diabetes was associated with lower LPC(C18:2) levels, suggesting a shift in phospholipid metabolism as type 2 diabetes develops. Lower LPC(C18:2) levels were markers for both prediabetes and type 2 diabetes in the KORA- and EPIC-cohorts, which comprised Caucasian/non-African individuals [16]. More recently, data from the PREDIMED study showed that one-year changes in lysophospholipid levels were inversely related to type 2 diabetes risk in a Mediterranean population including participants with high cardiovascular risk and many with established IGT [24]. Lysophospholipids, such as LPC and LPE, play an important role in glucose-mediated insulin secretion and insulin sensitivity in peripheral tissues. Indeed, LPCs enhance glucose-dependent insulin secretion via an orphan G-protein coupled receptor (GPR), such as the GPR119 receptor, in pancreatic beta-cells, but their specificity remains to be determined [25]. These lysophospholipid species may therefore act as lipokines that, via increased insulin secretion, induce beta-cell failure. Of note, previous

research has shown that black SA women, compared to Caucasians, hypersecrete insulin to maintain normoglycemia [26,27]. But with increasing age, insulin secretion decreases and is associated with IGT and type 2 diabetes [28]. However, lysophospholipid-related GPRs are also found in other tissues, such as skeletal muscle, where lysophospholipid-activation impairs fat and glucose oxidation and may thus induce metabolic inflexibility and thus infer tissue dysfunction and potentially IR [29]. Our finding of altered lysophospholipid metabolism could thus be a sign of both altered glucose-mediated insulin secretion and decreased metabolic flexibility associated with IR in skeletal muscle.

Similar to the lysophospholipids, we observed an altered bile acid profile between the glycemic groups, both at baseline and at follow-up. The primary function of bile acids is to aid fat absorption, but they can also influence metabolic flexibility by stimulating glucagon-like peptide-1 and insulin secretion [30]. After synthesis from cholesterol in the liver, primary bile acids are effectively re-absorbed and recycled in the distal ileum. However, when they are not re-absorbed, bile acid species can interact and mediate a wide-range of metabolic effects. Currently, the mechanisms underlying many of these effects remain unknown. We found that the levels of both a primary bile acid, chenodeoxycholic acid, which is easily recycled, and a secondary bile acid, ursodeoxycholic acid, which possesses anti-inflammatory properties [31], were lower at baseline in the NGT-T2D group, compared to the NGT-NGT group. At follow-up, we found higher levels of the secondary bile acid, deoxycholic acid. This bile acid has been shown to modulate cell death by disrupting the mitochondrial outer membrane [32]. However, ursodeoxycholic acid has been shown to counteract/inhibit the apoptotic effect of deoxycholic acid [33], which suggests that the NGT-T2D group had lower levels of "good" bile acids before type 2 diagnosis and higher levels of "bad" bile acids after the diagnosis. In line with this, previous studies have shown that both the size and composition of the bile acid pool are altered in rodents and humans with type 2 diabetes [34,35], potentially via impairments in insulin and glucose signaling.

Numerous studies have shown that elevated BCAAs (leucine, isoleucine, and valine) predict the development of type 2 diabetes, and it has been suggested that BCAAs might be involved in the pathogenesis of this disease [12,36]. This may be due to a reduced activity of branched chain keto-acid dehydrogenase (BCKDH), the rate-limiting step in BCAA catabolism, which is markedly lower in type 2 diabetes [36]. Notably, our study is the first to show that changes in leucine catabolism precede alterations in valine and isoleucine catabolism >10 years before the onset of type 2 diabetes. Although BCAAs are structurally similar, their catabolic pathways diverge after the BCKDH-step, which produces BCAA-specific CoA-intermediates with different metabolic fates. Leucine is exclusively ketogenic, because it is directly degraded into acetyl-CoA, and it cannot be converted into glucose. Thus, this may make leucine and its intermediates prone to accumulate when circulating glucose levels are higher. The accumulation of BCAAs and their BCKAs, such as leucine and ketoleucine, have been shown to inhibit the nutrient sensing pyruvate dehydrogenase complex (PDH), which controls glucose oxidation [37]. Indeed, inhibition of the PDH complex can result in the accumulation of toxic intermediates that confer tissue dysfunction via mitochondrial dysfunction/overload, which may ultimately lead to type 2 diabetes [38]. Leucine is also a potent activator of the mammalian target of rapamycin complex 1 signaling pathway, which enhances allosteric activation of PDH, altering TCA cycle activity, and potentially inducing metabolic inflexibility [36]. Thus, elevated leucine metabolites could be a key mediator in the development of

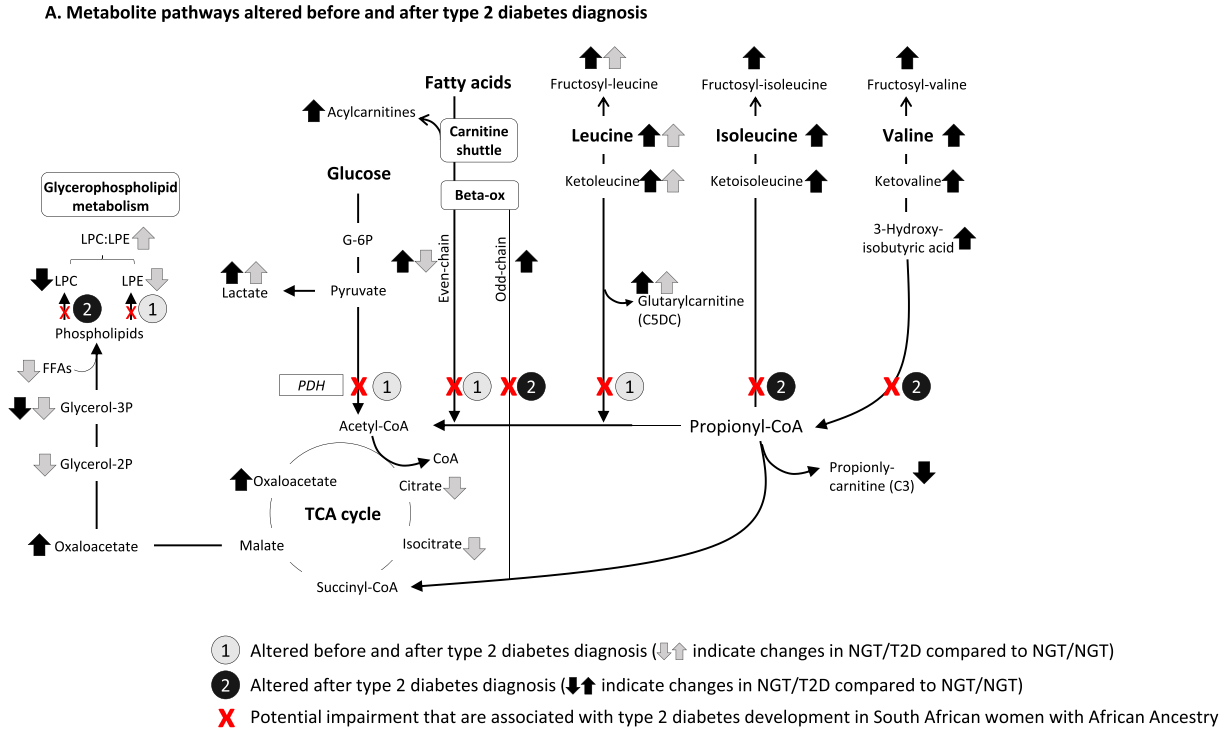

Fig. 3. Metabolite pathways altered before and after type 2 diabetes development in black South African women. Leucine metabolites (leucine, ketoleucine, fructosyl-leucine) accumulate in the circulation of women that are normal glucose tolerant (NGT), but who develop type 2 diabetes over the study period (NGT-T2D), compared to those that will remain NGT (NGT-NGT). Lactate also increases in the NGT-T2D group, presumably due to increased metabolic inflexibility, defined as inhibited glucose oxidation at the pyruvate dehydrogenase complex (PDH) [30]. The lower levels of glycerophospholipid precursors, i.e. glycerol 2- and 3-phosphate, in the NGT-T2D group suggests altered glycerophospholipid biosynthesis in the pre-type 2 diabetes state. This hypothesis is supported by the lower lyso-phospholipids in NGT-T2D compared to NGT-NGT. Impairments in branched-chain amino acid (BCAA) metabolism and glycerophospholipid metabolism form a putative link with impaired/lowered glucose oxidation, and thus, TCA intermediates, like malate, accumulate. Via the malate-aspartate shuttle, malate can be converted into oxaloacetate and glycerol-2-phosphate, which are consumed in glycerophospholipid synthesis, including the synthesis of lysophospholipids, like LPC and LPE. After disease development, all three BCAAs (leucine, isoleucine, and valine) and their catabolic intermediates are elevated in the NGT-T2D group compared to the NGT-NGT group, which suggests that this pathway is further impaired as disease progresses.

metabolic inflexibility and/or part of a feed-back mechanism of mitochondrial overload/dysfunction. This hypothesis is strengthened by our observation of elevated C5-carnitine levels in the NGT-T2D group at baseline. Our findings may indicate a "defensive response" since acylcarnitine-efflux has been suggested to serve as a detoxification process to prevent CoA-trapping and allow for CoA-dependent metabolic processes to continue, including the TCA cycle and β-oxidation [39].

Based on our findings we suggest that a series of alterations in the discussed metabolic pathways occur during the development of type 2 diabetes in overweight and obese black SA women. These are summarized in Fig. 3 and include: *1) Inhibition of lysophospholipid synthesis*; *2) Altered composition of the bile acid pool; 3) A reduced BCAA catabolism*. Analyses of metabolite patterns in specific pathways that are associated with insulin resistance and β-cell failure, can therefore be useful in increasing the understanding of the pathophysiology, as well as predicting and monitoring the development of prediabetes and type 2 diabetes in this high-risk population.

This study has some limitations including the low number of women that developed IGT and type 2 diabetes during the study period, which affected the study power. However, since the same individuals were sampled at both occasions, the inter-individual variability was reduced, with increased study power. Differences in storage time did not allow for comparison of metabolite concentrations between baseline and follow-up, we therefore interpreted the two sampling occasions separately. Of note, even though all women had normal fasting glucose levels at baseline, baseline fasting glucose levels were higher in the NGT-T2D group compared to the NGT-NGT and NGT-IGT groups. Nonetheless we found distinct alterations in specific metabolite pathways that can provide additional information over and above that obtained from classical risk factors such as fasting glucose concentrations. The circulating metabolite profile provides a global snapshot of the metabolome; future studies should investigate the dynamics of the highlighted pathways in relevant tissues such as the muscle and liver, relative to changes in hepatic and peripheral insulin sensitivity for a broader understanding of the underlying mechanisms. Further studies are warranted on phospholipid metabolism and the flux within the bile acid pool that, compared to BCAA metabolism, are less explored in relation to the pathophysiology of type 2 diabetes. It should be noted that our analyses did not capture the complete lipids metabolism, e.g., phosphatidylcholines/ethanolamines, ceramides and diacylglycerols, which are of interest and should be incorporated in future studies. Of note, we did not detect any consistent or prominent change in circulating fatty acids (precursors of ceramide metabolites) between baseline and follow-up. Importantly, circulating fatty acids as well as many other metabolites are highly associated with dietary intake. Future studies should therefore incorporate objective measures of dietary intake, including red blood cell lipid contents.

This is to the best of our knowledge the first longitudinal metabolomics study that has been conducted in overweight and obese black African women, a group at high risk of developing type 2 diabetes. Our results may serve as a basis for further exploration of the pathophysiology with ensuing targeted prevention and treatment of type 2 diabetes in this specific population.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request and with permission of Umeå University and the University of the Witwatersrand.

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Author Contributions

E.C., J.H.G., L.K.M. and T.O. were involved in the study conception and design. Y.Z. and E.C. were responsible for data analysis and wrote the manuscript draft. A.M. was responsible for sample collection, involved in data analyses and reviewed and edited the manuscript. J.H.G., L.K.M. and T.O. reviewed and edited the manuscript. E.C. is the guarantor of this work with full access to all included data and therefore takes responsibility for the integrity and accuracy of the data and data analyses.

Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.04.001.

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