

Exploring the Interactions between Obesity and Diabetes: Implications for Understanding Metabolic Dysregulation in a Saudi Arabian Adult Population

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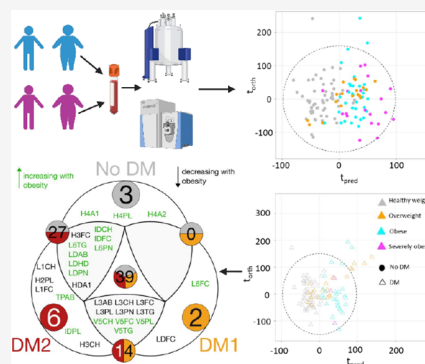
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Supporting Information

ABSTRACT: The rising prevalence of obesity in Saudi Arabia is a major contributor to the nation's high levels of cardiometabolic diseases such as type 2 diabetes. To assess the impact of obesity on the diabetic metabolic phenotype presented in young Saudi Arabian adults, participants ($n = 289$, aged 18–40 years) were recruited and stratified into four groups: healthy weight (BMI 18.5–24.99 kg/m²) with ($n = 57$) and without diabetes ($n = 58$) or overweight/obese (BMI > 24.99 kg/m²) with ($n = 102$) and without diabetes ($n = 72$). Distinct plasma metabolic phenotypes associated with high BMI and diabetes were identified using nuclear magnetic resonance spectroscopy and ultraperformance liquid chromatography mass spectrometry. Increased plasma glucose and dysregulated lipoproteins were characteristics of obesity in individuals with and without diabetes, but the obesity-associated lipoprotein phenotype was partially masked in individuals with diabetes. Although there was little difference between diabetics and nondiabetics in the global plasma LDL cholesterol and phospholipid concentration, the distribution of lipoprotein particles was altered in diabetics with a shift toward denser and more atherogenic LDL5 and LDL6 particles, which was amplified in the presence of obesity. Further investigation is warranted in larger Middle Eastern populations to explore the dysregulation of metabolism driven by interactions between obesity and diabetes in young adults.

KEYWORDS: obesity, diabetes, lipoprotein, metabolic profiling, metabolomic, metabonomic, NMR, LC–MS



INTRODUCTION

The global prevalence of obesity has increased dramatically in the past decade, imposing an impossible burden on the health and economy of almost every nation. This phenomenon is particularly evident in the Middle Eastern region, where the prevalence of obesity has reached epidemic proportions due to a higher degree of urbanization and shift in economic activities, leading to decreased physical activity and increased consumption of highly processed beverages and food.^{1–3} Between 70 and 88% of adults aged 30–60 years in Saudi Arabia, Kuwait, and Qatar are living with overweight or obesity, and six of the 10 countries with the highest adult diabetes prevalence are located in the Middle East, according to the International Diabetes Federation.^{4–6} Increased prevalence of both type 1 (DM1) and type 2 diabetes (DM2) has increased substantially since 1990.^{7,8} Childhood and adolescent obesity rates in Saudi Arabia are also among the highest in the world, and comorbidities such as diabetes, cardiovascular disease, and breast cancer attributable to high BMI are appearing earlier in life.^{9–11}

The development of an obesogenic and diabetic environment is the result of multiple factors, including overnutrition, physical inactivity, multiple pregnancies, lifestyle cultural restrictions, and an altered gut microbiome. These factors lead to increased inflammatory processes, oxidative stress, and altered energy substrate utilization, which contribute to the development of obesity and diabetes.^{1,3,12–17}

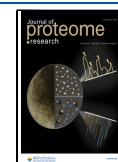
Improvement of individuals' metabolism via preventative interventions could help reduce the burden of chronic diseases on healthcare systems. Metabolic phenotyping via spectroscopic profiling of biofluids is an effective approach to characterizing molecular perturbations associated with obesity and diabetes and can be used to explore the underlying

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molecular connectivities between the two.^{18,19} However, most metabolic phenotyping studies have focused on populations in Western countries, and there is a lack of data from Middle Eastern populations where the expression of DM2 is often more aggressive.^{20,21} Moreover, population studies have tended to characterize cohorts with a mean age of >50 years,²² which are not necessarily comparable or relevant for the obese and diabetic phenotypes encountered in the Middle East. To address this gap, this study was conducted using a multimodal metabolic profiling strategy to characterize the plasma metabolic phenotype of Saudi Arabian men and women aged 18–40 years. The study used a 2 × 2 factorial design with healthy weight/obesity and nondiabetes/diabetes as stratification factors for type 1 and type 2 diabetes. This study aimed to provide insights into the metabolic drivers of obesity and diabetes in a young Middle Eastern population.

METHODS

Cohort Description and Sample Collection

The Biomedical Research Ethics Committees of the Faculty of Medicine at King Abdulaziz (KA) University and the Directorate of Health Affairs Jeddah, Ministry of Health, SA approved the study, and a written informed consent was obtained from all study participants. All procedures were carried out in accordance with the Declaration of Helsinki and the bioethical regulations as described in the “Regulations of Research Bioethics on the living Creature” by the National Bioethics Committee (King Abdulaziz City for Science and Technology).

A total of 289 participants with obesity and/or diabetes were recruited to the study between January 2015 and March 2016 and included Arabic or English-speaking individuals aged between 18 and 40 years: living with either overweight (BMI 25.0–30.0 kg/m²) or obesity (BMI > 30.0 kg/m²) with (*n* = 102) and without diabetes (*n* = 72) or healthy weight (BMI 18.5–24.99 kg/m²) with (*n* = 57) and without diabetes (*n* = 58). The following exclusion criteria were applied: individuals with malignant neoplasms, pregnant women, and underweight individuals (BMI < 18.5 kg/m²). Participants with diabetes (glucose concentration ≥ 7 mmol/L fasted and/or 11.1 mmol/L in 2 h postprandial state) were recruited from the outpatient diabetes clinics of the Center of Diabetes and Hypertension at KA Hospital, Mahjar District, Jeddah and King Abdulaziz (KA) University Hospital. Diabetes status and classification of diabetes type were made by the treating physicians in alignment with the guidelines of American Diabetes Association. Participants were stratified into groups (Table 1) based

Table 1. Study Groups and Participant Numbers

BMI	established diabetes			total
	no	type 1	type 2	
healthy weight	58	51	6	115
with overweight/obesity	72	52	50	174
total	130	103	56	289

on the presence of diabetes mellitus (DM) and WHO-guided body mass index (BMI) categories. The two diabetes groups included mixed types 1 and 2 DM forms, with ratio (DM type 1: type 2) of 9:1 and 1:1 in the healthy body weight and overweight to obese body weight group, respectively.

Each participant completed a medical history questionnaire including information on drug use. The metadata, including demographic variables, morphometric measurements, and diet/lifestyle factors, are provided in Table S1.

Fasted plasma samples were obtained through venipuncture using the Lithium-Heparin BD Vacutainer system. Blood glucose levels were determined through routine blood chemistry analysis using the glucose oxidase enzymatic reaction. Samples were centrifuged at 1200g for 10 min, and the supernatant was stored at −80 °C. Samples were transported frozen on dry ice to Imperial College London for metabolic profiling analysis.

Sample Processing and Measurement

Proton Nuclear Magnetic Resonance (¹H NMR) Spectroscopic Analysis of Plasma Samples. Sample processing was executed using a previously published high-throughput ¹H NMR protocol for analysis of human plasma samples.²³ Acquisition of ¹H NMR spectra at 600 MHz was achieved using a Bruker Avance III HD spectrometer equipped with a double resonance broadband probe (BBI), operating at 310 K. Three ¹H NMR experiments were performed for each sample: (a) standard 1D (90° pulse), (b) a spin-echo (Carr-Purcell Meiboom Gill: CPMG) pulse sequence to attenuate signals arising from macromolecules, and (c) a 2D homonuclear J-resolved (JRES) experiment using acquisition parameters as published previously.²³ Concentrations of plasma lipoprotein classes (VLDL, IDL, LDL, and HDL) and their subclasses were measured using a regression approach applied to standard 1D ¹H NMR spectra (Bruker B.I.-LISA).^{24,25} A list of the 112 lipoprotein subfractions and parameter annotations is provided in Table S2.

Untargeted Ultraperformance Liquid Chromatography Mass Spectrometry (UPLC-MS) Analysis of Plasma Samples. An aliquot of 200 μL of sample was treated with isopropanol (1:3 v/v), incubated at −20 °C for 24 h, and centrifuged at 2700g for 10 min, and the resulting supernatant was aliquoted into a glass vial (Waters Corp., Milford, USA). Sample separation was performed using a Waters Acquity Ultra-Performance LC system (Waters Corp., Milford, USA) with an established reversed phase (RP) chromatographic method.²⁶ Spectral data acquisition was performed in both positive and negative electrospray ionization modes using a Q-ToF Premier instrument (Waters Corp., Milford, USA) operating in centroid mode. Aliquots of the pooled QC sample were interspersed in the beginning and the end of each analytical run and at every fifth sample run-order position. Additionally, a five-step serial dilution of a QC sample was included at the beginning of the analytical run. Centroided UPLC-MS data were preprocessed using an in-house established pipeline based on the R package xcms (V1.48)²⁷ (see Supporting Information).

Targeted Quantification of Carnitine and Acyl Carnitines. A UPLC-MS assay using a tandem quadrupole mass spectrometer was used to quantify a series of 15 carnitines (acetylcarnitine, butyrylcarnitine, decanoylcarnitine, dodecanoylcarnitine, glutaryl carnitine, hexanoylcarnitine, isobutyrylcarnitine, isovalerylcarnitine, myristoylcarnitine, octanoylcarnitine, propionyl carnitine, palmitoylcarnitine, stearoylcarnitine, succinyl carnitine, and valerylcarnitine). A volume of 20 μL from each plasma sample was mixed and incubated for 20 min with 60 μL of ice-cold methanol and 20 μL of internal standard solution. The mixture was centrifuged at 10,300g for

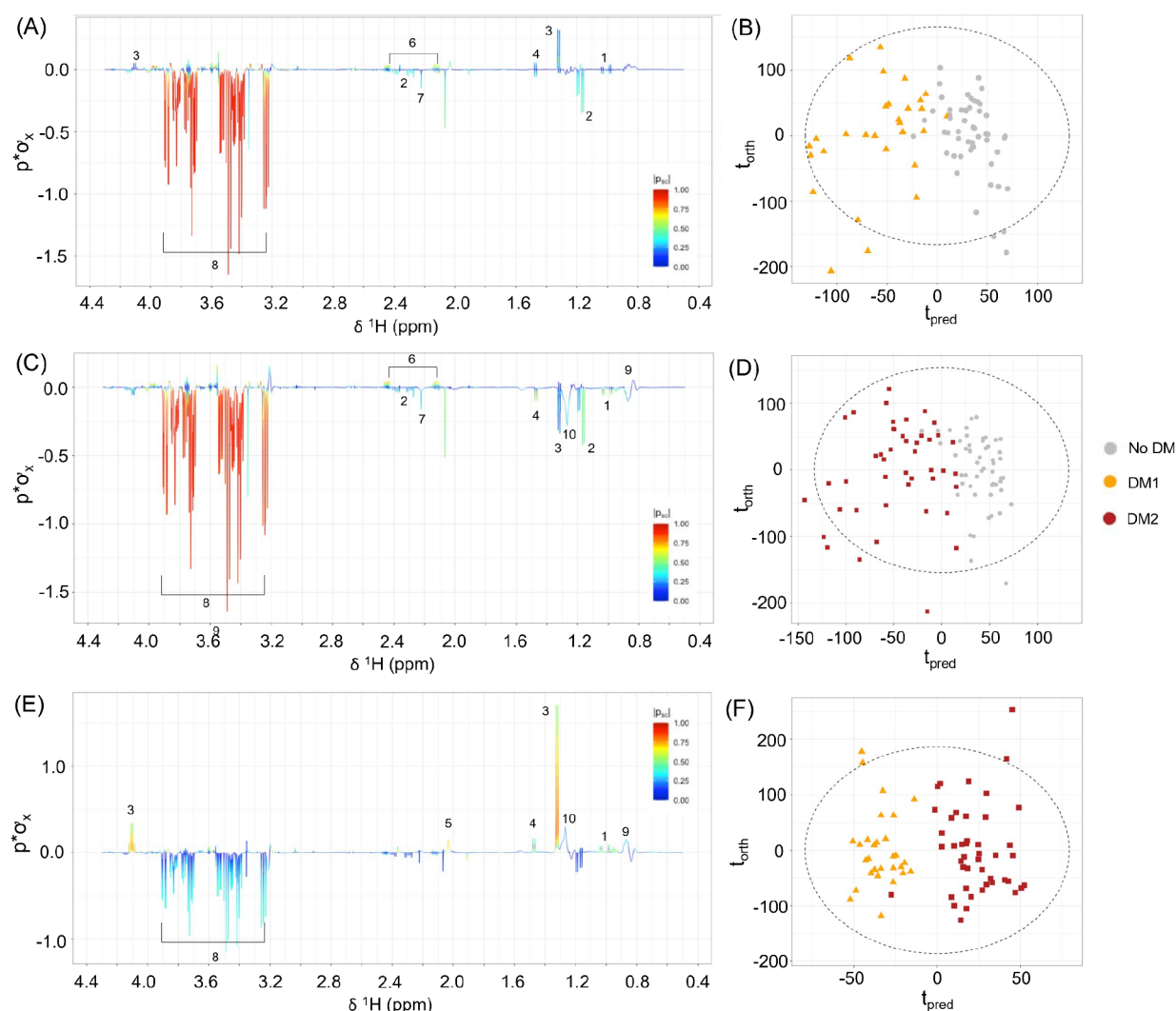


Figure 1. (A) O-PLS-DA loadings of obese individuals with type 1 diabetes vs obese individuals with no diabetes ($R^2X = 0.07$, CV-AUROC = 0.87), (B) corresponding score plot, (C) O-PLS-DA loadings of obese individuals with type 2 diabetes vs obese individuals with no diabetes ($R^2X = 0.07$, CV-AUROC = 0.83), (D) corresponding score plot, (E) O-PLS-DA loadings of obese individuals with type 1 diabetes vs obese individuals with type 2 diabetes ($R^2X = 0.03$, CV-AUROC = 0.65), and (F) corresponding score plot. Circle = no diabetes, triangle = type 1 diabetes, and square = type 2 diabetes. Key: 1, branched chain amino acids; 2, 3-hydroxybutyric acid; 3, lactate; 4, alanine; 5, GlycA; 6, glutamine; 7, acetoacetic acid; 8, glucose; 9, lipid CH_3 moiety; and 10, lipid CH_2 moiety.

10 min, and 20 μL of the supernatant was diluted with 180 μL of MS-grade water prior to being pipetted into MS glass vials for MS injection. A HSS T3 2.1 $\times$ 150 mm, 1.8 μm column (Waters, Milford, USA) running a linear solvent gradient from water and acetonitrile containing 0.1% formic acid was used to achieve LC separation. Samples were measured using an Acquity UPLC binary solvent manager, sample manager, and column heater (Waters, Milford, USA) coupled with a Xevo TQ (tandem quadrupole)-S mass spectrometer (Waters, Milford, USA) running in positive ESI mode.

NMR Spectral Processing. To enable quantification, all data points of the standard 1D and CPMG spectra were corrected using the ERETIC factor to account for variability within the instrument. Spectra were subsequently calibrated to the α anomeric proton signal of glucose at δ 5.23, and regions of noise and the TSP peak were removed using methods previously described.²⁸

Metabolite Identification

Structural elucidation of metabolites that were significant for any pairwise comparison (e.g., diabetes vs nondiabetes) was achieved using further NMR and MS assays. Statistical total correlation spectroscopy (STOCSY) was applied to the NMR data to aid metabolite identification,²⁹ and putative assignments were established by comparison of candidate structures with in-house and public spectral libraries (e.g., HMDB).³⁰ MS features were identified by matching retention time and m/z values to an internal database.

Statistical Modeling and Interpretation of the Spectral Data

Statistical group comparison was carried out using Mann–Whitney test for continuous variables and two proportions z -test for categorical variables. Where applicable, the alternative test hypothesis was two sided. Statistical significance was fixed at the level of $\alpha = 0.05$, and Cliff's delta was calculated as a nonparametric measure of effect size between two groups.³¹ Unsupervised and supervised multivariate statistical analyses

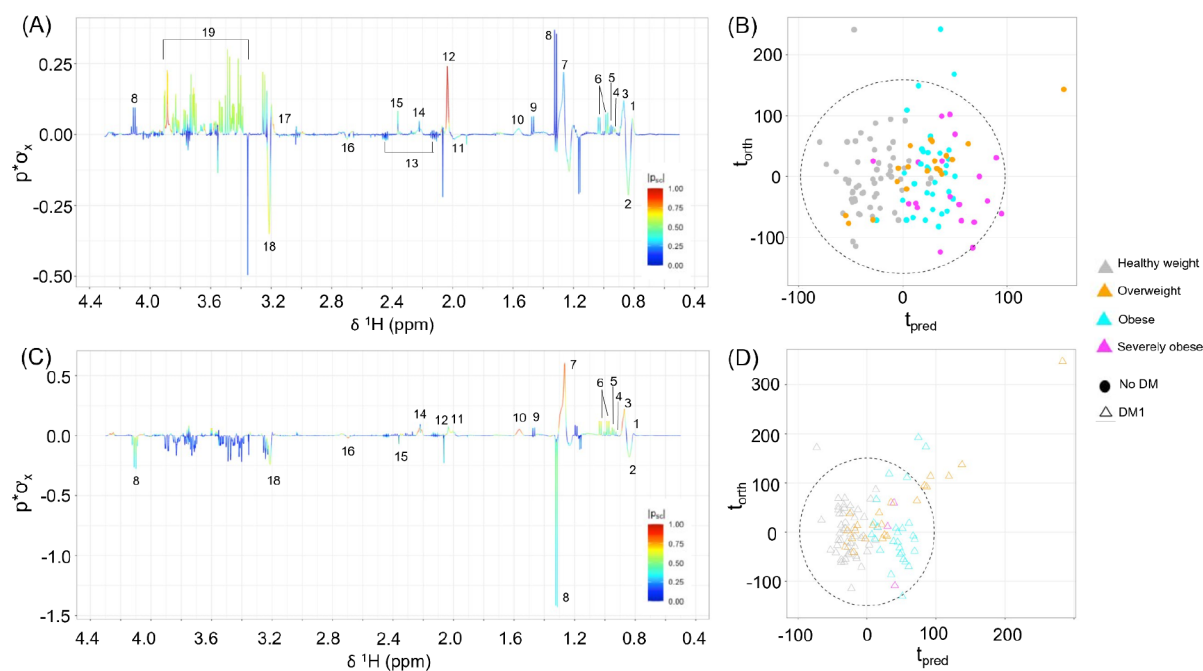


Figure 2. OPLS-DA models of 1D CPMG ¹H NMR spectra stratified by body weight in people with and without diabetes. (A) O-PLS-DA loadings of individuals at a healthy weight without diabetes vs obese weight without diabetes ($R^2X = 0.15$, $Q^2Y = 0.20$, CV-AUROC = 0.77) and (B) corresponding score plot, with overweight and severely obese individuals projected into the model. (C) O-PLS-DA loadings of individuals at a healthy weight with type 1 diabetes vs individuals who are obese with type 1 diabetes ($R^2X = 0.14$, $Q^2Y = 0.09$, CV-AUROC = 0.78) and (D) corresponding score plot, with overweight and severely obese individuals projected into the model. Key: 1, LDL; 2, HDL; 3, VLDL; 4, isoleucine; 5, leucine; 6, valine; 7, CH₂ moiety; 8, lactate; 9, alanine; 10, CH₂CH₂CO moiety; 11, CH₂C=C moiety; 12, GlycA; 13, glutamine; 14, CH₂CO moiety; 15, acetoacetate; 16, C=CCH₂C=C, 17, choline; 18, supramolecular phospholipid composite (SPC); and 19, glucose.

were performed with principal component analysis (PCA) and orthogonal-partial least-squares-discriminant analysis (OPLS-DA), respectively. Data were scaled to unit variance prior to modeling. The performance of the OPLS-DA models was assessed using area under the receiver operator characteristic curve, which was estimated using 7-fold cross-validation (CV-AUROC). Back-scaled OPLS-DA model loadings (using standard deviation of the original chemical shift variables) were represented as line-plots over $\delta^1\text{H}$, with the line color representing minmax-scaled absolute back-scaled loadings for the predictive component.

In order to assess the distribution of inherent metabolic phenotypes and to ascertain the impact of obesity and diabetes status on the plasma metabolome, self organizing maps (SOM) were generated using Kohonen³² R package version 3.0.12. The SOM^{33–35} method was applied to NMR spectral and lipoprotein data to visualize the spread of samples across and to map samples according to their biochemical phenotype. In this study, SOM clustered the data set into a map of 4-by-3 nodes. Each node represents a cluster of samples, and each small circle inside the node represents a sample. The samples were colored coded by the obesity and diabetes status the samples represent.

All computation was performed using the statistical programming language and environment R (Version 4.3.0). NMR data preprocessing was performed using the *NMR-spectra-processing* package (<https://github.com/phenological/nmr-spectra-processing>). Multivariate statistical analyses were performed using the *ropls* package (<https://bioconductor.org/packages/release/bioc/html/ropls.html>).

RESULTS

The median age of the participants was 27 years (18–40 years) with a male-to-female ratio of 0.8 and a median BMI of 27.8 kg/m² (18.5–69.3 kg/m²). The majority of study participants were born in Saudi Arabia and held Saudi Arabian citizenship (93% and 81%, respectively). The study demographics (Table S1) indicate some inherent differences between the groups, specifically a slightly lower participant age in the healthy weight categories (median ages of 23 vs 28 years and 24 vs 33 years for groups without and with DM, respectively, p value = 5.0×10^{-9}); minor but statistically significant elevated blood pressure (BP) levels in the overweight to obese study group when compared to the healthy body weight group (median systolic/diastolic BP 131/79 mmHg vs 122/76 mmHg for non-DM obese vs non-DM healthy weight groups, respectively, and 130/83 mmHg vs 118/75 mmHg for DM-obese vs DM-healthy weight groups, respectively) with p values of 5.3×10^{-8} and 5.5×10^{-7} for systolic and diastolic BP, respectively.

The Metabolic Signature of Type 1 and Type 2 Diabetes in an Obese Saudi Arabian Cohort

A comparison of the diabetic and control groups for participants with obesity showed that, as expected, both DM1 and DM2 diabetes were metabolically distinct from control participants with higher urinary glucose concentrations in the two diabetic groups (Figure 1). Other common features of the two diabetic groups in comparison to nondiabetics included higher concentrations of acute phase proteins (GlycA), 3-D-hydroxybutyrate, and acetoacetate, with lower concentrations of glutamine. Additionally, the DM2 group had higher concentrations of alanine and branched chain amino acids than the nondiabetic group. These observations are

reinforced in the comparison between the type 1 and type 2 diabetic groups (Figure 1E), where the DM2 group is characterized by higher levels of the GlycA associated with inflammation, alanine, and branched chain amino acids. Additionally lactate concentrations were higher in the DM2 group. Conversely, glucose concentrations were typically higher in participants with DM1. In terms of overall model strength, the model for DM1 was more strongly differentiated from the nondiabetic group (CV-AUROC = 0.87) than the DM2 model (CV-AUROC = 0.83). Since higher plasma concentrations of glucose dominate the models, the data were reanalyzed without the spectral areas containing glucose resonances (3.20–4.00 ppm) and anomeric regions (5.22–5.24 ppm), and the models generated retained a strong separation of the diabetic groups in comparison to the nondiabetic group (DM1 vs No DM CV-AUROC = 0.80; DM2 vs No DM CV-AUROC = 0.84) and in the differentiation of DM1 and DM2 (Figure S1). The key differential metabolites in the new loadings plot were ketone bodies (3-D-hydroxybutyrate and acetoacetate acetone) and acetate, which were present in higher concentrations in the plasma of participants with DM1 and glutamine, which was present in higher concentrations in the participants without DM1. The ketone bodies were still differential for the DM2 vs No DM model but additionally, alanine, the branched chain amino acids, particularly valine and mobile triglycerides were positively associated with the DM2 group (Figure 1E).

Quantified glucose concentrations were higher in individuals with diabetes across all BMI groups (Cliff's delta range 0.35–0.82) with a large spread of fasted plasma glucose concentrations, reflecting the drastic reduction of glycaemic control in individuals with diabetes (Figure S2).

The plasma profiles of participants without diabetes were influenced by sex (CV-AUROC = 0.80; $Q^2Y = 0.26$) (Figure S3), whereas sex had a lesser impact on the plasma biochemistry of participants with diabetes (DM1 CV-AUROC = 0.70, $Q^2Y = 0.03$; DM2 CV-AUROC = 0.72, $Q^2Y = 0.13$). In the nondiabetic and DM1 groups, women had higher concentrations of mobile triglycerides and the supra-molecular phospholipid composite peak³⁶ than men and lower concentrations of valine and alanine. Additionally, in the nondiabetic group comparison of men and women, plasma glutamine was associated with male samples. For the DM2 group, higher levels of branched chain amino acids were still characteristic for male participants, but glucose was also present in higher concentrations, whereas lactate and alanine were associated with women, and the triglyceride resonances were not significantly different between sexes (Figure S3F).

The Metabolic Signature of Weight in Individuals with and without Diabetes Based on the ¹H NMR Plasma Profiles

Stratification of participants without diabetes according to BMI indicated a progressive metabolic dysregulation with increasing BMI as seen in the OPLS-DA model based on the CPMG ¹H NMR data set (Figure 2). The BMI-associated metabolic variation was predominantly driven by GlycA, glucose, branched chain amino acids, acetoacetate, LDL, and VLDL, which were all positively associated with BMI. Conversely, the supra-molecular phospholipid composite peak (SPC) and HDL were inversely associated with BMI (Figure 2A).

In a similar comparison of the healthy weight versus obese groups of participants with type 1 diabetes, the metabolic

signature of obesity differed slightly (Figure 2C,D). Here, BMI was associated most strongly with VLDL and the CH₂CH₂CO moiety of lipids and branched chain amino acids. As with the model for participants without diabetes, HDL and SPC were inversely correlated with BMI, but, in addition, lactate was also the characteristic of the healthy weight group. Neither glucose nor GlycA were significantly associated with BMI in the type 1 diabetes participants. The peak representing the supra-molecular phospholipid composite (SPC) was higher in participants with healthy weight in both the nondiabetic and type 1 diabetic groups.

In terms of the impact of obesity on the metabolite profiles, both models (with and without type 1 diabetes) were comparable in strength, each with one predictive and one orthogonal component, with the nondiabetic ($R^2X = 0.15$, $Q^2Y = 0.20$, CV-AUROC = 0.77) and the diabetic groups ($R^2X = 0.14$, $Q^2Y = 0.09$, CV-AUROC = 0.78) returning similar model parameters. The score plots created from a pairwise comparison between obese and healthy weight participants included projections of the spectra that were not used for the training model (i.e., individuals who were classed as having overweight (BMI range: 25–29.99 kg/m²): the predictive score projections of individuals with overweight are roughly located between the healthy nutritional status and obesity groups in each model; however, the samples from participants overweight in the type 1 diabetes model were skewed toward the higher body weight groups. In the nondiabetic individuals, glucose showed the strongest association with obesity after GlycA. The absolute quantification of glucose through peak integration suggested an overall fasting plasma glucose range of 3.1–13.8 mM (Figure S2). A comparison of BMI vs waist-to-hip ratio as a measure of obesity (Figure S4) showed stronger correlation in men than that in women, with the correlation weakening in both sexes in participants with class 3 obesity, particularly for the women.

Since the healthy weight type 2 diabetes group was under-powered ($n = 6$), a valid OPLS-DA model could not be generated. A PCA model color coded by weight category for the type 2 diabetic group indicated that plasma concentrations of GlycA, triglycerides, and glucose drove the spectral variation in the score plot, but this variation was not associated with weight (Figure S5).

In the nondiabetic (Figure 2A,B) model, the highest magnitude loadings differentiating participants with and without obesity corresponded to GlycA. This was not apparent for the type 2 diabetes since the healthy weight group was under-powered ($n = 6$). Similarly, the CH₂ moieties of the lipoprotein signals at δ 1.31 and unsaturated lipoprotein component at δ 2.75, which were increased in the group with obesity (Figure 2A,C), were only significant in the nondiabetic and type 1 diabetic models. As expected, glucose was found to be of high importance in the nondiabetic OPLS-DA model for discrimination between individuals with healthy weight versus obesity but had low weightings for the predictive component in the diabetic model equivalents. Similarly, plasma lipoprotein resonances had high model importance for the differentiation of healthy weight from obese in individuals with type 1 diabetes and much lower weighting in the equivalent model comparison for individuals without diabetes. The branched chain amino acids showed a weak, direct association with obesity in the type 1 diabetes model and a trend toward a similar association in the nondiabetic model, which was not significant.

Obesity is Associated with Greater Dyslipidaemia than Diabetes

Statistical group comparison between the healthy weight individuals with and without type 1 diabetes highlighted moderate elevation of the triglyceride fraction of LDL-6 in the latter group (Cliff's delta = 0.44, adjusted p -value = 1.04×10^{-4}). All other compound comparisons for these groups were found statistically insignificant (Table S3). The same comparison for healthy weight individuals with and without type 2 diabetes (Table S4) showed a stronger class separation, with the multiple plasma lipid components being differentially expressed with moderate effect sizes of Cliff's delta > 0.4. Lipid components of the VLDL subfractions were significantly overexpressed in individuals with type 2 diabetes, together with IDAB (intermediate density apolipoprotein B). Conversely, LDL-2 lipoprotein subfractions were found in higher concentrations in the healthy participants. Comparison of the plasma lipoprotein profiles between nondiabetics and diabetics, regardless of weight category, showed that 10 lipoproteins were common to DM1 and DM2 in differentiating participants with and without diabetes (Figure 3), with 2 lipoproteins being unique to DM1 and 67 unique to DM2, highlighting the greater impact of DM2 than that of DM1 on the lipoprotein profile (Figure S6).

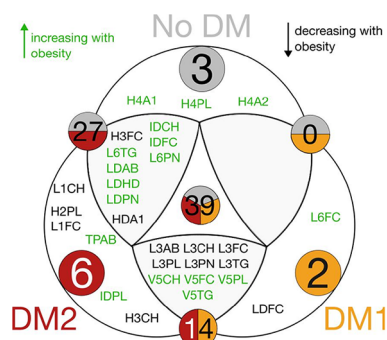


Figure 3. Venn diagram showing lipoproteins that are differential between the no diabetes healthy weight group and the overweight/obese groups for no diabetes, DM1, and DM2.

Obesity had a stronger impact on the lipoprotein profile than diabetes based on p -values for the OPLS-DA model and the Cliff's delta statistic (Tables S3–S7). Obesity was directly associated with IDAB, IDPN, LDL, particularly LDL5 and LDL6 subfractions, and VLDL particles and inversely associated with HDL1 particles. Although the metabolic signature of obesity dominated over diabetes, in individuals with type 1 and type 2 diabetes, amplification of some of the effects of obesity was found when comparing healthy weight nondiabetics with individuals having obesity and diabetes (Figure 3). The presence of type 1 and type 2 diabetes amplified the metabolic signature of obesity with VLDL5 cholesterol and triglycerides becoming significantly higher in both the type 1 and type 2 diabetes groups with obesity, along with lower plasma concentrations of LDL3 cholesterol and triglycerides (Figure 3). Additionally, in type 2 diabetics, a stronger inverse correlation between obesity and both H2PL and H2CH was noted.

While there was some overlap with the subfractions influenced by both obesity and diabetes (i.e., increased concentration of VLDL particles and decrease in LDL2

subfractions), additional lipoprotein parameters that differentiated the participants with obesity included increased IDCH and IDPN, L6 subfractions, and ABA1 (apolipoprotein-B100/apolipoprotein-A1 ratio) and decreased HDL1 subfractions.

The decrease in larger LDL particles, particularly LDL3, and increase in smaller, denser LDL particles with the diabetes–obesity interaction prompted an evaluation of the LDL1–3 (1.019–1.037 kg/L) to LDL4–6 (1.037–1.063 kg/L) ratio. Both obesity and diabetes impacted the distribution of LDL lipoprotein particles, with the healthy weight nondiabetic group being associated with the larger, less dense subfractions LDL1–3 (Table S8 and Figure S7).

A more detailed analysis of the plasma lipid subclasses (based on standard 1D ^1H NMR spectra) in study participants without diabetes and with healthy body weight (further referred to as healthy) revealed characteristic sex differences (Figure S8), where women had higher plasma concentrations of HDL1 and HDL2 particles, and men were characterized by higher concentrations of LDL subfractions 4 and 5 and VLDL subfractions. The impact of sex on the lipoprotein profiles diminished with increasing body weight (Figures S9 and S10). O-PLS-DA models were used to refine the differential lipoprotein signature for sex (Figure S11) and confirmed that HDL1 and HDL2 were present in relatively higher concentration in plasma obtained from women, along with LDL2 subfractions. Conversely, VLDL particles, LDL5, and HDL4 were present in higher concentrations in men regardless of weight category. Additionally, in the obese model, ABA1 was associated with the male group (Figure S11F).

Comparison of the Relative Impact of Obesity and Diabetes on Metabolic Profiles

Self-organizing maps (SOMs) were used to explore the inherent phenotypes in the population based on their NMR spectroscopic and lipoprotein data to ascertain the main driving force behind variation in the plasma metabolome in this population. The chosen configuration was a 4×3 node map, in which the spectra were reasonably evenly distributed indicating a high degree of variance within the data set and that there were more phenotypes (12) than classes (six classes corresponding to nondiabetes, type 1 diabetes, and type 2 diabetes stratified by weight: healthy/overweight/obese). For the lipoproteins, the clearest trend was observed for healthy weight versus overweight/obese with more participants with overweight/obesity (red) clustering on the left-hand side of the map (Figure 4A: specifically nodes 1, 2, 5, 6, and 9), whereas the trend for healthy weight, although less distinct and therefore indicating a wider variance, showed clustering toward the right-hand side of the map (particularly node 4). The same map recoloured for diabetes status did not show a clear trend (Figure S12), indicating that the plasma lipoprotein profile was more strongly influenced by BMI. Comparison of a high BMI-associated node (2) versus a healthy weight node (4) using OPLS-DA showed complete differentiation of the metabolic phenotypes associated with those nodes (Figure S12A), with node 2 (high BMI) being dominated by VLDL parameters, LDL4 parameters, ABA1, IDAB, and IDPN. In contrast, node 4 (lower BMI) was dominated by HDL parameters and LDL2 associated parameters (Figure S13B).

On the contrary, the distribution of participants for the SOM generated for the CPMG data set was mostly influenced by diabetes status (Figure 4B) with nondiabetic participants clustering at the left side of the map (nodes 1, 5, 6, 10) and

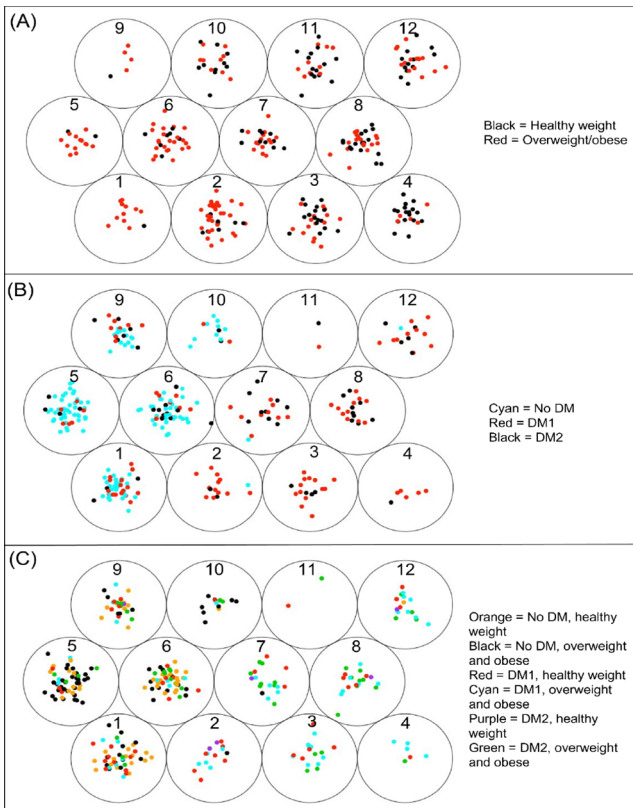


Figure 4. Self-organizing maps of the (A) lipoproteins colored by weight classification, (B) 1D CPMG ^1H NMR spectra colored by diabetes status, and (C) 1D CPMG ^1H NMR spectra colored by diabetes status and weight classification. Nodes are numbered to correspond with the loadings.

diabetics tending to cluster toward the right side of the map. However, the distribution of participants with type 2 diabetes was dispersed across all 12 nodes wherein several nodes indicated distinct clusters of type 1 diabetes (nodes 2, 3, and 4). Recoloring the map to include all six classes, stratifying by both BMI category and diabetes status (Figure 4C) emphasized the diabetes status-driven clustering with orange (no diabetes, healthy weight) and black (no diabetes, obesity) clusters in nodes 1, 5, 6, and 9. Certain nodes contained fewer participants with a distinctive profile, independent of weight or diabetes. For example, node 11 of the CPMG data stratified by diabetes status (Figure 4B) contains plasma spectra dominated by high concentrations of lactate (Figure S14). Within a given clinical phenotype, for example, nondiabetics, subphenotypes are distributed according to their similarities in plasma metabolite profiles, for example, five nodes 1, 5, 6, 9, and 10 all contain samples from participants with no diabetes. Deeper analysis of this clustering distribution using an OPLS-DA model to explore the differences only in nondiabetics between nodes 1 versus 6 ($R^2X = 0.03$, $Q^2Y = 0.06$, and $\text{CV-AUROC} = 0.67$) indicated that node 1 represented a phenotype that had higher lactate and formate with lower SPC and glutamine concentrations (Figure S14A, B). A similar comparison of nodes 1 versus 10 ($R^2X = 0.05$, $Q^2Y = 0.02$, $\text{CV-AUROC} = 0.66$) (Figure S15C,D) showed that node 10 was dominated by higher concentrations of glucose, glutamine, and branched chain amino acids and lower levels of lactate. In terms of the DM1 cohort, participants were dispersed across multiple nodes again indicating that there is more than one phenotype of

DM1. Selecting DM1 samples from nodes 2 and 12 ($R^2X = 0.07$, $Q^2Y = 0.13$, and $\text{CV-AUROC} = 0.62$) (Figure S15E,F), the diabetic phenotype attributed to node 2 contained greater relative proportions of glucose, triglycerides, and valine than node 12, which contained spectra with high levels of SPC.

UPLC-MS Plasma Lipid Profiles Stratify Individuals with Obesity and Healthy Weight without Diabetes but not in Diabetes

Plasma lipid constituents were further profiled using a reversed phase UPLC-MS approach. Modeling BMI categories using OPLS-DA resulted in 1 predictive + 1 orthogonal component models with moderate generalization capacity ($\text{ESI}(-)$: $\text{CV-AUROC} = 0.75$, $R^2X = 0.07$; $\text{ESI}(+)$: $\text{CV-AUROC} = 0.76$, $R^2X = 0.08$) and led to the identification of 14 different lipid species belonging to groups of phosphatidylcholines (PC) and lysophosphatidylcholines (LysoPC) (Table 2). Supervised

Table 2. Discriminatory UPLC-MS Derived Plasma Lipid Compounds for BMI Categories in Non-diabetics

ESI	compound identity	p value ^a	$d_{\text{BMI in DM}}$ ^b
(+)	LysoPC (18:2)	<0.01	−0.48
	LysoPC (20:2)	<0.01	−0.58
	LysoPC (P-18:0)	0.03	−0.40
	PC (18:2/18:2)	<0.01	−0.48
	PC (18:2/18:1)	0.01	−0.50
	PC (18:0/22:6)	0.01	0.48
	PC (P-18:0/16:0)	0.01	−0.44
	PC (36:5)	0.03	−0.40
	PC (38:6)	0.02	0.42
	LysoPC (18:2)	<0.01	−0.48
(−)	PC (18:2/18:2)	<0.01	−0.48
	PC (16:0/22:6)	<0.01	0.41
	PC (18:0/20:4)	<0.01	0.43

^aFDR adjusted. ^bCliff's delta comparing groups of diabetics with healthy weight vs overweight/obese (healthy weight group used as reference).

OPLS-DA models for pairwise comparison between BMI strata in nondiabetic group, as well as diabetes vs nondiabetes in individuals with healthy weight showed low generalization capabilities ($\text{AUROC}_{\text{CV}} < 0.6$), and therefore, these models were not further considered.

Obesity and Diabetes are Associated with Increased Plasma Carnitine Concentrations

Fifteen carnitine (CN) species were targeted for absolute quantification. Univariate statistical comparison of BMI groups in the nondiabetic group flagged isovalerylcarnitine (CN-isoC5) and propionylcarnitine (CN-C3) as being significantly higher in the overweight to obese group (Table 3). None of these trends were statistically significant in the equivalent comparison for the diabetic group. In individuals with healthy weight, a trend toward lower concentrations of medium chain carnitines CN-C6 and CN-C8 was found in lower concentrations in individuals with type 2 diabetes compared to individuals without diabetes, but these differences were not statistically significant and were not observed for the obese comparison equivalent. Higher plasma concentrations of stearoylcarnitine (C18) were observed in individuals within the healthy weight range with type 1 diabetes with a similar, but not statistically significant, trend for individuals with

Table 3. Expression Pattern of Plasma Carnitines in Overweight and Diabetes^a

comparison ^b	compound	median concentration in mM (range)		p value ^c	d ^{b,d}
		reference group	comparator group		
healthy weight vs obesity (without diabetes)	CN-isoC5	0.11 (0.03–0.48)	0.18 (0.06–0.48)	1.9×10^{-3}	0.53
	CN-C3	1.41 (0.67–3.88)	2.11 (0.88–6.00)	2.1×10^{-2}	0.40
nondiabetic vs diabetic (with healthy weight)	CN-C18	1.93 (1.44–12.00)	3.87 (1.52–12.00)	1.7×10^{-2}	0.44

^aStatistically significant differences in the carnitine profile were expressed in groups stratified for BMI and diabetes. Key: propionylcarnitine (CN-C3), isovalerylcarnitine (CN-isoC5), and stearyl carnitine (CN-C18). ^bThe group indicated first in column named comparison was used as reference for the calculation of Cliff's delta. ^cFDR adjusted. ^dEffect size measured as Cliff's delta.

healthy weight and type 2 diabetes and with type 1 diabetes and obesity.

Impact of Prescribed Drugs on Metabolic Profiles

Prescribed drugs were recorded for all participants, and the major classes of drug, stratified by BMI and diabetes categories (Table S9) showed, as expected, 100% insulin use in participants with DM1 irrespective of BMI category, with 80% of participants with overweight/obesity and DM2 taking metformin or biguanides with no difference in medication use between BMI categories. The impact of metformin, which was modeled separately for DM1 and DM2, was weaker than the impact of either BMI or diabetes and was not a major influence in the clustering of samples in the PCA score plot (Figures S16 and S17). Similar analysis for statins, angiotensin converting enzyme (ACE) inhibitors, and thyroxine/levothyroxine (Figure S18) also showed no systematic effect of these drugs on the plasma metabolite profile.

DISCUSSION

Given the prevalence of obesity in Middle Eastern populations, taking into account its potential role in the early onset of diabetes, we sought to explore the impact of obesity on the diabetic phenotype using a multimodal approach combining NMR spectroscopy and MS platforms. Distinctive metabolic phenotypes were identified for high BMI and for both type 1 and type 2 diabetes with some commonalities between the metabolic signatures of diabetes and obesity. As expected, high-level evaluation of the metabolic phenotypes associated with obesity and diabetes, based on the NMR profiles, indicated that glucose and plasma lipids were characteristic of obesity in blood plasma in individuals with and without diabetes. The results suggest that loss of plasma glucose homeostasis when comparing individuals with obesity to individuals with healthy weight is more obvious in nondiabetes state compared to individuals with either type 1 or type 2 diabetes, where glucose regulation is already lacking regardless of weight and is consistent with the reported strong association between body weight and presence of prediabetes in a Saudi Arabian population.³⁷

One of the most prominent features of obesity in the nondiabetes group was the significantly elevated GlycA identified in the full resolution NMR data coupled with lower plasma concentrations of SPC. GlycA has been reproducibly associated with chronic inflammatory conditions, including rheumatoid arthritis³⁸ and atherosclerosis, being described as an independent risk factor for cardiovascular disease.^{39–41} Higher plasma GlycA concentrations in the participants with obesity are consistent with low grade inflammation associated with adiposity. The pairing of higher plasma concentrations of GlycA together with lower concentrations of SPC in individuals in states of either acute

or chronic inflammation has been observed in several studies and is also apparent in the current study with the Glyc:SPC ratio being higher in individuals with obesity.^{42,43} The diminished weighting of GlycA in differentiating individuals with/without obesity in the diabetic groups reflects the fact that inflammation contributes to both diabetes and adiposity.^{44,45} A similar phenomenon is observed with respect to sex-related differences in the metabolic profile, which are more apparent in healthy weight individuals and in individuals without diabetes. Plasma obtained from women was characterized by relatively lower concentrations of amino acids and higher concentrations of triglycerides and HDL. The models characterizing sex differences in the obese and diabetic groups were not as strong, keeping with the fact that both obesity and diabetes introduce metabolic dysregulation that overshadows physiological differences attributed to sex. In terms of plasma lipoprotein composition, a sex effect was observed for most lipoprotein classes in healthy individuals with healthy weight, with higher concentrations in VLDL and cholesterol observed in men and higher levels of HDL subclass-1 found in women. These results are consistent with findings of the Framingham study, where higher VLDL and cholesterol in men was associated with a greater risk profile for coronary heart disease and 2-fold higher large HDL particles in women.⁴⁶ In a Japanese population, similar findings were reported for VLDL-cholesterol (higher in men) and HDL (higher in women).⁴⁷ The participants in the current study were significantly younger than those recruited for both the Framingham and Japanese studies.

Increased concentrations of small, dense LDL and HDL particles have been associated with poor cardiorespiratory fitness, as measured by VO_{2max} .⁴⁸ Increased serum LDL has been reported as a marker of obesity, and central adiposity in particular, in several populations.⁴⁹ Increased VLDL triglycerides and decreased mean LDL particle size, as observed in the current study, have been associated with inflammation.⁵⁰ Madssen et al. found an association between VLDL and IDL particles at baseline and later weight gain in a study on the impact of chemotherapy on blood parameters,⁵¹ which was correlated with downstream prognosis. The obesity-associated lipoprotein phenotype observed in this study was partially masked in individuals with diabetes, where both diabetes and obesity are associated with a similarly dysregulated lipoprotein profile. Of particular note was the amplification of the obese lipoprotein profile by both type 1 and type 2 diabetes, with a shift in the distribution of LDL particles as evidenced by the decreased ratio of LDL1–3 (1.019–1.037 kg/L) to LDL4–6 (1.037–1.063 kg/L) in both obese and diabetic groups compared to the healthy weight non diabetic group. A shift toward smaller, denser LDL particle sizes, as observed here, has been previously reported in type 2 diabetics,⁵² even though no difference in total cholesterol levels between diabetics and

nondiabetics was found.⁵³ The proportional increase in small dense LDL particles, which are more susceptible to glycation, has been proposed to contribute to the increased atherogenicity of circulating glycated apolipoprotein B in type 2 diabetes.⁵⁴ Of note, the ABA1 ratio, which was significantly higher in the group of individuals with obesity, has been associated with atherogenic risk⁵⁵ and represents the balance between atherogenic particles that are rich in apolipoprotein B and particles that are rich in the generally antiatherogenic apolipoprotein 1.

In addition to the lipoprotein subclasses, the effect of obesity on the diabetic lipidome was evaluated using a reversed phase UPLC-MS profiling method. The main findings were lower plasma concentrations of lysophosphatidylcholines C18:0, C18:2, and C20:0 in individuals with obesity without diabetes. As with other lipid parameters, the effect was not significant in individuals with diabetes suggesting that diabetes-induced dyslipidemia overlaps with the signature of obesity. Altered blood levels of lysophosphatidylcholines, including lysoPC (C18:2), have demonstrated an inverse relationship with BMI and with waist and hip circumferences.⁵⁶ Conversely, increased plasma lysophosphatidylcholine levels have been associated with atherogenic traits including visceral fat accumulation and oxidized LDL in metabolically unhealthy individuals with obesity.⁵⁷

In concordance with the observation of altered carnitine metabolism in DM2,^{58,59} we found higher concentrations of isovalerylcarnitine (CN-isoC5) and propionylcarnitine (CN-C3) in the plasma of the overweight to obese group, compared to individuals with healthy weight. Diabetes was associated with higher plasma concentrations of stearyl carnitine in healthy weight individuals. Stearyl carnitine has been shown to accumulate in β cells, causing excessive β -oxidation and energy deficiency via reduction of TCA cycle and oxidative phosphorylation metabolites. These effects have been associated with diminishment of insulin synthesis.⁶⁰ Other studies have proposed that increased fatty acylcarnitine levels observed in people with DM2 reflect incomplete fatty acid β -oxidation in diabetes, and that the increase in shortened chain acylcarnitine molecules activate proinflammatory pathways linked to insulin resistance.⁶¹ This same study showed an inverse association between propionylcarnitine, associated with obesity in the current study, and hemoglobin A1c.

As expected, higher plasma concentrations of ketone bodies were characteristic of participants with DM1 and have been previously reported as differential between DM1 and DM2, with 3-D-hydroxybutyrate showing temporal variation in response to the timing of meals and serum insulin levels.⁶²

Within each group, we noted several subphenotypes, most easily identified from the SOMs. Participants without diabetes inherently clustered into five subphenotypes (nodes 1, 5, 6, 9, and 10) based on the CPMG spectra, wherein these five main subphenotypes were characterized by differing relative concentrations of lactate, glucose, formate, glutamine, and branched chain amino acids (Figures 4 and S14). DM1 and DM2 were more dispersed and did not show such strong phenotypes based on the low molecular weight profiles, but distinct differences between nodes were still apparent based on triglycerides, glucose, and other metabolites. The AUROC values for the paired node comparisons were similar for the internode comparisons, but the Q^2Y value was stronger for the paired DM1 node comparisons than that for the node comparisons in the nondiabetic group, which suggests that

type 1 diabetes has distinct subphenotypes. Based on the number of samples in the current cohort, further exploration into the phenotypes is not appropriate but investigating the association of these subphenotypes with clinical characteristics in larger cohorts to identify whether glucose control, ketosis, or other factors are driving the metabolic patterns would be beneficial. Given the discordance of the number of individuals with type 1 diabetes in groups with or without obesity, we did not conduct extensive analysis of the metabolic impact of obesity on type 1 and 2 diabetes. However, although the metabolic signature of the two types of diabetes may differ, the impact of obesity on both was similar. Although there was no recorded inherent bias in the therapeutic management of the participants with obesity versus healthy weight, a limitation of the current study was that, given the relatively small group sizes, it was not feasible to stratify according to therapeutic management, which would warrant further investigation in larger cohorts. However, classifying individuals according to intake of drug or drug classes showed no systematic influence of any of the drugs on the metabolic profile.

A further confounder in the interpretation of the results presented here may be the use of BMI as the metric for obesity. It is becoming increasingly apparent that individuals classified as "normal" weight using the BMI criterion have excess adiposity.⁶³ In reality, metabolic syndrome represents a continuum of synergistic effects with multiple phenotypes, as captured by the SOMs here. Similarly, although obesity is a risk factor for type 2 diabetes and cardiovascular disease, there is a growing body of evidence suggesting that there are multiple obesity phenotypes such as the metabolically "healthy" obese who do not manifest risk factors such as elevated BP or high cholesterol.⁶⁴ Thus, in a relatively small cross-sectional study such as this, it is difficult to infer relationships between obesity and diabetes. Nevertheless, we find a complex interaction between obesity, diabetes, and sex and capture some of those interactive effects on the plasma metabolome.

There are several limitations within this study, first comorbidities have not been explored within this study as for 33% of the cohort no information is provided on their presence. Therefore, we could not investigate whether comorbidities may be further influencing sex differences. Second, the group of healthy weight individuals with type 2 diabetes is small ($n = 6$), therefore no multivariate analysis could be conducted on this phenotype. However, all the other groups are sufficiently powered.

In summary, we present one of the first metabolic characterizations of combined obesity and diabetes in a Saudi Arabian cohort and show convergence metabolic phenotypes for obesity and diabetes with obesity enhancing the metabolic dysregulation associated with diabetes for some metabolic pathways. The expression of such severely dysregulated phenotypes in this relatively young adult Saudi Arabian population reflects the metabolic consequences of the evolving obesogenic environment throughout the Middle East. In particular, the association between dysregulated plasma lipid parameters and the GlycA warrants further investigation including systematic consideration of covariates such as sex.

■ ASSOCIATED CONTENT

Data Availability Statement

The data that support the findings of this study are available at DOI: 10.5281/zenodo.10279109.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00717>.

Table S1, summary of study cohort metadata; Table S2, annotation of the keys used by the Bruker IVDr Lipoprotein Subclass Analysis; Table S3, OPLS loadings, Cliff's delta, and adjusted *p*-values of the lipoproteins for the comparison of the healthy weight group non-diabetics versus healthy weight group type 1 diabetics; Table S4, OPLS loadings, Cliff's delta and adjusted *p*-values of the lipoproteins for the comparison of the healthy weight group nondiabetics versus healthy weight group type 2 diabetics; Table S5, OPLS loadings, Cliff's delta, and adjusted *p*-values of the lipoproteins for the comparison of the healthy weight group nondiabetics versus obesity weight group nondiabetics; Table S6, OPLS loadings, Cliff's delta, and the adjusted *p*-values of the lipoproteins for the comparison of the healthy weight group nondiabetics versus obesity weight group type 1 diabetics; Table S7, OPLS loadings, Cliff's delta, and adjusted *p*-values of the lipoproteins for the comparison of the healthy weight group nondiabetics versus obesity weight group type 2 diabetics; Table S8, comparison of the different weight groups with and without diabetes of the ratio L1-L3CH:L4-L6CH; Table S9, main drugs/drug classes for study participants, stratified by diabetes type and BMI; Figure S1, OPLS-DA loading and score plots of ¹H CPMG spectra of obese individuals with and without diabetes; Figure S2, plasma glucose concentrations segmented by BMI classes and presence of diabetes; Figure S3, OPLS-DA models of 1D CPMG ¹H NMR spectra stratified by sex; Figure S4, comparison of participants BMI and corresponding waist to hip ratio; Figure S5, PCA model of 1D CPMG ¹H NMR spectra from participants with type 2 diabetes stratified by weight; Figure S6, Venn diagram of the number of differential lipoproteins in the comparison of no diabetes with type 1 or type 2 diabetes; Figure S7, box plots stratified by weight and diabetes status for the ratio L1-L3CH:L4-L6CH; Figure S8, PCA loading and score plots for individuals that are of a healthy weight, colored by sex; Figure S9, PCA loading and score plots of the lipoproteins for individuals that are overweight, colored by sex; Figure S10, PCA loadings and score plots of the lipoproteins for individuals who are obese, colored by sex; Figure S11, OPLS-DA scores and loadings of the lipoproteins for females versus males; Figure S12, self organizing maps (SOMS) of the lipoproteins colored by diabetes status and 1D ¹H NMR spectra colored by BMI; Figure S13, OPLS-DA score and loading plots of lipoprotein SOM node 2 versus SOM node 4; Figure S14, median 1D CPMG ¹H NMR spectrum from node 5 and node 11 and a comparison of nodes showing high concentrations of lactate in the spectrotpe associated with node 11; Figure S15, OPLS-DA scores and loadings for nodes 1 and 6 (nondiabetics only), nodes 1 and 10 (nondiabetics only), and nodes 2 and 12 (type 1 diabetics only);

Figure S16, PCA score plots of the plasma NMR CPMG data of participants with type 1 diabetes only, colored by type of medication; Figure S17, PCA score plots of the plasma NMR CPMG data of participants with type 2 diabetes only, colored by type of medication; Figure S18, PCA score plot of the plasma NMR CPMG data for all study participants, colored by thyroxine use (PDF)

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Author Contributions

M.S.A., Z.A.D., and E.H. conceived and planned the project. M.S.A., J.I.S.C., E.Y.S., M.K., C.B., S.A., and A.H.A. conducted experiments and collected data. M.S.A., J.I.S.C., E.Y.S., S.L.L., M.K., C.B., and N.M. performed data analysis and contributed to data interpretation. M.S.A., N.M., S.L., and E.H. wrote the manuscript. M.S.A., T.A., Z.A.D., S.L., and J.W. supervised the project. All authors reviewed and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

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