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Metformin Monotherapy Alters the Human Plasma Lipidome Independent of Clinical Markers of Glycemic Control and Cardiovascular Disease Risk in a Type 2 Diabetes Clinical Cohort

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Running Title: Metformin alters the lipidome independent of diabetes control.

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Abbreviations:
ACEi: Angiotensin-converting enzyme inhibitor
ARB: Angiotensin receptor blocker
BB: Beta-blocker
BMI: Body mass index
CVD: Cardiovascular disease
CRP: C-reactive protein
EMR: Electronic medical record
EPA: Eicosapentaenoic acid
ESR: Erythrocyte sedimentation rate
FAHFAs: Fatty acid esters of hydroxy fatty acids

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FFAs: Free fatty acids
FIE: Flow injection ionospray
FTICR: Fourier Transform Ion Cyclotron Resonance
HbA1c: Hemoglobin A1c
HOMA-IR: Homeostatic Model Assessment for Insulin Resistance
IRB: Institutional Review Board
LC: Liquid chromatography
Lp-PLA2: Lipoprotein-associated phospholipase A2
MS: Mass spectrometry
m/z: Mass/charge ratio
NaTFA: Sodium trifluoroacetate
ND: Non-diabetic
PAHSA: Palmitic acid esthers of hydroxystearic acid
PAI-1: Plasminogen activator inhibitor type 1
PC: Phosphatidyl choline
PGE_2: Prostaglandin E_2
PE: Phosphatidyl ethanolamine
SM: Sphingomyelin
T2D: Type 2 diabetes
T2D-DL: Type 2 diabetic treated with diet and lifestyle modifications
T2D-M: Type 2 diabetic treated with metformin monotherapy
UW: University of Wisconsin
UWHC: UW Hospitals and Clinics
WMA: World Medical Association

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Abstract: Type 2 diabetes (T2D) is a rising pandemic worldwide. Diet and lifestyle changes are typically the first intervention for T2D. When this intervention fails, the biguanide, metformin, is the most common pharmaceutical therapy. Yet, it's full mechanisms of action remain unknown. In this work, we applied an ultrahigh resolution, mass spectrometry-based platform for untargeted plasma metabolomics to human plasma samples from a case-control observational study of non-diabetic and well-controlled T2D subjects, the latter treated conservatively with metformin or diet and lifestyle changes only. No statistically significant differences existed in baseline demographic parameters, glucose control, or clinical markers of cardiovascular disease risk between the two T2D groups, which we hypothesized would allow the identification of circulating metabolites independently associated with treatment modality. Over 3000 blank-reduced metabolic features were detected, with the majority of annotated features being lipids or lipid-like molecules. Altered abundance of multiple fatty acids and phospholipids were found in T2D subjects treated with diet and lifestyle changes as compared to non-diabetic subjects: changes that were often reversed by metformin. Our findings provide direct evidence that metformin monotherapy alters the human plasma lipidome independent of T2D disease control and support a potential cardioprotective effect of metformin worthy of future study.

Significance Statement: This work provides important new information on the systemic effects of metformin in type 2 diabetic subjects. We observed significant changes in the plasma lipidome with metformin therapy, with metabolite classes previously associated with cardiovascular disease risk significantly reduced as compared to diet and lifestyle changes. While cardiovascular disease risk was not a primary outcome of our study, our results provide a jumping-off point for future work into the cardioprotective effects of metformin, even in well-controlled type 2 diabetes.

1. Introduction

Type 2 diabetes (T2D), often associated with obesity, is a rising pandemic world-wide, with 9.3% of adults estimated to have T2D globally: a number that is predicted to rise to 10.9% (700 million individuals) by 2045 (International Diabetes Federation, 2020). T2D comes at a high cost, both in terms of human life and economic damage due to loss of productivity and global health expenditures

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(Ng et al., 2014; International Diabetes Federation, 2017). In 2020, diabetes was the 8th leading cause of death in the US (Ahmad and Anderson, 2021). Individuals with diabetes also suffer from a host of co-morbidities whose severity increases the longer blood glucose remains inadequately controlled, including neuropathy, nephropathy, retinopathy, and microvascular and macrovascular complications (Nathan, 1993). Identifying ways to more effectively treat diabetes and its complications are of critical importance.

T2D is exemplified by chronically elevated blood glucose levels caused both by insufficient insulin release from the pancreatic β-cells and resistance of the body's tissues to the effects of insulin. Hemoglobin A1c (HbA1c) is a clinical test that quantifies the glycemic state over the previous 2-3 months. For T2D individuals, current HbA1c targets for vary from 6.5 – 8%, depending on age and comorbidities (Qaseem et al., 2018; Garber et al., 2020; American Diabetes Association Professional Practice Committee, 2021). T2D disease management typically focuses first on diet and lifestyle changes, progressing to pharmaceutical interventions once those fail. Diet and lifestyle management focuses on five principles: improving diet, increasing exercise, reducing smoking, reducing alcohol consumption, and reducing body weight (Schlesinger et al., 2020). Yet, in a vast majority of individuals, diet and lifestyle modifications are insufficiently effective in achieving blood glucose targets, particularly long term (Khunti et al., 2013; American Diabetes Association Professional Practice Committee, 2021).

Once pharmaceutical intervention is called for, the biguanide, metformin, is nearly always the first-line therapeutic (American Diabetes Association Professional Practice Committee, 2021). Metformin is generally accepted as a medication with few severe complications, and, besides initial gastrointestinal side effects, is usually well-tolerated (American Diabetes Association Professional Practice Committee, 2021). Metformin has also been suggested to have cardioprotective benefits in individuals with T2D (Eurich et al., 2005; Evans et al., 2006; Holman et al., 2008). With increasing evidence achieving HbA1c targets alone is insufficient to prevent adverse outcomes (The NICE-SUGAR Study Investigators, 2009), particularly with regards to cardiovascular events (Herman et al., 2005; Skyler et

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al., 2009; Giorgino et al., 2016), there has been a push for advancing pharmaceutical interventions in concert with diet and lifestyle changes (Altaf et al., 2015; Padhi et al., 2020). With nearly 70 years of clinical use and a well-established safety profile, metformin is a logical candidate (Holman et al., 2008; 2021).

Although there is still some debate in the field, metformin's primary blood glucose-lowering mechanisms are thought to be reduced glucose production and release by the liver and increased lactate production in the small intestine (Pernicova and Korbonits, 2014; McCreight et al., 2016; Song, 2016). Both of these mechanisms have been linked with alterations in circulating metabolites (Wang-Sattler et al., 2012; Floegel et al., 2013; Walford et al., 2014; Guasch-Ferre et al., 2016; Lu et al., 2016; Pallares-Méndez et al., 2016; Yu et al., 2016; Lai et al., 2020; Zhao et al., 2020). Metabolomics, the endpoint of the 'omics cascade, is well suited to study metabolic disorders like T2D (Dunn and Hankemeier, 2013; Guasch-Ferre et al., 2016; Pallares-Méndez et al., 2016). As high throughput and high sensitivity/specificity techniques are continually being developed, metabolomics has emerged as an approach well-suited to provide coverage of the changes that occur in clinical biosamples because of disease status and intervention.

In this study, we applied a high-throughput, ultrahigh resolution Flow Injection Electrospray (FIE) Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometry (MS) workflow to plasma samples from three groups—non-diabetic subjects, T2D subjects treated with diet and lifestyle modifications, and T2D subjects on metformin monotherapy—quantifying the relevant abundance of metabolic features among groups using an untargeted approach. Notably, all T2D subjects had good-to-excellent glucose control, and subjects were well-matched for potential confounders such as age and body mass index (BMI). Over 3000 discrete metabolic features were significantly expressed per group, with over 2000 being annotated by chemical name or structure. Family and pathway analyses revealed key changes in the lipidome, particularly with regards to fatty acids and related molecules, were strongly associated with metformin monotherapy, independent of commonly used measurements of T2D disease control and cardiovascular disease (CVD) risk.

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2. Materials and Methods

2.1. Human Subjects

All human subjects research was conducted in accordance with the standards set out by the World Medical Association (WMA) Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" as approved by the University of Wisconsin (UW) Health Sciences Institutional Review Board (IRB) protocol number UW 2013-1082. Study design and participant recruitment has been previously described (Truchan et al., 2021). Briefly, potentially eligible subjects were UW Hospitals and Clinics (UWHC) Endocrinology or Internal Medicine patients who met baseline inclusion and exclusion criteria by consent-waived electronic medical record (EMR) search. Briefly, inclusion criteria included age 18-74 years old, not pregnant or lactating, no anemia or grossly abnormal kidney or liver function tests, no known autoimmune diseases or inflammatory disorders, and, for T2D subjects, no diagnosis of diabetes besides T2D. Potentially-eligible subjects were contacted by phone. Those interested in participating provided written informed consent, and a fasting plasma sample was collected. The full clinical cohort comprised 35 non-diabetic and 132 T2D subjects, as described previously (Truchan et al., 2021; Fenske et al., 2022). For this work, plasma samples from 15 T2D subjects treated conservatively with diet/lifestyle modifications or metformin monotherapy and 14 ND controls matched for age and BMI were selected for downstream untargeted metabolomics.

2.2. Metabolite extraction

The extraction was performed as previously described (Zhu et al., 2021). In brief, a 30 µL aliquot was taken from thawed plasma samples, mixed with 60 µL of chilled liquid chromatography (LC)-MS grade methanol. The samples were then vortexed, mixed with a nutating mixer, and centrifuged. 50 µL of supernatant was mixed with 50 µL of water for flow injection electrospray FTICR MS analysis.

2.3. FTICR MS metabolomics analysis

FIE-FTICR MS experiments were performed using a Waters nanoACQUITY UPLC (Waters Corporation, Milford, MA, USA) coupled to a Bruker solariX 12 T FTICR mass spectrometer (Bruker

Daltonics, Bremen, Germany) without an LC column. 5 µL of each metabolite extract was directly injected in triplicate from the Waters nanoACQUITY UPLC into the FTICR MS via 100 µm x 40 cm PEEK tubing. The mobile phase was 50:50 methanol:water with 0.1% formic acid or 10 mM ammonium acetate added for positive or negative modes, respectively, with a flow rate of 20 µl/min. Ions were accumulated for 0.1 s, and an 8 M transient size applied, with 50 scans collected. The m/z (mass/charge ratio) range was set to 40-1500, with 50 m/z Q1 mass. 50 scans were collected for each mass spectrum. Dry gas flow was set to 4 L/min at 150 ºC. Largest frequency values for octopole (5 MHz), quadrupole (2 MHz), and transfer hexapole (6 MHz) were used to improve ion transition. Time of flight was set to 0.8 ms. Sweep excitation power was set to 27%. The estimated resolving power at 400 m/z was 190,000. The FTICR MS was calibrated with 1 mM Sodium trifluoroacetate (NaTFA) in both positive and negative modes before experiments.

2.4. Data Analysis

Mass spectra were processed and analyzed using DataAnalysis 4.3 (Bruker Daltonics, Bremen, Germany). Bucket (mass) lists in positive and negative ion modes were generated using the T-ReX 2D workflow in MetaboScape 4.0. The maximum Δm/z was set to 0.50 mDa. The maximum charge state was set to 3, and the intensity threshold was set to 0. The minimum number of features for the result was set to 5. Bucket lists in positive and negative ion modes were merged into one bucket list with 1.0 ppm m/z tolerance. Features with a ratio of sample average:blank average < 10 were deleted. The merged bucket list was annotated with the SmartFormula function in MetaboScape 4.0 (Bruker Daltonics, Bremen, Germany) with 2.0 ppm as the narrow Δm/z cutoff, 5.0 ppm as the wide Δm/z cutoff, 20 as the narrow mSigma cutoff, and 50 as the wide mSigma cutoff. Elements were set to CHNOPS, and element ratio filters were applied with common element ratio presets in MetaboScape 4.0, including 0.2-3.1 H/C ratio, 0-1.3 N/C ratio, 0-1.2 O/C ratio, 0-0.3 P/C ratio, 0-0.34 P/O ratio, and 0-0.8 S/C ratio. Electron configuration was set to Even. Heuristic element count probability check was applied. The putative metabolites were annotated by METLIN with a 2 ppm mass error cutoff. In most cases, the isomer with the lowest METLIN number was reported. Lipids and lipid-like molecules were reported as a combined general chemical name with total fatty acid carbon number:number of double bonds. In a few cases, more than one isomer with similar structures and chemical properties were reported for one mass. SmartFormula and Metlin annotations provide information on the chemical formulas and chemical names, respectively (Smith et al., 2005; Guijas et al., 2018). InChI keys, HMDB IDs, and KEGG IDs were acquired using CTS servers from the METLIN name. InChI keys were used to classify the METLIN annotations using "ClassyFire," and annotated metabolites categorized according to their class information (Djoumbou Feunang et al., 2016). PubChem IDs were gathered using the PubChem server translator. Statistical analysis was performed using MetaboScape 4.0 and the online software MetaboAnalyst (Chong et al., 2018). MetaboScape was used for PCA analysis. MetaboAnalyst was used for pathway analysis, enrichment analysis, and statistical analyses (e.g., heat map, significant features). All p-values are reported as false discovery rate (FDR)-corrected. ChemRich plots were made using the database information from CTS, PubChem IDs and p-values from MetaboAnalyst to generate a plot of chemical similarity and significance.

3. Results

3.1. Validation of the Patient Cohort to Detect Treatment-specific Changes in the Metabolome.

Biobanked plasma samples from seven T2D individuals treated with diet and lifestyle modifications (T2D-DL), eight T2D individuals treated with metformin monotherapy (T2D-M) and 14 non-diabetic (ND) subjects, well-matched for age and BMI, were selected from a larger clinical cohort described in Truchan and colleagues and Fenske and colleagues (Truchan et al., 2021; Fenske et al., 2022). The mean duration of disease was nearly identical between T2D-DL and T2D-M groups (~3 years). As expected, HbA1c and fasting blood glucose were both elevated in T2D subjects as compared to ND, with no statistically significant differences between the T2D groups, and no T2D individuals had HbA1c levels above 8.1%, indicating good-to-excellent glucose control (**Table 1**). Fasting glucose and fasting insulin were used to calculate insulin resistance using the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (Matthews et al., 1985). Neither fasting insulin nor HOMA-IR were significantly different among the groups (**Table 1**). Common CVD risk factors, including systolic blood pressure, diastolic blood pressure, total cholesterol, HDL, LDL, triglycerides levels, were also similar

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(**Table 1**). As these values can be influenced by supplements or pharmaceuticals commonly prescribed to T2D patients, the rates of daily omega-3/fish oil supplement, statin, and prophylactic (i.e., low-dose) aspirin use were recorded, and none were significantly different among the T2D groups, although more T2D subjects were using prophylactic aspirin than ND (**Table 1**). Significantly more T2D subjects had been prescribed an angiotensin-converting enzyme inhibitor (ACEi), angiotensin receptor blocker (ARB), or beta-blocker (BB) for high blood pressure than ND subjects, with no difference between the T2D groups (**Table 1**). Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a marker of arterial inflammation associated with CVD risk (Dada et al., 2002), and there was a trend (p=0.06) towards elevated Lp-PLA_2 in the T2D groups as compared to ND, with no difference between the T2D groups (**Table 1**). No subjects had clinically elevated erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP), indicating the absence of acute inflammation or infection (**Table 1**). Taken together, both T2D groups had nearly identical diabetes control and CVD risk as measured by well-accepted clinical markers.

3.2. "Lipids and Lipid-like Molecules" are the Largest Superclass of Plasma Metabolites Detected by an Untargeted FIE-FTICR MS Approach.

We employed a FIE-FTICR MS workflow previously developed and validated for high-throughput, ultrahigh resolution untargeted metabolomics analysis of pre-clinical plasma samples (Schaid et al., 2021; Zhu et al., 2021). Using MetaboScape, over 3840 discrete metabolic features, as defined by unique m/z, were detected above background, with most being shared among groups (**Figure 1A, hatched bars**). Of these features, 2925 could be annotated by chemical formula, again, with most (1956) being shared among groups (**Figure 1A, solid bars, and Figure 1B**). 251 were limited to the ND group, 11 limited to the T2D-DL group, and 10 limited to the T2D-M group (**Figure 1B**). Performing a PCA analysis, samples from the T2D-DL group clustered the most strongly together, with significant overlap between the ND and T2D-M groups, with only the ND group having samples outside of the 95% confidence limit (**Figure 1C**). A schematic of the analysis workflow can be found in **Figure**

S1, and a full list of significantly expressed metabolic features with can be found in **Supplementary File 1**.

Based on the chemical ontology of annotated metabolites, the "Lipids and lipid-like molecules" superclass comprised over 67% of annotated metabolites, with the combined "glycerophospholipids" and "fatty acyls" classes accounting for two-thirds (**Figure 1D)**. Of the polar metabolites, "Organic acids and derivatives" was the second most abundant superclass, with 48 annotations, and was primarily composed of the subclass "amino acids, peptides, and analogues" (**Figure 1D**). Unsupervised hierarchical clustering of the MetaboScape-annotated features revealed ND samples strongly segregated from the T2D groups, with some overlap between the two T2D groups (**Figure 1E**).

3.3. A Broad Shift to Steroidogenesis T2D groups

A wide range of metabolite sets were enriched in the combined T2D groups vs. ND group. The metabolite set with the highest fold enrichment was "Sphingolipid metabolism," while "Purine metabolism" had the highest statistical significance (**Figure 2A**): both components in steroidogenesis, which was also significantly enriched. In agreement with the metabolite set analysis, the purine metabolism pathway was the most significantly altered, while changes to the linoleic acid metabolism pathway were the most highly affected by T2D status **(Figure 2B**). A ChemRich analysis, which clusters based on chemical similarity, highlights the broad differences between the combined T2D group and ND (**Figure 2C**). Among these differences were increases in fatty acid abundance, including both saturated and unsaturated fatty acids. Important to note is the observed increase in hexoses, as would be expected in plasma samples from T2D subjects. There were also changes to multiple lipid classes, including phosphatidylethanolamines (PEs), phosphatidylcholines (PCs), and sphingomyelins (SMs), with most of them being increased (**Figure 2C**).

Separate pairwise analyses were also performed among the ND, T2D-DL, and T2D-M groups. Similarly, to the combined T2D analysis, "Sphingolipid Metabolism" was the most highly enriched and

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most significantly impacted metabolite set in the T2D-DL vs. ND group (**Figure S2A**), purine metabolism the most significantly altered pathway (**Figure S2B**), and linoleic acid metabolism was the most highly impacted pathway (**Figure S2B**). The T2D-DL group showed a broad shift in metabolism, with numerous fatty acid classes increased, including saturated, unsaturated, and hydroxy fatty acids (**Figure S2C**). PCs, PSs and amino acids were increased, while cholesterol esters and unsaturated triglycerides were decreased (**Figure S2C)**. Similar results for metabolite set and pathway impact analyses when comparing the T2D-M vs. ND groups (**Figures S3A, S3B**). Interestingly, the ChemRich analysis showed far fewer differences in chemical similarity between the T2D-M and ND as compared to either the combined T2D or T2D-DL analyses (**Figure S3C**). PEs, unsaturated triglycerides, and cholesterol ethers as compared to the ND group (**Figure S3C**). As compared to the ND group, the T2D-M group had SM and PC lipid species that were increased, while others were decreased. Complete lists of significantly altered metabolites from each analysis can be found in **Supplementary Tables S1 and S2**.

3.4. Changes in Fatty Acid Metabolism by T2D Treatment

Based on the significant differences observed in the abundance of fatty acids, their conjugates, and related metabolite groups such as phospholipids (**Table 2 and Supplementary Tables S1 and S2**), we honed in on a subset of these metabolites for a more detailed analysis. Even though there were no significant differences between the two T2D groups with regards to T2D control and other important biometric and clinical parameters, there was a clear alteration in fatty acid metabolism in the T2D-DL group as compared to the ND group, with many of these changes being ameliorated with metformin treatment. The longer chain acylcarnitines, octanoylcarnitine and palmitoylcarnitine, were increased in the T2D-DL group, with no difference between the ND and the T2D-M groups (**Figure 3A**). The building block, carnitine, was lowest in the T2D-M group, albeit not with statistical significance. Higher levels of palmitoyl carnitine have been linked to disorders of fatty acid oxidation (Bjørndal et al., 2018), and an analysis of fatty acid abundance among groups also showed increased levels in the T2D-DL group, with a return to ND levels in the T2D-M group (**Figure 3B**). This trend held true for both saturated fatty acids, including palmitic and stearic acid, as well as unsaturated fatty acids, including oleic, linoleic, and arachidonic acid. Saturated hydroxy fatty acids were not altered among the groups, including the precursor to fatty acid esters of hydroxy fatty acids (FAHFAs), 2-hydroxy stearic acid (**Figure 3C**). Unsaturated hydroxy fatty acids did not follow this trend, with ricinoleic acid being increased in both T2D groups as compared to ND (albeit with statistical significance only for T2D-DL), while the abundance of dimorphecolic acid and α-kamlolenic acid decreased (**Figure 3C**). Finally, FAHFA abundance was increased across the board in the T2D-DL group as compared to ND, with a normalization in plasma from individuals treated with metformin (**Figure 3D**).

3.5. Changes in Phospholipid Abundance with Metformin Therapy

A volcano plot analysis revealed several metabolic features had a statistically significant fold change of > 2 between the two groups (**Figure 4A**). In exploring the metabolites that were altered, we observed decreases in unsaturated fatty acids and phospholipids composed of these fatty acids, including PCs, PEs, and SMs, in the T2D-M group compared to T2D-DL group (**Figure 4B**). In the pro-inflammatory state, Lp-PLA$_2$ cleaves oxidized fatty acids from the sn2 position of phospholipids (Rosenson and Stafforini, 2012), and increased levels of Lp-PLA$_2$ are associated with increased risk of CVD (Garza et al., 2007). While no differences in Lp-PLA$_2$ levels were observed between the two T2D groups, the levels of oxidized PCs were significantly increased levels in the T2D-DL group as compared to ND and were normalized by metformin treatment (**Figure 4C**). The trend of increased lipid abundance in the T2D-DL group was particularly strong for lipids with higher peak intensity, including SM (d34:1) and PC (38:6) (**Figure 4D**). For lower intensity lipids, such as SM(d36:2) and PC(36:5), we also observed the same trend, indicating a global shift independent of any effect of lipid species abundance (**Figure 4E**).

4. Discussion

4.1. Summary of results

In this work, we compared the abundance of plasma metabolites in patients with well-controlled T2D treated conservatively with diet and lifestyle interventions or metformin monotherapy, thereby

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excluding any global effects of uncontrolled T2D on our results. Within the T2D groups, subjects had similar BMI, HOMA-IR, and fasting insulin levels, thereby excluding global effects of obesity and insulin resistance. Significant changes in a number of lipids and lipid-like molecules, including carnitines, FFAs, hydroxy fatty acids, FAHFAs, and phospholipids were found, with patients in the T2D-M group often having levels similar to ND controls.

4.2. Metabolites associated with CVD risk are reduced with metformin monotherapy

With recent studies debating the therapeutic benefits of intensive glycemic control in T2D (Rodriguez-Gutierrez et al., 2019), it is important to understand what mechanisms outside of glucose control may contribute to the benefits of T2D therapeutics. Previous studies have shown changes the levels of branched chain amino acids (BCAAs), phospholipids, fatty acids, triglycerides, acylcarnitines, and small molecular weight compounds in individuals with T2D (Wang-Sattler et al., 2012; Floegel et al., 2013; Walford et al., 2014; Guasch-Ferre et al., 2016; Lu et al., 2016; Pallares-Méndez et al., 2016; Yu et al., 2016; Lai et al., 2020; Zhao et al., 2020; Truchan et al., 2021). These shifts can occur prior to the development of hyperglycemia, highlighting the global changes of the disease (Tabák et al., 2009). Metformin has multiple known potential modes of action, including the inhibition of the mitochondrial enzymes, glycerol-3-phosphate dehydrogenase and complex 1 of the electron transport chain (Minamii et al., 2018). Inhibiting these enzymes results in a reduction of substrate utilization and manifests systemically as reducing hepatic gluconeogenesis (Minamii et al., 2018).

The first lipid class we investigated was acylcarnitines, which function in transporting fatty acids into the mitochondria. The T2D-DL group had the highest levels of acylcarnitines, with subjects in the T2D-M group having similar levels to those in the ND group. Acylcarnitines have previously been reported as a proxy for fatty acid metabolism, with increased levels in circulation indicating β-oxidation dysfunction (Strand et al., 2017; Flam et al., 2022). This result is consistent with previous studies indicating that individuals with T2D have increased levels of short, medium, and long-chain acylcarnitines (Sun et al., 2016; Strand et al., 2017), which are reflective of cardiac metabolism (Makrecka-Kuka et al., 2017). However, our results differ from these previous studies, as the T2D-M

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group had similar levels of acylcarnitines to the ND group. Some hypothesize that medium chain acylcarnitines are increased early in T2D pathogenesis, with an increase in long-chain at later time points as fatty acid oxidation is further disrupted (Schooneman et al., 2013). As all of our subjects had well-controlled and conservatively treated T2D, though, changes in acylcarnitines were specific to metformin treatment. Circulating acylcarnitines have been identified as correlating with CVD morbidity and mortality (Hosseinkhani et al., 2022; Paulin Beske et al., 2022; Storesund et al., 2022). Our results suggest metformin has beneficial effects on these outcomes independent of T2D disease status.

In the T2D state, a change in the abundance of fuel substrates exists, with a shift towards greater utilization of FFAs (Herman et al., 2005). FFAs and their metabolites are known to play a role in inhibiting insulin secretion and signaling, making them a crucial link between metabolic disorder and disease manifestation (Boden and Shulman, 2002; Bosma et al., 2022). Recently, higher circulating FFAs have also been linked with elevated risk of cardiovascular events, independent of T2D status and glycemic control (Yu et al., 2021; Hu et al., 2022; Lluesa et al., 2022; Thirumathyam et al., 2022). In the context of these previous studies, a reduction in circulating FFAs with metformin further support beneficial effects of metformin even in well-controlled T2D patients.

FAHFAs were first reported to correlate with insulin sensitivity, with evidence they are reduced in insulin-resistant individuals (Yore et al., 2014). Yet, circulating FAHFA levels were also found to correlate with several markers of cardiovascular function in healthy human subjects (Dongoran et al., 2020). In our study, individuals in the T2D-DL group had higher mean FAHFA abundance than those treated with metformin. As HOMA-IR was not statistically different between the T2D-DL and T2D-M groups, our data support an association of FAHFA levels with CVD risk independent of T2D status. Yet, few of our subjects had clinically-significant HOMA-IR values, and, in Yore and colleagues, the change in circulating palmitic acid esthers of hydroxystearic acid (PAHSA) levels occurred at a later time point in T2D disease progression than our cohort (Yore et al., 2014). Additionally, only some PAHSA isomers have been shown to elicit an anti-inflammatory response in mice. In this work, we measured total PAHSAs and not isomer-specific, which requires a targeted method (Moraes-Vieira et al., 2016; Kolar et al., 2018). An additional factor to consider is PAHSA has also been shown to increase

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with exercise (Brezinova et al., 2020), and we do not have exercise data for our clinical cohort. Taken together, these differences could explain some of the discrepancies in our data compared to previous reports and caveats of our study that will require additional investigation to deconvolute.

In our study, phospholipid abundance decreased with metformin treatment, indicating a change in nutritional overload. The acyl chains of phospholipids with higher degrees of unsaturation have previously reported to increase in diabetic patients by 45-64% compared to nondiabetic patients (Chuang et al., 2012). This is consistent with our findings of increased phospholipid levels in the T2D-DL group. However, we also observed these phospholipids decreased in the T2D-M group, indicating the potential that phospholipids are sensitive to overall energy state. Further, in T2D-M subjects, phospholipid levels were more similar to the ND group than the T2D-DL groups, demonstrating the effects of metformin on energy balance.

Oxidized phospholipids have previously been shown to increase in insulin resistant individuals and be associated with the pathogenesis of oxidative stress-related diseases, including CVD (Fruhwirth et al., 2007; Sun et al., 2016; Que et al., 2018). Yet, in previous work, increased circulating oxidized phospholipids correlated with Lp-PLA$_2$ levels (Pantazi et al., 2022). In our study, we found oxidized phospholipids were specifically elevated in the T2D-DL group, even though there was no statistically significant difference between the number of subjects in the T2D-DL and T2D-M groups who had clinically elevated Lp-PLA$_2$ levels. Taken together with the increased levels of hydroxy fatty acids, this finding indicates metformin improves the oxidized lipid profile independent of this common clinical marker of CVD risk.

Hydroxy fatty acids have been linked to a variety of diseases, including cancer, inflammatory bowel disease, and neurodegenerative diseases (Li et al., 2020). Additionally, a link has been demonstrated between insulin resistant states and increased vascular risk via hydroxy fatty acids and increased secretion of plasminogen activator inhibitor type 1 (PAI-1) (Marx et al., 1999; Vangaveti et al., 2010). Our data shows the two T2D groups displayed similar trends in hydroxy fatty acids as compared to the ND group. As hydroxy fatty acids can be a proxy for reactive oxygen species (Wang et al., 2009), this indicates a potential underlying mechanism of T2D that is not controlled by metformin

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treatment. Yet, in our study, we found reduced levels of oxidized fatty acids, suggesting further study with a more comprehensive, targeted panel of hydroxy fatty acids is warranted.

4.3. Improved pancreatic β-cell function may occur with metformin therapy.

Prostaglandin E_2 (PGE_2), an arachidonic acid metabolite, is elevated in pancreatic islets from T2D mice and human organ donors, actively contributing to the β-cell dysfunction of the disease (Kimple et al., 2013; Neuman et al., 2017; Schaid et al., 2021; Zhu et al., 2021; Bosma et al., 2022). Circulating PGE_2 is rapidly degraded, and a targeted lipidomics approach is required to detect arachidonic acid metabolites. Yet, in previous work, the abundance of arachidonic acid in membrane phospholipids was significantly elevated in pancreatic islets from T2D mice as compared to ND, correlating directly with the concentration of PGE_2 produced (Kimple et al., 2013; Neuman et al., 2017), and incubating islets from T2D mice with eicosapentaenoic acid (EPA), which competes with arachidonic acid for the same site in membrane phospholipids, significantly improved T2D β-cell dysfunction (Neuman et al., 2017). In this work, we found elevated levels of arachidonic acid and its precursor, linoleic acid, in T2D subjects treated with diet and lifestyle modifications as compared to ND controls, with metformin therapy reversing these changes. While we did not directly measure β-cell function in our study, others have found metformin augments insulin secretion, even in the context of reduced insulin demand (Vazquez Arreola et al., 2022). Therefore, it is possible decreased β-cell PGE_2 is at least partially responsible for metformin's effects.

4.5. Limitations and conclusions.

Our results demonstrate in a small population of well-controlled T2D patients, metformin treatment significantly improved the circulating profiles of a number of lipids and lipid-like molecules, some of which have been independently correlated with CVD risk. Yet, as these metabolites are not clinically validated, we are unable to unequivocally conclude metformin reduces CVD risk independent of T2D control. Another limitation of our study is its design did not include longitudinal sample collection or long-term follow-up: a design required in order to confirm a reduced CVD risk with metformin therapy.

Even so, our findings suggest an untargeted plasma metabolomics approach may provide a much richer set of biomarkers to quantify CVD risk. Future studies with larger populations and targeted approaches will be necessary to validate and advance our results into improved clinical care.

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Authorship Contributions:

Participated in research design: Brasier, A., Cox, E.D., Ge, Y., Belt Davis, D., and Kimple, M.E.

Conducted experiments: Wancewicz, B., Zhu, Y., Fenske, R.J., Weeks, A.M., Wenger, K. Pabich, S., Daniels, M.D., Punt, M., Peter, D.C., and Nall, R.

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Performed data analysis: Wancewicz, B., Zhu, Y., Fenske, R.J., Weeks, A.M, and Kimple, M.E.

Wrote or contributed to the writing of the manuscript: Wancewicz, B., Zhu, Y., Brasier, A., Cox, E.D., Belt Davis, D., Ge, Y., and Kimple, M.E.

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Figure Legends:

Figure 1. Overview of Metabolic Features, Distribution, and Classes Among Groups. **A.** Bar chart displaying the total number of accurate mass features (hatched bars) and features annotated by chemical formula in MetaboScape (solid bars). **B.** Venn diagram showing the number of shared and unique MetaboScape-annotated features among the three groups. **C.** PCA plot of MetaboScape-annotated features among the three groups. **D.** Chemical classification of Metlin-annotated metabolic features separated into nonpolar (yellow) and polar (blue) metabolites. **E.** Heat map of MetaboScape-annotated features generated by unsupervised hierarchical clustering.

Figure 2. Comparison of enriched metabolic pathways for the ND group versus the combined T2D groups. A. Pathway enrichment analysis showing pathways that are significantly altered at p =0.05 level. **B.** Pathway analysis with significantly altered metabolite groups highlighted. **C.** ChemRich analysis using chemical similarity to show the trend in features that are modified. In *C*, Blue is downregulated compared to control, red is up-regulated, and purple is mixed.

Figure 3. Fatty acid metabolism shifts in response to T2D and metformin treatment. A. Acylcarnitines are upregulated in T2D-DL group and return to baseline in the T2D-M group. **B.** Fatty acids, both saturated (left) and unsaturated (right) are increased in T2D-DL and similar between ND and T2D-M. **C.** Hydroxy fatty acids are similar between the groups with saturated (left) showing no difference and unsaturated (right) showing changes in the T2D groups compared to ND. **D.** FAHFAs are all upregulated in T2D-DL and significantly decrease in the T2D-M group. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Figure 4. Metformin impacts lipid metabolism in T2D. **A**. Volcano plot of T2D-DT2D-M/T2D-DL with 11 features increased in the T2D-M group and 39 decreased at cutoff of 2-fold change and p-value<0.05. **B.** ChemRich plot highlighting lipophilic compounds (Xlog P) values are greater than 7.

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C. Selected oxidized lipids. **D**. Selected highly abundant lipids. **E**. Lower abundant lipids. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Table 1. Demographic and clinical parameters of patient cohort. Unless otherwise indicated, data is presented as average ± standard deviation. BMI, body mass index; HbA1c, glycated hemoglobin; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ACEi, Angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BB, beta-blocker; ESR, erythrocyte sedimentation rate; Lp-PLA2, lipoprotein-associated phospholipase A2.

Demographic and Clinical Data	ND (n = 14)	T2D-DL (n=7)	T2D-M (N=8)	p, ND vs. T2D	p, T2D-DL vs. T2D-M
Race = White, non-Hispanic, n (%)	14 (100)	7 (100)	6 (75)	0.157	0.155
Female, n (%)	10 (71.4)	6 (85.7)	4 (50)	0.782	0.143
Age, y (Range)	48.4 ± 10.9 (31 – 70)	57 ± 8.7 (43 – 67)	54 ± 9.2 (41 – 64)	0.067	0.530
BMI, kg/m^2 (Range)	30.9 ± 5.3 (24.7 – 42.3)	35.0 ± 8.9 (23.4 – 47.3)	33.7 ± 5.0 (25.6 – 40.8)	0.149	0.744
Duration of Disease, y (Range)	-	2.9 ± 2.7 (0.5 – 7)	3.1 ± 4.0 (0.5 – 10)	-	0.884
HbA1c, % (Range)	5.27 ± 0.19 (4.8 – 5.5)	6.50 ± 0.80 (5.9 – 8.1)	6.64 ± 0.48 (6.1 – 7.5)	p < 0.0001*	0.688
Fasting Glucose, mg/dl (Range)	91.9 ± 7.1 (83 – 107)	115.1 ± 22.9 (91 – 194)	135.0 ± 55.0 (96 – 263)	0.012*	0.433

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Fasting Insulin, μIU/ml (Range)	3.92 ± 2.39 (0.64 – 7.96)	4.04 ± 1.53 (1.55 – 6.51)	8.32 ± 11.5 (1.38 – 35.87)	0.316	0.349
HOMA-IR > 2, n (%)	0 (0)	1 (14.3)	2 (25)	0.077	0.522
Total Cholesterol, mg/dl (Range)	179.6 ± 31.8 (124 – 225)	179.7 ± 43.0 (118 – 256)	178.6 ± 44.6 (110 – 223)	0.971	0.962
HDL, mg/dl (Range)	58.2 ± 8.5 (26 – 101)	54.3 ± 6.1 (37 – 85)	49.4 ± 6.7 (27 – 75)	0.323	0.602
LDL, mg/dl (Range)	93.9 ± 31.2 (40 – 153)	99.9 ± 10.2 (53 – 145)	86.3 ± 14.4 (32 – 154)	0.920	0.468
Triglycerides, mg/dl (Range)	91.9 ± 8.5 (56 – 523)	126.9 ± 22.7 (68 – 254)	209.4 ± 36.9 (82 – 396)	0.407	0.089
On Statin, n (%)	2 (14.2)	2 (28.6)	4 (50)	0.130	0.435
On Omega-3/Fish Oil, n (%)	0 (0)	2 (28.6)	1 (12.5)	0.082	0.474
BP, Systolic, mm/Hg	120.2 ± 12.6 (106 – 152)	121.6 ± 23.4 (90 – 159)	113.6 ± 10.6 (101 – 132)	0.283	0.874
BP, Diastolic, mm/Hg	76.4 ± 7.5 (62 – 90)	72.4 ± 13.0 (60 – 95)	73.3 ± 5.9 (65 – 84)	0.283	0.874

On ACEi/ARB/BB, n (%)	1 (7.1)	3 (42.9)	5 (62.5)	0.006*	0.483
On Aspirin, n (%)	1 (7.1)	3 (42.9)	6 (75.0)	0.002*	0.234
CRP > 2, n (%)	0 (0)	0 (0)	0 (0)	1.000	1.000
ESR > 20, male, or > 30, female, n	0 (0)	0 (0)	0 (0)	1.000	1.000
Lp-PLA2 > 200, n	2 (14.3)	4 (57.1)	3 (37.5)	0.060	0.447

*, difference in means is statistically significant.

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Table 2. Significantly altered metabolites between diet and lifestyle (T2D-DL) versus metformin (T2D-M) in diabetic patients.

Class/Pathway and Metabolites	InChI Key	*p*(FDR)	Ratio T2D-DL vs. T2D-M
Amino acids, peptides, and analogues			
4-Methylene-glutamate	RCCMXKJGURLWPB-BYPYZUCNSA-N	0.0390	1.30
Carbohydrates and carbohydrate conjugates			
Dulcitol	FBPFZTCFMRRESA-GUCUJZIJSA-N	0.0440	0.34
Cholestane steroids			
Cholesterol	HVYWMOMLDIMFJA-DPAQBDIFSA-N	0.0312	0.80
Diterpenoids			
Phytenic acid	WDWBNNBRPVEEOD-PFXVRADUSA-N	0.0499	0.77
Fatty acid esters			
Palmitoylcarnitine	XOMRRQXKHMYMOC-OAQYLSRUSA-N	0.0111	0.64
Fatty acids and conjugates			
10,13-nonadecadienoic acid	FLYBGKXSHCVONZ-HZJYTTRNSA-N	0.0312	0.34
17-methyl-6-octadecenoic acid	QWCJNFBLSZGETP-CLFYSBASSA-N	0.0313	0.67
2-heptadecylenic acid	GEHPRJRWZDWFBJ-FOCLMDBBSA-N	0.0076	0.63
Eicosanedioic acid	JJOJFIHJIRWASH-UHFFFAOYSA-N	0.0044	0.56
Gaidic acid	ZVRMGCSSSYZGSM-CCEZHUSRSA-N	0.0124	1.69

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Myristoleic acid	YWWVWXASSLXJHU-WAYWQWQTSA-N	0.0141	0.51
OAHSA	OCHJVQODRYVDAA-YPKPFQOOSA-N	0.0218	0.71
Oleic Acid	ZQPPMHVWECSIRJ-KTKRTIGZSA-N	0.0003	1.63
Oleic Acid	ZQPPMHVWECSIRJ-KTKRTIGZSA-N	0.0201	0.71
SAHSA	NQJLCZWOOVLQNP-UHFFFAOYSA-N	0.0146	0.76
Fatty alcohols			
Artemoin A	KKUONIIRIFHWJC-UHFFFAOYSA-N	0.0026	0.41
Glycerophosphocholines			
PC (34:2)	JLPULHDHAOZNQI-ZTIMHPMXSA-N	0.0013	0.67
PC (34:4)	PWFGSGJBCRORHV-DVHMRFIGSA-N	0.0055	3.28
PC (36:2)	ZRTZULWIAWDUBY-UXSLIEDSSA-N	0.0348	0.81
PC (36:5)	KLTHQSWIRFFBRI-CPFPVJFHSA-N	0.0440	0.59
PC (38:6)	IESVDEZGAHUQJU-ZLBXKVHBSA-N	0.0001	0.76
PC (40:6)	YYWYJAHZRFSIIU-MRFSEBLPSA-N	0.0113	0.75
PC (o-38:4)	IRWRFKUTKSUFST-MDYGELLQSA-N	0.0408	0.78
PC (o-38:5)	VJNPDLZENXBRLB-MQEDXBOASA-N	0.0313	0.57
PC (o-38:6)	QQQQNYAHSSIZBU-HIQXTUQZSA-N	0.0026	0.29
Glycerophosphoethanolamines			
PE (40:4)	MHUZXLUKTLNHIX-GUNPMBPGSA-N	0.0055	0.73
PE (p-36:4)	KDMBUUZGCXQNBE-XBICFDGKSA-N	0.0312	0.29

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PE (p-38:5)	VWNWYWMBTKYEEL-SXDACRMGSA-N	0.0119	0.30
PE (p-38:6)	WVGALBKSWOUIEZ-XNHMFJFDSA-N	0.0248	0.39
Glycerophosphoserines			
PS (o-36:0)	YPXDUVWMKVFZDW-RGULYWFUSA-N	0.0397	0.43
PS (o-36:1)	NHFUOLYNPLVMLN-BFCPHGQOSA-N	0.0440	0.40
Class/Pathway and Metabolites	**InChI Key**	***p*(FDR)**	**Ratio (T2D-DL/T2D-M)**
Lineolic acids and derivatives			
Linoleic acid	OYHQOLUKZRVURQ-HZJYTTRNSA-N	0.0296	0.73
Linolenic acid	DTOSIQBPPRVQHS-PDBXOOCHSA-N	0.0194	0.72
Monoacylglycerols			
MG (14:0)	TVIMZSOUQXNWHO-UHFFFAOYSA-N	0.0440	0.77
MG (16:0)	QHZLMUACJMDIAE-SFHVURJKSA-N	0.0125	0.75
MG (16:1)	CXUXMSACCLYMBI-FPLPWBNLSA-N	0.0026	0.63
Phosphosphingolipids			
SM (d32:1)	KYICBZWZQPCUMO-PSALXKTOSA-N	0.0055	0.75
SM (d34:1)	RWKUXQNLWDTSLO-GWQJGLRPSA-N	0.0001	0.72
SM (d36:2)	NBEADXWAAWCCDG-QDDWGVBQSA-N	0.0000	0.70
SM (d42:3)	TXFLWJQVQCDUDZ-BRUGZULGSA-N	0.0373	0.75
Pregnane steroids			

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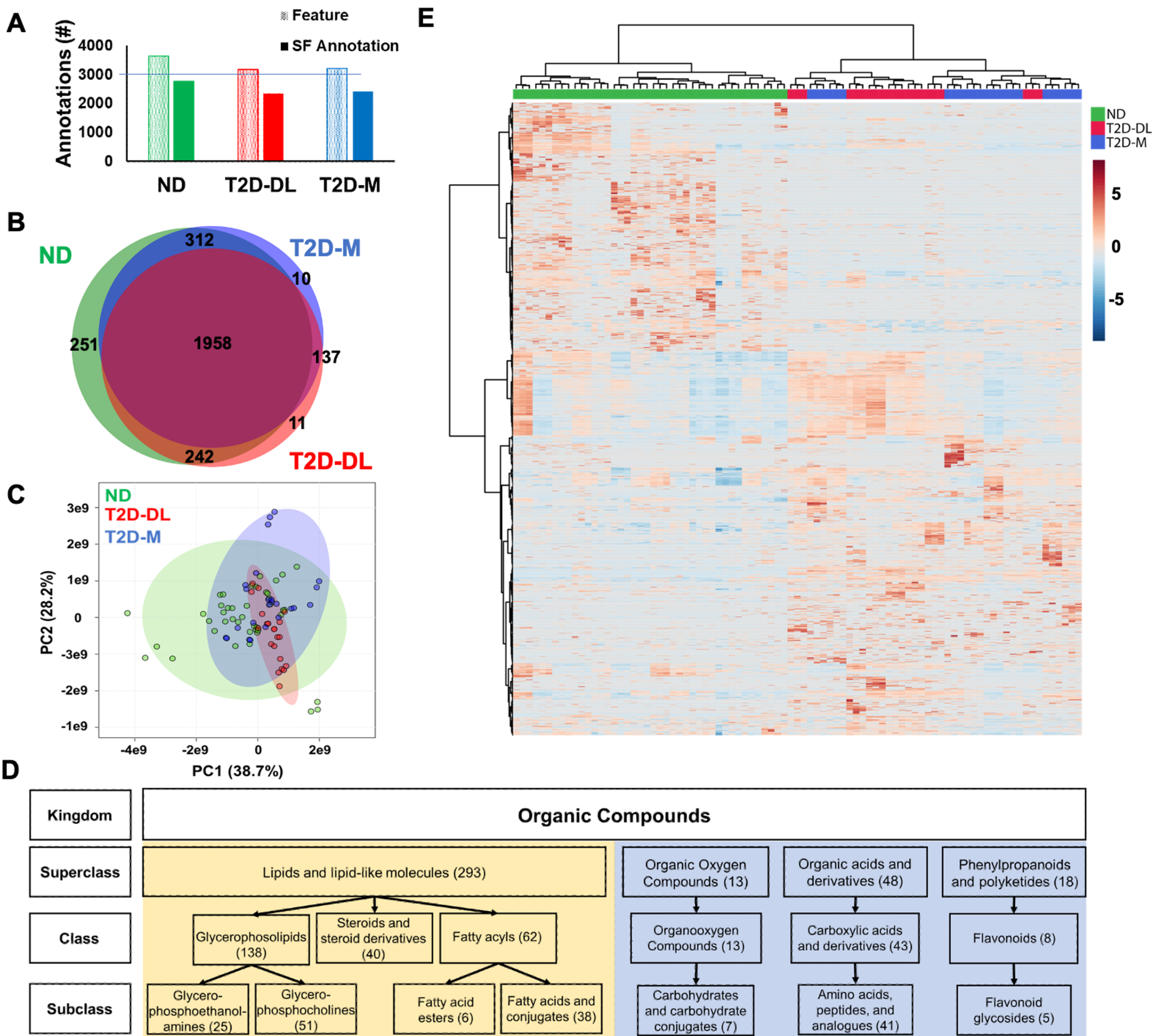
Allopregnanolone	AURFZBICLPNKBZ-SYBPFIFISA-N	0.0440	0.41
Stilbenes			
Dihydroresveratrol	HITJFUSPLYBJPE-UHFFFAOYSA-N	0.0440	0.39

FIGURE 1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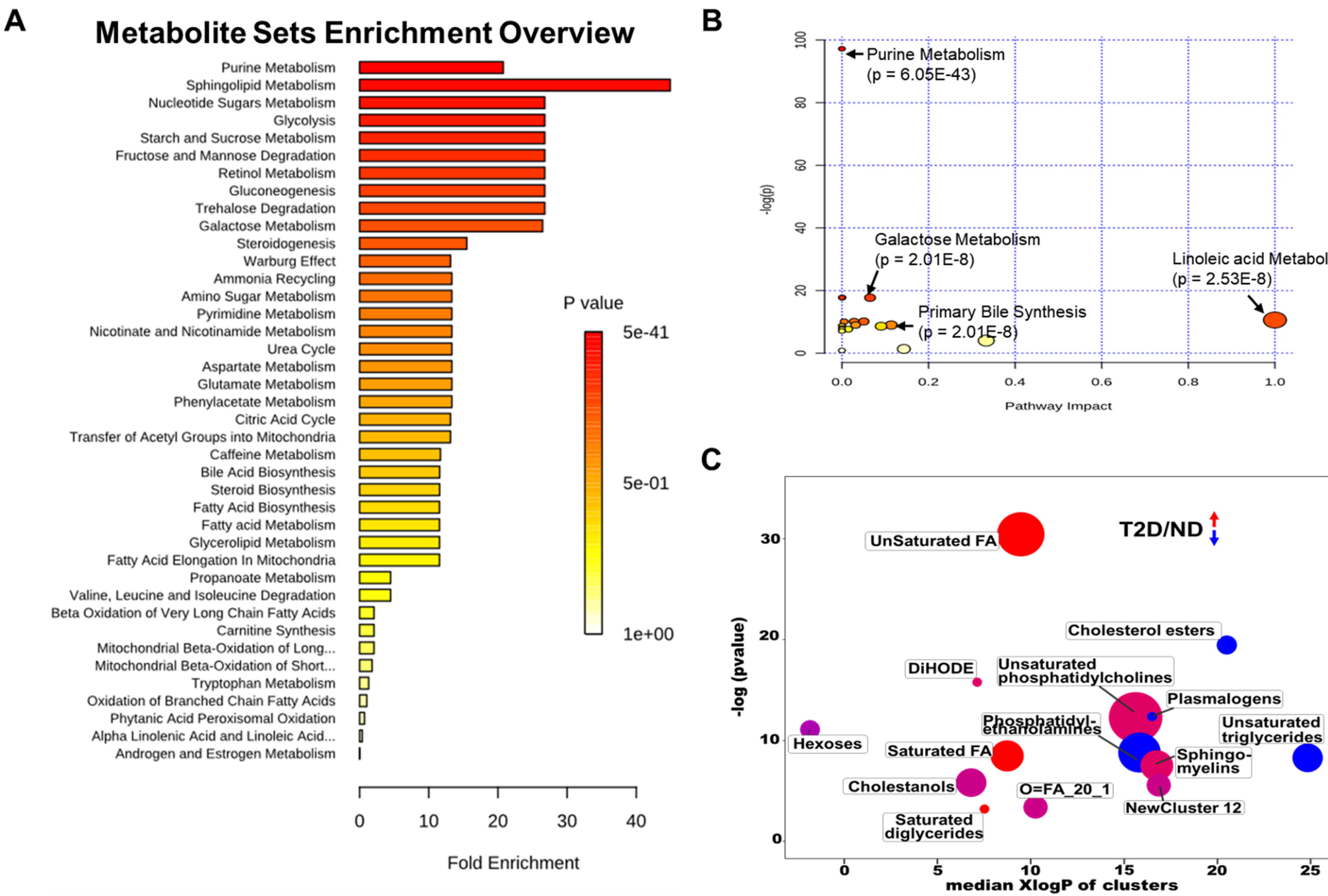
Feature
SF Annotation
Annotations (#)
4000
3000
2000
1000
0
ND
T2D-DL
T2D-M
B
ND
T2D-M
T2D-DL
312
10
251
1958
137
11
242
C
ND
T2D-DL
T2D-M
PC2 (28.2%)
PC1 (38.7%)
E
ND
T2D-DL
T2D-M
5
0
-5
D
Kingdom
Superclass
Class
Subclass
Organic Compounds
Lipids and lipid-like molecules (293)
Organic Oxygen Compounds (13)
Organic acids and derivatives (48)
Phenylpropanoids and polyketides (18)
Glycerophospholipids (138)
Steroids and steroid derivatives (40)
Fatty acyls (62)
Organooxygen Compounds (13)
Carboxylic acids and derivatives (43)
Flavonoids (8)
Glycero-phosphoethanol-amines (25)
Glycero-phosphocholines (51)
Fatty acid esters (6)
Fatty acids and conjugates (38)
Carbohydrates and carbohydrate conjugates (7)
Amino acids, peptides, and analogues (41)
Flavonoid glycosides (5)

FIGURE 2


A
Metabolite Sets Enrichment Overview
Purine Metabolism
Sphingolipid Metabolism
Nucleotide Sugars Metabolism
Glycolysis
Starch and Sucrose Metabolism
Fructose and Mannose Degradation
Retinol Metabolism
Gluconeogenesis
Trehalose Degradation
Galactose Metabolism
Steroidogenesis
Warburg Effect
Ammonia Recycling
Amino Sugar Metabolism
Pyrimidine Metabolism
Nicotinate and Nicotinamide Metabolism
Urea Cycle
Aspartate Metabolism
Glutamate Metabolism
Phenylacetate Metabolism
Citric Acid Cycle
Transfer of Acetyl Groups into Mitochondria
Caffeine Metabolism
Bile Acid Biosynthesis
Steroid Biosynthesis
Fatty Acid Biosynthesis
Fatty acid Metabolism
Glycerolipid Metabolism
Fatty Acid Elongation In Mitochondria
Propanoate Metabolism
Valine, Leucine and Isoleucine Degradation
Beta Oxidation of Very Long Chain Fatty Acids
Carnitine Synthesis
Mitochondrial Beta-Oxidation of Long...
Mitochondrial Beta-Oxidation of Short...
Tryptophan Metabolism
Oxidation of Branched Chain Fatty Acids
Phytanic Acid Peroxisomal Oxidation
Alpha Linolenic Acid and Linoleic Acid...
Androgen and Estrogen Metabolism
P value
5e-41
5e-01
1e+00
0
10
20
30
40
Fold Enrichment
B
Purine Metabolism
(p = 6.05E-43)
Galactose Metabolism
(p = 2.01E-8)
Linoleic acid Metabolism
(p = 2.53E-8)
Primary Bile Synthesis
(p = 2.01E-8)
-log(p)
0
20
40
60
80
100
0.0
0.2
0.4
0.6
0.8
1.0
Pathway Impact
C
T2D/ND
UnSaturated FA
Cholesterol esters
DiHODE
Unsaturated phosphatidylcholines
Plasmalogens
Phosphatidyl-ethanolamines
Unsaturated triglycerides
Hexoses
Saturated FA
Sphingo-myelins
Cholestanols
O=FA_20_1
NewCluster 12
Saturated diglycerides
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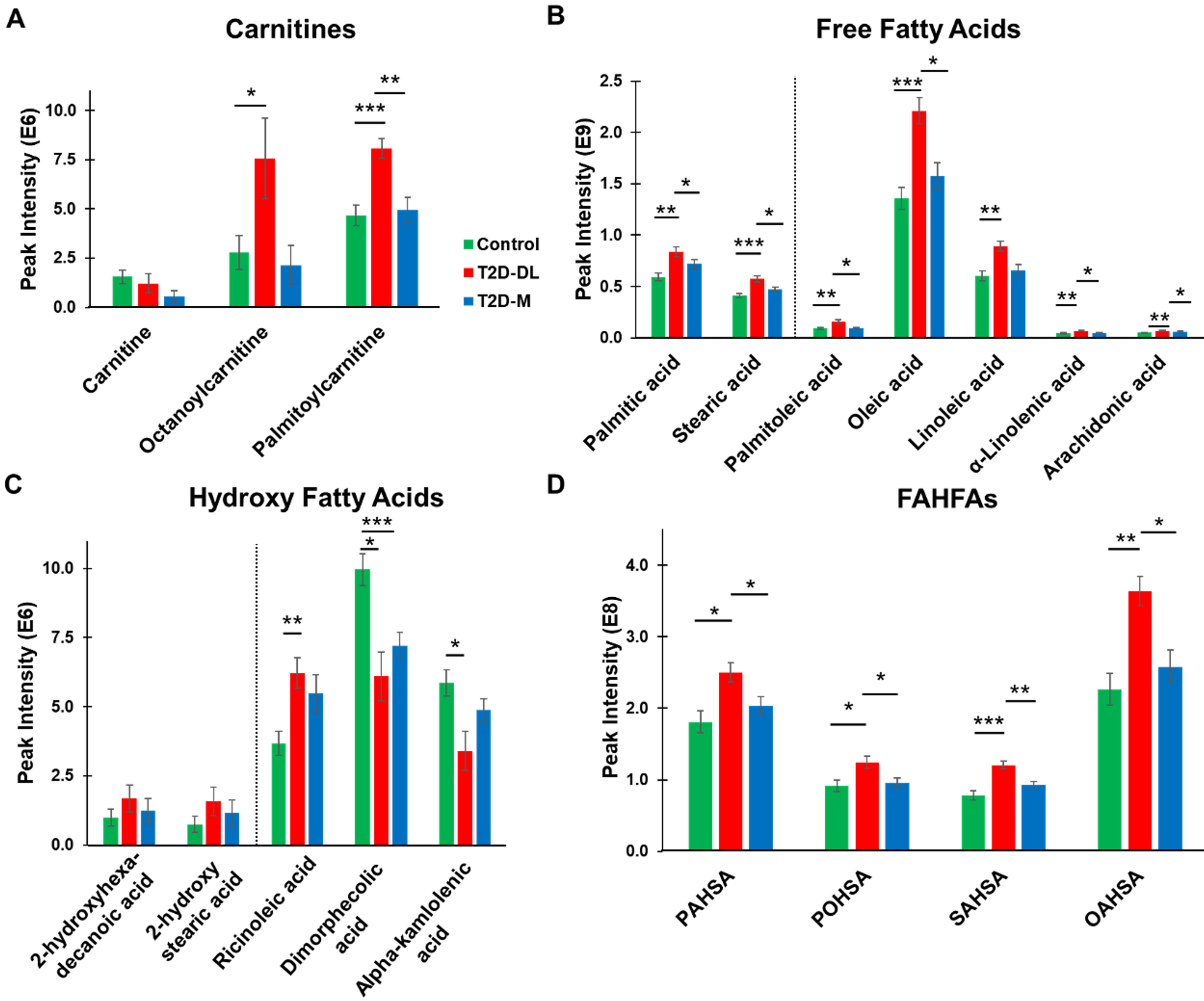
0
10
20
30
0
5
10
15
20
25
median XlogP of clusters

FIGURE 3


A
Carnitines
Peak Intensity (E6)
10.0
7.5
5.0
2.5
0.0
Carnitine
Octanoylcarnitine
Palmitoylcarnitine
Control
T2D-DL
T2D-M
B
Free Fatty Acids
Peak Intensity (E9)
2.5
2.0
1.5
1.0
0.5
0.0
Palmitic acid
Stearic acid
Palmitoleic acid
Oleic acid
Linoleic acid
α-Linolenic acid
Arachidonic acid
C
Hydroxy Fatty Acids
Peak Intensity (E6)
10.0
7.5
5.0
2.5
0.0
2-hydroxyhexa-decanoic acid
2-hydroxy stearic acid
Ricinoleic acid
Dimorphecolic acid
Alpha-kamlolenic acid
D
FAHFAs
Peak Intensity (E8)
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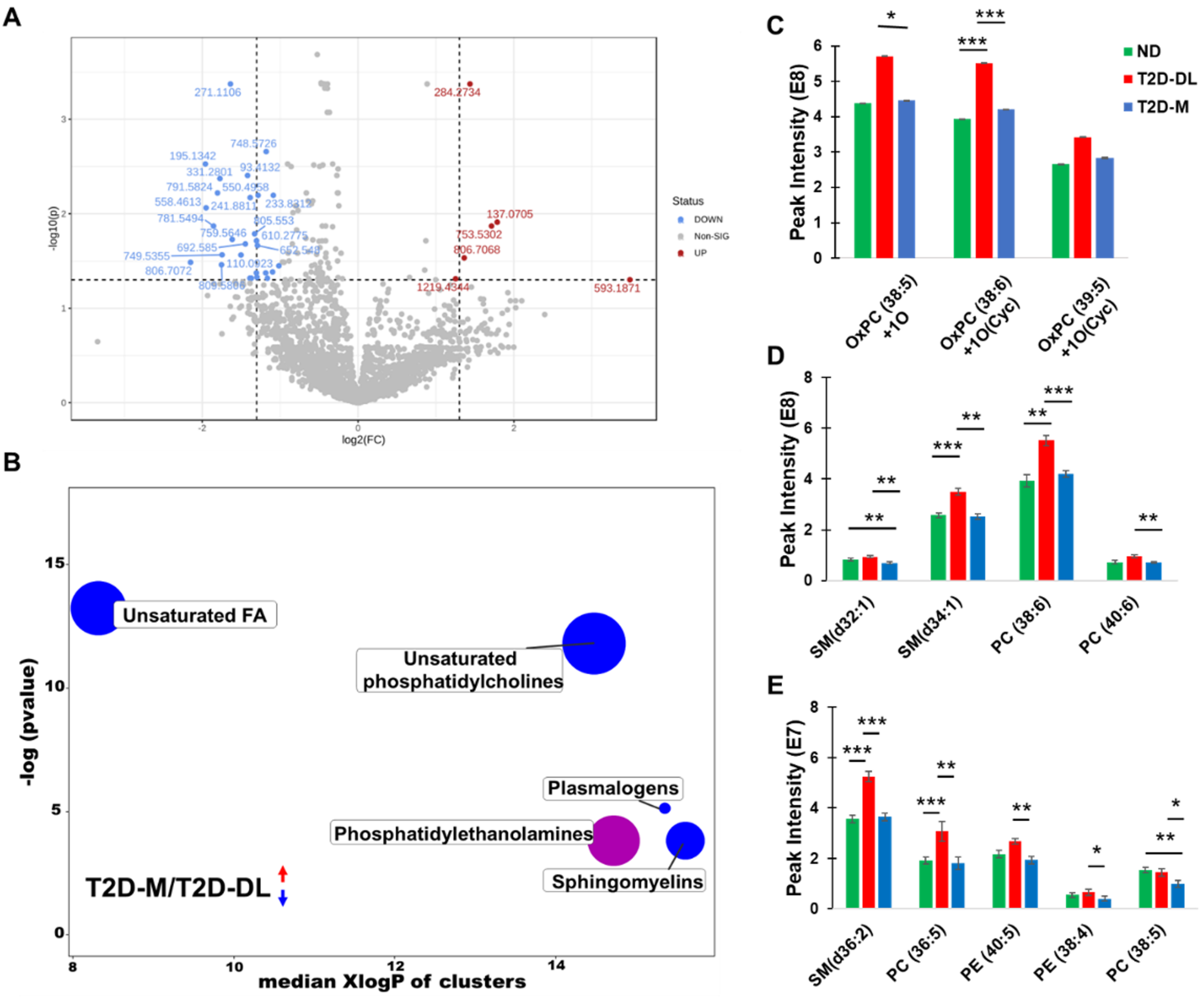
2.0
1.0
0.0
PAHSA
POHSA
SAHSA
OAHSA

FIGURE 4



A
B
C
D
E
log2(FC)
-log10(p)
Status
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Non-SIG
UP
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748.5726
195.1342
93.4132
331.2801
791.5824
550.4958
558.4613
241.8811
233.8312
781.5494
605.553
759.5646
610.2775
692.585
652.548
749.5355
110.0923
806.7072
809.5866
137.0705
753.5302
806.7068
1219.4344
593.1871
-log (pvalue)
median XlogP of clusters
Unsaturated FA
Unsaturated phosphatidylcholines
Plasmalogens
Phosphatidylethanolamines
Sphingomyelins
T2D-M/T2D-DL
Peak Intensity (E8)
ND
T2D-DL
T2D-M
OxPC (38:5) +1O
OxPC (38:6) +1O(Cyc)
OxPC (39:5) +1O(Cyc)
SM(d32:1)
SM(d34:1)
PC (38:6)
PC (40:6)
Peak Intensity (E7)
SM(d36:2)
PC (36:5)
PE (40:5)
PE (38:4)
PC (38:5)