

Non-Targeted Metabolomic Analysis Reveals Serum Phospholipid Alterations in Patients with Early Stages of Diabetic Foot Ulcer

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ABSTRACT: Diabetic foot ulcer (DFU) is a common complication of type 2 diabetes mellitus (T2DM) characterized by ulcer formation, which can lead to the amputation of lower extremities. However, the metabolic alterations related to this complication are not completely elucidated. Therefore, we carried out a metabolomic analysis of serum samples obtained from T2DM adult patients diagnosed with diabetic foot ulcer in a cross-sectional, observational, and comparative study. Eighty-four volunteers were classified into the following groups: without T2DM (control group, $n = 30$) and with T2DM and different stages of diabetic foot ulcer according to Wagner-Meggitt classification system: DFU G0 ($n = 11$), DFU G1 ($n = 14$), DFU G2 ($n = 16$), and DFU G3 ($n = 13$). The non-target metabolomic profile followed by chemometric analysis revealed that lysophosphatidylethanolamine (16:1) could be proposed as key metabolite related to the onset of diabetic foot ulcer; however, this phospholipid was not affected by diabetic foot ulcer progression. Therefore, further studies are necessary to validate these phospholipids as biomarker candidates for the early diagnosis of diabetic foot ulcer in T2DM patients.

KEYWORDS: Diabetic foot ulcer, type 2 diabetes mellitus, metabolomic analysis, mass spectrometry, phospholipids

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Introduction

In 2019, approximately 463 million adults had diabetes mellitus (DM) in the world (9.3%), leading to a health expenditure of about USD 760 billion. An uncontrolled DM causes multiple complications, including diabetic foot and lower limb complications (International Diabetes Federation, IDF, 2019). Diabetic foot affects 40 to 60 million people with DM worldwide (IDF, 2019). The mortality associated with diabetic foot is about 16.7% in 12 months and 50% in 5 years.¹

The high rates of morbidity and mortality of this complication has been associated to the elevated number of missed diagnoses (IDF, 2019). The most commonly used classification tool used for diabetic foot ulcer is the Wagner-Meggitt (WM) wound classification system, which classifies the patients into 6 grades (G0 to G5) depending of the ulcer depth, the presence of abscess, osteomyelitis or joint sepsis, and the presence of partial and total gangrene.²

Wound classification tools are used for the planification of corrective treatment strategies in terms of healing and amputation of lower limbs; however, to the best of our knowledge, there are no tools for the early diagnosis of diabetic foot ulcer, since all the classification tools rely on the presence of visible wounds. It is noteworthy that foot ulcers are the most common type of foot wounds and are precursors to the amputation of lower limbs in patients with DM, since they promote infection and tissue necrosis.³

The etiology of ulcers is associated with the presence of peripheral neuropathy and repetitive trauma. Therefore, the identification of diabetic persons at risk of diabetic foot ulcer rely in the diagnosis of peripheral neuropathy. In this regard, sensory evaluation has been proposed for the detection of neurological alterations such as Achilles reflex, and vibration and pressure evaluations; however, controversial results have been reported regarding their sensitivity, specificity, and predictive value.³

Metabolomics have been recently used to characterize the metabolomic alterations that occur during the onset and development of several chronic degenerative diseases, including type 2 DM (T2DM) and its complications. In this regard, genomics and proteomics have been directed to identify biomarkers associated with the progression from wounds to ulcers.⁴ However, to the best of our knowledge, omics sciences have not been applied to identify candidate biomarkers for the diagnosis of diabetic foot prior the development of foot wounds. Therefore, this study aimed to carry out a non-targeted metabolomic profile of serum samples obtained from adult patients without T2DM and with T2DM with different grades of diabetic foot ulcer.

Materials and methods

Study design

An observational, cross-sectional, and comparative study was designed. This study was conducted according to the guidelines



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of the Declaration of Helsinki and all the procedures involving human subjects were approved by the Bioethics' Committee of the Natural Sciences School of the Universidad Autónoma de Querétaro (UAQ, no. 9832, 2018) and the Health Research State Committee of the Secretaría de Salud del Estado de Querétaro (SESEQ) and the Research Local Committee of the Hospital General de Querétaro (HGQ) (no. 1013/09-08-2018).

Participant recruitment

Participants were recruited at the Wound Care Clinic of the Hospital General de Querétaro (Querétaro, México) from January to December 2018 during their evaluation appointments.

All participants signed the informed consent. Afterwards, participants were asked about their clinical history (including age, sex, scholary, occupation, medical history, socioeconomic details, anthropometric characteristics, and lifestyle habits). Then, foot assessment was carried out by diabetic foot ulcer specialists (MHEQ María Eugenia García-Aguilar and MHEQ Alicia Barajas-Pozos) at the Wound Care Clinic of the Hospital General de Querétaro.

Volunteers (n = 84) with T2DM and DFU were distributed according to their diagnosis into the following groups according to the WM classification system²: no open lesion (DFU G0), superficial ulcer (DFU G1), deep ulcer to tendon or joint capsule (DFU G2), and deep ulcer with abscess, osteomyelitis or joint sepsis (DFU G3). In addition, a control group was included with participants without T2DM, which were recruited from the Hospital General de Querétaro. Finally, blood pressure was assessed and fasting blood was collected from the antecubital vein (which was carried out by MHEQ María Rosario Arreola-Morales) for biochemical and metabolomic analysis.

The inclusion criteria for all groups were: Mexican ethnicity aged between 45 to 64 y, and DFU patients with G0, G1, G2, and G3 classified according to the WM system. The exclusion criteria for all groups included pregnancy or nursing, diagnosis of cancer and acute infections. Patients diagnosed with local gangrene-fore foot or heel (DFU G4) or gangrene of entire foot (DFU G5) were excluded from this study, since these patients have advanced stages of diabetic foot ulcer and were candidates for partial and total limb amputation, respectively.

Foot sensory assessment

Foot sensory assessment was carried out by DFU specialists ((MHEQ María Eugenia García-Aguilar and MHEQ Alicia Barajas-Pozos) at the Wound Care Clinic of the Hospital General de Querétaro. Achilles reflex was assessed by a neurological hammer, and results were expressed as sensitive (with reflex) or insensitive (with no reflex) in the Achilles tendon. Vibration was evaluated by a 128 Hz tuning fork on the dorsal bony prominence of the big toe. Results were expressed

as sensitive (vibration perceived) or insensitive (vibration not perceived). Pressure was assessed by a Semmes-Weinstein monofilament 7.5/10g in 5 points on the plantar surface of each foot (first toe, first, third, and fifth metatarsal heads, and heel) and 1 point on the dorsum surface of each foot (first web space). Results were expressed as sensitive (pressure perceived in all sites) or insensitive (pressure not perceived in one or more sites).

Biochemical analysis

Venous blood samples were collected (5 mL in serum tubes with gel and 4 mL in tubes with potassium EDTA) from volunteers with 12 hours fast at the Wound Care Clinic of the Hospital General de Querétaro. The serum samples were used for the biochemical and metabolomic analysis, whereas whole blood samples were used for Hb1ac analysis. Biochemical analysis was carried out immediately after blood sampling in the Clinical Services Unit (USC) of the Chemistry School of the UAQ.

HbA1c was determined using an enzymatic-colorimetric kit (Spinreact, Girone, Spain). Serum glucose, urea, creatinine, uric acid, cholesterol, and triglycerides were determined using enzymatic-colorimetric kits (Pointe Scientific, MI, USA) according to the manufacturers' instructions by an automated analyzer (BS-200 Chemistry Analyzer, Mindray Medical International Co., Shenzhen, China). Glomerular filtration rate (GFR) was estimated using the CKD-EPI (chronic kidney disease epidemiology collaboration) equation: $GFR = 141 \times \min(Scr/\kappa, 1)^\alpha \times \max(Scr/\kappa, 1) - 1.209 \times 0.993 \text{Age} \times 1.018$ (if female) $\times 1.159$ (if black), where Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/ κ or 1, and max indicates the maximum of Scr/ κ or 1.

Metabolomic analysis

Sample preparation. Serum samples (80 μ L) were mixed with 200 μ L of chloroform and 360 μ L of methanol for 60 seconds in a vortex. Then, 200 μ L of chloroform and 200 μ L of water were added and samples were mixed for 60 seconds using a vortex. Samples were centrifuged at $1800 \times g$ for 30 minutes at 4°C and 150 μ L of the upper-phase was recovered. Then, solvent was removed in a vacuum concentrator (Speedvac SC210A-115 Savant, Thermo Fisher Scientific, MA, USA) and samples were stored at -80°C until analysis.⁵ All solvents used for sample and mobile phase preparation were of LC/MS grade (Optima, Thermo Fisher Scientific).

Ultra-Performace Liquid Chromatography/Quadrupole/Time-of-Flight Mass Spectrometer analysis. Samples were reconstituted in 300 μ L of methanol, sonicated for 10 minutes, centrifuged at $16000 \times g$ for 10 min at 4°C, and filtered through PVDF syringe filters (13 mm, 0.45 μ m).⁵ Quality control (QC) samples were prepared by pooling an identical volume (10 μ L) of the samples of all the participants. Five QC samples were injected prior the

analytical sequence for system conditioning, and every 10 samples to ensure the stability of analytical conditions. Four technical replicates were run for each sample. The metabolomic analysis was carried out in an Ultra-Performance Liquid Chromatograph (UPLC) coupled to a Quadrupole/Time-of-Flight Mass Spectrometer (QTOF MS) using an Electrospray Ionization (ESI) source (Vion, Waters Co., MA, USA).

Samples were maintained at 4°C in the autosampler and were injected (10 µL) in full-loop mode into a BEH C18, 1.7 µm (2.1 mm × 100 mm) column at 35°C. The mobile phase consisted of water with 1% of formic acid (A) and acetonitrile with 1% formic acid (B). A gradient was applied linearly from 95% to 5% A for 22 minutes, followed by a washing step with 95% A for 5 minutes, and a re-equilibration step for 3 minutes. The mobile phase flow rate was set at 400 µL/min.

The MS conditions were as follows: mass range, 100–1500 Da; ionization mode, negative (ESI⁻); capillary voltage, 2 kV; source temperature, 120°C; desolvation gas (N₂), 800 L/h at 450°C; cone gas (N₂), 50 L/h; low collision energy, 5 eV; high collision energy, 15–45 eV. All data were collected in centroid mode with a duty cycle time of 0.2 seconds. Lock mass correction was carried out by the infusion of a leucine-enkephalin solution (50 pg/mL) at 10 µL/min.

Data processing. Raw data was acquired using the UNIFI software (Waters Co.) and was exported to the Progenesis QI software (Waters Co.) for processing and analysis. Retention time alignment was carried out as compared to QC samples. For peak selection, noise elimination level was set at 10.00 and minimum intensity was set to 15% of the base peak intensity. Automatic peak deconvolution was assessed to combine identical molecules at different charge states, which was further reviewed and fine-tuned. Normalization was carried out using total ion abundance. Then, data were exported for statistical analysis through a multivariate approach and key compounds were then identified in the Progenesis QI software. Putative identification was carried out by comparison of the precursor mass similarity (mass error <5 ppm), isotope distribution similarity, and fragmentation score (as compared to theoretical fragmentation). Peak identity was accepted with a confidence score ≥48.0. Lipids were labeled according to the nomenclature used by the Lipid Maps database (<http://lipidmaps.org>).

Statistical analysis. Data are described using mean ± standard deviation for continuous variables and frequencies as number of cases (%) for categorical variables. Kolmogorov-Smirnov's goodness of fit test was used to evaluate Normality and Levene's test for variance homogeneity assessment. Tukey's test was used to make comparisons when Gaussian assumption was valid and Kruskal-Wallis test was used for non-Gaussian cases. The univariate statistical analysis was carried out with IBM Statistical Package for the Social Sciences (SPSS) V.20 with a reliability >95%. The raw metabolomic data was scaled and centered. The

classification task was carried out by Partial Least Squares-Discriminant Analysis (PLS-DA) using Variable Importance in the Projection (VIP) plots, using the mixOmics package of the R software. Statistical analysis was adjusted by covariates age, gender, DM duration, Hb1Ac, glucose, urea, creatinine, uric acid, triglycerides, and cholesterol levels.

Results

Clinical and biochemical characteristics of the study population

The characteristics of the study participants is shown in Table 1. Eighty-four adults were recruited for this study, 30 were included in the no T2DM (control) group and 54 had clinical diagnosis of T2DM and DFU. These latter were subclassified according to their DFU grade using the WM classification tool.

The age range of the participants of this study was of 45 to 67 years, no significant difference was observed between groups (Table 1), and a higher prevalence of all stages of diabetic foot ulcer was found in the male population (Table 1). Regarding T2DM duration, no significant differences were observed between the T2DM groups with different stages of diabetic foot ulcer (Table 1).

The HbA1c and glucose mean levels of the no T2DM (control) group were of 5.1% and 92.1 mg/dL, respectively, whereas the mean value of HbA1c and fasting glucose of all the DFU groups was ≥7.7% and ≥158.4 mg/dL, respectively (Table 1), which is according to the diagnosis criteria for DM (≥6.5% and ≥126 mg/dL, respectively) (ADA, 2020). Interestingly, no significant differences were observed in the HbA1c and glucose values among DFU groups (Table 1).

The serum levels of urea, creatinine, and uric acid are shown in Table 1. No significant differences were observed between the no T2DM (control) group and the DFU G0 and G1 groups in both urea and creatinine levels, suggesting normal renal function, whereas the DFU G2 and G3 groups showed significantly ($P < .05$) higher levels. The no T2DM (control) group and DFU G0 groups showed a high prevalence of patients with normal and low GFR; whereas the DFU G1, G2, and G3 groups showed a high prevalence of patients with low and moderate GFR (Table 1). Interestingly, no prevalence of kidney failure was found in all of the study groups.

Serum uric acid levels were found non-significant between the no T2DM (control) group and the DFU G0 group, whereas the DFU G1, G2, and G3 groups showed increased uric acid levels (Table 1). Accordingly, the DFU groups with visible foot lesions (G1, G2, and G3) showed a high prevalence of hyperuricemia (>50%; Table 1) as compared to the DFU group without visible lesions (DFU G0).

Serum triglycerides and cholesterol levels are shown in Table 1. Interestingly, the mean triglyceride levels increased as the progression of diabetic foot ulcer augmented up to DFU G2, and then decreased in the DFU G3 group (Table 1).

Table 1. Characteristics of study participants.

PARAMETERS	NO T2DM (N = 30)	DFU G0 (N = 11)	DFU G1 (N = 14)	DFU G2 (N = 16)	DFU G3 (N = 13)
Age (y)	51.3 ± 5.1 ^a	51.2 ± 6.3 ^a	53.6 ± 5.8 ^a	55.8 ± 4.8 ^a	54.4 ± 4.5 ^a
Gender distribution					
Women	19 (63.3%)	4 (36.4%)	4 (28.6%)	7 (43.8%)	2 (15.4%)
Men	11 (35.7%)	7 (63.6%)	10 (71.4%)	9 (56.3%)	11 (84.6%)
Clinical history					
DM duration (y)	–	10.7 ± 10.1 ^a	12.4 ± 6.9 ^a	15.8 ± 9.1 ^a	13.0 ± 7.9 ^a
No DM drug treatment (%)	–	1 (9.1%) ^a	2 (14.3%) ^a	1 (6.3%) ^a	0 (0%)
Insulin treatment (%)	–	3 (27.3%) ^a	3 (21.4%) ^{a,b}	2 (12.5%) ^b	4 (30.8%) ^a
Oral hypoglycemic treatment ¹ (%)	–	5 (45.5%) ^a	4 (28.6%) ^b	6 (37.5%) ^{a,b}	5 (38.5%) ^{a,b}
Combined treatment ² (%)	–	2 (18.2%) ^c	5 (35.7%) ^b	7 (43.8%) ^a	4 (30.8%) ^b
Previously amputated right foot (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Previously amputated left foot (%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	2 (15.4%)
Biochemical analysis					
HbA1c (%)	5.1 ± 0.5 ^b	8.8 ± 1.8 ^a	7.7 ± 2.6 ^a	8.6 ± 1.9 ^a	8.5 ± 2.4 ^a
Glucose (mg/dL)	92.1 ± 11.3 ^b	216.5 ± 94.1 ^a	177.1 ± 92.3 ^a	158.4 ± 63.9 ^a	178.8 ± 105.9 ^a
Urea (mg/dL)	31.6 ± 7.0 ^a	33.6 ± 8.9 ^a	40.3 ± 19.0 ^{a,b}	48.8 ± 15.4 ^b	44.0 ± 22.3 ^b
Creatinine (mg/dL)	1.0 ± 0.2 ^a	0.9 ± 0.2 ^a	1.2 ± 0.5 ^{a,b}	1.1 ± 0.2 ^a	1.4 ± 0.7 ^b
Uric acid (mg/dL)	5.2 ± 1.6 ^a	5.0 ± 1.4 ^a	7.2 ± 2.1 ^b	8.0 ± 2.2 ^b	6.8 ± 2.1 ^b
Triglycerides (mg/dL)	122.8 ± 73.2 ^a	141.5 ± 61.7 ^a	154.9 ± 71.2 ^a	203.4 ± 106.1 ^a	170.8 ± 108.2 ^a
Cholesterol (mg/dL)	188.0 ± 36.6 ^a	180.4 ± 36.1 ^a	167.1 ± 52.2 ^a	173.4 ± 46.9 ^a	151.8 ± 37.2 ^a
Blood pressure					
Systolic blood pressure (mm Hg)	116.9 ± 12.8 ^a	123.1 ± 15.3 ^a	126.3 ± 17.6 ^a	130.0 ± 12.9 ^a	125.5 ± 22.7 ^a
Diastolic blood pressure (mm Hg)	81.1 ± 13.4 ^a	83.2 ± 7.8 ^a	81.9 ± 9.5 ^a	83.0 ± 10.3 ^a	80.8 ± 11.7 ^a
DM2-related comorbidities					
Renal dysfunction					
Normal GFR ³ (%)	5 (16.7%) ^b	4 (36.4%) ^a	2 (14.3%) ^b	3 (18.8%) ^b	1 (7.7%) ^c
Low GFR ⁴ (%)	22 (73.3%) ^a	6 (54.5%) ^b	8 (57.1%) ^b	8 (50%) ^b	7 (53.8%) ^b
Moderate GFR ⁵ (%)	3 (10.0%) ^c	1 (9.1%) ^c	3 (21.4%) ^b	5 (31.3%) ^a	4 (30.8%) ^a
Severe GFR ⁶ (%)	0 (0.0%)	0 (0.0%)	1 (7.1%) ^a	0 (0.0%)	1 (7.7%) ^a
Kidney failure ⁷ (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Hyperuricemia ⁸ (%)	4 (13.3%) ^c	1 (9.1%) ^c	7 (50.0%) ^b	12 (75.0%) ^a	7 (53.8%) ^b
Dyslipidemia					
Hypertriglyceridemia ⁹ (%)	7 (23.3%) ^c	4 (36.4%) ^b	7 (50%) ^a	7 (43.8%) ^a	6 (46.2%) ^a
Hypercholesterolemia ¹⁰ (%)	10 (33.3%) ^a	4 (36.4%) ^a	2 (14.3%) ^b	5 (31.3%) ^a	1 (7.7%) ^c
Mixed hyperlipidemia ¹¹ (%)	5 (16.7%) ^b	2 (18.2%) ^b	2 (14.3%) ^b	5 (31.3%) ^a	0 (0%)
Hypertension ¹² (%)	11 (36.7%) ^b	5 (45.5%) ^{a,b}	6 (42.9%) ^{a,b}	11 (68.8%) ^a	6 (46.2%) ^{a,b}

Data are showed as mean ± standard deviation or n (%).

Abbreviations: DFU, diabetic foot ulcer; GFR, glomerular filtration rate; HbA1c, hemoglobin A1c; T2DM, type 2 diabetes mellitus.

^{a,b,c}Indicate significant ($P < .05$) differences between groups.

¹Oral hypoglycemic agents: metformin and/or glibenclamide; ²combined treatment of insulin and hypoglycemic agents. DM2-related comorbidities were diagnosed as follows: ³GFR >90; ⁴GFR = 60–89; ⁵GFR = 30–59; ⁶GFR = 15–29; ⁷GFR <15; ⁸uric acid > 6.8 mg/dL; ⁹triglycerides >150 mg/dL; ¹⁰cholesterol >200 mg/dL;

¹¹triglycerides >150 mg/dL and cholesterol >200 mg/dL; ¹²blood pressure > 30/85 mmHg.

Table 2. Foot sensory assessment of study participants.

PARAMETERS		NO T2DM (N = 30)	DFU G0 (N = 11)	DFU G1 (N = 14)	DFU G2 (N = 16)	DFU G3 (N = 13)
Achilles reflex¹						
Right foot	Sensible limb	30 (100%) ^a	11 (100%) ^a	14 (100%) ^a	10 (62.5%) ^b	12 (92.3%) ^a
	Insensible limb	0 (0%)	0 (0%)	0 (0%)	6 (37.5%) ^a	1 (7.7%) ^b
Left foot	Sensible limb	30 (100%) ^a	10 (90.9%) ^a	13 (92.9%) ^a	11 (68.8%) ^b	10 (76.9%) ^b
	Insensible limb	0 (0%)	0 (0%)	1 (7.1%) ^b	5 (31.3%) ^a	1 (7.7%) ^b
Pressure sensation²						
Right foot	Sensible limb	30 (100%) ^a	11 (100%) ^a	14 (100%) ^a	14 (87.5%) ^b	12 (92.3%) ^{a,b}
	Insensible limb	0 (0%)	0 (0%)	0 (0%)	2 (12.5%) ^a	1 (7.7%) ^a
Left foot	Sensible limb	30 (100%) ^a	10 (90.9%) ^a	13 (92.9%) ^a	15 (93.8%) ^a	8 (61.5%) ^b
	Insensible limb	0 (0%)	0 (0%)	1 (7.1%) ^b	1 (6.3%) ^b	3 (23.1%) ^a
Vibration sensation³						
Right foot	Sensible limb	30 (100%) ^a	9 (81.8%) ^b	9 (64.3%) ^c	6 (37.5%) ^d	4 (30.8%) ^d
	Insensible limb	0 (0%)	2 (18.2%) ^c	5 (35.7%) ^b	10 (62.5%) ^a	9 (69.2%) ^a
Left foot	Sensible limb	30 (100%) ^a	9 (81.8%) ^b	8 (57.1%) ^c	7 (43.8%) ^c	3 (23.1%) ^d
	Insensible limb	0 (0%)	1 (9.1%) ^c	6 (42.9%) ^b	9 (56.3%) ^a	8 (61.5%) ^a

Data are showed as n (%).

Abbreviations: DFU, diabetic foot ulcer; T2DM, type 2 diabetes mellitus.

^{a,b,c,d}Indicate significant ($P < .05$) differences between groups.

¹Sensitivity assessed by a neurological hammer in the Achilles tendon; ²sensitivity assessed by a 128 Hz tuning fork on the dorsal bony prominence of the big toe;

³sensitivity assessed by a Semmes-Weinstein monofilament 7.5/10 g in 6 points.

However, no significant differences were observed between groups, which could be related to the high variability of the data. Regarding serum cholesterol levels, no significant differences were observed between the study groups. Accordingly, no clear trend was found in the prevalence of hypertriglyceridemia, hypercholesterolemia, and mixed hyperlipidemia (Table 1).

Regarding blood pressure, no significant differences were found in systolic and diastolic blood pressure between all the study groups and no clear trend was observed in the prevalence of diagnosed hypertension between groups (Table 1).

Sensory evaluation of the study population

As expected, the no T2DM (control) group reported 100% of sensitivity in both feet by the 3 sensory tests (Table 2). No clear trend was observed between the diabetic foot ulcer progression and the loss of sensitivity according to Achilles reflex and pressure sensitivity. In this study, all the T2DM patients with no visible foot lesions (DFU G0) reported foot sensitivity according to the Achilles reflex and pressure tests, thus are considered at very low risk of ulceration, whereas only 1 T2DM patient with superficial ulcers (DFU G1) reported an insensible limb. Interestingly, the prevalence of T2DM patients with affected vibration increased with the progression of diabetic foot ulcer (Table 2).

Metabolomic profile of the study population

A non-targeted metabolomic profile was assessed in the serum samples collected from all the study participants. After data pre-processing, 1139 peaks were subjected to a multivariate analysis. A PLS-DA score plot was constructed to identify if the metabolomic profile allowed the discrimination between the study groups (Figure 1).

The scores of the 2 major components indicated an explained variance of 19% (component 1) and 8% (component 2). As shown in Figure 1, there is no clear separation between all the study groups. Nevertheless, the no T2DM (control) group is clearly discriminated from the T2DM group with visible foot lesions (DFU G1, G2, and G3). The T2DM group with no visible foot lesions (DFU G0) was overlapped with the no T2DM (control) group and the T2DM groups with visible foot lesions (DFU G1, G2, and G3).

Altogether, these results indicate that ulceration in the lower limbs are associated with alterations in the serum global metabolome. However, the progression of the disease, regarding the depth and infection of foot ulcers, did not affect the metabolomic profile, since all the T2DM groups with visible foot lesions (DFU G1, G2, and G3) were found overlapped in the PLS-DA model plot.

The VIP score values of the metabolites responsible for the clustering observed in the PLS-DA model plot were obtained. VIP score values >1.0 are considered to be influential on the discrimination between groups in PLS-DA models. The top 10 discriminants obtained from the PLS-DA model and their putative identification is included in Table 3. Metabolite identification was assessed using the Progenesis Q1 software. A single compound could be assigned to multiple lipid isomers because the position of the fatty acid chain in the glycerol

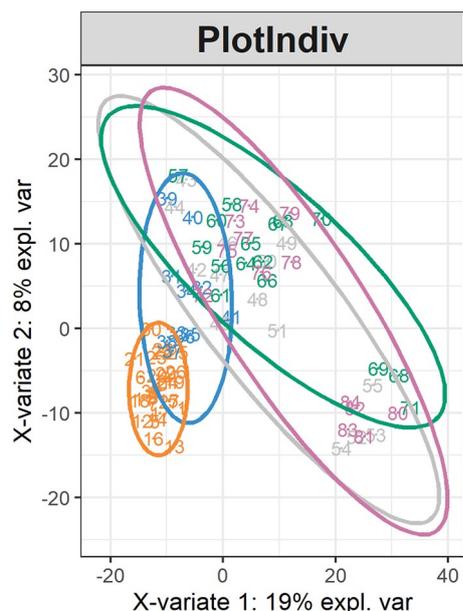


Figure 1. Partial Least Square-Discriminant Analysis (PLS-DA) of the serum non-targeted metabolomic profile of the study participants. Blue: no T2DM or control group; orange: DFU G0 group; gray: DFU G1 group; green: DFU G2 group; purple: DFU G3 group. Abbreviations: DFU, diabetic foot ulcer; T2DM, type-2 diabetes mellitus.

molecule (SN1 or SN2 isomers) could not be differentiated by the fragment pattern. Moreover, the identity assigned to the phospholipids identified in this study does not specify the position of the carbon-carbon double bonds (C=C).

Seven phospholipids were identified as major responsible of the metabolic differences associated with the onset of diabetic foot ulcer in T2DM adult patients, which included 2 phosphatidylinositols (PI, compounds 2 and 5), 1 phosphatidylethanolamine (PE, compound 3), 1 lysophosphatidylethanolamine (LysoPE, compound 10), 2 phosphatidylcholines (PC, compounds 6 and 8), and 1 lysophosphatidylserine (LysoPS, compound 7), whereas the fragmentation pattern did not allow the identification of 3 metabolites (compounds 1, 4, and 9) (Table 3). It is noteworthy that the associations between these metabolites and the development of diabetic foot ulcer are independent of clinical factors, including age, gender, DM duration, glucose and HbA1c levels, and T2DM-related comorbidities (renal dysfunction and hyperlipidemia).

Figure 2 shows the unsupervised hierarchical clustering of the top 10 discriminant metabolites. Interestingly, 2 major clusters were observed, 1 cluster included only the compound 10, whereas the second cluster included the other 9 discriminants. This clustering is due to the differential trends observed between the study groups: the serum levels of the compound 10 increased with the progression of diabetic foot ulcer in T2DM patients, whereas an inverse relationship was found with the serum levels of the other compounds.

Figure 3 shows the individual box-plots of the top 10 discriminant metabolites with their corresponding univariate statistical analysis. The selected discriminant metabolites showed a VIP score >2.4 according to the PLS-DA analysis. Interestingly, the compounds 1, 3, 6, and 8 were significantly ($P < .05$) lower whereas compound 10 was significantly higher

Table 3. VIP score values of the 10 major discriminants obtained from the PLS-DA.

NO. COMPOUND	PUTATIVE IDENTITY ¹	CODE (RT_M/Z)	VIP SCORE
Compound_1	Not identified	22.05_1013.4176	2.4096
Compound_2	PI (18:0)/22:6), PI (22:6/18:0)	22.07_895.1519	2.4240
Compound_3	PE (16:0/18:1), PE (18:1/16:0)	22.07_1065.4303	2.4260
Compound_4	Not identified	22.07_1036.4533	2.4558
Compound_5	PI (22:1/22:6), PI (22:6/22:1)	22.05_963.1376	2.4745
Compound_6	PC (20:1/22:2), PC (22:2/20:1)	22.07_866.1744	2.5008
Compound_7	LysoPS (18:0/0:0), LysoPS (0:0, 18:0)	5.88_510.3114	2.5179
Compound_8	PC (22:1/22:6), PC(22:6/22:1)	20.46_886.3930	2.5295
Compound_9	Not identified	22.07_882.1473	2.5727
Compound_10	LysoPE (16:1/0:0), LysoPE (0:0/16:1)	20.46_451.1625	2.6320

Abbreviations: DM2, type 2 diabetes mellitus; m/z, mass/charge; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLS-DA, Partial Least Squares-Discriminant Analysis; PS, phosphatidylserine; Rt, retention time; VIP, Variable Importance in the Projection; WM, Wagner-Meggiit classification system.

¹Identification was carried out with the Progenesis Q1 software, peak identity was accepted with a confidence score ≥ 48.0 .

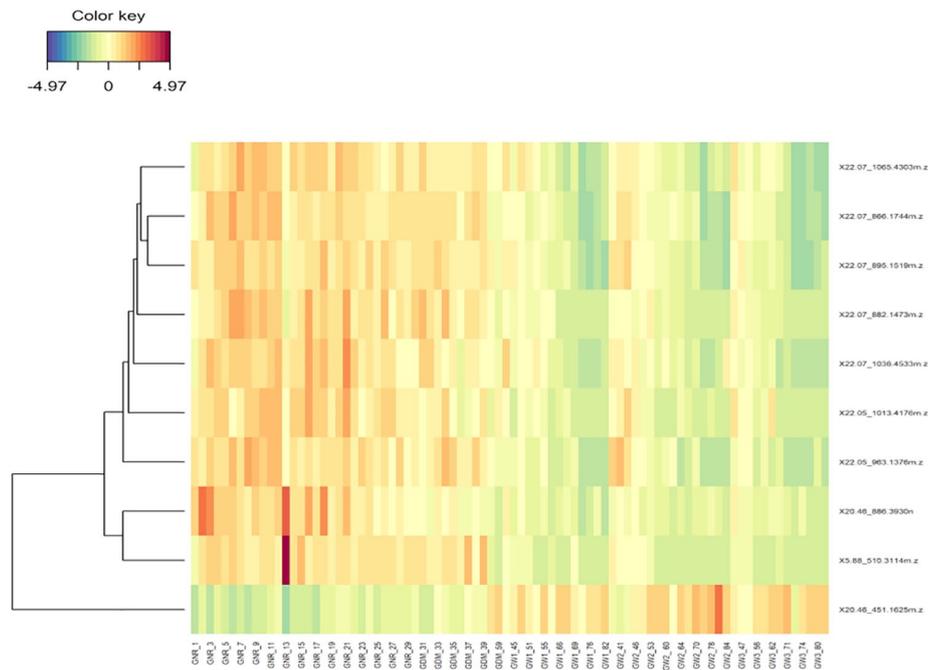


Figure 2. Unsupervised hierarchical clustering of the top 10 features that discriminate the study groups obtained from the PLS-DA model. Abbreviation: PLS-DA, partial least square-discriminant analysis.

between the no T2DM (control) group and the groups with different stages of diabetic foot ulcer (DFU G0, G1, G2, and G3). However, compounds 1 (not identified) and 8 (PC [22:1/22:6] or PC [22:6/22:1]) were not significantly ($P < .05$) different between the group with no visible foot lesions (DFU G0) and the groups with visible foot lesions (DFU G1, G2, and G3). Therefore, these metabolites are associated with the onset and development of T2DM, but not with the pathogenesis of diabetic foot ulcer.

Interestingly, compounds 3 (PE [16:1/18:0] or PE [18:0/16:1]), 6 (PC [20:1/22:2] or PC [22:2/20:1]), and 10 (LysoPE [16:1/0:0] or LysoPE [0:0/16:1]) were significantly ($P < .05$) different between the group with no visible foot lesions (DFU G0) and the groups with visible foot lesions (DFU G1, G2, and G3). These results suggest that these phospholipids are associated with the onset of diabetic foot ulcer in T2DM patients. However, the lack of significant differences between the T2DM groups with visible foot lesions (DFU G1, G2, and G3) indicate that these phospholipids can only be considered as biomarkers for early diagnosis of diabetic foot ulcer, but not for the assessment of the progression of the disease.

Discussion

This study aimed to identify the metabolic alterations associated with the onset and development of diabetic foot ulcer in adult patients with T2DM through a non-targeted metabolomic approach. Firstly, we analyzed the clinical characteristics of the study participants. Regarding age, no statistical differences were observed between the study groups. These results are not according with the reported by Boulton et al,⁶ who reported that the risk of ulcer development and lower limb

amputation increases by 2- to 4-fold with age. On the other hand, as observed in this study, male gender is associated with an increased risk of foot ulcer development (1.6-fold) as compared to female gender.⁶

It has been reported that increasing DM duration is positively associated with an increased risk of diabetic foot ulcer; however, no significant differences were found in this parameter between the DFU groups. Similarly, no significant differences were found on HbA1c and glucose levels between the DFU groups.

Conversely, previous studies have reported that a poor glucose control increases the risk of lower limb amputations,^{6,7} since hyperglycemia leads to an increased production of reactive oxygen species (ROS) and advanced glycation end (AGEs) products, inhibiting the activity of glucose 3-phosphate dehydrogenase (G3PDH), thus activating the polyol pathway, which leads to the accumulation of sorbitol in the Ranvier nodules, and thus the onset of diabetic neuropathy.⁸

The presence of DM-related comorbidities is common in patients with diabetic foot ulcer.⁹ Hyperglycemia leads a decreased glomerular filtration capacity, leading to a decreased excretion of urea and creatinine, and thus, their accumulation in blood. Therefore, diabetic nephropathy is a common comorbidity in patients with diabetic foot ulcer. The results of serum urea, creatinine, and uric acid found in this study are similar to those reported by Al-Shammaree et al,¹⁰ who reported that these parameters increase with diabetic foot ulcer progression.

Hyperuricemia is associated with the development of foot lesions. Oxidative stress derived from chronic hyperglycemia increases the accumulation of uric acid, which promotes the activation of the renin-angiotensin-aldosterone and the nitric

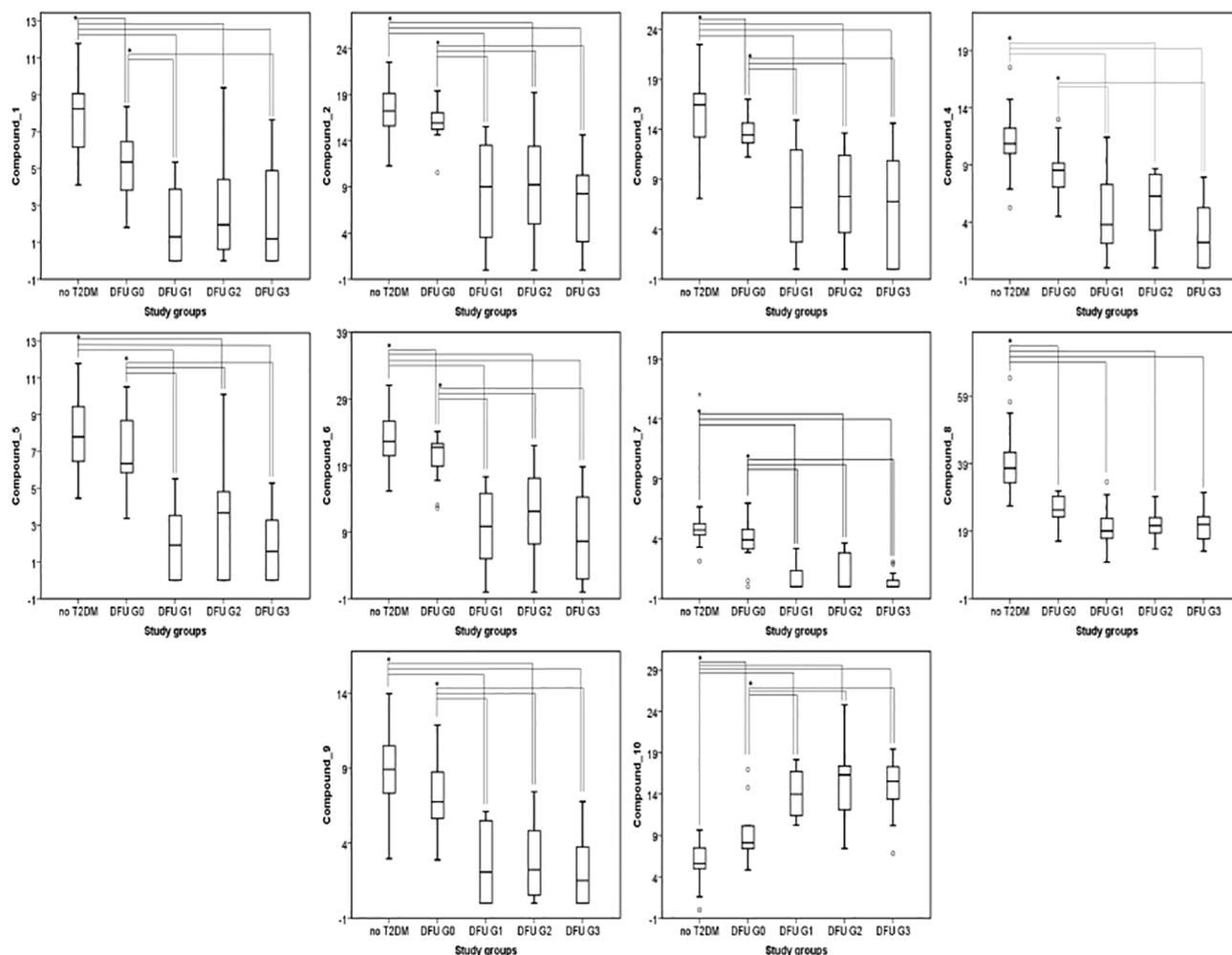


Figure 3. Box-plots of the major discriminant metabolites obtained from the PLS-DA.

Abbreviations: DFU, diabetic foot ulcer; PLS-DA, Partial Least Squares-Discriminant Analysis; T2DM, type-2 diabetes mellitus.

*Indicate significant ($p < 0.05$) differences between groups.

oxide systems, leading to an augmented vasoconstriction and vasodilation, respectively. These simultaneous events damage the blood vessels, leading to ischemia, which is a macrovascular complication associated with the development and progression of diabetic foot ulcer.¹¹

On the other hand, T2DM is commonly associated with the alteration of serum lipids, leading to dyslipidemia. In this study, no clear trend was observed regarding serum triglycerides and cholesterol levels. In this regard, controversial results have been previously reported. For instance, Al-Shammaree et al¹⁰ reported an association between serum triglycerides and the progression of diabetic foot ulcer in T2DM patients, whereas Yusof et al¹² and Atozona and Larbie¹³ reported no association. Accordingly, no association was found between hypercholesterolemia and diabetic foot ulcer progression.^{10,12} Conversely, Atozona and Larbie¹³ reported hypercholesterolemia as a risk factor for the development of ulcers.

Finally, similar systolic and diastolic blood pressure values were found in all groups. Accordingly, Almobarak et al¹⁴ reported no association between the prevalence of hypertension and diabetic foot ulcer, whereas a meta-analysis carried

out by Zhang et al¹⁵ indicated higher blood pressure in patients with diabetic foot ulcers.

The loss of foot sensitivity is considered an early stage of diabetic neuropathy, which is characterized by peripheral nervous dysfunction. Chronic hyperglycemia leads to an excessive accumulation of sorbitol in the Ranvier nodules, which leads to a decreased nervous conduction and neuronal sensitivity. Therefore, when a T2DM patient suffers a lesion in the lower extremities, no stimuli are perceived, which in combination with extrinsic factors, contribute to ulceration.¹⁶

One approach for the early diagnosis of diabetic foot ulcer is the evaluation of foot sensitivity to decrease the development of foot lesions. According to the International Working Group on the Diabetic Foot (IWGDF) guidelines, the absence of symptoms in a T2DM patient does not exclude foot disease due to the high probability of asymptomatic neuropathy and peripheral artery disease (PAD), thus these patients must be classified with low risk and should be annually examined for loss of sensation and PAD.¹⁷

The sensory evaluation is considered a practical, simple, low-cost, unpainful, and non-invasive approach for the assessment

of diabetic neuropathy.⁹ In this study, foot sensitivity was assessed by 3 methods: Achilles reflex, pressure, and vibration, since each method evaluate a different somatosensory function that are progressively lost during T2DM.¹⁸

According to the results found in this study, foot sensitivity assessed by neurological hammer and tuning fork can be considered as low predictors for the development of foot ulcers, since no clear trend was observed between diabetic foot ulcer progression and the loss of sensitivity assessed with these neurological tests. Conversely, foot insensitivity measured with the monofilament test was increased with the progression of diabetic foot ulcer. In this regard, Abraham et al¹⁶ compared 4 screening methods for diabetic peripheral neuropathy and reported that the monofilament test was one of the most reliable sensory tests.

The non-targeted metabolomic serum profile of the study groups was analyzed using a chemometric approach, which allowed the identification of the 10 major discriminants between the adults without T2DM (control group) and the adults with visible lesions of diabetic foot ulcer (DFU G1, G2, and G3). Interestingly, these latter groups were found overlapped, indicating similar global serum metabolomic profiles.

Accordingly, it has been proposed that the onset of diabetic foot ulcer is associated with the development of peripheral neuropathy and/or peripheral artery disease, whereas the progression of foot lesions is associated to extrinsic factors (ADA, 2019), which could be associated to the lack of differences in the global serum metabolome profile between the patients with different grades of visible foot lesions.

The major discriminants between the no T2DM (control) group and the DFU G1, G2, and G3 groups were phospholipids, which are considered the major component of cell membranes. A disruption of phospholipid metabolism results in the loss of cell membrane integrity, leading to cell dysfunction and death. Interestingly, all the phospholipids identified as discriminants in this study belong to the subclass of glycerophospholipids. Accordingly, glycerophospholipids have been previously associated with insulin resistance, and have been proposed as biomarkers for T2DM. Moreover, alterations in the fatty acid composition of glycerophospholipids has been associated to decreased insulin sensitivity.¹⁹

The association between non-bilayer lipids, like palmitoyl-oleoyl PE (POPE, compound 3) and glucose metabolism has been previously demonstrated, since POPE increases GLUT4 activity, increasing glucose uptake.²⁰ Accordingly, this phospholipid was significantly ($P < .05$) lower in all DFU groups as compared to no T2DM (control) group (Figure 3).

On the other hand, Semba et al²¹ reported that insulin resistance is associated with lower plasma levels of several polyunsaturated PC. Moreover, Zhao et al²² reported that elevated plasma PC (22:6/20:4) levels are associated with reduced risk of T2DM, whereas Suhre et al²³ reported lower polyunsaturated PC levels in T2DM groups. Accordingly, in this study, all the DFU groups showed lower serum PC levels (compounds 6 and 8) as compared to the no T2DM (control) group (Figure 3).

Finally, LysoPE (16:1) (compound 10) was found in higher levels in all DFU groups (Figure 3). Similarly, Wallace et al²⁴ reported that low levels of LysoPE (18:1) was associated with increased insulin resistance and García-Fontana et al²⁵ reported LysoPE (18:2) as a discriminant metabolite between T2DM patients and healthy adults, which was found in lower amount in the T2DM patients. Decreased LysoPE levels are associated with increased levels of phospholipase A1 (PLA1) in DM patients, which catalyzes phospholipids hydrolysis into fatty acids. However, the effect of altered lysoPE composition on diabetic foot ulcer onset on progression has not been reported.

To the best of our knowledge, an altered phospholipid metabolism has not been previously associated with the onset of diabetic foot ulcer, including diabetic peripheral neuropathy. Therefore, further studies must be undertaken to further understood the implication of phospholipids on the development of diabetic foot ulcer, mainly PE, PC, and LysoPE, which were the phospholipid classes associated to the onset of diabetic foot ulcer (Figure 3).

It is noteworthy that the large variance of compounds 3 and 6 in the no T2DM (control) group and the DFU G0 group does not allow a clear difference between these groups; whereas a better discrimination was found for compound 10. Therefore, we proposed that this latter metabolite (LysoPE [16:1]) should be further studied in a larger cohort to carry out an analytical and clinical validation as a biomarker for the early diagnosis of diabetic foot ulcer.

The following limitations of our study must be considered. (1) The non-targeted metabolomic results are expressed as relative abundance, which are highly correlated to absolute concentrations. (2) All statistical analyses were adjusted by known risk factors and T2DM-related comorbidities. However, the possibility of other potential confounding variables, like diet, physical activity, gut microbiota, and T2DM-unrelated comorbidities are not excluded. (3) The participants included in this study are Mexican adults who may have a high propensity for the development of T2DM and its complications; therefore, the findings of this study should be cautiously extended to other ethnic groups. (4) The design of this study is considered as exploratory due to the relatively low sample size and the non-targeted approach. Therefore, a targeted metabolomic study must be undertaken in a large-scale prospective study in the same ethnic group to confirm the results obtained in this study, and then should be extended to other ethnic groups. Nevertheless, it is noteworthy that this is the first study that assess a non-targeted metabolomic profiling of T2DM adults with different stages of diabetic foot ulcer.

Conclusion

In this study, metabolic alterations were observed in T2DM patients with no foot lesions (DFU G0) as compared with T2DM patients with visible foot ulcers (DFU G1, G2, and G3), mainly in the metabolism of phosphatidylethanolamines and phosphatidylcholines. The combined non-targeted metabolomics

and chemometrics approach allowed the identification of the phospholipid LysoPE (16:1) associated to the onset of diabetic foot ulcer. Further studies must be undertaken to evaluate its potential as a biomarker for the early diagnosis of diabetic foot ulcer, prior the development of foot wounds, as well to further understand its implication in the onset of diabetic foot ulcer. Interestingly, similar global metabolomic profiles were observed at different stages of diabetic foot ulcers, suggesting that the progression of diabetic foot ulcer relays mainly on extrinsic factors and not in global metabolic alterations. However, further studies are necessary to further understand the pathophysiology underlying diabetic foot ulcer progression.

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Authors' Contributions

IIAR carried out the recruitment of the participants, analyzed all biochemical, sensory, and clinical data, and carried out sample processing. ECT carried out the multivariate analysis and statistical interpretation. DGGG, RRC, and JEEP collaborated in the interpretation of the results. ABP supervised the recruitment of the participants and carried out the classification of the diabetic foot ulcer stages. IFPR carried out the conceptualization and design of the study, supervised the project and acquired the financial support for the study in collaboration with RRC and ECT. The manuscript was written by IIAR and IFPR and all the authors contributed to the final version of the manuscript.

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