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ORIGINAL ARTICLE

Plasma carnitine, choline, γ-butyrobetaine, and trimethylamine-N-oxide, but not zonulin, are reduced in overweight/obese patients with pre/diabetes or impaired glycemia

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Abstract

Background and aims Zonulin, carnitine, choline, γ-butyrobetaine (γ-BB), and trimethylamine-N-oxide (TMAO) are intricately involved in metabolic anomalies and type 2 diabetes mellitus (T2D). This study aimed to compare and correlate the plasma levels of zonulin, carnitine, choline, γ-butyrobetaine, and TMAO, along with the adiposity, atherogenicity, surrogate insulin resistance (sIR), and proinflammatory hematological indices of newly diagnosed drug-naive prediabetic and diabetic patients vs. apparently healthy normoglycemic controls.

Methods In a cross-sectional study, 30 normoglycemic subjects (controls) and 16 prediabetic (preDM) and 14 type 2 diabetes (T2D) cases, that were gender and age-matched, were enrolled. Zonulin, carnitine, choline, γ-BB, and TMAO plasma levels were appraised using colorimetric assays. A comparison between the study groups was conducted by ANOVA while Spearman rank correlations between the metabolic risk biomarkers and between the risk markers and adiposity, sIR, atherogenicity, and proinflammatory hematological indices were also examined.

Results Significant intergroup discrepancies in plasma carnitine, choline, γ-BB, and TMAO (but not zonulin) could be recognized in the cases vs. controls. Fasting blood glucose (FPG), glycated hemoglobin (A1C), triglycerides (TGs), body mass index (BMI), lipid accumulation product (LAP), visceral adiposity index (VAI), atherogenic index of plasma (AIP), and all sIR were outstandingly higher in the cases vs. controls. Blood indices lacked a scoring value to discriminate cases from controls. Inadvertently, no relation was found between plasma carnitine, choline, γ-BB, TMAO, or zonulin in cases. Among the rest of the markers and sIR indices, the triglyceride glucose-body mass index (TyG*BMI) related reciprocally to zonulin. Noticeably, among adiposity indices, TyG*BMI, triglyceride glucose-waist circumference (TyG*WC), and metabolic score for insulin resistance (MetS-IR) positively associated with waist circumference (WC), hip circumference (HC), BMI, body adiposity index (BAI), and waist-to-height ratio (WHtR). Exceptionally, LAP proportionally correlated with all sIR. TyG*WC and MetS-IR correlated directly with the conicity index (CI). WHR directly associated with triglyceride glucose (TyG) index and TyG*WC. Remarkably, the TyG index (but not TyG*BMI, TyG*WC, or MetS-IR) positively associated with all atherogenicity indices and RDW (but none of other blood indices). TMAO correlated inversely ($p < 0.05$) and moderately with choline. Distinctively, carnitine associated negatively with TC ($p < 0.05$). Both choline and carnitine related similarly and directly with PLR but inversely with lymphocytes ($p<.05$). Effectively, γ-butyrobetaine associated with both WC and the TyG-WC index equally negatively ($p < 0.05$). Substantially, γ-butyrobetaine correlated inversely with both atherogenic LDL-C/HDL-C ratio and MPV ($p < 0.05$). No pronounced relations were detected between the five microbiome signature determinants and glycemic control parameters (FBG and A1C%), sIR (TyG, TyG-BMI, or MetS-IR), adiposity (WHR, WHtR, CI, BAI, LAP, or VAI), atherogenicity indices (TC/HDL-C ratio, non-HDL-C/HDL-C ratio, or AIP), or blood indices (NLR or MLR).

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Conclusion Given the intergroup discrepancies in sIR, plasma zonulin, carnitine, choline, γ-BB, and TMAO along with their elective correlations with indices and clinical parameters of metabolic dysregulations, our study cannot rule out any possible molecular crosstalk and interplay of the biomarkers studied with the pathophysiology of prediabetes/diabetes. All in all, plasma zonulin, carnitine, choline, γ-BB, and TMAO with sIR can be putative surrogates for molecular cardiometabolic risk biomarkers to use as prognostic/predictive tools for the diagnosis/prevention and potential targets for prediabetes/diabetes management modalities.

Keywords Zonulin trimethylamine-N-oxide · Choline · Carnitine · γ-Butyrobetaine (γ-BB) · Trimethylamine-N-oxide (TMAO) · Flavin monooxygenase 3 · Atherogenic index of plasma · Intestinal barrier integrity · Metabolomics

Introduction

The human gut is inhabited by more than 100 trillion microorganisms that form the controlled, complex, and dynamic microenvironment inside the host gastrointestinal tract. Generally, human intestinal microbiota is composed of five phyla: Bacteroidetes and Firmicutes that form the majority (> 90%) in addition to Proteobacteria, Actinobacteria, and Cerrucomicrobia [1]. Recent studies have reported that the homeostasis of gut microbiota is crucial for conserving human health from any disturbance [2, 3]. Accumulated evidence reveals that gut microbiota dysbiosis may cause the intestinal microenvironment to modify into different states where new bacterial species will thrive while other bacteria species will be inhibited. Eventually, diseases such as inflammation, allergy, obesity, cardiovascular diseases (CVDs), autoimmune diseases, and type 2 diabetes mellitus (T2D) may emerge [3–10].

Trimethylamine-N-oxide (TMAO) is a phosphatidylcholine-related metabolite recently identified among several other metabolites derived from the gut microbiota. It can be generated from trimethylamine (TMA)-related precursors of carnitine and choline, and phosphatidylcholine richly found in Western diet. Besides, lifestyle interventions [11] can reduce its precursors' levels as well as TMAO renal clearance. Its microbiota-related intermediate of γ-butyrobetaine (γ-BB) correlated with visceral adiposity. Most importantly, γ-BB is involved in the conversion of carnitine to the pro-atherogenicity metabolite TMAO with detectable acceleration of atherosclerosis in mice [12] thereby triggering heart attacks and stroke [13] via foam cell formation. In effect, the increase in levels of γ-BB and its precursor trimethyllysine significantly associated with the prediction of CVD risk stratification [12], and development of renal insufficiency [14]. TMAO can worsen chronic inflammation and endothelial dysfunction in T2D-CKD patients via increase substantial increase in TMAO-generating gut microbiota [15–19]. Furthermore γ-butyrobetaine-producing *Bifidobacterium* and *Akkermansia*, *Bacteroides*, *Faecalibacterium*, and *Roseburia* negatively associated with T2D patients, while the genera of *Ruminococcus*, *Fusobacterium*, *Dorea*, and *Blautia* positively associated with T2D [15–19]. Thus, gut microbiota-based classifiers to identify individuals with a high risk for T2D were founded via amino-acid enrichment (isoleucine, proline, valine, cysteine, glutamine, and aminobutyrate) [15–21]. Zonulin protein, a "47 kDa protein also known as prehaptoglobin-2," of hepatocytes and eneterocytes has become presently a detrimental biological marker of mucosal barrier integrity, in modulating the permeability of tight junctions between cells located in the walls of digestive tract [22]. It presents the only measurable blood protein biomarker for controlling gut permeability; as blood elevated zonulin levels can mirror defects in the intestinal barrier [23]. Zonulin is derived from *Vibrio cholerae* phosphorylated zonula occludens toxin, which can disassemble small intestine tight junctions. Release of zonulin is prompted via enteric enterotoxins-releasing pathogens such as commensal *Eschericha coli*, virulent *E. coli*, lab *E. coli*, and *Salmonella typhi* and gluten-associated glycoprotein "gliadin." Subsequent induction of gut microbiota dysbiosis; zonulin release can trigger luminal antigen influx with interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) release and activation of T cells that would augment gut permeability and immune response [22–24]. Taken together, chronic inflammatory diseases such as IBD (inflammatory bowel disease), IBS (irritable bowel syndrome), or systemic CID (chronic inflammatory disease) [25] can be determinants of this pathophysiology. Given this particular emphasis on the development of beneficial microbiome-targeted therapies and interventions for obesity prevention and treatment, a multitude of approaches and accompanying challenges can be encountered.

Diabetes mellitus (DM) has become a threat to the worldwide populations since numbers of morbidity and mortality of type 1 diabetes (T1D) and type 2 diabetes (T2D) have soared dramatically in the last few decades. The World Health Organization (WHO) reported that 422 million individuals are living with diabetes, mostly in low-income countries; the number of patients diagnosed with prediabetes (pre-DM) is predicted to grow significantly and culminate into 482 million people by 2040 [26–29] (https://www.who.int/health-topics/diabetes). T1D, known as juvenile diabetes, is characterized by autoimmune β-cell devastation that eventually causes an absolute insulin deficiency [29] while T2D, that develops later in adulthood, can be considered to be a highly prevalent metabolic disorder recognized by a disorganized response to increased glucose levels in blood circulation, disturbance in lipid profile, endothelial dysfunction,

visceral adiposity, and raised blood pressure [30, 31]. The core pathological mechanism of T2D is insulin resistance (IR), a rapidly growing complication in various geographical regions of the world; it is characterized by insensitivity to the insulin hormone resulting in compensatory hyperinsulinemia. IR is known to be a major risk factor for metabolic syndrome, T2D, and CVDs. IR is known to be the core pathological mechanism for T2D. Principally, the atherogenic index of plasma (AIP) (derived from triglycerides and high density lipoprotein cholesterol: Log (TG/HDL-C)) is a biomarker to quantify atherogenicity-related blood lipid levels in plasma; it is usually indicative of CVD, dyslipidemia, and other related diseases [32, 33]. Heredity, high fat food, and a sedentary lifestyle are three major factors that contribute to the high risk of T2D [30, 34, 35]. Patients neglecting appropriate treatment and close glycemic control follow-up will experience CVDs, nephropathy, retinopathy, and neuropathy [36]. Metabolomics in the last few years has emerged as a new powerful technique aimed at comprehensive analysis, identification, and detection of large diverse physicochemical properties of endogenous metabolites (biomarkers) [26, 27, 37, 38]. Hence, any disruption in metabolite concentration or configuration may give an indication of disease development. This may allow diagnosing the disease at an early stage before causing any complications or damage. Metabolomics have been successfully and comprehensively examined via high technologies such as liquid chromatography with tandem mass spectrometry (LC-MS/MS) in order to detect and quantify entire or specific molecules of small endogenous metabolites [36].

Objectives

The objective of the present study is to compare serum metabolic biomarkers of prediabetic (preDM) and DM microbiome dysbiosis (further abbreviated as "metabolic biomarkers": zonulin, carnitine, choline, γ-BB, and TMAO) in preDM/T2D Jordanian patients vs. those of normoglycemic controls. Further relationships are explored—for the first time-between five microbiome signature determinants and indices of adiposity, atherogenicity, proinflammatory hematological indices, and surrogate insulin resistance (sIR).

Subjects, materials, and methods

Study design

This cross-sectional study was conducted to examine the comparison and relation between plasma metabolic biomarkers in two groups in the Jordanian population:

1. In controls, 30 participants were apparently healthy and normoglycemic (A1C < 5.7% and FPG < 100 mg/dL) [28, 39, 40]. These participants were mainly considered for comparison purposes.
2. In the preDM/T2D group, there were 30 participants who were either preDM or T2D patients (5.7% < A1C < 6.4% or > 6.5%, respectively; 110 mg/dL < FPG < 125 mg/dL or > 126 mg/dL, respectively) and were necessarily defined as drug-naïve subjects (Fig. 1).

All participants who attended the family medicine clinics at the JUH and the Diabetes Endocrinology clinics at KHMC were screened for potential recruitment, which took place over the period from April 2019 to March 2020. Adult patients of both genders 28–76 years old were included in the study. Subjects were defined as preDM/T2D according to the American Diabetes Association (2020) criteria [28]. Control participants were normoglycemic. In this study, patients with acute complications of diabetes, acute renal or hepatic dysfunction, chronic inflammatory or autoimmune disease, cancer, infectious diseases, tuberculosis, or neuromuscular diseases or were pregnant or breast feeding were excluded. Patients who received any of the medications such as oral antibiotics, proton-pump inhibitors, metformin, heart disease medications, laxatives, systemic corticosteroids, or oral contraceptives were also excluded from the study. The details of the participant recruitment process are shown in Fig. 1. Height and weight were measured with standardized techniques. Demographic data and anthropometric measurements and lab tests were obtained from each participant. Consequently, adiposity, atherogenicity, sIR, and inflammatory hematological indices are calculated as in Table 1. After a 10-h overnight fasting, venous blood samples were collected in the morning into 2-mL EDTA tubes by using Vacuette® Standard tube holder and BD Vacutainer® 21 G × 1.2 inch, multisample needle (Vacuette®, Weihai Hongyu Medical Device Co., Ltd., China) for a complete blood count (CBC) analysis (Beckman, Coulter Inc., California, USA). The glycated hemoglobin (HbA1c) percentage was measured by turbidimetric inhibition immunoassay and analyzed on a Roche Cobas 6000 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Glucose concentration was tested by glucose oxidase-based assay as blood was collected in 6-mL serum gel separator tubes (Vacuette®Tube Serum Gel Separator Clot Activator 6 mL, Jordan).

For biochemistry analyses, heparinized blood was allowed to clot and then immediately centrifuged at 4,000 rpm for 10 min at 4 °C. Fasting blood glucose and lipid profile including total cholesterol (TC), triglycerides (TGs), high density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations were analyzed via enzymatic assays (Roche Cobas 6000 autoanalyzer, Roche

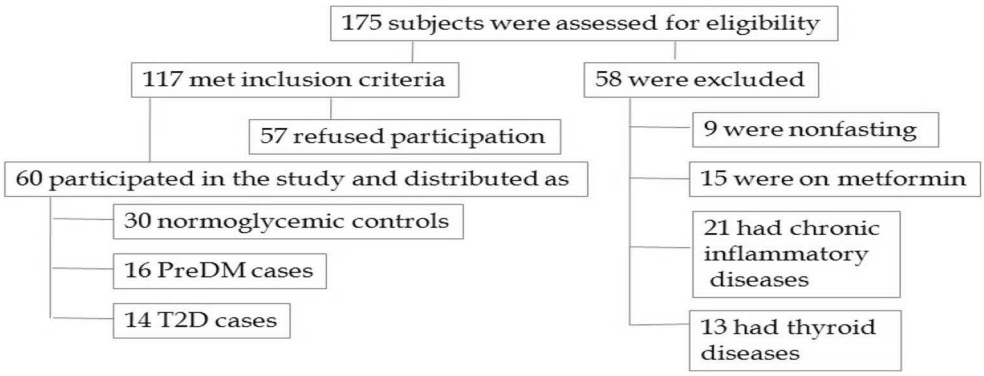

Fig. 1 Recruitment process flow chart

Diagnostics, Mannheim, Germany). For metabolomics biomarker testing, blood was added to lithium heparin tubes (Vacuette®Tube, Jordan) centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatant was carefully extracted, then transferred into a 1.5-mL sterile centrifuge tube and frozen at -20 °C before metabolomics testing. A zonulin ELISA kit was procured for determination of their plasma levels and implemented according to the manufacturer's instructions (MyBioSourse, San Diego, CA, USA).

Carnitine, choline, TMAO, and γ-BB HPLC determinations

Reagents Choline chloride, TMAO, and GBB were obtained from Sigma-Aldrich Chemicals (St. Louis, Missouri, USA) and D9-Choline chloride from Sigma-Aldrich Chemical. Methanol, acetonitrile, and formic acid were HPLC grade and purchased from Merck Chemicals (400 Summit Drive, Burlington, Massachusetts 1803, USA).

Sample collection and processing Plasma or serum samples (30 μL) were mixed with three volumes of acetonitrile; to be centrifuged for 2 min at 5,800 *g*. The supernatant was then transferred to sealed autosampler glass vials (Chromacol). Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was carried out on a Shimadzu series HPLC system (Shimadzu Corp.) equipped with a thermostated autosampler and a degasser for solvent delivery and sample introduction. Serum samples deproteinized with acetonitrile were placed in a cooled (4 °C) sample tray and injected (2 μL) into a normal-phase column (10 × 2.1 cm) packed with 5-μm diameter particles of Hypersil silica (Shimadzu Corp.) and equilibrated with 25% of solution A (15 mmol/L ammonium formate, pH 3.5) and 75% of solution B (acetonitrile). The column was eluted at ambient temperature at a flow rate of 0.6 mL/min and developed with gradient elution as follows: 0–0.1 min, 25% A and 75% B; 3.5 min, 80% A and 20% B; 3.6 min, 25% A and 75% B; and 5.6 min, 25% A and 75% B. All gradient steps were linear. The column effluent was split at a ratio of 1:4, delivering the eluate at a flow rate of 150 μL/min into the mass spectrometer. The injection interval was 6 min. We used a Shimadzu Lab Solution triple-quadrupole tandem mass spectrometer with Turbo Ion Spray TM interface in the positive-ion mode. Nitrogen was used as the drying gas at a flow rate of 6 L/min and for collision-activated dissociation. The collision energy was 28 eV, the declustering potential was 31 V, and the ion source temperature was 350 °C. For development work, the product-ion spectra for choline were acquired in the continuous flow injection mode, with use of a Harvard Model 11 syringe pump connected directly to the ion source by PEEK tubing. For signal optimization, we dissolved each compound at a concentration of 10 μmol/L in a mixture of 15 mmol/L ammonium formate and acetonitrile (75:25 by volume), infused at a rate of 10 μL/min choline, m/z 104 > 60; GBB, m/z 146.2 > 87.1; and TMAO, m/z 76.1 > 58.1. Analyst software (Shimadzu Lab Solution) was used for the HPLC system control, data acquisition, and data processing.

Reagents L-carnitine

Reagents L-carnitine were purchased from Sigma. All other chemicals and solvents were from Merck (Darmstadt, Germany) or Sigma and were of analytical grade. The LC-MS/MS equipment used was the HPLC system consisting of the Shimadzu Sil-zoc, column-oven (CTO-20A), a quaternary pump (LC-20AD), and a system controller (CBM-20A) from Shimadzu. The HPLC system was coupled to a Shimadzu triple quadrupole mass spectrometer with an electrospray ionization source. Samples were loaded on an Oasis MCX trapping column (30 μm, 2.1 × 20 mm; Waters Corporation, Milford, MA, USA) and separated on a Luna C8 5-μm column (150 × 2 mm) equipped with a C8 (4 × 2.0 mm) precolumn (Phenomenex, Torrance, CA, USA).

Sample collection and processing

The HPLC condition for the chromatographic separation was done with a binary flow at 50 °C. Phase A was an aqueous solution containing 5 mmol/L heptafluorobutyric acid and 5 mmol/L ammonium acetate and phase B was

Table 1 Indices of anthropometric adiposity, atherogenicity, inflammatory hematological indices, and surrogate insulin resistance (sIR) used in this study, reference, and equation for calculation

Measure	Author (year), clinical relevance, and impact	Equations
Surrogate IR (sIR) indices		
Triglyceride glucose (TyG) index	Simental-Mendía et al. (2008); Navarro-González et al. (2016); Kim et al., (2017) [41–43]	TyG = Ln [TG (mg/dL) * fasting glucose (mg/dL) / 2]
TyG-BMI	Er et al.(2016) [44]	TyG-BMI = TyG index * BMI
TyG-waist circumference	Okosun et al. (2020) [45]	TyG-WC = TyG index * WC (m)
MetS-IR	Bello-Chavolla et al. (2018)[46]	MetS–IR= ln[2 * Glucose (mg/dL) + Triglycerides (mg/dL)] * BMI (kg/m^2) / ln[HDL-C (mg/dL)]
Adiposity indices		
Waist-to-hip ratio (WHR)	Lockie et al. (2020) [47]	WHR = waist circumference (cm) ÷ hip circumference (cm)
Waist-to-height ratio (WHtR)	Filgueiras et al. (2019) [48]	WHtR = waist circumference (cm) ÷ height (cm)
Conicity index (CI)	Filgueiras et al. (2019) [48]	CI = WC (m) ÷ 0.109 √weight (kg) ÷ height (m)
Body adiposity index (BAI)	Cerqueira et al. (2018) [49]	BAI = [HC (cm) / (height $(m)^{1.5}$)] − 18
Lipid accumulation product (LAP)	Mazidi et al. (2018) [50]	LAP = (WC [cm] − 65) × (TG [mM]) for men LAP = (WC[cm] − 58) × (TG [mM]) for women
Visceral adiposity index (VAI)	Mazidi et al. (2018) [50]	VAI = (WC (cm) ÷ (39.68 + (1.88 * BMI))) * (TG mM/1.03) * (1.31/HDL-C mM) for males VAI = (WC (cm) ÷ (36.58 + (1.89 * BMI))) * (TG mM/0.81) * (1.52/HDL-C mM) for females
Atherogenicity indices		
Atherogenic index of plasma (AIP)	Khanduker et al. (2018) [33]	AIP = Log_{10} (triglyceride concentration/HDL-C)
Total cholesterol/HDL-C (TC/HDL-C) ratio	Gupta et al. (2018) [51]	Total cholesterol/HDL-C = Total cholesterol ÷ HDL-C
LDL-C/HDL-C ratio		LDL-C/HDL-C = LDL-C ÷ HDL-C
Non-HDL-C/HDL-C ratio	Morikawa et al. (2018) [52]	Non-HDL.C = total cholesterol − HDL.C
Blood indices		
Platelet-to-lymphocyte ratio (PLR)	Chrom et al. (2018) [53]	PLR = Platelets ÷ Lymphocytes
Neutrophil-to-lymphocyte ratio (NLR)	Chrom et al. (2018) [53]	NLR = Neutrophils ÷ Lymphocytes
Monocyte-to-lymphocyte ratio (MLR)	Antwi-Baffour et al. (2018) [54]	MLR = Monocytes ÷ Lymphocytes

methanol with the same additives. During 1.5 min, the analytes were loaded on the trapping column using 0.1% formic acid in water (V/V) as a mobile phase (flow, 0.5–1.0 mL/min) whereas the analytical column was conditioned with 10% phase B (flow, 0.35 mL/min). After valve switching, the analytes were transferred to the analytical column starting at 10% phase B with a linear increase of the gradient to 95% phase B within 4 min. After a plateau of 2 min at 95% phase B, the analytical column was re-equilibrated for 2 min with 10% B. This resulted in a total run time of 8 min. The chromatographic conditions for the mass spectrometry of carnitine were analyzed in the positive multiple reactions monitoring mode. A first transition was used for quantification, and a second one for qualification. Following transitions (m/z) were used: carnitine, 162→103 and 162→60; and carnitine-d3, 165→103 and 165→63. The ion spray voltage was 5,500 eV, the probe temperature was 450 °C, and the dwell time was 50 ms for each analyte. The plasma samples were deproteinized with 200 μL of methanol. The samples were extracted for 10 min at full speed on a Multi-Tube Vortex and centrifuged at 3,220 × *g* for 30 min. For the determination of total carnitine hydrolyzed under alkaline conditions, 25 μL of plasma was mixed with 25 μL of KOH 0.5 M (pH 13) and incubated at 40 °C for 30 min. The mixture was then neutralized with 50 μL of 1% formic acid in water (V/V) and centrifuged for 10 min at 1,811 × *g*.

Statistical analysis

All study participants were organized according to the study arm that they belonged to. Data were entered and tested through IBM SPSS© Statistics version 23 (SPSS, Inc., USA). The biomarker levels, indices, and clinical parameters

were presented as mean (±SD) and compared between the two study groups. Categorical data were presented as frequencies (%). Gender variation between the groups was analyzed utilizing the chi-square test. The Shapiro-Wilk test was used to assess the normality of distribution of the independent variables. Independent sample *t*-test was used for the contrasting of continuous dependent variables across the study arms when its assumptions were met; i.e., no significant outliers, approximately normally distributed data and homogeneity of variances. Otherwise, the non-parametric independent sample Mann-Whitney *U* test was used when the assumptions were not met. To assess the strength and direction of association between continuous variables in the two study groups, we used Spearman rank correlation, wherein continuous variables. p-value < 0.05 is considered statistically significant.

Results

There were no outliers in the data, as assessed by inspection of a boxplot for values greater than 1.5 box-lengths from the edge of the box.

Comparisons of cases vs. controls (N = 30 each) and correlations in cases (N = 30) of biochemistry parameters, markers, and metabolism indices (Tables 2, 3, and 4)

Collectively, carnitine, choline, γ-BB, and TMAO were substantially lower in cases vs. controls. Zonulin lacked any pronounced intergroup variance. Moreover, FPG, A1C, TG, BMI, LAP, VAI, and AIP proved significantly higher in cases in comparison to controls. Markedly, all surrogate insulin resistance indices (sIR) of cases were most substantially higher than in controls. Inflammatory blood indices hardly had any discrepancy in cases when compared to controls (N = 30; Table 2).

Exceptionally, TyG-BMI reciprocally related to zonulin among the rest of markers and sIR indices (Table 3). The TyG index had proportional correlations with FBG, A1C, TG, and non-HDL-C, but an inverse relation with HDL-C was evident. Among adiposity indices, TyG-BMI, TyG-WC, and MetS-IR positively associated with WC, HC, BMI, BAI, and WHtR. LAP proportionally correlated with all sIR. TyG-WC and MetS-IR correlated directly with CI. WHR directly associated with the TyG index and TyG-WC. Remarkably, the TyG index (but not TyG-BMI, TyG-WC, or MetS-IR) positively associated with all atherogenicity indices and RDW (but none of other proinflammatory blood indices; Table 3). No pronounced relations were detected between the five microbiome signature determinants and glycemic control parameters (FBG and A1C%), sIR (TyG, TyG-BMI, or MetS-IR), adiposity (WHR, WHtR, CI, BAI, LAP, or VAI), atherogenicity indices (TC/HDL-C ratio, non-HDL-C/HDL-C ratio, AIP), or blood indices (NLR or MLR; Table 4). TMAO exceptionally correlated inversely and moderately ($p < 0.05$) with choline, so did zonulin and carnitine with age ($\rho = -0.39$, $p < 0.05$). Conversely γ-butyrobetaine related proportionally with age. Distinctively, carnitine associated negatively with TC ($p < 0.05$). Both choline and carnitine related similarly and directly with PLR but inversely with lymphocytes ($p < 0.05$). Effectively γ-butyrobetaine associated with both WC and the TyG-WC index equally negatively ($p<.05$). Substantially, γ-butyrobetaine correlated inversely with both atherogenic LDL-C/HDL-C ratio and MPV ($p < 0.05$; Table 4).

Discussion

In this pioneering Jordanian cross-sectional study, we compared adiposity, atherogenicity, sIR, and proinflammatory hematological indices between newly diagnosed drug-naive prediabetic and diabetic patients vs. apparently healthy and normoglycemic controls matched by gender and age. Expectedly, FBG, A1C, TG, BMI, LAP, VAI, and AIP along with sIR were higher in cases vs. controls; our results are powered by several previous studies where significant correlations between A1C and TG, TC, LDL-C, and FPS were in accordance with our results but without significant correlations between A1C with HDL-C to report [55, 56]. Unlike the inverse correlation between glycosylated A1C with HDL-C in different reports [56], the correlation of LAP as a novel biomarker of central lipid accumulation related to the risk of diabetes and CVDs, with FPG, insulin, IR index, and lipid and lipoprotein levels, was investigated. Highly significant positive correlations were principally delineated [57] between LAP index and fasting glucose ($\rho = 0.39$, $p < 0.001$), LAP-total cholesterol ($\rho = 0.45$, $p < 0.001$), and triglycerides-to-HDL-C ratio ($\rho = 0.89$, $p < 0.001$). The best indicator of the relationship between obesity and T2D is waist-to-stature ratio (WSR) for men and BMI for women in randomly eligible male and female Chinese residents aged ≥ 50 years, though still valid for ethnicity relevance. Moreover, the prevalence of T2D was higher for men (19.3%) than for women (15.3%). Furthermore, diabetic subjects were noticeably older and heavier, with higher HC, WC, BMI, WHR, WSR, SBP, FPG, TG, and TC measurements, and had a higher prevalence of hypertension and obesity residing within cases than those in the non-diabetic group ($p < 0.05$). Also, our results are in the agreement with their results [58]. Importantly, this cross-sectional study provides novel information about the plasma levels of zonulin, L-carnitine, choline, γ-BB, and TMAO in preDM/T2D Jordanian patients. T2D can be molecularly connected to alterations in the gut permeability of tight intestinal junctions that are

Table 2 Comparisons of anthropometric data, biochemistry parameters, and markers and metabolism indices in cases vs. controls ($N = 30$ each)

		Gender	
Gender	**Controls**	**Cases**	***p*-value***
Female, *N* (%)	16 (53.3%)	16 (53.3%)	0.602
Male, *N* (%)	14 (46.7%)	14 (46.7%)	
Total	30 (100%)	30 (100%)	
Age (years, mean ± SD)	**Controls, *N* = 30**	**Cases, *N* = 30**	***p*[1]-value**
	49.53 ± 9.19	51.2 ± 9.11	0.483
Clinical characteristics			
	Controls, *N* = 30, mean (SD)	**Cases, *N* = 30, mean (SD)**	***p*[1]-value**
Weight (kg)	80.97(16.54)	88.4(15.78)	0.08
Height (m)	1.69(0.097)	1.68(0.09)	0.736
SBP (mmHg)	125.20(11.49)	129.87(17.67)	0.23
DBP (mmHg)	79.43(6.53)	85.83(20.90)	0.115
FBG (mg/dL)	**89.29(7.63)**	**130.23(80.07)**	**0.007**
A1C (%)	**5.37(0.24)**	**7.09(1.69)**	**0.000**
TG (mg/dL)	**160.68(99.78)**	**230.75(111.18)**	**0.013**
LDL-C (mg/dL)	144.18(29.51)	146.11(33.43)	0.814
HDL-C (mg/dL)	48.03(13.32)	46.31(11.98)	0.602
TC (mg/dL)	216.71(42.94)	212.81(33.87)	0.697
Non-HDL-C (mg/dL)	168.68(42.31)	166.5(33.59)	0.825
Adiposity indices			
WC (cm)	102.03(14.97)	109.48 ± 16.47)	0.072
HC (cm)	111.1(14.03)	116.2(16.54)	0.203
BMI (kg/m^2)	**28.49(5.51)**	**31.53(5.89)**	**0.044**
WHR	0.92(0.07)	0.94(0.055)	0.174
WHtR	0.61(0.09)	0.66(0.12)	0.072
CI	1.35(0.13)	1.39(0.20)	0.377
BAI	33.04(7.93)	35.95(10.31)	0.225
LAP	**77.48(56.34)**	**122.94(66.82)**	**0.006**
VAI	**2.81(2.12)**	**4.25(2.75)**	**0.027**
Atherogenicity indices			
TC/HDL-C	4.78(1.32)	4.85(1.31)	0.833
LDL-C/HDL-C	3.22(1.02)	3.33(1.04)	0.686
Non-HDL-C/HDL-C	3.78(1.32)	3.85(1.31)	0.833
TG/HDL-C ratio	**3.83(3.2)**	**5.57(3.39)**	**0.046**
AIP	**0.47(0.32)**	**0.66(0.28)**	**0.015**
Inflammatory hematological indices			
RDW-CV (%)	14.26(1.31)	14.27(1.39)	0.977
MPV	9.07(1.09)	9.38(0.96)	0.164
PLT count (× 10^9/L)	262.47(65.69)	265.9(64.73)	0.839
Monocytes %	6.31(2.46)	5.97(1.84)	0.543
Neutrophils %	60.25(9.14)	55.68(10.31)	0.074
Lymphocytes %	31.94(8.92)	38.61(12.95)	**0.024**
MLR	0.22(0.12)	0.17(0.095)	0.104
NLR	2.3(1.87)	1.69(0.89	0.115
sIR			
TyG-index	**8.72(0.60)**	**9.4(0.54)**	**0.000**
TyG-BMI	**248.90(52.37)**	**295.78(55.65)**	**0.001**
TyG-WC	**8.93(1.59)**	**10.30(1.65)**	**0.002**
MetS-IR	**43.37(9.7)**	**51.05(9.73)**	**0.003**

Table 2 (continued)

Metabolic risk biomarkers			
Zonulin (ng/mL)	10.76(5.22)	12.37(5.19)	0.227
Carnitine (nmol/mL)	**67.25(6.77)**	**46.90(10.35)**	**0.000**
Choline (μmol/L)	**12.42(1.37)**	**8.04(0.64)**	**0.000**
γ-BB (μmol/L)	**0.99(0.13)**	**0.47(0.08)**	**0.000**
TMAO (μM)	**5.19(0.52)**	**2.29(1.14)**	**0.000**

*p-value by chi-square

AIP, atherogenicity index of plasma; *BAI*, body adiposity index; *C-index*, conicity index; *DBP*, diastolic blood pressure; *FPG*, fasting plasma glucose; *A1C%*, percent glycosylated hemoglobin; *HC*, hip circumference; *HDL-C*, high density lipoprotein-cholesterol; *LAP*, lipid accumulation product; *LDL-C/HDL-C*, low density lipoprotein cholesterol-to-high density lipoprotein cholesterol ratio; *LDL-C*, low density lipoprotein-cholesterol; *MLR*, monocyte-to-lymphocyte ratio; *NLR*, neutrophil-to-lymphocyte ratio; *non-HDL-c/HDL*, non-high density lipoprotein-to-high density lipoprotein ratio; *non-HDL-C*, non-high density lipoprotein cholesterol; *PLT*, platelet; *RDW*, red cell width; *SBP*, systolic blood pressure; *TC/HDL-C*, total cholesterol-to-high density lipoprotein cholesterol ratio; *TC*, total cholesterol; *TG*, triglyceride; *TMAO*, trimethylamine-N-oxide; *WC*, waist circumference; *WHR*, waist-to-hip ratio; *WHtR*, waist-to- height ratio; and *γ-BB*, γ-butyrobetaine

dysregulated by elevated zonulin levels [23, 24]. It is a detrimental and valuable predictive non-invasive biomarker correlated appreciably with the pathogenesis of gestational diabetes mellitus (GDM). The level of plasma zonulin proportionally correlated to BMI, creatinine, FPS, baseline, and first-hour and two-hour glucose levels along with the OGTT, hemoglobin A1C, HOMA-IR, and alanine aminotransferase (ALT) levels [59]. It exponentially increased with a longer T2D history [60]. Zonulin levels positively correlated with WHR, BMI, fasting triglycerides, fasting insulin, uric acid, and IL-6 and negatively associated with HDL-C and insulin sensitivity via circulating IL-6 [61]. In "Okinawan-based diets" characterized by lower carbohydrate content and higher contents of fat, fiber, and protein, the levels of zonulin in serum and feces were found to be elevated after 12 weeks up to 28 weeks [24]. Presently, TyG-BMI reciprocally related to zonulin in preDM/T2D cases among the rest of other biomarkers and sIR indices. Importantly, TyG-BMI measurement was found to be superior to other obesity parameters for sIR assessing [62] followed by TyG-WC and then the TyG index [44]. Additionally, the TyG index showed positive significant correlations with A1C and IR in long-term-controlled diabetics patients. The TyG index, TyG-WC, and TyG-BMI were elevated in uncontrolled diabetic patients. Besides, the TyG index showed significant correlations with all cardiometabolic risk factors (HDL-C, non-HDL-C, TC, and IR) [63, 64].

We found out that L-carnitine, choline, γ-BB, and TMAO concentrations were obviously lower in preDM/T2D patients when compared to normoglycemic control. Recent studies support our findings that the L-carnitine plasma level was found to be lower in IR patients than in controls [65]. Furthermore, L-carnitine supplementation in addition to a low-calorie diet intake had beneficial influence on multiple diabetic risk parameters such as plasma cholesterol, lipids, and lipoprotein, in addition to decreasing TGs synthesizing and enhancing fatty acids mitochondrial oxidation in obese T2D women [66]. Moreover, in collectively randomized controlled trials (RCTs), the supplementation of L-carnitine resulted in considerable reduction in FPG, homeostatic model assessment for insulin resistance (HOMA-IR), and HbA1C concentrations [67]. Furthermore, L-carnitine administration had a beneficial impact on the HOMA-IR score [68] via oxidative stress reduction in T2D patients, increasing long-chain acyl-CoAs oxidation in mitochondria which was clearly thought of as the most important pathways by which L-carnitine could enhance blood glucose hemostasis [69, 70]. Thus, L-carnitine-based therapeutics can be tailored as the best-in class approach for managing metabolism health [71]. Choline is essentially extracted from dietary phosphatidylcholine and subsequently converted by intestinal microbiota to produce TMA, which in turn is absorbed and oxidized in the liver to form trimethylamine oxide (TMAO). Potential correlation between higher dietary phosphatidylcholine supplementation "precursor to the generation of choline and TMAO" and T2D growing risk was delineated. Therefore, choline deficiency in a diet decreased fat mass and improved glucose tolerance in obese and diabetic mice [72]. Conversely, excessive choline-rich food intake, particularly phosphatidylcholine, conclusively correlated with lower T2D prevalence among 2,332 men involved in that study [73]. In our study, we found that lower choline levels showed statistically significant negative correlation with glycosylated hemoglobin (HbA1c), without any obvious relation with any other demographic or clinical parameters in preDM/T2D cases.

Betaine acts as a methyl donor in the methionine cycle, with an improvement in blood glucose control. Inhibition of carnitine biosynthesis induces an increase in gamma-butyrobetaine contents and cardioprotection in isolated rat heart infarction [74]. Betaine concentrations were experimentally found to be reduced in IR-related MetS patients vs. a healthy population, which correlates appreciably with our results [74]. Interestingly, betaine supplementation to mice with diet-induced obesity resulted in preventing the development of impaired glucose control and

Table 3 Spearman's correlations of biochemistry parameters and markers and metabolism indices in cases ($N = 30$)

		TyG index	TyG-BMI	TyG-WC	MetS-IR
Metabolic risk biomarkers					
Zonulin (ng/mL)	ρ	−0.270	**−0.398***	−0.166	−0.357
	p-value	0.150	**0.029**	0.380	0.053
Carnitine (nmol/mL)	ρ	0.241	0.202	0.099	0.139
	p-value	0.199	0.284	0.602	0.464
Choline (μmol/L)	ρ	0.010	−0.051	0.155	−0.077
	p-value	0.960	0.790	0.413	0.685
γ-BB (μmol/L)	ρ	0.087	0.152	0.161	0.157
	p-value	0.647	0.424	0.396	0.408
TMAO (μM)	ρ	0.343	−0.171	−0.190	−0.111
	p-value	0.063	0.367	0.315	0.559
Clinical parameters					
SBP (mmHg)	ρ	−0.255	0.094	0.086	0.061
	p-value	0.173	0.623	0.650	0.749
DBP (mmHg)	ρ	−0.111	**0.578****	0.166	**0.478****
	p-value	0.561	**0.001**	0.380	**0.008**
FBG (mg/dL)	ρ	**0.539****	0.064	0.138	0.028
	p-value	**0.002**	0.738	0.466	0.882
A1C (%)	ρ	**0.385***	0.001	0.112	0.023
	p-value	**0.036**	0.997	0.555	0.904
TG (mg/dL)	ρ	**0.604****	−0.029	0.120	0.064
	p-value	**0.000**	0.878	0.526	0.736
LDL-C (mg/dL)	ρ	0.192	−0.097	0.094	−0.166
	p-value	0.310	0.610	0.622	0.379
HDL-C (mg/dL)	ρ	**−0.418***	0.011	−0.010	−0.301
	p-value	**0.021**	0.955	0.957	0.107
TC (mg/dL)	ρ	0.217	−0.157	0.111	−0.247
	p-value	0.248	0.408	0.559	0.188
Non-HDL-C (mg/dL)	ρ	**0.368***	−0.162	0.116	−0.142
	p-value	**0.045**	0.393	0.543	0.454
Adiposity indices					
WC (cm)	ρ	0.007	**0.477****	**0.939****	**0.416***
	p-value	0.972	**0.008**	**0.000**	**0.022**
HC (cm)	ρ	−0.162	**0.550****	**0.811****	**0.514****
	p-value	0.393	**0.002**	**0.000**	**0.004**
CI	ρ	0.043	−0.043	**0.794****	−0.096
	p-value	0.821	0.822	**0.000**	0.613
BMI (kg/m^2)	ρ	−0.178	**0.953****	**0.382***	**0.879****
	p-value	0.347	**0.000**	**0.037**	**0.000**
BAI	ρ	−0.266	**0.502****	**0.686****	**0.419***
	p-value	0.155	**0.005**	**0.000**	**0.021**
WHR	ρ	**0.469****	−0.135	**0.367***	−0.191
	p-value	**0.009**	0.478	**0.046**	0.313
WHtR	ρ	−0.096	**0.467****	**0.860****	**0.376***
	p-value	0.614	**0.009**	**0.000**	**0.041**
LAP	ρ	**0.487****	**0.376***	**0.694****	**0.406***
	p-value	**0.006**	**0.041**	**0.000**	**0.026**
VAI	ρ	**0.528****	0.001	0.201	0.164
	p-value	**0.003**	0.994	0.287	0.388

Table 3 (continued)

		TyG index	TyG-BMI	TyG-WC	MetS-IR
Atherogenicity indices					
Non-HDL-C/HDL-C ratio	ρ	**0.433***	−0.125	0.095	0.122
	p-value	**0.017**	0.511	0.619	0.520
TG/HDL-C ratio	ρ	**0.574****	−0.064	0.077	0.125
	p-value	**0.001**	0.735	0.685	0.509
TC/HDL-C ratio	ρ	**0.433***	−0.125	0.095	0.122
	p-value	**0.017**	0.511	0.619	0.520
LDL-C/HDL-C ratio	ρ	**0.366***	−0.103	0.097	0.103
	p-value	**0.047**	0.587	0.609	0.587
AIP	ρ	**0.642****	0.004	0.144	0.210
	p-value	**0.000**	0.982	0.449	0.265
Proinflammatory blood indices					
RDW	ρ	**−0.415***	0.336	−0.125	0.296
	p-value	**0.022**	0.069	0.512	0.113
MPV	ρ	0.068	0.106	0.190	0.070
	p-value	0.720	0.578	0.315	0.712
PLT count	ρ	−0.217	0.021	0.027	−0.033
	p-value	0.248	0.911	0.887	0.862
Monocytes %	ρ	0.221	−0.045	−0.047	−0.017
	p-value	0.240	0.813	0.804	0.928
Neutrophils %	ρ	0.203	0.279	−0.172	0.356
	p-value	0.281	0.135	0.364	0.054
Lymphocytes %	ρ	0.108	−0.290	0.246	−0.301
	p-value	0.570	0.120	0.189	0.106
MLR	ρ	0.224	0.015	−0.146	0.036
	p-value	0.235	0.935	0.440	0.848
NLR	ρ	0.106	0.216	−0.115	0.224
	p-value	0.578	0.252	0.545	0.234

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

$^{*}p < 0.050$, $^{**}p < 0.01$, $^{***}p < 0.001$

AIP, atherogenicity index of plasma; *BAI*, body adiposity index; *C-index*, conicity index; *DBP*, diastolic blood pressure; *FPG*, fasting plasma glucose; *A1C%*, percent glycosylated hemoglobin; *HC*, hip circumference; *HDL-C*, high density lipoprotein-cholesterol; *LAP*, lipid accumulation product; *LDL-C/HDL-C*, low density lipoprotein cholesterol-to-high density lipoprotein cholesterol ratio; *LDL-C*, low density lipoprotein-cholesterol; *MLR*, monocyte-to-lymphocyte ratio; *NLR*, neutrophil-to-lymphocyte ratio; *non-HDL-c/HDL*, non-high density lipoprotein-to-high density lipoprotein ratio; *non-HDL-C*, non-high density lipoprotein cholesterol; *PLT*, platelet; *RDW*, red cell width; *SBP*, systolic blood pressure; *TC/HDL-C*, total cholesterol-to-high density lipoprotein cholesterol ratio; *TC*, total cholesterol; *TG*, triglyceride; *TMAO*, trimethylamine-N-oxide; *WC*, waist circumference; *WHR*, waist-to-hip ratio; *WHtR*, waist-to-height ratio; and *γ-BB*, γ-butyrobetaine

decreasing hepatic lipid concentration but increasing energy utilizing, enhancing white adipose oxidative capacity, and robustly increasing hepatic fibroblast growth factor (FGF) 21 levels [74–77] with new understandings of how utilizing betaine can improve T2D-obesity management outcomes. In our study, betaine lacked statistically significant correlation with any other biomarkers or demographic and clinical parameters. Notably, increased levels of betaine are strongly associated with reduction in diabetes prevalence for up to 10 years while a reduction in betaine levels can be detrimentally linked to ineffective betaine intestinal absorption, impaired or defects in metabolism of betaine-related metabolites by gut microbiota, or osmotic dilution in IR patients [72].

TMAO is an independent gut microbiota–derived factor mostly determined by genetic makeup, configuration of

Table 4 Spearman's correlations of microbiome signature biomarkers and clinical, anthropometric, and diverse indices in cases ($N = 30$)

	Zonulin	Carnitine	Choline	γ-Butyrobetaine	TMAO
Age	ρ **−0.39**	ρ **−0.44**	-	ρ **0.45**	-
	p-value **0.033**	*p*-value **0.015**		*p*-value **0.014**	
FBG	-	-	-	-	-
A1C%	-	-	-	-	-
WC	-	-	-	ρ **−0.38**	-
				p-value **0.036**	
TyG-WC	-	-	-	ρ **−0.38**	-
				p-value **0.041**	
TC	-	ρ **−0.41**	-	-	-
		p-value **0.025**			
LDL-C/HDL-C ratio	-	-	-	ρ **−0.42**	-
				p-value **0.02**	
MPV	-	-	-	ρ **−0.38**	-
				p-value **0.042**	
Neutrophils	-	ρ **0.47**	-	-	-
		p-value **0.009**			
Lymphocytes	-	ρ **−0.49**	ρ **−0.36**	-	-
		p-value **0.006**	*p*-value **0.05**		
PLR	-	ρ **0.37**	ρ **0.42**	-	-
		p-value **0.044**	*p*-value **0.022**		
Choline	-	-	-	-	ρ **−0.502**
					p-value **0.005**

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

*$p < 0.050$, **$p < 0.01$, ***$p < 0.001$

AIP, atherogenicity index of plasma; *BAI*, body adiposity index; *C-index*, conicity index; *DBP*, diastolic blood pressure; *FPG*, fasting plasma glucose; *A1C%*, percent glycosylated hemoglobin; *HC*, hip circumference; *HDL-C*, high density lipoprotein-cholesterol; *LAP*, lipid accumulation product; *LDL-C/HDL-C*, low density lipoprotein cholesterol-to-high density lipoprotein cholesterol ratio; *LDL-C*, low density lipoprotein-cholesterol; *MLR*, monocyte-to-lymphocyte ratio; *NLR*, neutrophil-to-lymphocyte ratio; *non-HDL-c/HDL*, non-high density lipoprotein-to-high density lipoprotein ratio; *non-HDL-C*, non-high density lipoprotein cholesterol; *PLT*, platelet; *RDW*, red cell width; *SBP*, systolic blood pressure; *TC/HDL-C*, total cholesterol-to-high density lipoprotein cholesterol ratio; *TC*, total cholesterol; *TG*, triglyceride; *TMAO*, trimethylamine-N-oxide; *WC*, waist circumference; *WHR*, waist-to-hip ratio; *WHtR*, waist-to- height ratio; and *γ-BB*, γ-butyrobetaine

human intestinal microbes, and diet composition [68]. Evidently, it is produced by gut microbiota from a diet rich in betaine, L-carnitine, γ-BB, and choline oxidized via liver flavin monooxygenase 3 enzymes [11, 22, 78–84]. Unlike our results, a dose-dependent association between TMAO levels and increased diabetes risk was concluded as circulating plasma levels of TMAO in T2D patients were higher vs. subjects without diabetes [23].

Study limitations

- Small sample size of cases
- Restricted to regions and race which may cause bias
- Lack of data on the diet, lifestyle, and intestinal microbiota configuration of the subjects, in addition to genetic variation among individuals.

Conclusions

Our study examined, for the first time to our knowledge, the associations between zonulin, L-carnitine, choline, γ-BB, and TMAO in preDM/T2D Jordanian patients. Also, our study investigated the correlations between those metabolic biomarkers and clinical parameters, atherogenicity, and adiposity indices along with inflammatory hematological indices in preDM/T2D Jordanian patients. Identifying metabolic biomarkers of human IR before the emerging of a disease is crucial in the prediction/prevention and treatment modalities. However, further large scale and longevity animal and human studies are demanded.

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Declarations

Ethics approval This article does not contain any studies with animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

Ethics statement Written informed consent was obtained from all subjects. The experimental protocol was reviewed and approved by the Ethical Committee of Jordan University. Approval for the study was obtained from the Institutional Review Board affiliated with the Jordan University Hospital (JUH; 7/2019/IRB) and King Hussein Medical City (KHMC; 271/2019/2) and was conducted according to the principles expressed in the Declaration of Helsinki (World Medical Association, 2008).

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