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Original Research

The relationship of selected biomarkers of gut microbiota dysbiosis with adiposity and metabolic risk factors in nascent metabolic syndrome patients

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Abstract

Background: This study aimed to compare and correlate between non-diabetic MetS, newly diagnosed drug naive pre-diabetic MetS patients vs. lean, apparently healthy and normoglycemic controls the plasma levels of cardiometabolic risk biomarkers' of pharmacotherapy (appraised using colorimetric and chromatography assays of gut dysbiosis carnitine, choline, γbutyrobetaine, TMAO, Zonulin, survivin, Leukocyte cell-derived chemotaxin 2 (LECT2) and antioxidative stressors (catalase, superoxide dismutase (SOD) and Trolox total antioxidative capacity), adiposity, and atherogenicity with non-insulin based surrogate insulin resistance (sIR) indices. **Methods**: ANOVA comparisons and Spearman's rank correlations were conducted in this cross-sectional study of 30 normoglycemic lean subjects (control), 30 nonprediabetic MetS subjects and 30 MetS/pre-diabetic (PreDM) enrolled. **Results**: MetS-PreDM group presented significantly higher values of FPG (P^2<0.001,P^3 =0.009) and A1C (P values <0.001) than both normoglycemic MetS and control groups. However, MetS-PreDM and normoglycemic MetS recruits had appreciably higher values of DBP, SBP, TG, and non-HDL-C but significantly lower values of HDL-C (P values <0.001) than the controls. Explicitly no significance in variance was noticeable among any of the study arms (P value < 0.05) for any of the hematological indices. Nevertheless, Both MetS groups (nonprediabetic and PreDM) had substantially higher values for each of adiposity, atherogenecity and surrogate insulin resistance (non insulin based) indices (P^2<0.001) vs. controls' respectively. Both Survivin and LECT2 levels were significantly higher in PreDM MetS group (P value < 0.05 vs. nondiabetic MetS participants). Conversely all 5 gut dysbiosis biomarkers (carnitinine, choline, γBB, TMAo and Zonulin) which proved significantly lower vs. those of either controls (nondiabetic lean or MetS). Surprisingly, a significant variation in all tested 7 biomarkers' plasma levels were found between nondiabetic MetS and PreDM-MetS groups (P^3 < 0.05). Interestingly all 3 antioxidative stressors were on the decline as anticipated; where catalase, SOD % inhibitions and trolox total antioxidative capacities were significantly lower in both MetS recruits vs. controls. Importantly the discrepancy between normoglycemic nonprediabetic MetS vs. the MetS-PreDM (P^3 < 0.05) may have not ranked up to significance in indices, clinical parameters or biomarkers. Notably in pooled MetS (both normoglycemic and pre-diabetics participants (N =60)). Most exquisitely survivin with dysbiosis choline and γBB correlated positively and pronouncedly with carnitine in pooled MetS participants. Also in a striking similarity, cardiometabolic LECT2 has a marked direct relation with each of dysbiosis carnitine and γBB. TMAO, nevertheless, related inversely and significantly with all 3 dysbiosis biomarkers, likewise Zonulin associated disproportionally with both choline and γBB. Exceptionally TMAO- TYG and Zonulin-TYG-WHpR paired in substantial and inverse relations in pooled normoglycemic and preDM MetS participants (n=60). To superbly signify the anticipated deterioration in metabolism via gut microbiota-insulin insensitivity interconnectivity; all dysbiosis biomarkers (carnitine, choline, γBB, TMAO, Zonulin and survivin) correlated highly remarkably and proportionally with all non insulin based surrogate insulin resistance indices in 60 MetS recruits (both normoglycemic and prediabetic; equally). Unequivocally γBB associated directly and pronouncedly with almost all adiposity indices. Surprisingly VAI correlated negatively with Zonulin in the same MetS population. FBG associated exceptionally with carnitine and γButyrobetaine (γBB). Substantially A1c correlated proportionally (P values <0.05) with MetS pooled cases dysbiosis' carnitine, choline, γBB, and cardiometabolic surviving. Outstandingly both SBP and DBP had direct and marked linkage to LECT2 and so did DBP with choline's plasma levels. Remarkably TMAO related negatively and pronouncedly with MetS cases levels of FBG, A1c, TG, LDL-C, and so did also zonulin with both A1c and LDL-C. **Conclusions:** Given the intergroup discrepancies in dysbiosis and cardiometabolic biomarkers along with their elective correlations with MetS-related indices and clinical parameters; our study cannot rule out any potentiality in molecular crosstalk and interplay of those biomarekers with the pathophysiology of MetS and preDM with their related dysregularities. Carnitine, choline, γbutyrobetaine, TMAO, Zonulin, survivin, and LECT2 can be putatively surrogate biomarkers to use as prognostic/predictive tools for the diagnosis/prevention and potential targets for MetS treatment.

Keywords:

1. Microbiome Dysbiosis Signature Determinants: Carnitine; Choline; γButyrobetaine; TMAO; Zonulin; Survivin; LECT2
2. Antioxidative Stressors: CATALASE; SOD %Inhibition; Trolox Equivalent Total Antioxidant Capacity;
3. Adiposity, Surrogate (Non Insulin Based) Insulin Resistance and Atherogenecity Indices
4. Cardiometabolic risk factors of nascent metabolic syndrome and prediabetes

Abbreviations: Adiposity indices (BMI, WHR (waist/Hip ratio), WtHR (waist/Height ratio), Conicity-index, BAI (Body adiposity index), LAP (Lipid accumulation Product), VAI (Visceral adiposity Index)) and atherogenicity indices (AIP (atherogenecity index of plasma); WAT (White adipose tissue), T2D (type 2 diabetes mellitus), TMAO (trimethylamine N-oxide), LECT2 (Leukocyte cell-derived chemotaxin-2), SOD (Superoxide dismutase), SCFAs (short chain fatty acids), PPIs (proton pumps inhibitors), LPS/TLR4 (lipopolysaccharide/toll like receptors4), HOMA-IR (homeostatic model assessment for insulin resistance), sIR (surrogate insulin resistance.

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INTRODUCTION

Microbiome And Prediabetes/Metabolic syndrome (MetS): Any Significant Linkage!

Diabetes and associated comorbidities as cumulatively incremental global health threats are on the rise.[1] Reportedly of the gut microbiome features in patients with prediabetes (PreDM) and type 2 diabetes (T2D); low gut microbial diversity was generally observed in preDM and newDM when compared to nonDM; *Proteobacteria* were significantly higher in the PreDM vs. nonDM controls. *Prevotella* and *Alloprevotella* relative abundance was significantly higher in the T2DM, and *Paraprevotella* relative abundance of in T2DM and PreDM groups was lower vs. nonDM controls. *Bacteroides* relative abundance of in the T2DM was significantly lower vs. both PreDM and nonDM controls.[2] Explicitly In a healthy population; diet can rapidly and differentially affects the gut microbiota and host lipid mediators.[3] Dietary interventions (as in berberine) and metformin exerted additive modulation of gut microbiota in high-fat diet-induced obesity in rats.[1,4] Moreover Principally putatively short-chain fatty acids (SCFA)-producing bacteria, including *Allobaculum, Bacteriodes, Blautia, Butyricoccus,* and *Phascolarctobacterium,* were markedly enriched by both treatments meanwhile microbial diversity was subject to their therapeutic reductions of dysbiosis. Interestingly berberine was proven for slowing progression of prediabetes to diabetes in Zucker diabetic fatty rats by improving intestinal permeability, and the structure of the gut microbiota along with enhancing intestinal secretion of Glucagon-Like Peptide-2.[5] Evidently Metformin exerts anti-obesity effect via oral/gut microbiome modulation in prediabetics.[1,6] Additionally probiotics as an adjunctive to metformin treatment could reduce HbA1c, insulin resistance, and zonulin but, meanwhile, could generate of enrichment of microbial butyrate producing pathways in the collective enhancement of metabolism of glucose.[7] Substantially microbiota imbalance induced by dietary sugar could disrupt immune-mediated protection from MetS.[8] Obviously Fat, but not sugar, was taken for a signature determinant for gut microbiota changes, obesity, and related metabolic disorders in mice.[9] Interestingly probiotics, in mitigation of PPI-induced dysbiosis with leaky gut, and metabolically unhealthy obesity along with systemic low-grade inflammation, and reduced amounts of SCFAs in obese subjects, could alleviate hallmarks of MetS (hypertension, dyslipidemia, hyperglycemia, abdominal obesity).[10]

Importantly adverse intestinal microbiome dysbiosis is related to higher insulin resistance, longer diabetes duration, in medicated diabetes patients.[11] and prevalence of dysglycemia (prediabetes plus diabetes.[12] In effect alterations in gut microbiota and microbial metabolism can be intricately implicated in cardiometabolic disease- early risk prediction.[13] Moreover potential modulation of both via novel therapeutic/preventive strategies can impact cardiometabolic/ cardiovascular disease phenotypes.[14] Pharmacomicrobiomics explores essentially interactions and cross talks of gut microbiome-drug response variability or drug toxicity. As the second genome; manipulating composition of gut microbiome can improve drug efficacy and safety. In personalized antidiabetes treatments, modulating microbiome response to antidiabetic drugs can prove innovative in augmenting drug efficacy or reducing drug toxicity and predicting response to treatment.[15] foreseeing perspectives of microbial-based therapeutics, aiming to provide novel preventative strategies and personalized therapeutic targets in T2D.[16] In the frame of this reference; pharmacomicrobiomics of the adverse effect for Hydrochlorothiazide were delineated. It was a proof of concept of metabolic disorders initiated by its pharmacology via a significant increase in gut microbiota Gram-negative *Enterobacteriaceae*, with elevations of LPS levels thereby activating LPS/TLR4 pathway, promoting inflammation and macrophage polarization.[17] Furthermore blueberries were found to abrogate target pathophysiologies of gut microbiota dysbiosis and hepatic dysmetabolism that proceed before pharmacological interventions of prediabetes development and progression ensue.[18] Strikingly gut microbiota demonstrated regulatory impact on pancreatic growth, exocrine function, and gut hormones.[19] Phlorizin, phytonutrients in apples, could alleviate obesity-associated endotoxemia and insulin resistance in high-fat diet-fed mice by targeting the gut microbiota and intestinal barrier integrity.[20] Most recently reports signified the distinctive dysbiosis signature taxa in prediabetes and diabetes during the disease development and progression.[21]

Carnitine

Remarkably carnitine, among the rest, proved to the biomarker or the risk factor for gastric cancer in males in a study aimed to evaluate the association between gastric cancer (GC) and higher concentrations of the metabolites L-carnitine, γ-butyrobetaine (GBB) and gut microbiota-mediated trimethylamine N-oxide (TMAO) in the circulation.[22] Recent studies found lower L-carnitine plasma level in IR patients than in controls.[23] Furthermore, L-carnitine supplementation in obese T2D women with 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. In effect it proved collectively to contribute to synthesizing and enhancing mitochondrial oxidation of fatty acids.[24] Also, further considerably appreciable reductions in

HbA1C concentrations, FPG, as well as HOMA-IR score in similar interventional RTCs were obtained.[25] Such beneficial impacts were gained via oxidative stress reductions in T2D patients, increasing mitochondrial long-chain acyl-CoAs oxidation and enhancing blood glucose hemostasis.[26] In effect, L-carnitine based therapeutics can be tailored as the best-in class approach for managing metabolism health.[27]

Choline

Choline is essentially extracted from dietary phosphatidylcholine and subsequently converted by intestinal microbiota to produce trimethylamine (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.[28] Conversely, excessive choline-rich food intake, particularly phosphatidylcholine, conclusively correlated with lower T2D prevalence among 2,332 men involved in that study.[29] 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.

γButyrobetaine (γBB)

γBB acts as a methyl donor in the methionine cycle. Inhibition of carnitine biosynthesis induced an increase in γBB contents and cardioprotection in isolated rat heart infarction.[30] γBB concentrations were experimentally found to be reduced in IR-related MetS patients vs. a healthy population.[30] As it had a definitive role in blood glucose control improvement, substantially increased levels of γBB associated with reduction in diabetes prevalence for up to 10 years. Conversely a reduction in betaine levels detrimentally linked to ineffective γBB intestinal absorption, impaired or defects in metabolism of γBB -related metabolites by gut microbiota, or osmotic dilution in IR patients.[28] Interestingly, γBB supplementation to mice with diet-induced obesity resulted in preventing the development of impaired glucose control. Further decrease in hepatic lipid concentration but an increase in energy utilizing, enhancement in white adipose oxidative capacity, with a robust increase in hepatic fibroblast growth factor (FGF) 21 levels were remarkable findings.[30, 31] Taken together new understanding of how utilizing γBB can improve T2D-obesity management outcomes were required

LECT2

Leukocyte cell-derived chemotaxin 2 (LECT2) is a circulating hepatokine with higher levels detected in newly diagnosed diabetics vs. controls.[32] Importantly being associated with metabolic disorders, it was enrolled as a risk factor of arteriosclerosis.[33] LECT2, as an energy-sensing hepatokine, is a link between obesity and skeletal muscle IR. Lan et al.[34] found that circulating LECT2 positively correlated with the severity of both obesity and IR, BMI, WC, WHR, and W/Ht ratio.[35] Remarkably patients with acute myocardial infarction with elevated levels of LECT-2 had a higher risk of major adverse cardiovascular events vs. those with lower LECT2 levels.[33] Unequivocally it was linked to promoting inflammation and insulin resistance in adipocytes.[36] LECT2 was reported as a potential biomarker linking visceral obesity to dyslipidemia.[37] In a study by Zhang et al.[32] the results showed that LECT2 negatively associated with HDL-C levels in patients with T2DM and obese subjects without T2DM.

Survivin

Earlier Survivin was assigned as a target for myocardial regeneration. It was reported as a highly profoundly impactful on both cardiomyocyte replication and apoptosis.[38] Survivin was also a requirement for beta-cell mass expansion with a preference for proliferation of preexisting beta cells and increases in obesity to protect adipocyte stem cells from apoptosis.[39] Survivin also attenuates DNA damage and inhibits TNFα-induced lipolysis.[40] Survivin is a protein that inhibits apoptosis via blocking caspase activation and promotes cellular proliferation. Its over-expression is strongly linked to autoimmune disease, hyperplasia, and tumors. Survivin is highly expressed in most human cancers, such as lung, pancreatic and breast cancers, relative to normal tissues. Henceforth it was taken for as a biological biomarker for diagnosis and therapy of cancer.[41] Also Survivin is expressed in the intestinal epithelial stem and progenitor cells. Therefore survivin-deficient cells demonstrate cell-cycle defects and signs of mitotic catastrophe.[42]

TMAO

Evidently, TMAO, being a gut microbiota–derived factor,[25b] is generated from a diet rich in betaine, L-carnitine, γ-BB, and choline.[43] Besides, lifestyle interventions[44] can reduce its precursors' levels as well as TMAO renal clearance. Its microbiota-related intermediate of γ-butyrobetaine (γBB) correlated with visceral adiposity. TMAO was found to mediate the crosstalk between the gut microbiota and hepatic vascular niche to alleviate liver fibrosis in nonalcoholic steatohepatitis.[45] Obviously high animal protein/L-carnitine diets may result in TMAO generation by gut microbiome thereby contributing to atherosclerosis via increased foam cell formation, decreased reverse cholesterol transport and pro-thrombotic actions.[46] Remarkably a dose-dependent cross correlation between TMAO levels and increased diabetes risk was concluded as circulating plasma levels of TMAO in T2D patients were higher vs. subjects without diabetes.[47] More effectively microbiome inhibition of IRAK-4 by trimethylamine mediates metabolic and immune benefits in high-fat-diet-induced IR.[48]

Zonulin

It is a protein that is synthesized in intestinal and liver cells and regulates intestinal permeability. Also zonulin modulates the permeability of GI tight junctions.[49] Among the 5 gut dysbiosis signature determinants is Zonulin. Its levels negatively associated with HDL-C and insulin sensitivity via circulating IL-6 but positively correlated with WHR, BMI, TG, fasting insulin, and Uric Acid.[50] High Zonulin concentrations were inadvertently associated with adverse pregnancy and perinatal outcomes.[51]

Zonulin level was enrolled as new metabolic biomarker in diabetes mellitus patients and associated complications.[52] Most exquisitely zonulin has emerged as a potential therapeutic target in microbiota-gut-brain axis disorders.[53]

Aims and objectives of the study

The objective of the present study is to compare the selected plasma nascent MetS- related cardiometabolic biomarkers (survivin and LECT2 (Leukocyte cell-derived chemotaxin-2)), gut microbiome dysbiosis plasma biomarkers (zonulin, carnitine, choline, γ--ButyroBetaine, and TMAO), and oxidative stress related markers (catalase, superoxide dismutase (SOD) and trolox total antioxidative capacity); collectively further abbreviated as "cardimetabolic biomarkers", in Jordanian **nascent** metabolic syndrome (**nascent** MetS) patients with and without prediabetes (preDM) or T2D vs. those of non-MetS and normoglycemic controls. Further relationships are explored –for the first time-between the seven microbiome signature determinants and adiposity, atherogenicity, proinflammatory hematological indices, and (non insulin based) surrogate insulin resistance (sIR).

SUBJECTS, MATERIALS AND METHODS

Ethics Statement

The study was conducted according to the principles expressed in the Declaration of Helsinki (World Medical Association, 2008).[54]Approval for the study was obtained from the Institutional Review Board affiliated with the Jordan University Hospital (JUH; 7/2019). All ethical principles for research related to human race were considered. The objectives of the study were explained to all eligible participants in their native language and a written consent was obtained from all those who agreed to participate in the study. The candidates were clearly informed that the participation in the study is voluntary and their approval or denial to be part of the study will not cause him/her any type of rewards or penalty. Also, they were informed that their participation in the study does not mean they have to pay any expense related to it. Participants' privacy, safety and confidentiality were ensured throughout the conduct of the study

Study Design

This cross sectional study was conducted to compare and correlate plasma cardiometabolic biomarkers in three groups of the Jordanian population. The groups are classified as follows (Recruitment Flow chart with the details of the participants' recruitment process shown in Figure 1):

1. Normoglycemic lean controls: 30 apparently healthy participants (A1C < 5.7 % and FPG < 100 mg/dL). These participants were mainly considered for comparison purposes.

2. Normoglycemic patients with **nascent MetS (**Table 1a [55]**)**: 30 participants who met the definition of MetS but had no preDM or T2DM (i.e. normoglycemic individuals) (A1C < 5.7 % and FPG < 100 mg/dL).

3. PreDM/T2D patients with **nascent MetS (**Table 1a [55]**)**: 30 participants who met the definition of MetS and who were either pre/DM or T2D patients and were necessarily defined as drug naive subjects (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

All participants who attended the JUH/Family Medicine outpatient clinics were screened for potential recruitment, which took place over the period from April 2019 to March

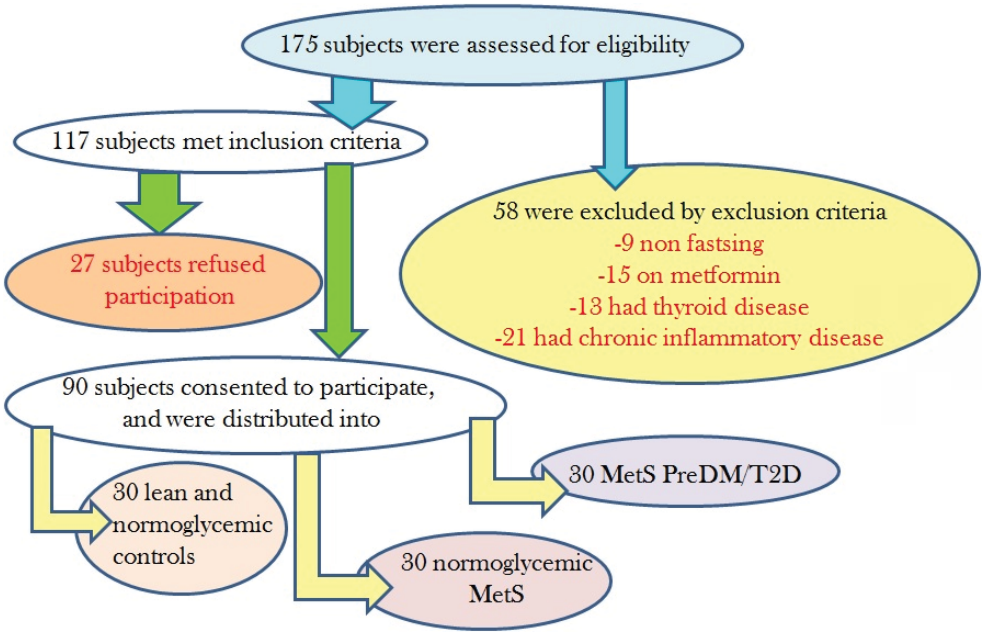


Figure 1. Recruitment Process Flow Chart

2020. Adult patients of both genders 18-76 years -old were included in the study. Subjects were defined as preDM/T2D according to the American Diabetes Association ADA criteria. Control participants were normoglycemic. Demographic data as well as anthropometric measurements and lab tests were obtained from each participant. Consequently, adiposity, atherogenicity, Non insulin based- surrogate insulin resistance (sIR), and inflammatory and hematological indices were calculated as in Table 1b.[56]

Table 1a. IDF Metabolic Syndrome (MetS) World-Wide Definition[55]	
For Eastern Mediterranean and Middle East population, the measure of central obesity include waist circumference of ≥ 94 cm for males and ≥ 80 cm in females. If body mass index is > 30 kg/m^2 then central obesity can be assumed, and waist circumference does not need to be measured.	
Raised triglycerides	≥ 1.7 mmol/l (150 mg/dL)or specific treatment for this lipid abnormality
Reduced HDL-cholesterol	< 1.03 mmol/l (40 mg/dL) in males < 1.29 mmol/l (50 mg/dL) in females or specific treatment for this lipid abnormality
Raised blood pressure	Systolic: ≥ 130 mmHg Or Diastolic: ≥ 85 mmHg or treatment of previously diagnosed hypertension
Raised fasting plasma glucose	Fasting plasma glucose ≥ 5.6 mmol/l (100 mg/dL) or previously diagnosed Type 2 diabetes If > 5.6 mmol/l or 100 mg/dL, oral glucose tolerance test is strongly recommended but is not necessary to define presence of the syndrome

Table 1b. Indices of Anthropometric Adiposity, Atherogenicity, Inflammatory Hematological Indices and Surrogate non insulin based insulin Resistance (sIR) Used in this Study, and Equations for Calculation [56]	
Measure	**Equations**
Surrogate non insulin IR (sIR) indices [56a,b]	
Triglyceride Glucose (TyG) index	TyG = Ln [TG (mg/dL) * fasting glucose (mg/dL)/2]
TyG-BMI	TyG-BMI = TyG index * BMI
TyG-Waist Circumference	TyG-WC = TyG index * WC (m)
MetS-IR	MetS–IR= ln[2 * Glucose (mg/dL) + Triglycerides (mg/dL)] * BMI (kg/m^2)/ln[HDL-C (mg/dL)]
Adiposity indices [56c]	
Waist-to-hip ratio (WHR)	WHR = waist circumference (cm) ÷ hip circumference (cm)
Waist-to-height ratio (WHtR)	WHtR = waist circumference (cm) ÷ height (cm)
Conicity index (CI)	CI = WC (m) ÷ 0.109 √weight (kg) ÷ height (m)
Body adiposity index (BAI)	BAI = [HC (cm) / (height (m)$^{1.5}$)] - 18
Lipid accumulation product (LAP)	LAP = (WC [cm] − 65) × (TG [mM]) for men LAP = (WC[cm] − 58) × (TG [mM]) for women
Visceral adiposity index (VAI)	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 [56d]	
Atherogenic index of plasma (AIP)	AIP = Log_{10} (triglyceride concentration/HDL-C)
Total cholesterol/ HDL-C (TC/HDL-C) ratio	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	Non-HDL.C = total cholesterol - HDL.C
Blood indices [56e]	
Platelet-to-lymphocyte ratio (PLR)	PLR = Platelets ÷ Lymphocytes
Neutrophil-to-lymphocyte ratio (NLR)	NLR = Neutrophils ÷ Lymphocytes
Monocyte-to-lymphocyte ratio (MLR)	MLR = Monocytes ÷ Lymphocytes

Exclusion criteria

- Non-fasting individuals, Pregnant or breast feeding/ lactating women
- Patients with acute complications of diabetes, acute renal or hepatic dysfunction
- Patients who received any of the following medications: such as oral antibiotics, proton-pump inhibitors, metformin, cardiovascular medications, laxatives, systemic corticosteroids
- Patients with chronic inflammatory or autoimmune disease, cancer, infectious diseases, tuberculosis, or neuromuscular diseases

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 x 1.2 inch, multi sample 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 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 its plasma levels and implemented according to the manufacturer's instructions (MyBioSourse, San Diego, CA, USA). Catalase, SOD (superoxide dismutase) and TROLOX total

antioxidative capacity, survivin and LECT2 were procured from Abcam (Cambridge, MA, USA). Markers' plasma levels were assayed according to manufacturers' instructions (intra- and inter-assay precisions of <10-<12%). UV-VIS spectrophotometer used was Spectro Scan 80D UV-VIS spectrophotometer (Sedico Ltd., Nicosia, Cyprus). Harvested plasma (from lithium heparin collection tubes centrifuged at 4000 rpm for 10 minutes) were immediately stocked at -80°C until analysis.

Carnitine, Choline, TMAO, and γ-BB LC/MS/MS Determinations

Reagents: **Choline chloride, TMAO, and GBB** were obtained from Sigma-Aldrich Chemicals (St. Louis, Missouri, United States) 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 X 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 for 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 conditions 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 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 was 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 belong to. Data were entered and tested through IBM SPSS© Statistics version 23 (SPSS, Inc., USA). The biomarker levels, indices as well as clinical parameters were presented as mean (±SD) and compared between the three studied groups. Categorical data were presented as frequencies (%). Gender variation between the groups was analyzed utilizing the Chi-square test. One Way Analysis of variance (ANOVA) test was used for the contrasting of continuous dependent variables across the study arms. Pairwise posthoc comparisons were done through Bonfferoni. To assess the strength and direction of association between continuous variables in MetS groups which contain both normoglycemic and Prediabetic MetS subjects, we used Spearman rank correlation, wherein continuous variables were not normality distributed as assessed by Shapiro Wilk test. *P*-value <0.05 is considered statistically significant.

RESULTS

Group Comparisons and Correlations of Biochemistry Parameters, Markers, and Metabolism Indices (Table 2)

Table 2 explains the clinical characterizes of each study arm. Nascent MetS-PreDM group presented significantly higher values of FPG (P^2 value <0.001,P^3 value =0.009) and A1C ($P^{2and\ 3}$ values <0.001) than both normoglycemic MetS and control group. However, MetS-PreDM and normoglycemic MetS groups had significantly higher values of DBP (P^1 value =0.002, P^2 value<0.001), SBP ($P^{1\ and\ 2}$ values<0.001), TG ($P^{1and\ 2}$ values <0.001), and non-HDL-C (P^1 value =0.006, P^2 value =0.001) than the control group and significantly lower values of HDL-C($P^{1and\ 2}$ values <0.001) than the control group. MetS-PreDM group shows significant higher values of DBP (P^3 value =0.019) and TG (P^3 value =0.008) than the normoglycemic MetS, while no significant difference (P^3< value 0.05) was seen between the MetS groups for the following variables (SBP, non-HDL-C, LDL-C, and HDL-C). MetS-PreDM had significantly higher values of LDL-C (P^2 value=0.028) than the control group, while no difference was seen between normoglycemic MetS and control groups (P^1 value =0.493). Interestingly, TC failed to demonstrate significant difference between all of the study arms ($P^{1,2,and\ 3}$ values < 0.05).

Table 2. Comparison of clinical and anthropometric parameters, adiposity, atherogenecity, surrogate insulin resistance and hematological indices as well as metabolic risk biomarkers in the three study arms

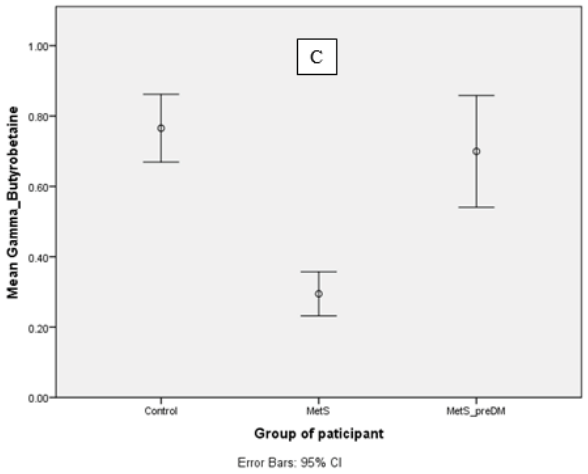
Parameters	Total Sample Proportions (N=90)	Control Group Proportions# (N=30)	MetS/normoglycemic Group Proportions# (N=30)	MetS/preDM Group Proportions# (N=30)		#P-value
Gender						
Female, N (%)	46(51.1%)	16(53.3%)	19(63.3%)	11(36.7%)		0.113
Male, N (%)	44(48.9%)	14(46.7%)	11(36.7%)	19(63.3%)		
Total	90 (100%)	30 (100%)	30 (100%)	30 (100%)		
	Total Sample Mean±SD (N=90)	**Control Group Mean ± SD# (N=30)**	**MetS/normoglycemic Group Mean ±SD# (N=30)**	**MetS/preDM Group Mean ± SD# (N=30)**		***P-value**
Age (years)	48.75±12.87	37.83±11.12	54.13±10.72	53.73±9.55		**<0.001**
Clinical characteristics						
	Controls (N=30) Mean±SD#	**MetS group (N=30) Mean±SD#**	**MetS/preDM, group (N=30) Mean±SD#**	**P^1-value**	**P^2-value**	**P^3-value**
SBP (mmHg)	111.23±9.05	132.20±11.61	138.17±11.55	**<0.001**	**<0.001**	0.106
DBP (mmHg)	73.77±6.82	81.43±9.22	87.50±8.93	**0.002**	**<0.001**	**0.019**
FPG (mg/dL)	88.18±8.68	101.07±15.95	124.31±47.38	0.275	**<0.001**	**0.009**
A1c%	5.13±0.3	5.43±0.243	6.65±1.32	0.471	**<0.001**	**<0.001**
TG (mg/dL)	76.33±23.61	168.74±47.14	221.84±102.69	**<0.001**	**<0.001**	**0.008**
LDL-C (mg/dL)	117.1±36.57	129.7±36.57	141.03±43.12	0.493	**0.028**	0.632
HDL-C (mg/dL)	58.4±10.21	45.87±8.68	45.40±13.03	**<0.001**	**<0.001**	1
TC (mg/dL)	185.2±24.82	204.22±45.81	208.43±45.74	0.207	0.081	1
Non-HDL-C (mg/dL)	126.8±26.62	158.35±43.134	163.03±43.13	**0.006**	**0.001**	1
Adiposity indices						
WC (cm)	77.37±7.74	111.03±10.5	115.2±10.26	**<0.001**	**<0.001**	0.287
HC (cm)	90.43±10.7	116.4±8.36	116.63±12.57	**<0.001**	**<0.001**	0.996
BMI (kg/m^2)	22.91±1.86	32.74±4.33	32.87±3.82	**<0.001**	**<0.001**	1
WHR	0.86±.11	0.95±.06	0.99±.14	**0.007**	**<0.001**	0.396
WHtR	0.45±.03	0.68±.06	0.69±.06	**<0.001**	**<0.001**	1
C-index	44.38±.6.43	74.53±.11.46	78.44±.10.68	**<0.001**	**<0.001**	**0.375**
BAI	23.23±9.61	38.23±5.97	36.34±8.4	**<0.001**	**<0.001**	0.886
LAP	14.6±8.36	93.68±20.98	132.63±66.82	**<0.001**	**<0.001**	**0.001**
VAI	19.29±22.16	49.90±67.24	131.34±137.43	0.563	**<0.001**	**0.002**
Atherogenicity indices						
AIP (mM)	-0.26±0.182	0.20±0.147	0.30±0.26	**<0.001**	**<0.001**	0.156
TC/HDL-C ratio	3.26±0.71	4.51±0.94	4.82±1.29	**<0.001**	**<0.001**	0.752

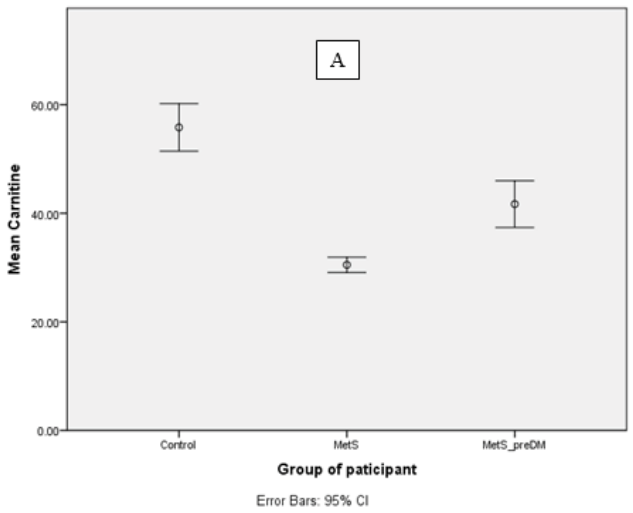
LDL-C/HDL-C ratio	2.06±.53	2.86±0.73	3.28±1.15	**<0.001**	**0.01**	0.188
Non-HDL-C/HDL-C ratio	2.26±0.71	3.51±0.94	3.82±1.29	**<0.001**	**<0.001**	0.752
TG/HDL-C ratio	1.35±0.51	3.82±1.36	5.39±3.19	**<0.001**	**<0.001**	**0.011**
Surrogate insulin resistance (sIR) indices						
MetS-IR	31.35±3.56	50.87±6.81	53.28±7.08	**<0.001**	**<0.001**	0.380
TyG Index	8.06±.39	9.0±.24	9.38±.62	**<0.001**	**<0.001**	**0.005**
TyG-BMI	185.14±21.01	294.66±37.93	308.38±39.81	**<0.001**	**<0.001**	0.365
TyG-WC	625.31±82.47	999.07±87.59	1081.63±127.91	**<0.001**	**<0.001**	**0.007**
TYG-WHpR	6.99±1.12	8.59±0.53	9.35±1.36	**<0.001**	**<0.001**	**0.019**
TYG-WHtR	3.68±0.41	6.14±0.53	6.48±0.73	**<0.001**	**<0.001**	0.067
TYG/HDL-C	0.14±0.05	0.21±0.05	0.22±0.07	**<0.001**	**<0.001**	1
Putatively surrogate Cardiometabolic risk identification biomarkers						
Carnitine (nmol/mL)	55.81±11.72	30.50±3.78	41.69±11.51	**<0.001**	**<0.001**	**<0.001**
Choline (µmo/L)	12.97±3.08	5.67±1.79	8.56±2.17	**<0.001**	**<0.001**	**<0.001**
γButyrobetaine (µmo/L)	0.77±0.26	0.29±0.17	0.70±0.43	**<0.001**	1	**<0.001**
TMAO (µM)	3.85±1.41	7.03±1.40	3.57±1.76	**<0.001**	1	**<0.001**
Zonulin (ng/mL)	8.01±2.02	8.21±3.65	5.23±1.45	1	**<0.001**	**<0.001**
Survivin (pg/mL)	3318.62±1476.4	3768.97±1626.65	5482±2779.79	1	**<0.001**	**0.006**
LECT2 (ng/mL)	24.23±6.39	25.21±4.64	29.03±5.16	1	**0.003**	**0.026**
CATALASE activity* **10^{-3}**of H2O2 µmol/min	7.70±4.53	5.52±3.99	2.76±3.10	**0.035**	**<0.001**	0.127
SOD %Inhibition	39.96±21.21	45.16±14.49	55.32±12.15	0.065	**0.002**	0.723
Trolox equivalent total antioxidant capacity (mM)	0.99±0.14	0.83±0.19	0.76±0.21	0.372	**<0.001**	**0.009**
Hematological indices						
RDW-CV % (%)	14.45±1.07	14.78±1.54	14.67±0.9	0.874	1	1
PLT count (× 10^9/L)	263.27±63.63	276.67±65.31	271.0±69.35	1	1	1
Monocytes %	5.81±1.93	5.51±1.52	5.12±0.97	1	0.225	0.992
Neutrophiles %	60.18±8.73	58.65±8.83	57.05±9.85	1	0.568	1
Lymphocytes %	31.07±7.79	31.68±7.42	34.23±10.08	1	0.461	0.748
MLR	0.21±0.13	0.18±0.06	0.16±.05	0.673	0.092	0.997
NLR	2.15±0.96	2.05±0.99	1.89±0.86	1	0.853	1
PLR	9.03±3.28	9.27±3.57	8.46±3.07	1	1	1

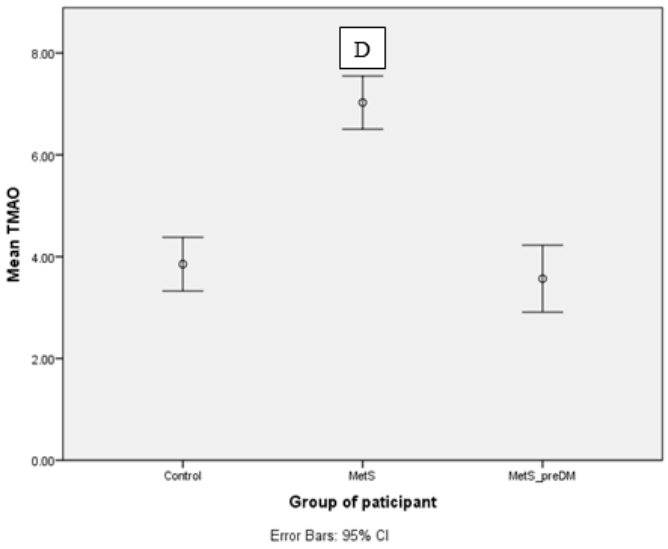
*P-value obtained by ANOVA test
For gender we obtained #P-value by Chi-Square test.
Pair wise comparisons were done through Bonfferoni adjustment.
P-value <0.05 was highlighted bold.
P^1 MetS/normoglycemic group vs. controls,
P^2 MetS/preDM group vs. controls,
P^3 MetS/preDM group vs. MetS group.
Comparisons of mean and P-value obtained by ANOVA test.
CATALASE activity *10^{-3} µmol of H2O2 per min at pH 4.5 at 25 °C.

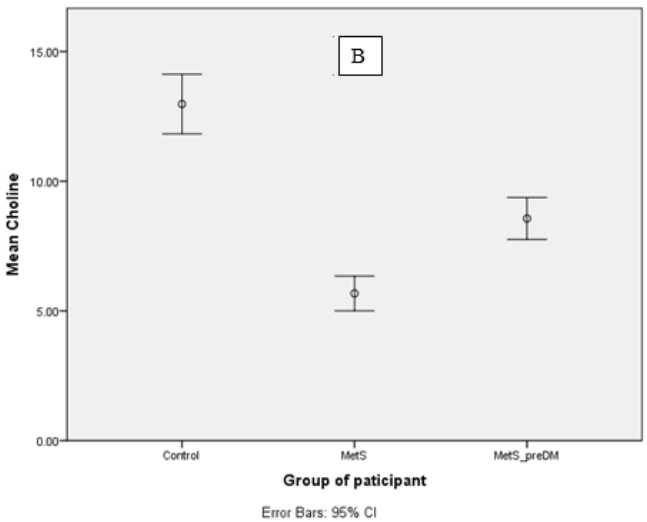
It is worth mentioning that no significance in variance was noticeable among any of the study arms ($P^{1, 2 \text{ and } 3}$ values < 0.05) for any of the hematological indices. Nevertheless, Both MetS groups (normoglycemic and PreDM) had a significantly higher value of WC, HC, BMI, WHtR, BAI, LAP, VAI ($P^{1 \text{and } 2}$ values <0.001) and WHR (P^1 value =0.007, P^2 value <0.001) than the control group. Meanwhile, the significance of variation in CI was lacked for both MetS groups ($P^{1 \text{ and } 2}$values <0.05) in comparison to control group. Notably, all adiposity indices could not exhibit the significance of discrepancy between normoglycemic MetS and the MetS-PreDM (P^3 value< 0.05) except for LAP (P^3 value= 0.001). Significant difference were observed between both MetS groups (normoglycemic and PreDM) for all atherogenicity indices ($P^{1 \text{and } 2}$ values< 0.05) and the control group, where the MetS groups have higher values. In contrast, no significant differences were found between normoglycemic MetS and MetS-PreDM (P^3 value <0.05) except for TG/HDL-C ratio (P^3 value=0.011).

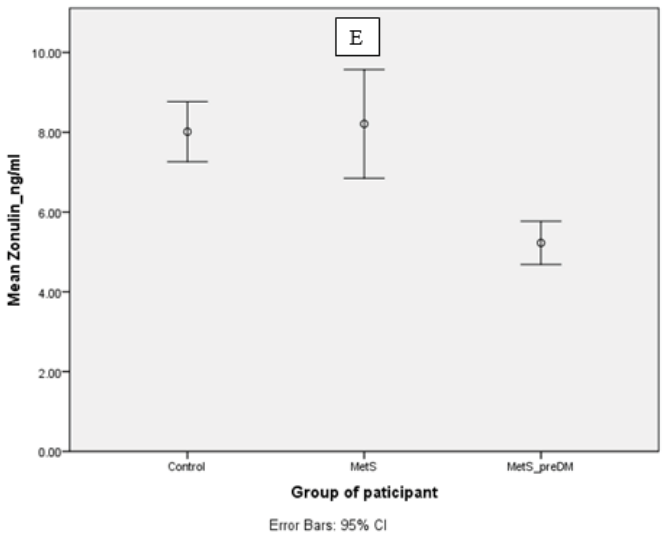
Both Survivin and LECT2 levels were significantly higher in PreDM MetS group (P^2 value< 0.05) in comparison to the controls' highly unlike all 5 gut dysbiosis biomarkers (carnitinine, choline, γBB, TMAO and Zonulin**)** which proved significantly lower vs. those of either controls (Figures 2A-G). Surprisingly, a significant variation in all tested 7 biomarkers' plasma levels were found between normoglycemic MetS and PreDM-MetS groups (P^3 value< 0.05). Interestingly antioxidative stressors were on the favorable decline as catalase, SOD and trolox total antioxidative capacities were significantly lower in both MetS (non prediabetic and prediabetic) recruits vs. controls. Both MetS groups (normoglycemic and PreDM) had a significantly higher value of MetS-IR, TyG Index, TyG-BMI and TyG-WC ($P^{1and\ 2}$ values <0.001) than the control group. Interestingly, no significant variations were found between MetS groups (normoglycemic and PreDM) in MetS-IR and TyG-BMI indices (P^3 value =0.06) (P^3 value = 0.365) respectively. In contrast, significant variations were found between MetS groups in TyG Index and TyG-WC (P^3 value < 0.05).











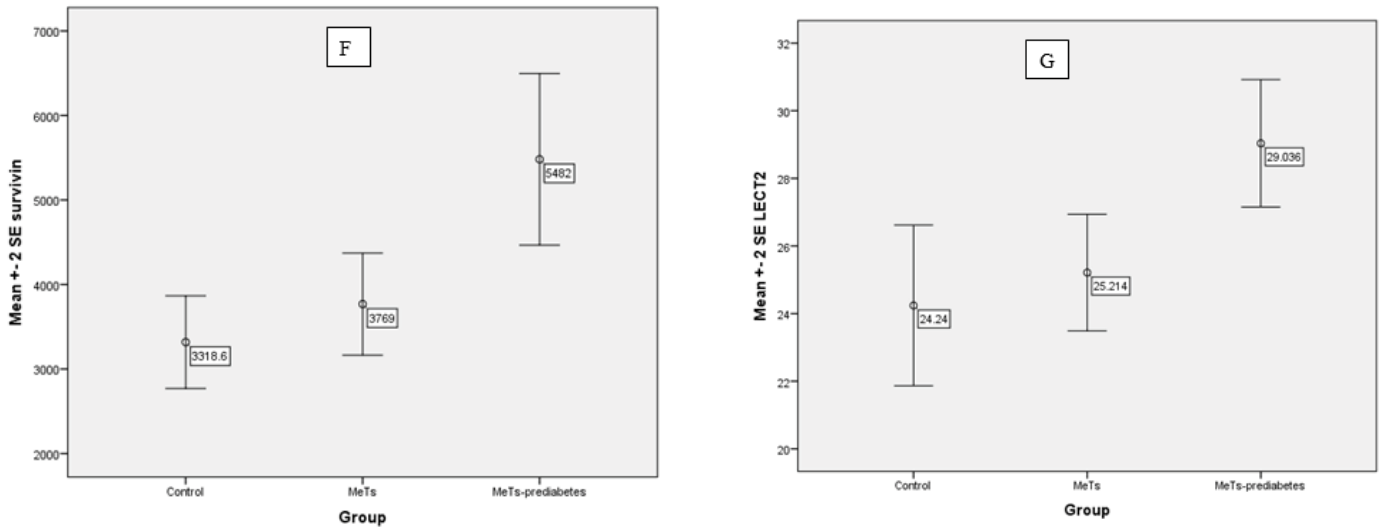


Figure 2A-G. Plasma Levels of biomarkers in Each Study Group

Spearman's Correlations of Biomarkers in pooled MetS (both normoglycemic and pre-diabetics participants) with each other and diverse indices (Tables 3-6)

Notably Tables 3-6 illustrate Spearman's Correlations of 5 Dysbiosis signature determinants in pooled MetS (both normoglycemic and pre-diabetic participants (N =60)) and their clinical parameters, atherogenecity, adiposity, proinflammatory hematology and Non insulin based – surrogate insulin resistance (sIR) Indices, respectively. Table 3 displays that survivin with gut dysbiosis choline and γBB positively and pronouncedly correlated with carnitine in 60 pooled MetS participants. Most exquisitely cardiometabolic LECT2 had a marked direct relation with each of dysbiosis carnitine and γBB. TMAO, nevertheless, related inversely and significantly with all 3 dysbiosis biomarkers. Also in a striking similarity, Zonulin associated disproportionally with both choline and γBB in all 60 MetS pooled cases.

In Table 4 exceptionally TMAO- TYG and Zonulin-TYG-WHpR paired in substantial and inverse relations in pooled normoglycemic and preDM-MetS participants (n=60). To superbly signify the anticipated deterioration in metabolism via gut microbiota-insulin insensitivity interconnectivity; all dysbiosis biomarkers (carnitine, choline, γBB, TMAO, Zonulin and survivin) correlated highly remarkably and proportionally with all non insulin based surrogate insulin resistance (sIR) indices in MetS recruits (both normoglycemic and prediabetic; equally). To further complement this outcome of metabolic dysregularity unequivocally; in Table 5, γBB associated directly and pronouncedly with almost all adiposity indices (WC, BMI, Conicidy index, and WHR). Surprisingly VAI (visceral adiposity index) correlated with Zonulin negatively in the same MetS population. Table 6 demonstrates that FBG associated exceptionally with carnitine and γButyrobetaine (γBB). Substantially A1c correlated proportionally (P values <0.05) with MetS pooled cases dysbiosis' carnitine, choline, γBB, and cardiometabolic survivin. Outstandingly both SBP and DBP had direct and marked linkage to LECT2 and so did DBP with choline's plasma levels. Remarkably TMAO related negatively and pronouncedly with MetS cases levels of FBG, A1c, TG, LDL-C, and so did also zonulin with both A1c and LDL-C (Table 6).

Table 3. Spearman's Correlations of Microbiota Biomarkers of Dysbiosis, Cardiometabolic survivin and LECT2 with oxidative stress markers in pooled MetS (both normoglycemic and pre-diabetics participants (N =60) and each others

	Choline		γButyrobetaine		TMAO		Zonulin		Survivin		LECT2	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
Carnitine	0.540**	0.04	0.326*	0.011	-0.697**	0.04			0.261*	0.046	0.283*	0.03
Choline			0.315*	0.014	-0.576**	0.04	-0.400**	0.002				
γButyrobetaine					-0.358**	0.005	-0.327*	0.011			0.265*	0.042

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
We used Spearman correlation coefficient; r, correlation coefficient;
r=0.1-.0.29 means low relationship, r=0.3-0.49 means moderate relationship and r>0.5 means high relations

Table 4. Spearman's Correlations of Metabolic Biomarkers of Dysbiosis in pooled MetS (both normoglycemic and pre-diabetics participants (N =60) and Non insulin based - sIR Indices

Non insulin based- sIR Indices Biomarker	TYG Index		TYG-BMI		TYG-WC		TYG-WHpR		TYG-WHtR	
	r	P value	r	P value	r	P value	r	P value	r	P value
Carnitine	0.288*	0.026								
γButyrobetaine			0.278*	0.031	0.425**	0.001	0.402**	0.001	0.318*	0.013
TMAO	-0.365**	0.004								
Zonulin							-0.283*	0.029		
Survivin					0.263*	0.044			0.323*	0. 012

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
We used Spearman correlation coefficient; r, correlation coefficient;
r=0.1-.0.29 means low relationship, r=0.3-0.49 means moderate relationship and r>0.5 means high relationship

Table 5. Spearman's Correlations of Metabolic Biomarkers of Dysbiosis in pooled MetS (both normoglycemic and pre-diabetics participants (N =60) and Adiposity with Hematologic Indices

Adiposity and Hematologic Indices Biomarker	VAI		WC		BMI		C-index		WHR	
	r	P value	r	P value	r	P value	r	P value	r	P value
Zonulin	-0.333**	0.009								
Carnitine	0.307*	0.017								
γButyrobetaine			0.368**	0.004	0.262*	0.043	0.385**	0.002	0.339**	0.008

Adiposity and Hematologic Indices Biomarker	Neutrophils		Lymphocytes		NLR		PLR	
	r	P value	r	P value	r	P value	r	P value
Choline	-0.293*	0.035						
Zonulin	0.331*	0.016	-0.345*	0.012	0.346*	0.012	0.32*	0.02

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
We used Spearman correlation coefficient; r, correlation coefficient;
r=0.1-.0.29 means low relationship, r=0.3-0.49 means moderate relationship and r>0.5 means high relationship

Table 6. Spearman's Correlations of Metabolic Biomarkers of Dysbiosis in pooled MetS (both normoglycemic and pre-diabetics participants (N =60) and Clinical Parameters with Atherogenecity Indices

Clinical Parameters and Atherogenecity Indices Biomarkers	SBP		DBP		FBG		A1C		TG		LDL-C	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
Carnitine					0.320*	0.013	0.545**	0.000				
Choline			0.341**	0.008			0.496**	0.000				
γButyrobetaine					0.342**	0.007	0.600**	0.000				
TMAO					-0.344**	0.007	-0.639**	0.000	-0.264*	0.041		
Zonulin							-0.421**	0.001			-0.286*	0.027
Survivin							0.375**	0.003				
LECT2	0.265*	0.042	0.327*	0.012			0.336**	0.009				

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
We used Spearman correlation coefficient; r, correlation coefficient;
r=0.1-.0.29 means low relationship, r=0.3-0.49 means moderate relationship and r>0.5 means high relationship

DISCUSSION

Microbiome and T1D: is it the perfect modality for a gut dysbiosis study intervention[57]

Type 1 diabetes (T1D) is a multifactorial autoimmune destruction of pancreatic islet insulinogenic β cells in the disease trajectory triggered by the crucial interactions between predisposing genes and environmental changes.[58] Evidence so far has evidenced that the incrementally soaring incidence and predisposition to T1D worldwide, which can be, at best, very likely attributed by the increasingly growing impact of environmental factors, mostly the gut microbiome dysbiosis, amongst the rest.[59] The most common bacterial alterations in T1D patients included *Bacteroides* spp., *Streptococcus* spp., *Clostridium* spp., *Bifidobacterium* spp., *Prevotella* spp., *Staphylococcus* spp., *Blautia* spp., *Faecalibacterium* spp., *Roseburia* spp., and *Lactobacillus* spp. The decreased diversity of gut microbiome occurs before disease onset and remains after the diagnosis of T1D.[60] Moreover the microbiota was ascribed increased lipopolysaccharide biosynthesis and bile acid metabolism in newly onset T1D in children.[61] Gut dysbiosis was additionally attributed epithelial barrier disruption, and microbiome-derived toxins spreading across the "leaky gut" thereby giving rise collectively to systemic inflammation and insulin resistance before the clinical onset of T1D and its prediabetes phase.[62] Furthermore Microbiota transplantation in animal models in association with obesity phenotype transferability is yet to be well characterized. Of note; Decreased butyrate (decreased short-chain fatty acids) production, plant-based low protein diets and certain anti-diabetic drugs could be consistently linked with marked microbiome influential outcomes.[63] Most remarkably *Parabacteroides distasonis* presence in the gut microbiome generates T1D in a mouse model thereby predicting the onset of the disease in humans. This *P. distasonis* secretes most likely an insulinomemaitic peptide that triggers insulin-targeted autoantibodies generations hence, priming the immune system to launch an attack against insulin and its secretory cells.[64] Taken together powerful prognostic and therapeutic tools can be resourcefully formulated into the insightful and substantial cross associations of gut microbiome dysbiosis and T1D.[57,61-64]

Carnitine

Carnitine supplementation seemingly reduced WC and BP (as MetS biomarkers) with further reductions in FBS and TG and augmentation in HDLc.[65] Nevertheless; with a concerning progression of carotid plaque stenosis in participants with metabolic syndrome on carnitine supplementation for 6 months in RCT; Carnitine association with pro-atherogenic metabolites has definitely raised concerns for its further use as a potential therapy.[66] Additional conflicting reports shed light on ameliorating of MetS via L-carnitine dietary supplementation in high-fat diet-induced obese mice –mainly independent of gut microbes produced TMAO.[67] In 60 MetS (nonprediabetic and prediabetic) participants; carnitine cross-correlated significantly inversely with gut dysbiosis TMAO but directly with ELISA levels of choline, γBB, survivin and LECT2, sIR index TYG, adiposity index VAI, clinical A1c and FBG. Intergroup substantial variance in carnitine plasma levels proved them lower in normoglycemic MetS subjects than those in both prediabetic MetS recruits and lean non prediabetic controls.

Choline

Gut microbial metabolism of choline genertaes trimethylamine (TMA), and following its absorption by the host, it is maybe converted in the liver into trimethylamine-N-oxide (TMAO). A high accumulation of TMA and TMAO can be closely linked to development of CVD, IBD, NAFLD and CKD.[68] Hence Choline deprivation was found to aggravate hyperglycemia and fatty liver in non-obese streptozocin-induced diabetic rats.[69] Interestingly Choline supplementation was reported as a modulator of gut microbiome diversity, gut epithelial activity, and the cytokine gene expression in representative animal models.[70] In 60 MetS (nonprediabetic and prediabetic) participants; Choline cross-correlated proportionally with γBB, A1c and DBP but inversely with gut dysbiosis TMAO and Zonulin as well as neutrophils. Intergroup significant discrepancy in choline plasma levels proved them evidently lower in normoglycemic MetS subjects than those in both prediabetic MetS recruits and lean non prediabetic controls.

γBB

Elevated vascular γ-butyrobetaine levels attenuate the development of high glucose-induced endothelial dysfunction.[71] Remarkably serum levels of γBB could predict long-term risk of T2D independently of traditional risk factors in patients with suspected stable angina pectoris possibly reflecting dysfunctional fatty acid metabolism.[72] Principally γBB associated directly and pronouncedly with A1c, FBG, adiposity indices (WC, BMI, Conicidy index, and WHR) as well as surrogate (non insulin based) insulin resistance indices in MetS recruits (both normoglycemic and prediabetic; equally) (TYG-WC, TYG-WHpR, TYG-WHtR and TYG-BM). In a cohort of 4442 participants; γ-butyrobetaine was reported of association with insulin resistance, and of greater association with insulin sensitivity but lacked for any similar significant association with T2D among older adults.[73] Outstandingly both dysbiosis signature determinants TMAO and Zonulin associated negatively and exceptionally with γBB in the same population of MetS cases. Patients with carotid atherosclerosis had increased serum levels of Carnitine and γBB, but not TMAO and trimethyllysine (TML). Additionally higher serum levels of γBB and TML were independently associated with cardiovascular death.[74]

LECT2

With an anticipated excursion rise in blood ahead of MetS.[75] Considerably LECT2 was emerging as an attractive therapeutic target for obesity-induced IR providing critical and crucial insights into metabolic disorders.[76] Impressively green tea extracts, including catechins such as epigallocatechin gallate and epicatechin gallate, had a beneficial effect on obesity, hyperglycemia, insulin resistance, endothelial dysfunction, and inflammation via reduction hepatokines of LECT2 and selenoproteins in livers of high fat fed mice.[77] Among the rest of gut dysbiosis signature determinants, carnitine and γBB

proportionally correlated with LECT2 highly substantially in the nascent MetS 60 participants. Surprisingly unlike the rest of evaluated plasma biomarkers; LECT2 lacked any pronounced cross correlations with surrogate insulin resistance (non insulin based) indices or any of adiposity, atherogenicity or proinflammtory hematologic indices. It had neither any relation with cross sectional clinical parameters except for SBP and DBP. In a striking similarity to surviving, intergroup substantial variance in LECT2 plasma levels proved them higher in prediabetic MetS subjects than those in both nonprediabetic MetS recruits and lean normoglycemic controls.

Survivin

In the pooled MetS 60 participants surviving lacked any considerably appreciable cross correlations with adiposity, atherogenecity or proinflammatory hematology indices. In contrary unprecedented highly substantial proportional relations of survivin with each of -A1c; surrogate insulin resistance indices (sIR) -TYG-WC and -TYG-WHtR and -carnitine were established. Remarkably Survivin-positive circulating tumor cells were designated as metastasis biomarkers for of hepatocellular carcinoma.[78] In our study its ELISA plasma levels were significantly increased in MetS (prediabetic) recruits in comparison to normoglycemic lean controls. More surprisingly weight loss could normalize enhanced expression of the oncogene survivin in visceral adipose tissue and blood leukocytes from obese individuals.[79] Intergroup substantial variance in surviving plasma levels proved them higher in prediabetic MetS subjects than those in both nonprediabetic MetS recruits and lean normoglycemic controls.

TMAO

As an amine oxide generated from choline, betaine, and carnitine by gut microbial metabolis.[80] Its atherogenic effects were further associated with alterations in cholesterol acid metabolism, hypertention and hyperlipidemia. Drastically TMAO levels increase with deteriorating kidney function. Accumulating evidence on positive correlations between elevated plasma levels of TMAO and an increased risk for major adverse cardiovascular events were obtained.[47] As endothelial dysfunction modulated by TMAO is largely determined by inflammation and oxidative stress, there are also the activation of foam cells; the upregulation of cytokines and adhesion molecules; the increased production of reactive oxygen species (ROS); the platelet hyperreactivity; and the reduced vascular tone.[47] Thus functional food items with dietary bioactive ingredients modulate the gut microbiota-derived metabolite TMAO can prove as promising preventive against atheromatous cardiovascular events.[81] Significantly in 60 MetS (nonprediabetic and prediabetic) enrolled subjects; TMAO cross-correlated inversely with gut dysbiosis signature determinants: γBB, choline and carnitine. TMAO related directly and highly substantially with sIR index TYG but with none of either adiposity or atherogenecity indices. Proatherogenic TMAO associated strongly with TG, A1c and FBG in the same pool of MetS recruits of prediabetic and nonprediabetic subjects. Intergroup significant discrepancy in TMAO plasma levels proved them evidently higher in normoglycemic MetS subjects than those in both prediabetic MetS recruits and lean non prediabetic controls.

Zonulin

Importantly, this cross-sectional study delineates the plasma levels of dysbiosis signature determinants of zonulin, L-carnitine, choline, γ-BB, and TMAO in nascent MetS subjects with/without prediabetes in Jordan. T2D can be molecularly connected to alterations in the gut permeability of tight intestinal junctions that are dysregulated by elevated zonulin levels.[49] Exclusively in this present study VAI (visceral adiposity index) distinctively correlated negatively with Zonulin only. Most exquisitely Zonulin as well as TMAO associated significantly and disproportionally with choline and γBB but proportionally both A1c and LDL-C in pooled 60 nascent MetS participants. Highly Exceptional Zonulin-TYG-WHpR and TMAO- TYG (the sIR indices) pairings in substantial and inverse rankings were evident. Zonulin exponentially increased with a longer T2D history.[50] As the level of plasma zonulin proportionally correlated to BMI, creatinine, FPS, along with the OGTT, hemoglobin A1C, and HOMA-IR; [50] It can be a detrimental and valuable predictive non-invasive biomarker correlated appreciably with the pathogenesis of gestational diabetes mellitus (GDM).[51]

Study limitations

- As a onetime point assessment experimental design; Lack of data on intestinal microbiota configuration, the daily habitual lifestyle and the diet of the study participants, in addition to genetic variation among individuals.
- Small sample size of enrolled lean nondiabetic controls, nondiabetic and prediabetic MetS subjects
- One single massive referral medical center was involved, whilst the preferences would be typicaly expanding the nationwide survey beyond Restricted regions and races to avert us any plausible bias

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Our unprecedented study examined, for the first time to our knowledge, the associations between 5 gut dysbiosis signature determinants zonulin, L-carnitine, choline, γ-BB, and TMAO, surviving with LECT2 in nascent MetS patients with/without prediabetes in Jordan. Also, our study investigated the correlations between those metabolic biomarkers and clinical parameters, atherogenicity, and adiposity indices along with inflammatory hematological indices in the same pool of nascent MetS subjects. Identifying metabolic biomarkers of clinical utility and candidacy with strong causality in emerging of a disease is instrumental. Further translational evidence of possible personalized medicine intertwining with indices of sIR-adiposity-atherogenecity- is crucial in the prediction/prevention and intervention modalities in *T1D, T2D, prediabetes and MetS*. However, further large scale and longevity animal and human studies are demanded inevitably for interrelations to be delineated.

AUTHORS CONTRIBUTION

Authors contributed equally towards conceiving and study

design, conducting research and providing research materials, along with data collection and organizing. Authors analyzed and interpreted data. All authors have contributed towards initial drafting, critical reviewing and approval of the final draft.

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***ABBREVIATIONS:** Adiposity indices (BMI, WHR (waist/Hip ratio), WtHR (waist/Height ratio), Conicity-index, BAI (Body adiposity index), LAP (Lipid accumulation Product), VAI (Visceral adiposity Index)) and atherogenicity indices (AIP (atherogenecity index of plasma); WAT (White adipose tissue), T2D (type 2 diabetes mellitus), TMAO (trimethylamine N-oxide), LECT2 (Leukocyte cell-derived chemotaxin-2), SOD (Superoxide dismutase), SCFAs (short chain fatty acids), PPIs (proton pumps inhibitors), LPS/TLR4 (lipopolysaccharide/toll like receptors4), HOMA-IR (homeostatic model assessment for insulin resistance), sIR (surrogate insulin resistance).

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