Impaired Amino acid and TCA Metabolism and Cardiovascular Autonomic Neuropathy Progression in Type 1 Diabetes

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Abstract

While diabetes is characterized by hyperglycemia, nutrient metabolic pathways like amino acid and tricarboxylic acid cycle (TCA) are also profoundly perturbed. As glycemic control alone does not prevent complications, we hypothesized that these metabolic disruptions are responsible for the development and progression of diabetic cardiovascular autonomic neuropathy (CAN). We performed standardized cardiovascular autonomic reflex tests and targeted fasting plasma metabolomic analysis of amino acids, and TCA cycle intermediates in type 1 diabetes and healthy controls subjects followed for three years. Forty-seven type 1 diabetes participants (mean age 35±13 years, 60% females, duration 13±7 years, HbA1c 7.9±1.2%) had lower fumarate levels and higher threonine, serine, proline, asparagine, aspartic acid, phenylalanine, tyrosine, and histidine levels compared to 10 age-matched healthy controls. Higher baseline fumarate levels and lower baseline amino acids- asparagine and glutamine correlate with CAN (lower baseline SDNN). Baseline glutamine and ornithine levels also associated with the progression of CAN (lower SDNN at 3-years) and change in SDNN respectively after adjustment for baseline HgA1C, blood glucose, BMI, cholesterol, urine microalbumin/ creatinine ratio, eGFR, and years of diabetes. Therefore, significant changes in the anaplerotic flux into the TCA cycle could be the critical defect underlying CAN progression.

Introduction

Cardiovascular autonomic neuropathy (CAN) is a widely prevalent chronic diabetic complication re that is characterized by impaired autonomic control of the cardiovascular system (1). Although the initial prevalence of CAN in newly diagnosed type 1 diabetes patients is low, later prevalence after 15 years of diabetes increases to 35% in type 1 diabetes and 60% in type 2 diabetes patients (1-4). CAN is an independent predictor of chronic kidney disease progression and of cardiovascular disease (CVD) morbidity and mortality in patients with diabetes (5-8). CAN is also associated with an increased risk of cardiac arrhythmias, silent myocardial ischemia, myocardial dysfunction, and sudden death (1; 2; 4; 8; 9). The earliest clinical manifestations of CAN are insidious and include reduced heart rate variability (HRV) at rest and during several challenges such as standing, deep breathing, and the Valsalva maneuver. Currently, objective measures of HRV using recommended standard cardiovascular autonomic testing for research studies or clinical care remain the gold-standard diagnostic (1). Given the critical prognostic consequences of CAN, its targeted and timely diagnosis is paramount. However, the current cardiovascular autonomic testing and other tests such as the baroreflex or imaging studies remain both cumbersome and expensive.

Several risk factors play essential roles in the development of CAN including chronic hyperglycemia, diabetes duration, hypertension, hyperlipidemia, chronic inflammation, oxidative stress, and more recently glucose variability (1; 2; 4; 10). While CAN progression is prevented with tight glucose control in type 1 diabetes (2; 10) and possibly with combined multifactorial interventions in type 2 diabetes (11), to date there are no specific disease-modifying therapies for of CAN. Thus, a deeper understanding of the mechanisms that modulate CAN development, and progression is crucial for both risk assessment and therapeutic interventions.

In addition to the changes in carbohydrate metabolism, diabetes is characterized by profound alterations in amino acid and lipid metabolism. In fact, elevated levels of branched chain amino acids (BCAA) are a well-characterized risk predictor of future type 2 diabetes risk and insulin resistance (12-14). While glycemic control as documented by hemoglobin A1c (HbA1c) has been the primary goal for diabetes management, a broader understanding of nutrient metabolism can offer both a mechanistic as well as a potential risk modifier in the care of diabetic patients. Indeed, microvascular complications like neuropathy, diabetic kidney disease, and retinopathy are also associated with increased oxidative stress and alterations in the levels of intermediary metabolites (15). There are tissue-specific patterns of altered metabolic flux through the glycolytic and tricarboxylic cycles in diabetic mouse models (16; 17). Importantly, diabetic neuropathy and retinopathy show a distinctly different metabolic signature compared with diabetic kidney disease in animal models (15; 17; 18). Recent advancement in mass spectrometry techniques- both targeted and untargeted approaches have made simultaneous large-scale assessments of various metabolic pathways possible. Many studies have demonstrated that these intermediary metabolites can predict the progression and severity of disease in type 1 and type 2 diabetic kidney disease (17; 19-21) and diabetic retinopathy (22). These studies have clarified the pathophysiological mechanisms, highlighted biomarkers, and identified potential therapeutic targets. However, such targeted metabolomic profiling has not been used to characterize the metabolic perturbations associated with CAN in diabetes. Therefore, the primary objective of this study was to evaluate the association between perturbations in metabolic intermediates (TCA cycle metabolites and amino acids) and measures of CAN in subjects with type 1 diabetes. The discovery of distinct metabolomic biomarker signature associated with CAN may provide insight into the pathogenic pathways that are currently unknown and may allow for the clinical stratification of these patients early in the course of the disease so that interventions can be targeted to specific vulnerable subjects.

Methods

Subjects

Forty-seven subjects with type 1 diabetes and ten age-matched healthy controls were enrolled in a 3-year longitudinal observational study. Main inclusion criteria for diabetic subjects were: age 18–65 years, presence of type 1 diabetes, with a minimum of 5 years' diabetes duration, and no signs of microvascular complications or uncontrolled hypertension at baseline. All subjects had normal resting electrocardiogram and normal exercise treadmill test results before enrolling in the study. Patients with a history of cardiovascular disease were excluded from the study. Healthy controls were age-matched with normal weight, normal glucose tolerance, and normal blood pressure (BP). Forty type 1 diabetes subjects completed the study. Demographic and anthropometric measures were collected through questionnaires and physical examination; fasting blood and urine samples were obtained for the measurement of various metabolic parameters including HbA1c, lipid panel, and renal function tests. The University of Michigan Institutional Review Board approved the study and written informed consent was obtained from all subjects.

CAN assessments

Standardized CAN evaluations were performed on all subjects after an overnight fast. Subjects were asked to avoid caffeine and tobacco products for 8 h before testing and to hold any medication (except for basal insulin) until CAN testing was completed. Subjects who experienced a hypoglycemic episode after midnight (blood glucose ≤50 mg/dL [2.77 mmol/L]) before the testing were rescheduled. The electrocardiogram recordings were obtained in the supine position using a physiologic monitor (Nightingale PPM2, Zoe Medical Inc.), and data were collected during a resting study (5 min), and during several standardized cardiovascular autonomic reflex tests (CARTs) obtained under paced breathing (R-R response to deep

breathing, Valsalva maneuver, and postural changes). Indices of CAN were derived using the ANX 3.1 (ANSAR Inc.) as previously described (23). All CAN variables were assessed for the entire cohort at baseline and for 40 type 1 diabetes subjects (out of 47) that completed the study at three years of follow-up.

CAN outcome measures: The following measures of CAN were predefined as outcomes of interests and analyzed: standard deviation of normal RR interval (SDNN), root-mean square differences of successive R-R intervals (RMSSD), expiration-to-inspiration ratio (E:I) during deep breathing, Valsalva ratio (average of two measures), 30:15 ratio, low-frequency (LF) power (0.04 to 0.15 Hz), high-frequency (HF) power (0.15 to 0.4 Hz), and LF/HF at rest and during CARTs.

Metabolites measurements:

Amino acids were measured after purification and derivatization of 100-µL samples of plasma via gas chromatography-mass spectrometry (Agilent 6890) using a modified EZ:faast kit (Phenomenex); Norvaline used as internal standard (17; 21). TCA metabolites were extracted from 100 ul of plasma with a mixture of methanol, chloroform, and water (8:1:1) containing C13 isotope-labeled internal standards for citrate, succinate, fumarate, malate, alpha-ketoglutarate, lactate and pyruvate. Liquid chromatography-mass spectrometry (LC/MS) analysis was performed on an Agilent system consisting of a 1260 UPLC module coupled with a 6520 Quadrupole-Time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Santa Clara, CA). Data were processed using MassHunter Quantitative analysis version B.07.00. Metabolites were normalized to the nearest isotope labeled internal standard and quantitated using a linear calibration curve (17).

Statistical analysis

Data integrity check. Metabolomic variables were examined for evidence of problematic signal detection based on PCA-based inspection of metabolite-level and subject-level outliers, batch effect, and detectability. All variables were checked for normality, and appropriate data transformation (natural log transformation or another scale) was performed to satisfy the assumption of normality for the various statistical technique used. Optimal data normalization and scaling were performed to ensure appropriate data format for the subsequent statistical analysis.

Analysis Plan: The differences in clinical characteristics, CAN measures, TCA cycle metabolites, and amino acid levels between subjects with T1D and the healthy controls were analyzed by using Student's t-test or Fischer's exact test for continuous variable and Chi-square test for categorical variables. Bonferroni correction was applied to account for multiple comparisons. Pearson's correlation coefficient was used to assess the relationship between the metabolic intermediates (TCA cycle metabolites and amino acids) and CAN parameters to detect strong trends in a biological context that were confirmed with subsequent regression models that account for the effects of clinical variables. Linear regression was used to predict the association with baseline metabolites, and baseline CAN parameters and predict CAN measures at the 3-year follow up. Dimension reduction of variability in biologically related metabolites with principal component analysis was used to account the high correlation between metabolites and the principal component accounting for the most variance was used for further analysis. All statistical analysis was performed using the software SPSS (Version 24, IBM Corp).

Results

Baseline clinical characteristics, metabolite measures, and CAN measures in all participants

Table 1 shows the baseline clinical characteristics of this cohort comparing the 47 subjects with type 1 diabetes (mean age 34±13 years, 61% females, 4% current smokers, duration 13±6 years, HbA1c 8±1.2%) and the age- and gender-matched healthy controls. There were no significant differences between patients with type 1 diabetes and healthy controls except for fasting blood glucose (153.7±76 vs. 86.2±14; p-value 0.007) and HbA1c which were the criteria used to define the type 1 diabetes group. The type 1 diabetes subjects were slightly heavier with no evidence of microvascular and macrovascular complications at baseline (no retinopathy, normal serum creatinine and no microalbuminuria as per study design). While the healthy controls were on no medication, all type 1 diabetes subjects were on insulin, and among these 24 (51%) were using continuous subcutaneous insulin infusion via a pump, seven (15%) were on statins, five (11%) were on ACE inhibitors (inspite of no prior history of diabetic nephropathy), and none were on beta blockers. There were no differences between in any CAN measures between the type 1 diabetes subjects and healthy controls at baseline (Table 1).

Forty type 1 diabetes subjects returned at the end of three years for follow up CAN and laboratory measures (Table 1). During the follow-up there were no significant changes to the glycemic control or changes to their BP, body mass index (BMI) or lipid control. However, there was a significant decline in the SDNN indicating worsening CAN ($p<0.05$). As shown in Table 2, at baseline amino acids-threonine, serine, proline, asparagine, aspartic acid, phenylalanine, tyrosine, and histidine were elevated in the type 1 diabetes subjects as compared to their healthy counterparts. Among the TCA cycle metabolites, fumarate was lower in subjects with type 1 diabetes (Table 2). We stratified our cohorts based on the highest daily insulin requirements and found no differences in the levels of BCAA or CAN measures between these subgroups (Supplementary Table 1).

Correlation of metabolites with measures of CAN in type 1 diabetes at baseline and 3-year follow-up

Table 3 and Figure 1 show the Pearson's correlations between measures of CAN and baseline metabolites levels in participants with type 1 diabetes. Figure 2 shows the Pearson's correlations between the baseline metabolites levels and change in CAN measures at baseline and at 3 year follow up of significant metabolites in a pathway-specific pattern. As observed, higher levels of baseline fumarate were associated with worsening baseline CAN parameters (SDNN: r= - 0.46, P =0.003; RMSSD: r= - 0.40, P= 0.01; pNN50: r=-0.45, P=0.003). Similarly, higher baseline citrate levels were associated with worse baseline CAN parameters (RMSSD: r=-0.51, P=0.003) (Table 3). Meanwhile, lower levels of both amino acids- asparagine (SDNN: r=0.42, P=0.007) and glutamine (SDNN: r=0.51, P=0.001) were positively correlated with baseline SDNN. The correlation of the entire metabolite panel and all baseline CAN measures are represented in Supplementary Tables 2. Baseline eGFR and microalbumin ratio have no relationship with baseline, year three and change in CAN measures.

Baseline glutamine and ornithine levels were also correlated with SDNN at the three years follow up (glutamine r=0.60, P=0.005; ornithine r=0.45, P=0.005) (Table 3 and Figure 1). The association of baseline glutamine levels with baseline SDNN and three-year follow up remained significant even after adjustment for years of diabetes, history of smoking, baseline HbA1c, blood glucose, BMI, total cholesterol, urine albumin-creatinine ratio, and eGFR (p=0.014 and p=0.005 respectively). In other words, increased citrate and fumarate levels and decreased glutamine and asparagine levels at baseline were associated with lower SDNN levels at baseline indicating worsening CAN. The relationship with baseline glutamine and ornithine with SDNN was maintained at 3-year follow up. The correlation of the entire metabolite panel and 3-year CAN measures are represented in Supplementary Tables 3.

As observed in Table 3, lower levels of baseline ornithine were associated with worsening of several CAN parameters from baseline to 3-year follow up (SDNN: r= 0.37, P =0.024; RMSSD: r= 0.466, P= 0.003; HF power: r=0.342, P=0.036; 30:15 ratio r=0.353, P=0.03;

E:I ratio r=0.365, P=0.024). The association of baseline ornithine levels with change in RMSD, 30:15 ratio and E:I ratio over 3 years remained significant even after adjustment for age, years of diabetes, history of smoking, BMI, baseline HgA1C, blood glucose, Cholesterol, urine microalbumin-creatinine, and eGFR). The correlation of the entire metabolite panel and change in CAN measures are represented in Supplementary Table 4.

To account for the close correlation between many of the metabolites, we reduced the dimensions of groups of similar metabolites based on biological context using principal component analysis. We found that the principal component representing glutamine, asparagine, and alpha-ketoglutarate (anaplerotic pathway/gluconeogenesis) and the principal component representing glutamine and ornithine (ornithine synthesis) both positively correlated with baseline SDNN and 3-year SDNN indicating worsening CAN measures (Table 3). Similarly, the principal component representing the altered TCA metabolites citrate, alpha-ketoglutarate, and fumarate negatively correlated with baseline SDNN. Hence, the metabolite changes associated with CAN parameters are pathway-specific and reveal systematic changes in metabolite patterns (Figure 2).

Discussion

This is the first study to explore the association between the CAN and intermediates of central carbon metabolism (TCA cycle metabolites and amino acids) using a targeted metabolomics approach. In this study, we demonstrate that subjects with type 1 diabetes had baseline perturbations in levels of several amino acids and lower fumarate levels in the plasma when compared to the healthy controls. Higher baseline levels of TCA cycle metabolites such as fumarate and citrate were also associated with lower SDNN measures in type 1 diabetes indicating worsening CAN measures. Furthermore, lower baseline glutamine levels associated with lower SDNN measures at baseline and at three year follow up indicating worsening CAN

240 measures after adjustment for other traditional risk factors. Similarly, baseline ornithine levels
241 are associated with changes in SDNN and RMSDD measured at the three year follow up after
242 adjustment of clinical variables.

243 The metabolic profiles linked with diabetes risk and diabetes itself include metabolites
244 beyond glucose metabolism. Type 1 diabetes risk is traditionally associated with autoimmunity
245 and lysophosphatidylcholine (18:0/0:0), BCAA, and glutamic acid are increased along with
246 decreased glutamine and methionine levels before and after seroconversion with autoantibodies
247 (24; 25). Whereas, type 2 diabetes risk is associated with insulin resistance (26) and is
248 associated with elevated amino acids- isoleucine, leucine, valine, tyrosine, and phenylalanine
249 and decreased asparagine, glycine, and glutamine levels (26; 27). Although this study only
250 included participants with type 1 diabetes, given the contemporary changes in the phenotypes
251 of patients with type 1 diabetes and that insulin resistance may be present in some participants,
252 we stratified our cohorts based on highest daily insulin requirements and found no differences
253 between these subgroups. In C-peptide negative type 1 diabetes patients, levels of leucine,
254 isoleucine, valine, phenylalanine, and tyrosine are increased, whereas levels of glycine,
255 glutamate, and threonine are decreased compared to both matched controls and insulin-treated
256 patients (28); However, treatment with insulin with a euglycemic clamp removed these
257 metabolic differences. Thus the metabolome is very sensitive to the presence or absence of
258 insulin in type 1 diabetes, and this could be the driving force for diabetic complications. In this
259 study, type 1 diabetes subjects demonstrated increased levels of threonine, serine, proline,
260 histidine, asparagine, aspartic acid, phenylalanine, and tyrosine compared to healthy controls.
261 Aromatic amino acids -phenylalanine and tyrosine have been previously associated with insulin
262 resistance, obesity and future diabetes risk along with increased BCAA levels (26). The
263 increase in these glucogenic amino acids compared to controls raises the possibility of altered
264 metabolism of the metabolites in type 1 diabetes despite control of diabetes.

Our work on the *db/db* mouse model of diabetes demonstrated tissue-specific metabolic reprograming and mitochondrial dysfunction that is associated with microvascular diabetic complications (17). Peripheral nerves are dependent on glycolysis independent of insulin action. However, diabetic db/db mice demonstrated decreased glucose metabolic flux and increased fatty acid oxidation in peripheral nerves (17; 29). In a similar type 2 diabetes mouse model, decreased glycolytic and tricarboxylic acid intermediates in sural, sciatic and dorsal root ganglion were observed (15). Whereas in other animal models of diabetic neuropathy, a striking upregulation of mitochondrial oxidative phosphorylation and perturbation of lipid metabolism was found in the distal sciatic nerve unlike the corresponding cell bodies of the dorsal root ganglion and the cranial trigeminal nerve (30). While these studies indicate the perturbed metabolism in diabetic peripheral neuropathy in mouse models, there is no evidence linking metabolomic profiles with the development or progression of CAN which involves the autonomic nervous system. Our data in circulating plasma levels in direct contradiction to the peripheral nerves in animal models of diabetes indicate elevated TCA metabolites and decreased glutamine, ornithine, and asparagine levels associated with worsening CAN measures. The data suggests anaplerotic flux into the TCA cycle in patients with worsening CAN which needs to be confirmed with more definitive metabolic flux studies.

Diabetic retinopathy – a closely related microvascular complication has elevated serum tryptophan metabolites-kynurenine, kynurenic acid, and 3-hydroxykynurenine (22). Diabetic kidney disease also demonstrates perturbed metabolic signatures (19-21). Serum leucine and phospholipids are altered in diabetic kidney disease compared to diabetic and healthy controls (31). Elevated amino acid-derived acyl-carnitines, essential amino acids, and their derivatives are associated with progression to end-stage renal disease in type 2 diabetes patients over many decades (21). Similarly, serum levels of seven modified amino acids (C-glycosyl tryptophan, pseudouridine, O-sulfo tyrosine, N-acetyl threonine, N-acetyl serine, N6-carbamoyl threonyl adenosine, and N6-acetyl lysine) were associated with renal function decline

291 independent of the relevant clinical covariates in type 1 diabetes (20). In our study lower levels
292 of glutamine, asparagine, and ornithine associated with worsening CAN measures. In addition
293 to amino acids, several TCA metabolites are increased in the urine of type 2 diabetes patients,
294 and urinary fumarate levels predicted chronic kidney disease progression in men (16). Mouse
295 models also support the influence of higher fumarate levels in the kidney and urine as a result of
296 NADPH oxidase 4 (NOX4) activity leading to decreasing renal function (32). In line with these
297 changes in other diabetic complications, high circulating fumarate, citrate, and alpha-
298 ketoglutarate levels were associated with low SDNN indicating worsening CAN in our study.
299 Hyperglycemia is generally acknowledged as the driving factor for most of the diabetic
300 complications. In our study, the metabolite associations with the CAN measures remained
301 unaffected by the concurrent blood glucose in the same sample and long term blood glucose
302 control in the form of HgBA1C. Increased glucose flux can result in the downstream production
303 of advanced glycation end products, polyol, hexosamine, protein kinase C, and poly(ADP-
304 ribose) polymerase pathways. Oxidative stress, apoptosis, and inflammation are also the
305 consequences of the above-increased flux (33). Diabetic glucotoxicity along with these altered
306 TCA metabolites can contribute to protein modifications include glycation, carbonylation,
307 nitration, cysteine S-nitrosylation, acetylation, sumoylation, ADP-ribosylation, O-GlcNAcylation,
308 and succinylation (34). In addition to this succinate and other TCA derivatives act on specific
309 receptors and pathways to effect oxidative stress and inflammation (35; 36). Hence the
310 preserved Krebs cycle intermediates that negatively associate with CAN measures might be
311 driving altered posttranslational protein modifications, binding to receptors, oxidative stress, and
312 inflammation in type 1 diabetes.

313 Glutamine is the most abundant amino acid in the circulation and both glutamine and
314 asparagine depending on conditions can both feed in to or be derived from the TCA cycle
315 metabolites- alpha-ketoglutarate and oxaloacetate (3). Certain cells rely solely on glutamine for

316 critical cellular functions and other metabolic inputs are unable to replace them. Glutamine itself
317 is the source of many metabolites including glucose, alpha-ketoglutarate, glutamate, ornithine
318 (therefore arginine, urea, and nitric oxide production), and glutathione (3). Glutamine is
319 responsible for gluconeogenesis and ammonia production in the kidney and liver,
320 neurotransmitter synthesis in the brain (γ-amino butyrate, GABA), NADPH, antioxidant
321 defenses, and DNA and protein synthesis in cells of the immune system (3). In our study, lower
322 levels of amino acids- glutamine, asparagine, and ornithine were positively related to worsening
323 CAN parameters- SDNN and RMSD both at baseline and at follow up. Glutamine metabolism is
324 known to be perturbed in diabetes, and increased glutamine levels are associated with
325 decreased risk of both type 2 diabetes and coronary artery disease; Also, glutamine levels are
326 also increased with rosiglitazone treatment in these patients (37; 38). Glutamine
327 supplementation was beneficial in preventing neuronal loss and development of experimental
328 diabetic cardiomyopathy (39; 40). Similarly, glutamine was shown to increase insulin sensitivity
329 and cause overnight hypoglycemia post exercise in adolescents with type 1 diabetes by
330 decreasing glucose production (41). Thus glutamine and glutamine metabolism play crucial
331 roles in the cardiovascular burden, insulin sensitivity and microvascular complications in
332 diabetes, and data from our study indicate that glutamine and its metabolism is central to CAN
333 progression in type 1 diabetes. Glutamate product GABA acts on GABA receptors present in
334 sympathetic ganglia causing diminished ganglion blockade – thus glutamate byproducts could
335 influence the autonomic nervous system (42). Similarly, ornithine, a product of glutamate plays
336 a central part of the urea cycle, polyamine synthesis, and collagen formation. Ornithine
337 supplementation promotes weight loss in rats by increasing sympathetic nerve activity in white
338 and brown adipose tissue and modulating lipid metabolism (43). Therefore, the relationship
339 between decreased ornithine levels in our study and changes in CAN measures at three year
340 follow up is possibly due to the modulation of autonomic nerve activity.

High baseline fumarate and citrate levels are linked to worse CAN measures at baseline along with the low glutamine levels indicating breaks in the citric acid cycle in CAN. In diabetic nephropathy, NADPH oxidase 4 induced decrease in kidney fumarate hydratase levels (the enzyme that converts fumarase to malate) caused increased urinary fumarate levels (44). Fumarate levels in the diabetic kidney stimulated endoplasmic reticulum stress, matrix gene expression, and expression of hypoxia-inducible factor-1α and Transforming growth factor -β. So, oxidative stress could reduce fumarate hydratase levels leading to fumarate accumulation (44). Inherited fumarate hydratase deficiency results in increased fumarate levels along with severe neurological deficits and failure to thrive. Therefore, it is possible that the increased oxidative stress associated with CAN could decrease fumarate hydratase levels and increase fumarate levels leading to neurotoxicity. However, there is no published connection linking fumarate levels to neuropathy. Fumarate levels and glutamine levels are also linked via the urea cycle. Glutamate generates carbamoyl phosphate from ammonia as the first step of the urea cycle, while fumarate is a product of the urea cycle. Clearly, quantifying the entire plasma metabolome, including the metabolites of the urea cycle will uncover the possible link between these metabolites.

Both SDNN and RMSSD are indices of heart rate variability over time. Though correlated, these indices provide information on different aspects of autonomic modulation. It is widely accepted that SDNN is a broad measure of both the sympathetic and parasympathetic modulation of HRV, while the RMSSD mainly characterizes the parasympathetic effect (45). In diabetic CAN, the interactions between the sympathetic/parasympathetic tone and function are complex and the changes in various indices may not be fully synchronized, with alterations in some measures preceding others. SDNN and RMSSD are very early indicators of CAN and changes in these indicators usually precede manifest forms of CAN by many years. Given that the participants enrolled in this observational study had no evidence of complications at

baseline (as per study design), and thus in a very early, pre-clinical stage of CAN, it is conceivable to observe selectivity in the relationship between certain HRV indices and specific metabolites. Our findings could also suggest differentiated mechanisms contributing to the modulation of various aspects of the autonomic nervous system in early stages of disease that could be targeted.

Lower baseline glutamine and higher baseline fumarate, citrate, and pyruvate levels are related to worse CAN measures at baseline and possibly related autonomic nerve dysfunction. Ornithine levels are associated with worsening CAN measures at follow up possibly indicating an early deficiency associated with causation. This distinct metabolite pattern may underlie unique aspects of the pathophysiology of the presence and development of CAN. Additionally, they could serve as a biomarker for autonomic dysfunction in type 1 diabetes patients and as an outcome in exploratory trials of therapies for early CAN. Although these patterns are very informative, these findings need to be confirmed in a larger, adequately powered, independent cohort.

CAN is an independent predictor of progression of diabetic kidney disease and patients with more advanced chronic kidney disease are also more likely to have CAN (5-7). Per study design, none of the patients had evidence of diabetic kidney disease as evidenced by normal creatinine and absence of microalbuminuria. Also, there were no relationships between the baseline eGFR and microalbumin/creatinine ratio and CAN measures at baseline, year three and change during follow up period respectively. Both eGFR and urine microalbumin/ creatinine ratio did not diminish the relationship between baseline metabolites and CAN measures. Therefore, we can reasonably conclude that the relationship of these metabolites and CAN measures is independent of renal function.

In summary, this study elucidates specific patterns amongst TCA metabolites and amino acids that associate with baseline CAN and changes in CAN measures. Specifically, lower levels of alpha-keto amino acids- asparagine and glutamine, that feed into and/or can be synthesized from oxaloacetate and alpha-ketoglutarate of the TCA cycle are associated with CAN measures (Figure 2). This pattern might indicate either a block in the flux of these metabolites out of the TCA cycle and/or increased flux in synthesizing other downstream derivatives. The data provided utilize only static metabolomic analysis, and metabolic flux data can only be inferred, which is a limitation. For accurate flux analysis, isotopically labeled tracers documenting the flux of nutrients along various pathways, need to be followed to estimate metabolic fluxes. Our cohort lacked information of diet and physical activity but smoking status did not influence the relationship of the metabolites with the CAN measures in our models. The sample size was also limited, and we are unable to pinpoint the origin of the circulating markers without tissue-specific metabolomics, thus the study is underpowered to detect many strong correlations. Our findings need to be followed by larger, well powered studies to confirm these findings. However, the strengths of this study include standardized, accurate and repeated CAN measures and associated metabolomic profiling. To mitigate the diurnal variation of the metabolites in relation to the circadian rhythm , meal time and physical activity (46; 47), only fasting samples obtained in a standardized fashion were used in this study. Day to day metabolite variation in fasting samples is consistent with variation in plasma glucose in healthy subjects and our analysis included adjustment for blood glucose at time of the sample collection (48). In conclusion, we present a novel study profiling the metabolic perturbations associated with type 1 diabetes and progression of CAN measures. These findings, when expanded to a larger cohort, will test the biomarker potential of these altered metabolites and potentially open therapeutic avenues for the prevention of CAN. Furthermore understanding these alterations will shed light on the pathways involved in CAN development and progression.

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Guarantor's statement

Drs. Rodica Pop-Busui and Pennathur are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest: None

Data and resource availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

No applicable resources were generated or analyzed during the current study.

References

1. Pop-Busui R, Boulton AJ, Feldman EL, Bril V, Freeman R, Malik RA, Sosenko JM, Ziegler D: Diabetic Neuropathy: A Position Statement by the American Diabetes Association. Diabetes Care 2017;40:136-154
2. Martin CL, Albers JW, Pop-Busui R, Group DER: Neuropathy and related findings in the diabetes control and complications trial/epidemiology of diabetes interventions and complications study. Diabetes Care 2014;37:31-38
3. Newsholme P, Procopio J, Lima MM, Pithon-Curi TC, Curi R: Glutamine and glutamate--their central role in cell metabolism and function. Cell biochemistry and function 2003;21:1-9
4. Spallone V, Ziegler D, Freeman R, Bernardi L, Frontoni S, Pop-Busui R, Stevens M, Kempler P, Hilsted J, Tesfaye S, Low P, Valensi P, Toronto Consensus Panel on Diabetic N: Cardiovascular autonomic neuropathy in diabetes: clinical impact, assessment, diagnosis, and management. Diabetes Metab Res Rev 2011;27:639-653
5. Orlov S, Cherney DZI, Pop-Busui R, Lovblom LE, Ficociello LH, Smiles AM, Warram JH, Krolewski AS, Perkins BA: Cardiac Autonomic Neuropathy and Early Progressive Renal Decline in Patients with Nonmacroalbuminuric Type 1 Diabetes. Clinical Journal of the American Society of Nephrology 2015;10:1136
6. Chandra P, Sands RL, Gillespie BW, Levin NW, Kotanko P, Kiser M, Finkelstein F, Hinderliter A, Pop-Busui R, Rajagopalan S, Saran R: Predictors of heart rate variability and its prognostic significance in chronic kidney disease. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2012;27:700-709
7. Wheelock KM, Jaiswal M, Martin CL, Fufaa GD, Weil EJ, Lemley KV, Yee B, Feldman E, Brosius FC, 3rd, Knowler WC, Nelson RG, Pop-Busui R: Cardiovascular autonomic neuropathy associates with nephropathy lesions in American Indians with type 2 diabetes. Journal of diabetes and its complications 2016;30:873-879
8. Pop-Busui R, Evans GW, Gerstein HC, Fonseca V, Fleg JL, Hoogwerf BJ, Genuth S, Grimm RH, Corson MA, Prineas R, Action to Control Cardiovascular Risk in Diabetes Study G: Effects of cardiac autonomic dysfunction on mortality risk in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial. Diabetes Care 2010;33:1578-1584
9. Orlov S, Cherney DZ, Pop-Busui R, Lovblom LE, Ficociello LH, Smiles AM, Warram JH, Krolewski AS, Perkins BA: Cardiac autonomic neuropathy and early progressive renal decline in patients with nonmacroalbuminuric type 1 diabetes. Clin J Am Soc Nephrol 2015;10:1136-1144
10. Pop-Busui R, Low PA, Waberski BH, Martin CL, Albers JW, Feldman EL, Sommer C, Cleary PA, Lachin JM, Herman WH, Group DER: Effects of prior intensive insulin therapy on cardiac autonomic nervous system function in type 1 diabetes mellitus: the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications study (DCCT/EDIC). Circulation 2009;119:2886-2893
11. Gaede P, Vedel P, Larsen N, Jensen GV, Parving HH, Pedersen O: Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. N Engl J Med 2003;348:383-393
12. Felig P, Marliss E, Cahill GF: Plasma Amino Acid Levels and Insulin Secretion in Obesity. New England Journal of Medicine 1969;281:811-816
13. Newgard CB: Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell metabolism 2012;15:606-614
14. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE: Metabolite profiles and the risk of developing diabetes. Nature medicine 2011;17:448-453

15. Hinder LM, Vivekanandan-Giri A, McLean LL, Pennathur S, Feldman EL: Decreased glycolytic and tricarboxylic acid cycle intermediates coincide with peripheral nervous system oxidative stress in a murine model of type 2 diabetes. The Journal of endocrinology 2013;216:1-11
16. Liu JJ, Liu S, Gurung RL, Ching J, Kovalik JP, Tan TY, Lim SC: Urine tricarboxylic acid (TCA) cycle metabolites predict progressive chronic kidney disease in type 2 diabetes. The Journal of clinical endocrinology and metabolism 2018;
17. Sas KM, Kayampilly P, Byun J, Nair V, Hinder LM, Hur J, Zhang H, Lin C, Qi NR, Michailidis G, Groop PH, Nelson RG, Darshi M, Sharma K, Schelling JR, Sedor JR, Pop-Busui R, Weinberg JM, Soleimanpour SA, Abcouwer SF, Gardner TW, Burant CF, Feldman EL, Kretzler M, Brosius FC, 3rd, Pennathur S: Tissue-specific metabolic reprogramming drives nutrient flux in diabetic complications. JCI Insight 2016;1:e86976
18. Sas KM, Lin J, Rajendiran TM, Soni T, Nair V, Hinder LM, Jagadish HV, Gardner TW, Abcouwer SF, Brosius FC, 3rd, Feldman EL, Kretzler M, Michailidis G, Pennathur S: Shared and distinct lipid-lipid interactions in plasma and affected tissues in a diabetic mouse model. J Lipid Res 2017;
19. Sharma K, Karl B, Mathew AV, Gangoiti JA, Wassel CL, Saito R, Pu M, Sharma S, You YH, Wang L, Diamond-Stanic M, Lindenmeyer MT, Forsblom C, Wu W, Ix JH, Ideker T, Kopp JB, Nigam SK, Cohen CD, Groop PH, Barshop BA, Natarajan L, Nyhan WL, Naviaux RK: Metabolomics reveals signature of mitochondrial dysfunction in diabetic kidney disease. J Am Soc Nephrol 2013;24:1901-1912
20. Niewczas MA, Mathew AV, Croall S, Byun J, Major M, Sabisetti VS, Smiles A, Bonventre JV, Pennathur S, Krolewski AS: Circulating Modified Metabolites and a Risk of ESRD in Patients With Type 1 Diabetes and Chronic Kidney Disease. Diabetes Care 2017;40:383-390
21. Niewczas MA, Sirich TL, Mathew AV, Skupien J, Mohney RP, Warram JH, Smiles A, Huang X, Walker W, Byun J, Karoly ED, Kensicki EM, Berry GT, Bonventre JV, Pennathur S, Meyer TW, Krolewski AS: Uremic solutes and risk of end stage renal disease in type 2 diabetes. Kidney international 2014;85:1214-1224
22. Munipally PK, Agraharm SG, Valavala VK, Gundae S, Turlapati NR: Evaluation of indoleamine 2,3-dioxygenase expression and kynurenine pathway metabolites levels in serum samples of diabetic retinopathy patients. Arch Physiol Biochem 2011;117:254-258
23. Jaiswal M, McKeon K, Comment N, Henderson J, Swanson S, Plunkett C, Nelson P, Pop-Busui R: Association Between Impaired Cardiovascular Autonomic Function and Hypoglycemia in Patients With Type 1 Diabetes. Diabetes Care 2014;37:2616-2621
24. Orešič M, Simell S, Sysi-Aho M, Näntö-Salonen K, Seppänen-Laakso T, Parikka V, Katajamaa M, Hekkala A, Mattila I, Keskinen P, Yetukuri L, Reinikainen A, Lähde J, Suortti T, Hakalax J, Simell T, Hyöty H, Veijola R, Ilonen J, Lahesmaa R, Knip M, Simell O: Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. The Journal of Experimental Medicine 2008;205:2975-2984
25. Pflueger M, Seppänen-Laakso T, Suortti T, Hyötyläinen T, Achenbach P, Bonifacio E, Orešič M, Ziegler A-G: Age- and Islet Autoimmunity–Associated Differences in Amino Acid and Lipid Metabolites in Children at Risk for Type 1 Diabetes. Diabetes 2011;60:2740-2747
26. Guasch-Ferre M, Hruby A, Toledo E, Clish CB, Martinez-Gonzalez MA, Salas-Salvado J, Hu FB: Metabolomics in Prediabetes and Diabetes: A Systematic Review and Meta-analysis. Diabetes Care 2016;39:833-846
27. Urpi-Sarda M, Almanza-Aguilera E, Llorach R, Vazquez-Fresno R, Estruch R, Corella D, Sorli JV, Carmona F, Sanchez-Pla A, Salas-Salvado J, Andres-Lacueva C: Non-targeted metabolomic biomarkers and metabotypes of type 2 diabetes: A cross-sectional study of PREDIMED trial participants. Diabetes & metabolism 2018;

28. Lanza IR, Zhang S, Ward LE, Karakelides H, Raftery D, Nair KS: Quantitative Metabolomics by 1H-NMR and LC-MS/MS Confirms Altered Metabolic Pathways in Diabetes. PLOS ONE 2010;5:e10538
29. Greene DA, Winegrad AI: In vitro studies of the substrates for energy production and the effects of insulin on glucose utilization in the neural components of peripheral nerve. Diabetes 1979;28:878-887
30. Freeman OJ, Unwin RD, Dowsey AW, Begley P, Ali S, Hollywood KA, Rustogi N, Petersen RS, Dunn WB, Cooper GJ, Gardiner NJ: Metabolic Dysfunction Is Restricted to the Sciatic Nerve in Experimental Diabetic Neuropathy. Diabetes 2016;65:228-238
31. Zhu C, Liang QL, Hu P, Wang YM, Luo GA: Phospholipidomic identification of potential plasma biomarkers associated with type 2 diabetes mellitus and diabetic nephropathy. Talanta 2011;85:1711-1720
32. You YH, Quach T, Saito R, Pham J, Sharma K: Metabolomics Reveals a Key Role for Fumarate in Mediating the Effects of NADPH Oxidase 4 in Diabetic Kidney Disease. J Am Soc Nephrol 2016;27:466-481
33. Edwards JL, Vincent AM, Cheng HT, Feldman EL: Diabetic neuropathy: mechanisms to management. Pharmacol Ther 2008;120:1-34
34. Zheng H, Wu J, Jin Z, Yan LJ: Protein Modifications as Manifestations of Hyperglycemic Glucotoxicity in Diabetes and Its Complications. Biochem Insights 2016;9:1-9
35. Peti-Peterdi J, Kang JJ, Toma I: Activation of the renal renin–angiotensin system in diabetes—new concepts. Nephrology Dialysis Transplantation 2008;23:3047-3049
36. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, Cervantes-Barragan L, Ma X, Huang SC-C, Griss T, Weinheimer CJ, Khader S, Randolph GJ, Pearce EJ, Jones RG, Diwan A, Diamond MS, Artyomov MN: Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. Cell metabolism 2016;24:158-166
37. Cha SA, Yun JS, Lim TS, Min K, Song KH, Yoo KD, Park YM, Ahn YB, Ko SH: Diabetic Cardiovascular Autonomic Neuropathy Predicts Recurrent Cardiovascular Diseases in Patients with Type 2 Diabetes. PLoS One 2016;11:e0164807
38. Ottosson F, Smith E, Melander O, Fernandez C: Altered Asparagine and Glutamate Homeostasis Precede Coronary Artery Disease and Type 2 Diabetes. The Journal of clinical endocrinology and metabolism 2018;103:3060-3069
39. Pereira RV, Tronchini EA, Tashima CM, Alves EP, Lima MM, Zanoni JN: L-glutamine supplementation prevents myenteric neuron loss and has gliatrophic effects in the ileum of diabetic rats. Digestive diseases and sciences 2011;56:3507-3516
40. Badole SL, Jangam GB, Chaudhari SM, Ghule AE, Zanwar AA: L-Glutamine Supplementation Prevents the Development of Experimental Diabetic Cardiomyopathy in Streptozotocin-Nicotinamide Induced Diabetic Rats. PLOS ONE 2014;9:e92697
41. Mauras N, Xing D, Fox LA, Englert K, Darmaun D: Effects of Glutamine on Glycemic Control During and After Exercise in Adolescents With Type 1 Diabetes: A pilot study. Diabetes Care 2010;33:1951-1953
42. Vemulapalli S, Barletta M: The role of the sympathetic nervous system in the cardiovascular effects of systemically administered gamma-aminobutyric acid. Archives internationales de pharmacodynamie et de therapie 1984;267:46-58
43. Konishi Y, Koosaka Y, Maruyama R, Imanishi K, Kasahara K, Matsuda A, Akiduki S, Hishida Y, Kurata Y, Shibamoto T, Satomi J, Tanida M: L-Ornithine intake affects sympathetic nerve outflows and reduces body weight and food intake in rats. Brain research bulletin 2015;111:48-52
44. You Y-H, Quach T, Saito R, Pham J, Sharma K: Metabolomics Reveals a Key Role for Fumarate in Mediating the Effects of NADPH Oxidase 4 in Diabetic Kidney Disease. Journal of the American Society of Nephrology 2016;27:466

585 45. Heart rate variability: standards of measurement, physiological interpretation and clinical
586 use. Task Force of the European Society of Cardiology and the North American Society of
587 Pacing and Electrophysiology. Circulation 1996;93:1043-1065
588 46. Sato S, Parr EB, Devlin BL, Hawley JA, Sassone-Corsi P: Human metabolomics reveal daily
589 variations under nutritional challenges specific to serum and skeletal muscle. Molecular
590 Metabolism 2018;16:1-11
591 47. Dallmann R, Viola AU, Tarokh L, Cajochen C, Brown SA: The human circadian
592 metabolome. Proceedings of the National Academy of Sciences 2012;109:2625
593 48. Kim K, Mall C, Taylor SL, Hitchcock S, Zhang C, Wettersten HI, Jones AD, Chapman A,
594 Weiss RH: Mealtime, temporal, and daily variability of the human urinary and plasma
595 metabolomes in a tightly controlled environment. PloS one 2014;9:e86223-e86223

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598 Table 1. Clinical characteristics of type 1 diabetes subjects and healthy controls.

Variable, units	Type 1 Diabetes	Healthy controls	P value	Type 1 Diabetes (N=40)		P value
	N=47	N=10		Baseline	Follow up	
Age, years	34 ± 13	34 ± 12	0.85	35 ± 13	38 ± 13	-
BMI, kg/m2	26 ± 5	23 ± 3	0.08	27 ± 5	27 ± 4	0.89
Systolic BP, mm Hg	116 ± 11	115 ± 8	0.63	117 ± 11	117 ± 11	0.99
Diastolic BP, mm Hg	72 ± 8	69 ± 8	0.29	73 ± 8	69 ± 11	0.08
Heart rate, beats/min	67 ± 10	71 ± 8	0.16	67 ± 10	67 ± 11	0.97
HbA1c, %	8.0 ± 1.2	5.4 ± 0.3	<0.0001	8 ± 1	8 ± 1	0.99
Total Cholesterol, mg/dl	166 ± 28	162 ± 30	0.66	165 ± 29	173 ± 27	0.98
LDL-c, mg/dl	89 ± 23	86 ± 23	0.7	89 ± 24	90 ± 21	0.81
HDL-c, mg/dl	64 ± 19	58 ± 13	0.37	64 ± 20	67 ± 19	0.42
Triglycerides, mg/dl	70 ± 31	87 ± 33	0.12	67 ± 33	78 ± 41	0.24
LF power	2.96 ± 3.01	2.72 ± 2.59	0.81	3.23 ± 3.27	2.35 ± 4.05	0.07
HF power	2.95 ± 3.47	2.05 ± 2.02	0.44	2.85 ± 3.39	3.25 ± 7.38	0.68
LF:HF ratio	2.08± 1.89	1.43 ± 0.24	0.29	2.32± 2.03	2.65 ± 3.57	0.62
Valsalva ratio	1.36 ± 0.27	1.36 ± 0.20	0.99	1.35 ± 0.31	1.34 ± 0.33	0.81
30:15 ratio	1.24 ± 0.15	1.23 ± 0.17	0.9	1.22 ± 0.15	1.21 ± 0.14	0.93
E:I ratio	1.23 ± 0.12	1.25 ±0.13	0.71	1.23 ± 0.13	1.21 ±0.14	0.29
SDNN, msec	53 ± 21	54 ± 31	0.87	51 ± 19	43.62 ± 22	0.02
RMSSD, msec	40 ± 27.6	39 ± 31	0.91	36 ± 24	34 ± 31	0.47

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600 All data are shown as mean ±SD, median (interquartile range) or n (%). BMI: body mass index, BP: blood pressure,
601 LDL: low-density lipoprotein, HDL: high density lipoprotein, LF: low-frequency power, HF: high-frequency power, E:I
602 expiration inspiration ratio, SDNN: standard deviation of normal RR interval, RMSSD: root mean square of difference
603 of successive normal RR interval.

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Table 2. Baseline Tricarboxylic Acid Cycle (TCA) Intermediates and Amino Acids in Study Participants

Variable	Type 1 diabetes n= 47	Healthy Controls n = 10	P-value	q value
Amino acids				
Alanine	433.0±181.1	361.9±94.2	0.48	0.58
Glycine	321.4±124.3	266.4±55.4	0.21	0.32
Threonine	186.1±71.2	127.9±34.8	**0.01**	**0.05**
Serine	197.6±77.0	127.1±20.6	**0.0015**	**0.02**
α-aminoisobutyric acid	22.9±9.6	22.6±9.3	0.97	0.97
Valine	242.1±76.1	204.4±54.5	0.20	0.32
Leucine	103.2±43.8	102.6±32.4	0.80	0.84
Isoleucine	72.2±22.8	62.5±21.2	0.17	0.31
Phenylalanine	58.6±17.1	40.5±10.0	**0.0007**	**0.02**
Tyrosine	51.2±21.4	35.8±13.2	**0.015**	**0.05**
Tryptophan	49.5±18.7	44.3±14.2	0.49	0.58
Asparagine	75.5±25.9	53.6±11.9	**0.01**	**0.05**
Aspartic acid	6.2±3.5	3.9±1.0	0.11	0.22
Glutamic acid	55.4±30.6	66.6±28.4	0.18	0.31
Glutamine	1002.7±537.0	597.9±191.1	**0.02**	**0.07**
Ornithine	48.2±24.7	54.1±25.3	0.40	0.53
Proline	213.6±69.8	162.8±54.2	**0.01**	**0.05**
4-Hydroxyproline	19.9±12.2	14.3±7.4	0.07	0.17
Lysine	224.3±93.3	182.8±51.2	0.30	0.42
Histidine	86.5±25.6	67.5±19.2	**0.01**	**0.05**
TCA metabolites				
Citrate/Isocitrate	19.50±3.72	21.09±3.12	**0.04**	**0.19**
α-ketoglutarate	1.80±0.25	1.78±0.14	0.57	0.88
Succinate	2.87±0.66	2.84±0.54	0.85	0.88
Fumarate	0.46±0.04	0.54±0.04	**<0.0001**	**<0.0001**
Malate	2.07±0.62	2.02±0.35	0.88	0.88
Lactate	180.17±82.31	204.52±57.17	0.61	0.88
Pyruvate	3.91±1.58	4.43±1.72	0.44	0.88
Flavin adenine dinucleotide	0.46±0.001	0.46±0.001	0.73	0.88

All data are presented as Mean ± SD and in microM

Table 3. Correlation between baseline and three-year cardiovascular autonomic neuropathy parameters with baseline metabolites and principal components

	SDNN (r)			RMSSD (r)		
Metabolites	*Base*	*3 yr.*	*Diff*	*Base*	*3 yr.*	*Diff*
Fumarate	**-0.38**	-0.18	0.2	**-0.32**	-0.2	0.06
Pyruvate	**-0.34**	-0.19	0.19	**-0.31**	**-0.19**	0.08
Citrate/Isocitrate	-0.28	-0.06	0.3	-0.43	-0.24	0.2
α-ketoglutarate	-0.26	-0.09	0.21	-0.14	-0.04	0.09
Asparagine	0.44	**0.3**	-0.04	0.21	0.19	0.1
Glutamine	0.52	0.6	0.16	0.26	0.24	0.1
Ornithine	0.11	0.45	**0.37**	0.24	0.44	0.47
Principal Components						
Glutamine-Asparagine-α-ketoglutarate	0.44	0.62	0.02	0.32	**0.4**	0.08
Ornithine-Glutamine	**0.38**	0.61	0.31	0.3	**0.41**	**0.34**
α-ketoglutarate-Fumarate-Citrate	**-0.38**	-0.14	0.3	**-0.37**	-0.2	0.15

Values represent r values of Pearson's correlation. Bold font -P < 0.05, Red font -P< 0.001, SDNN: standard deviation of normal RR interval, RMSSD: root mean square difference of successive RR intervals.

638 **Figures**

639 Figure 1: Correlation of baseline SDNN with baseline glutamine levels (A), asparagine levels
640 (B), and fumarate levels (C). Correlation of three-year SDNN with baseline glutamine levels (D),
641 baseline ornithine levels (E). Panel F demonstrates the correlation between the change in
642 SDNN over three year follow up with baseline ornithine levels. All metabolite levels in micro M
643 and SDNN in seconds. Pearson's' correlation represented as r and * represents p-value less
644 than 0.05. SDNN: standard deviation of normal RR interval.

645 Figure 2: Panel A: Schematic diagram of the altered metabolites that associate with
646 cardiovascular autonomic neuropathy (CAN) parameters. Metabolites highlighted in red ovals
647 are increased in type 1 diabetes and positively related to baseline SDNN and RMSSD; green
648 filled ovals are decreased in diabetics and positively related to SDNN and RMSSD at baseline
649 and follow up (r values by Pearson's correlation to baseline CAN parameters represented in
650 parenthesis as –(SDNN/RMSSD)); yellow filled oval are decreased in diabetics and positively
651 related to SDNN and RMSSD at follow up and the difference in SDNN and RMSSD from
652 baseline and follow up (r values by Pearson's correlation to 3 year follow up CAN parameters
653 represented in parenthesis as –(SDNN/RMSSD)). SDNN: standard deviation of normal RR
654 interval; RMSSD: root mean square difference of successive RR intervals; Diff – change in CAN
655 parameters over three years; P5CS-Pyrroline 5-carboxylate synthetase; OAT-ornithine amino
656 transferase.

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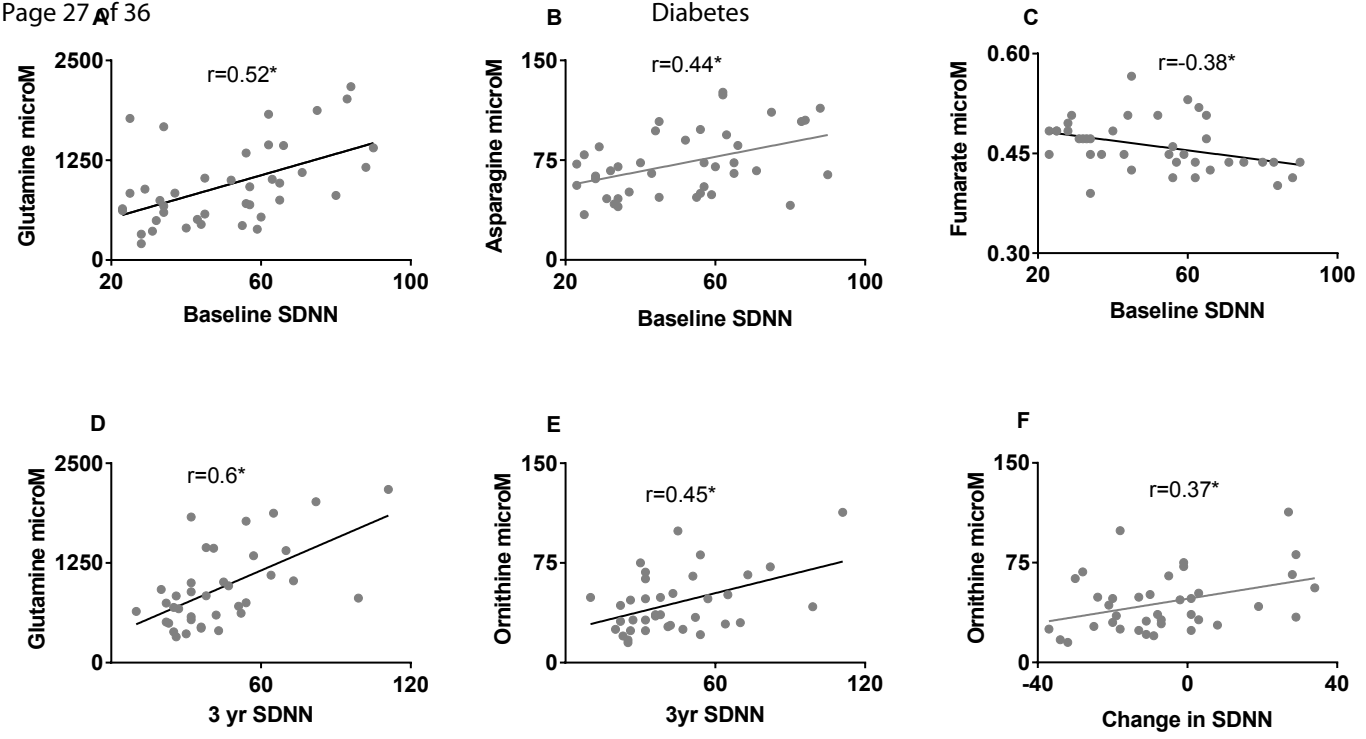
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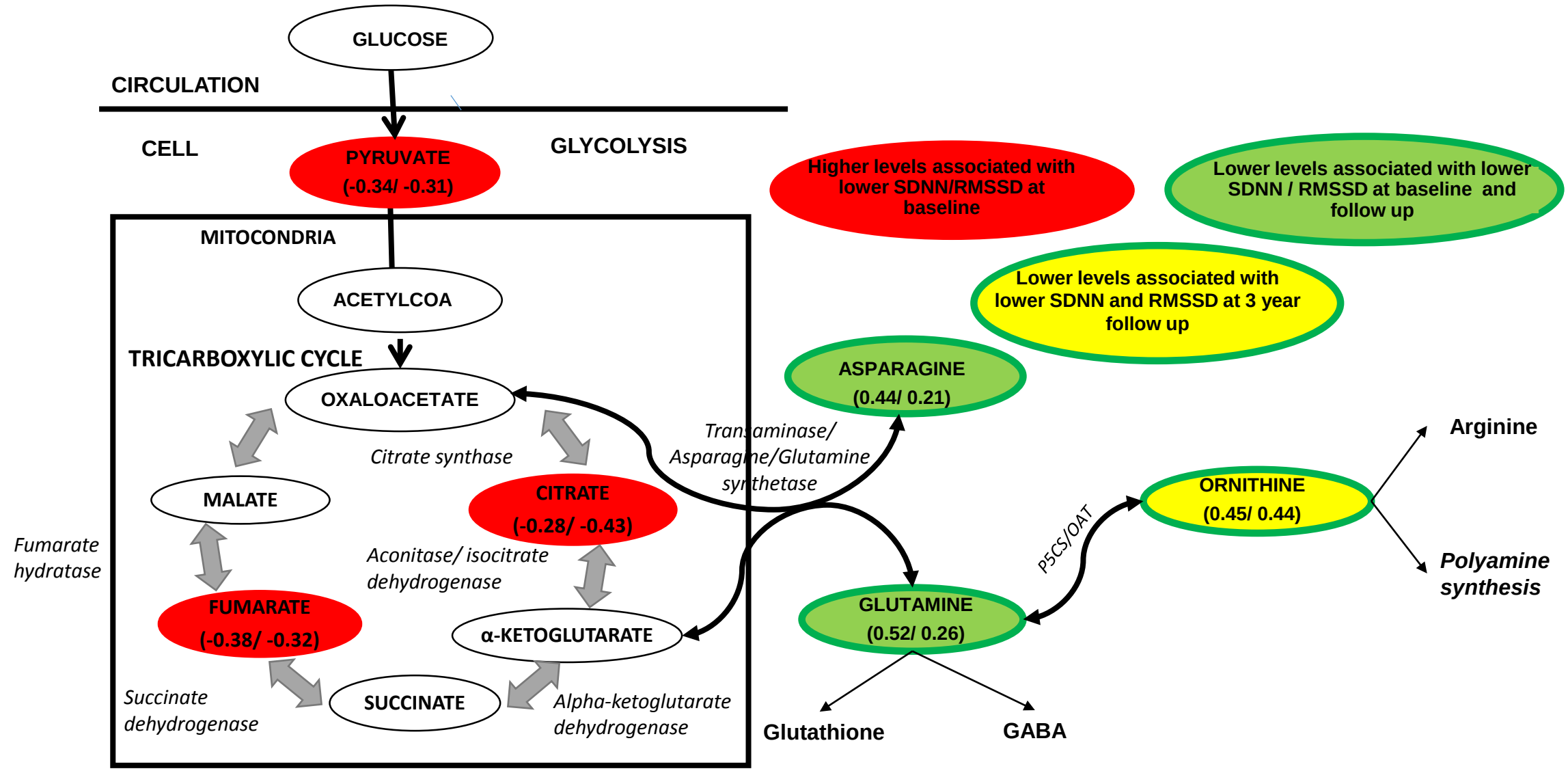
Change in SDNN


GLUCOSE
CIRCULATION
CELL
GLYCOLYSIS
PYRUVATE
(-0.34/ -0.31)
MITOCONDRIA
ACETYLCOA
TRICARBOXYLIC CYCLE
OXALOACETATE
Citrate synthase
CITRATE
(-0.28/ -0.43)
MALATE
Fumarate hydratase
Aconitase/ isocitrate dehydrogenase
FUMARATE
(-0.38/ -0.32)
α-KETOGLUTARATE
Succinate dehydrogenase
SUCCINATE
Alpha-ketoglutarate dehydrogenase
Higher levels associated with lower SDNN/RMSSD at baseline
Lower levels associated with lower SDNN / RMSSD at baseline and follow up
Lower levels associated with lower SDNN and RMSSD at 3 year follow up
ASPARAGINE
(0.44/ 0.21)
Transaminase/ Asparagine/Glutamine synthetase
GLUTAMINE
(0.52/ 0.26)
P5CS/OAT
ORNITHINE
(0.45/ 0.44)
Arginine
Polyamine synthesis
Glutathione
GABA

Online Supplemental Material

Impaired Amino acid and TCA Metabolism and Cardiovascular Autonomic Neuropathy Progression in Type 1 Diabetes

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Supplementary Materials

Supplementary Table 1: Comparison of cardiovascular autonomic neuropathy parameters between groups with top quartile Insulin dose compared to the rest of the cohort.

	High Insulin dose (N=10)		**Others (N=30)**		
Metabolite	Mean	SD	Mean	SD	p-value
BMI	27.19	4.35	26.44	4.87	0.67
TG	76.78	51.32	68.41	26.24	0.52
B-SDNN	54.50	24.35	49.80	18.12	0.52
B-RMSSD	38.00	24.73	37.00	24.52	0.9
F-SDNN	51	31.97	41.25	18.16	0.26
F-RMSSD	38.2	36.61	33.1	29.12	0.67
D-SDNN	1.5	12.38	5.9	33.04	0.68
D-RMSSD	1.11	17.5	-2.9	16.6	0.53
Valine	224.84	97.39	261.76	65.32	0.29
Leucine	98.61	46.93	105.76	46.56	0.68
Isoleucine	66.77	20.48	73.76	25.71	0.44

B-baseline, F-three-year follow-up, D- change in CAN measure. SDNN: standard deviation of normal RR interval, RMSSD: root mean square difference of successive RR intervals, BMI: Body Mass Index, SD: standard deviation.

Supplementary Table 2: Correlation of metabolites to baseline cardiovascular autonomic neuropathy measures

Metabolites	Variable	SDNN	RMSSD	pNN50	30:15	E:I Ratio	LF	HF	LF:HF ratio	Valsal-va ratio
Alanine	r	-0.16	-0.28	-.395*	-0.27	-0.23	0.06	-0.20	0.07	-0.02
	p-value	0.33	0.08	0.01	0.09	0.15	0.70	0.22	0.67	0.92
Glycine	r	0.02	-0.15	-0.20	0.10	-0.08	-0.03	-0.03	0.16	-0.08
	p-value	0.91	0.37	0.23	0.55	0.63	0.84	0.84	0.31	0.63
Alpha-aminoisobutyric acid	r	-0.29	-0.18	-.336*	**-.447****	-0.04	0.14	-0.11	-0.14	0.06
	p-value	0.07	0.28	0.03	**0.00**	0.81	0.40	0.49	0.38	0.73
Valine	r	-0.19	-0.22	-.374*	-0.30	-0.18	0.07	-0.17	-0.03	-0.03
	p-value	0.23	0.17	0.02	0.06	0.28	0.68	0.30	0.87	0.85
Leucine	r	-0.03	-0.01	-0.13	-0.17	-0.08	0.16	-0.09	.317*	0.04
	p-value	0.86	0.98	0.42	0.29	0.61	0.32	0.57	0.05	0.80
Isoleucine	r	-0.17	-0.08	-0.20	-0.29	-0.06	0.08	-0.18	0.10	-0.10
	p-value	0.30	0.64	0.21	0.07	0.70	0.64	0.28	0.53	0.54
Threonine	r	0.30	0.13	0.22	0.25	0.23	0.04	0.00	0.23	-0.02
	p-value	0.06	0.41	0.17	0.12	0.15	0.80	0.99	0.16	0.90
Serine	r	0.27	0.02	0.14	0.25	0.00	-0.09	0.01	0.10	-0.10
	p-value	0.09	0.91	0.40	0.12	1.00	0.58	0.98	0.53	0.55
Proline	r	-0.08	-0.17	-0.25	-0.14	-0.23	0.01	-0.18	-0.03	-0.02
	p-value	0.64	0.30	0.12	0.40	0.16	0.93	0.26	0.84	0.89
Asparagine	r	**.436****	0.21	0.28	.317*	0.21	0.21	0.22	0.11	-0.06
	p-value	**0.01**	0.20	0.08	0.05	0.20	0.19	0.17	0.50	0.70
Aspartic acid	r	-0.01	-0.12	-0.16	-0.02	-0.01	0.04	-0.03	0.10	0.05
	p-value	0.94	0.46	0.32	0.91	0.97	0.80	0.87	0.53	0.78
Methionine	r	-0.06	-0.07	-0.01	0.09	0.01	-0.01	-0.13	0.25	0.01
	p-value	0.70	0.66	0.97	0.59	0.96	0.95	0.44	0.12	0.95
4-Hydroxyproline	r	0.27	0.15	0.22	0.03	0.03	-0.08	-0.10	0.05	0.03
	p-value	0.10	0.35	0.18	0.87	0.85	0.63	0.54	0.74	0.84
Glutamic acid	r	0.14	0.22	0.28	0.19	0.06	0.10	0.14	0.09	0.01
	p-value	0.38	0.18	0.08	0.25	0.72	0.53	0.41	0.59	0.97
Phenylalanine	r	0.16	0.00	0.10	0.01	0.00	-0.04	-0.08	0.27	0.06
	p-value	0.31	0.99	0.54	0.97	0.98	0.81	0.64	0.09	0.72
Glutamine	r	**.516****	0.26	**.433****	**.472****	0.31	0.10	**.409****	-0.18	-0.09
	p-value	**0.00**	0.11	**0.01**	**0.00**	0.05	0.54	**0.01**	0.27	0.59
Ornithine	r	0.11	0.24	0.17	0.08	0.06	0.27	.326*	0.23	-0.11
	p-value	0.49	0.14	0.29	0.63	0.70	0.09	0.04	0.16	0.49
Lysine	r	0.05	-0.03	0.02	-0.08	-0.02	-0.04	-0.11	0.31	0.09
	p-value	0.74	0.84	0.91	0.63	0.93	0.82	0.52	0.06	0.60
Histidine	r	0.11	0.02	0.15	0.09	0.08	-0.06	0.11	-0.11	0.05
	p-value	0.51	0.92	0.35	0.57	0.64	0.70	0.51	0.49	0.77
Tyrosine	r	0.28	0.09	0.15	-0.07	-0.06	0.07	-0.03	0.31	0.26
	p-value	0.08	0.59	0.35	0.69	0.72	0.68	0.88	0.06	0.11
Tryptophan	r	0.19	0.20	.327*	0.07	0.07	-0.01	0.04	0.12	0.21
	p-value	0.24	0.21	0.04	0.65	0.65	0.97	0.81	0.47	0.20

Metabolites	Variable	SDNN	RMSSD	pNN50	30:15	E:I Ratio	LF	HF	LF:HF ratio	Valsal-va ratio
α-ketoglutarate	r	-0.26	-0.14	-0.22	-0.22	-0.14	-0.06	-0.21	-0.01	0.05
	p-value	0.10	0.38	0.18	0.17	0.38	0.73	0.20	0.95	0.75
Fumarate	r	-.379*	-.322*	**-.479****	-.391*	-0.29	0.08	-.317*	0.24	-0.10
	p-value	0.02	0.04	**0.00**	0.01	0.07	0.61	0.05	0.14	0.54
Lactate	r	-0.16	-0.12	-0.11	-0.16	-0.23	0.02	-0.02	0.15	-0.14
	p-value	0.34	0.47	0.52	0.34	0.16	0.92	0.90	0.36	0.41
Pyruvate	r	-.336*	-0.31	-0.31	-0.19	-0.29	-0.18	-0.24	0.12	-0.15
	p-value	0.03	0.05	0.05	0.23	0.07	0.28	0.13	0.47	0.36
Citrate/ Isocitrate	r	-0.28	**-.427****	**-.480****	**-.330***	-0.14	-0.03	-0.30	0.16	-0.21
	p-value	0.09	**0.01**	**0.00**	**0.04**	0.41	0.87	0.06	0.33	0.20
Flavin Adenine Nucleotide	r	0.23	0.06	0.08	-0.13	0.05	-0.13	-0.03	-0.19	0.31
	p-value	0.16	0.71	0.62	0.42	0.77	0.41	0.84	0.25	0.05
Hexose-6-Phosphate	r	0.08	-0.06	-0.03	0.16	0.04	0.10	.323*	0.01	-0.20
	p-value	0.64	0.73	0.86	0.34	0.79	0.55	0.04	0.95	0.21
Malate	r	-0.18	-0.19	-0.23	-0.17	-0.21	0.08	-0.13	0.01	-0.18
	p-value	0.27	0.24	0.16	0.30	0.19	0.64	0.43	0.96	0.27
Succinate	r	0.15	0.10	0.07	0.20	-0.02	0.22	0.14	0.04	-0.06
	p-value	0.36	0.54	0.66	0.22	0.90	0.18	0.38	0.81	0.71

N=40 patients; r- pearson Correlation Coefficient; yellow cells are significant negative correlation; orange cells are significant positive correlation. ** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed). LF: low-frequency power, HF: high-frequency power, E:I expiration inspiration ratio, SDNN: standard deviation of normal RR interval, RMSSD: root mean square of difference of successive normal RR interval.

Supplementary Table 3: Correlation of metabolites to cardiovascular autonomic neuropathy measures at three year follow up.

Baseline Metabolites	Variables	SDNN	RMSSD	pNN50	30-15	E:I Ratio	LF power	HF power	LF:HF ratio	Valsalva ratio
Alanine	r	-0.30	-0.26	-.346*	-0.12	-0.20	-0.16	-0.12	0.07	-0.25
	p-value	0.07	0.12	0.03	0.48	0.23	0.35	0.46	0.67	0.12
Glycine	r	-0.05	-0.07	-0.12	-0.05	-0.06	-0.07	0.00	-0.15	0.18
	p-value	0.78	0.69	0.46	0.76	0.74	0.70	1.00	0.39	0.29
Alpha-aminoisobutyric acid	r	-0.17	0.03	-0.17	0.07	-0.03	0.08	0.13	-0.02	-0.19
	p-value	0.32	0.85	0.32	0.69	0.86	0.63	0.45	0.88	0.26
Valine	r	-0.24	-0.20	-.325*	-0.02	-0.10	-0.02	-0.02	0.11	-0.08
	p-value	0.16	0.22	0.05	0.89	0.55	0.89	0.89	0.53	0.63
Leucine	r	0.03	0.05	-0.14	0.19	0.12	0.15	0.11	0.03	0.20
	p-value	0.84	0.79	0.39	0.26	0.47	0.38	0.52	0.86	0.23
Isoleucine	r	-0.05	-0.02	-0.18	0.08	0.10	0.10	0.08	0.27	-0.04
	p-value	0.79	0.89	0.29	0.62	0.54	0.54	0.65	0.11	0.83
Threonine	r	0.23	0.08	0.15	-0.05	0.24	-0.01	-0.04	0.31	0.29
	p-value	0.16	0.62	0.38	0.75	0.14	0.96	0.80	0.06	0.08
Serine	r	0.18	-0.02	0.06	-0.17	0.04	-0.11	-0.09	**.425****	.378*
	p-value	0.28	0.91	0.71	0.32	0.83	0.51	0.59	**0.01**	0.02
Proline	r	-0.06	-0.18	-0.23	-0.15	-0.19	-0.11	-0.13	.389*	-0.04
	p-value	0.75	0.29	0.17	0.37	0.26	0.50	0.44	0.02	0.79
Asparagine	r	0.30	0.19	0.22	0.08	0.27	0.08	0.06	0.19	0.15
	p-value	0.07	0.25	0.19	0.64	0.11	0.65	0.73	0.25	0.38
Aspartic acid	r	0.18	0.06	-0.10	0.12	0.15	0.19	0.05	0.01	0.06
	p-value	0.28	0.72	0.55	0.47	0.39	0.27	0.76	0.97	0.70
Methionine	r	0.15	-0.05	-0.07	-0.08	0.07	-0.04	-0.11	0.03	0.11
	p-value	0.39	0.79	0.67	0.62	0.66	0.80	0.52	0.86	0.51
4-Hydroxyproline	r	0.17	0.05	-0.01	-0.06	0.03	0.06	0.04	.330*	**.512****
	p-value	0.31	0.78	0.97	0.71	0.85	0.73	0.82	0.04	**0.00**
Glutamic acid	r	0.29	0.19	0.14	0.18	0.07	0.21	0.17	0.21	0.23
	p-value	0.09	0.25	0.40	0.27	0.68	0.22	0.31	0.21	0.17
Phenylalanine	r	0.18	-0.03	-0.08	0.02	0.01	-0.01	-0.09	0.29	0.27
	p-value	0.29	0.87	0.63	0.89	0.97	0.95	0.60	0.08	0.10
Glutamine	r	**.598****	0.24	.407*	0.14	0.26	0.08	0.04	.334*	0.18
	p-value	**0.00**	0.14	0.01	0.40	0.11	0.62	0.81	0.04	0.28
Ornithine	r	**.451****	**.442****	0.29	**.508****	.332*	**.448****	**.429****	0.11	0.08
	p-value	**0.01**	**0.01**	0.08	**0.00**	0.04	**0.01**	**0.01**	0.49	0.62
Lysine	r	0.17	-0.01	-0.11	0.08	0.04	0.00	-0.04	0.03	0.22
	p-value	0.32	0.95	0.50	0.62	0.79	0.99	0.80	0.86	0.18
Histidine	r	.328*	0.07	0.12	0.09	-0.01	-0.01	-0.08	0.15	0.16
	p-value	0.05	0.69	0.46	0.60	0.95	0.96	0.64	0.36	0.35
Tyrosine	r	0.27	0.02	0.05	0.00	-0.03	-0.13	-0.13	0.09	0.24
	p-value	0.11	0.92	0.79	0.99	0.88	0.45	0.43	0.58	0.15
Tryptophan	r	.363*	0.10	0.20	-0.08	0.07	-0.11	-0.08	0.09	0.30
	p-value	0.03	0.57	0.23	0.62	0.69	0.53	0.62	0.60	0.07

Baseline Metabolites	Variables	SDNN	RMSSD	pNN50	30-15	E:I Ratio	LF power	HF power	LF:HF ratio	Valsalva ratio
α-ketoglutarate	r	-0.09	-0.04	-0.11	-0.08	-0.10	-0.02	-0.02	0.04	-0.17
	p-value	0.59	0.81	0.50	0.63	0.55	0.93	0.93	0.82	0.30
Fumarate	r	-0.18	-0.20	-.346*	-0.11	-0.09	-0.06	-0.07	0.13	-0.16
	p-value	0.28	0.23	0.03	0.52	0.60	0.71	0.68	0.45	0.35
Lactate	r	0.07	-0.06	-0.06	-0.12	-0.03	-0.03	0.00	**.473****	-0.16
	p-value	0.69	0.73	0.70	0.48	0.84	0.85	1.00	**0.00**	0.35
Pyruvate	r	-0.19	-0.19	-0.15	-0.15	-0.22	-0.13	-0.14	0.21	-0.32
	p-value	0.26	0.25	0.37	0.39	0.19	0.44	0.41	0.20	0.05
Citrate/ Isocitrate	r	-0.06	-0.24	-.323*	-0.04	-0.02	-0.04	-0.15	**.463****	-0.19
	p-value	0.71	0.15	0.05	0.82	0.91	0.82	0.38	**0.00**	0.25
Flavin Adenine Nucleotide	r	-0.01	-0.13	-0.07	-0.15	-0.06	-0.29	-0.25	0.07	-0.04
	p-value	0.97	0.44	0.69	0.36	0.71	0.08	0.13	0.69	0.84
Hexose-6-Phosphate	r	0.26	0.14	0.09	.382*	0.20	.325*	0.18	-0.08	-0.17
	p-value	0.12	0.40	0.59	0.02	0.22	0.05	0.29	0.65	0.32
Malate	r	0.10	-0.08	-0.12	-0.01	-0.05	0.06	-0.03	**.573****	-0.28
	p-value	0.54	0.63	0.49	0.94	0.76	0.71	0.84	**0.00**	0.10
Succinate	r	0.15	0.11	0.07	0.10	0.08	0.12	0.08	0.07	-0.15
	p-value	0.38	0.52	0.66	0.57	0.65	0.46	0.65	0.66	0.38

N=40 patients; r- pearson Correlation Coefficient; yellow cells are significant negative correlation; orange cells are significant positive correlation. ** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed). LF: low-frequency power, HF: high-frequency power, E:I expiration inspiration ratio, SDNN: standard deviation of normal RR interval, RMSSD: root mean square of difference of successive normal RR interval.

Supplementary Table 4: Correlation of metabolites to change in cardiovascular autonomic neuropathy measures from baseline to three year follow up.

Baseline Metabolite	Variable	SDNN	RMSD	PNN50	LF Power	HF Power	LF:HF ratio	30:15 Ratio	E:I Ratio	Valsalva ratio
Alanine	r	-0.21	-0.12	-0.09	-0.23	-0.01	0.04	0.11	-0.02	-0.06
	p-value	0.22	0.48	0.59	0.17	0.94	0.80	0.49	0.89	0.75
Glycine	r	-0.05	0.06	0.01	-0.01	0.03	-0.20	-0.09	-0.03	-0.20
	p-value	0.77	0.72	0.97	0.97	0.85	0.24	0.59	0.85	0.25
Alpha-aminoisobutyric acid	r	0.07	0.26	0.17	-0.01	0.24	0.06	.363*	-0.02	-0.14
	p-value	0.69	0.11	0.31	0.96	0.15	0.72	0.03	0.90	0.41
Valine	r	-0.08	-0.06	-0.01	-0.08	0.08	0.11	0.19	0.04	-0.13
	p-value	0.65	0.71	0.95	0.65	0.65	0.50	0.25	0.79	0.47
Leucine	r	0.03	0.09	-0.03	0.02	0.19	-0.13	0.26	0.26	-0.07
	p-value	0.84	0.58	0.85	0.93	0.26	0.43	0.11	0.11	0.67
Isoleucine	r	0.11	0.10	0.08	0.03	0.19	0.17	0.25	0.23	-0.09
	p-value	0.52	0.57	0.64	0.85	0.26	0.30	0.13	0.16	0.62
Threonine	r	-0.02	0.00	-0.04	-0.08	-0.06	0.15	-0.22	0.05	-0.01
	p-value	0.92	0.98	0.83	0.61	0.72	0.37	0.19	0.78	0.96
Serine	r	0.04	-0.01	-0.07	-0.05	-0.12	0.31	-0.30	0.02	-0.15
	p-value	0.83	0.93	0.68	0.77	0.48	0.06	0.07	0.92	0.40
Proline	r	0.00	-0.12	-0.09	-0.13	-0.04	.363*	-0.02	0.00	0.00
	p-value	0.99	0.47	0.60	0.43	0.81	0.03	0.92	0.98	1.00
Asparagine	r	-0.04	0.10	0.01	-0.16	-0.07	0.10	-0.15	0.06	-0.17
	p-value	0.83	0.54	0.96	0.34	0.69	0.55	0.36	0.72	0.33
Aspartic acid	r	0.31	0.31	0.11	0.22	0.08	-0.04	0.12	0.16	-0.09
	p-value	0.07	0.06	0.53	0.19	0.62	0.79	0.48	0.35	0.60
Methionine	r	0.22	0.04	-0.07	-0.07	-0.07	-0.11	-0.14	0.13	-0.10
	p-value	0.18	0.83	0.67	0.66	0.68	0.52	0.41	0.43	0.55
4-Hydroxyproline	r	0.04	0.03	-0.06	0.08	0.08	0.22	-0.10	0.05	0.00
	p-value	0.84	0.86	0.71	0.61	0.62	0.19	0.53	0.76	0.98
Glutamic acid	r	0.13	0.05	-0.15	0.12	0.12	0.13	0.01	0.08	0.00
	p-value	0.43	0.77	0.38	0.47	0.47	0.45	0.97	0.64	0.99
Phenylalanine	r	0.09	0.05	-0.12	-0.03	-0.08	0.09	-0.01	0.08	-0.09
	p-value	0.61	0.77	0.46	0.85	0.62	0.59	0.97	0.64	0.61

Baseline Metabolite	Variable	SDNN	RMSD	PNN50	LF Power	HF Power	LF:HF ratio	30:15 Ratio	E:I Ratio	Valsalva ratio
Glutamine	r	0.16	0.09	0.08	-0.04	-0.20	.368*	-0.22	0.01	-0.03
	p-value	0.35	0.59	0.64	0.79	0.22	0.02	0.18	0.96	0.86
Ornithine	r	.371*	.466**	0.25	0.30	.342*	-0.02	.353*	.365*	-0.04
	p-value	0.02	0.00	0.14	0.07	0.04	0.92	0.03	0.02	0.84
Lysine	r	0.12	0.07	-0.13	-0.01	0.00	-0.14	0.10	0.16	-0.01
	p-value	0.47	0.68	0.44	0.94	0.98	0.39	0.55	0.35	0.94
Histidine	r	0.26	0.11	0.02	0.02	-0.17	0.18	-0.01	-0.04	-0.05
	p-value	0.12	0.53	0.92	0.91	0.30	0.27	0.96	0.82	0.79
Tyrosine	r	0.08	-0.07	-0.13	-0.25	-0.15	-0.07	0.04	0.04	0.20
	p-value	0.66	0.67	0.44	0.12	0.36	0.66	0.80	0.82	0.26
Tryptophan	r	0.21	-0.12	-0.15	-0.17	-0.14	0.01	-0.13	0.05	0.12
	p-value	0.21	0.47	0.38	0.30	0.41	0.96	0.43	0.75	0.48
α-ketoglutarate	r	0.21	0.09	0.03	0.11	0.12	0.05	0.11	-0.02	-0.04
	p-value	0.22	0.61	0.86	0.53	0.46	0.75	0.51	0.91	0.84
Fumarate	r	0.20	0.06	0.06	-0.11	0.13	0.00	0.21	0.19	-0.11
	p-value	0.24	0.71	0.72	0.50	0.43	1.00	0.20	0.25	0.55
Lactate	r	0.28	0.03	-0.04	-0.02	0.02	.344*	0.02	0.19	-0.09
	p-value	0.09	0.86	0.83	0.89	0.88	0.03	0.89	0.25	0.59
Pyruvate	r	0.19	0.08	0.15	0.08	-0.01	0.13	0.03	0.02	-0.04
	p-value	0.26	0.64	0.37	0.65	0.94	0.42	0.85	0.89	0.82
Citrate/ Isocitrate	r	0.30	0.20	0.19	0.01	0.00	.327*	0.20	0.09	-0.13
	p-value	0.07	0.22	0.25	0.94	0.98	0.04	0.22	0.60	0.47
Flavin Adenine Nucleotide	r	-0.04	-0.24	-0.18	-0.20	-0.29	0.16	-0.01	-0.25	0.06
	p-value	0.82	0.15	0.29	0.23	0.08	0.33	0.97	0.14	0.73
Hexose-6-Phosphate	r	0.31	.368*	0.24	.335*	0.03	-0.07	0.21	0.19	-0.06
	p-value	0.07	0.02	0.14	0.04	0.86	0.67	0.21	0.27	0.74
Malate	r	.412*	0.15	0.16	0.02	0.04	.495**	0.11	0.14	-0.07
	p-value	0.01	0.36	0.34	0.88	0.81	0.00	0.49	0.39	0.68
Succinate	r	0.13	0.07	0.01	-0.06	0.02	0.05	-0.04	0.05	-0.23
	p-value	0.46	0.66	0.95	0.72	0.93	0.78	0.79	0.76	0.18

N=40 patients; r- pearson Correlation Coefficient; yellow cells are significant negative correlation; orange cells are significant positive correlation. ** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed). LF: low-frequency power, HF: high-frequency power, E:I expiration inspiration ratio, SDNN: standard deviation of normal RR interval, RMSSD: root mean square of difference of successive normal RR interval.