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Exploratory metabolomics of nascent metabolic syndrome

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ABSTRACT

Introduction: Metabolic syndrome (MetS) is a disorder defined by having three of five features: increased waist circumference (WC), hypertriglyceridemia, decreased high-density lipoprotein-cholesterol, hypertension and an elevated blood glucose (BG). Metabolic Syndrome (MetS) affects 35% of American adults and significantly increases risk for Atherosclerotic cardiovascular disease (ASCVD) and type-2 diabetes (T2DM). An understanding of the metabolome will help elucidate the pathogenesis of MetS and lead to better management. We hypothesize that the metabolites, gamma-aminobutyric acid (GABA), D-pyroglutamic acid (PGA) and *N*-acetyl-D-tryptophan (NAT) will be altered in nascent MetS patients without the confounding of ASCVD or T2DM. We also correlated these metabolites with biomarkers of inflammation.

Patients and methods: This was an exploratory study of 30 patients with nascent MetS and 20 matched controls undertaken in 2018.

Metabolites were evaluated from patient's frozen early morning urine samples and were correlated with biomarkers of inflammation and adipokines. They were assayed by the NIH Western Metabolomics Center using liquid chromatography/mass spectrometry and standardized to urinary creatinine. All patients had normal hepatic and renal function.

Results: GABA and PGA levels were significantly increased in MetS patients compared to controls: 2.8-fold and 2.9-fold median increases respectively with $p < 0.0001$ and $p = 0.004$, possibly deriving from glutamate. NAT was significantly decreased by 90% in MetS patients compared to controls, $p < 0.001$. GABA correlates significantly with cardio-metabolic (CM) features including WC, blood pressure systolic (BP-S) while NAT correlated inversely with WC, BP-S, blood glucose (BG) and triglycerides (TG). GABA correlated positively with chemerin, leptin, Fetuin A and endotoxin. NAT correlated inversely with WC, BP-S, BG, TG, high sensitivity C - reactive protein (hsCRP), toll-like receptor-4 (TLR-4), lipopolysaccharide binding protein (LBP), chemerin and retinol binding protein-4 (RBP-4).

Conclusions: We make the novel observation of increased GABA and PGA with decreased NAT in patients with MetS. While GABA and PGA correlates positively with CM features and biomediators of inflammation, the metabolite NAT correlated inversely. Thus, GABA and PGA could contribute to the pro-inflammatory state of MetS while NAT could mitigate this pro-inflammatory response.

1. Introduction

Metabolic syndrome (MetS) is a common disorder defined by having three of five features including increased waist circumference, hypertriglyceridemia (>150 mg/dL), decreased high-density lipoprotein (HDL)-cholesterol, elevated blood pressure (>130/85) and on antihypertensive medication and an elevated blood glucose (>100 mg/dL).[1,2] This disorder affects 35% of American adults and confers an increased risk of both ASCVD and diabetes, which makes the underlying dysfunctions in MetS significant to elucidate. Studies have shown that low grade inflammation and insulin resistance are pivotal players in the pathogenesis of MetS.[3,4] In previous studies we have carefully documented that MetS is a pro-inflammatory state manifest by both circulating and cellular biomarkers of inflammation.[3,4] Also, to better understand the dysregulation of the metabolome we have examined amino acid levels and have recently shown increased levels of Isoleucine and a profound decrease in lysine in these patients, both correlating with biomarkers of inflammation.[5] In this report we present data on 3 additional metabolites, NAT, GABA, PGA.

Conflict of Interest Statement: The authors have no financial or other conflicts of interest to disclose.

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2. Methods

2.1. Patients and methods

Subjects' with age ranges from 24 to 72 years were recruited from Sacramento County, CA via public advertisements such as newspapers. MetS (n = 30) without confounding factors such as CVD and T2DM and healthy controls (n = 20) were evaluated.[3–5] The Adult Treatment Panel 3 criteria defines MetS as having 3 of the 5 following features: increased triglycerides, low HDL-cholesterol, plasma glucose of 100–125 mg/dL, increased waist circumference and hypertension. The selected control patients had <2 features of MetS, a fasting plasma glucose below 100 mg/dL, triglycerides lower than 200 mg/dL, and were not taking blood pressure medications. Both groups met the following exclusion criteria: diabetes (fasting plasma glucose <126 mg/dL and HbA1c <6.5%), clinical atherosclerosis (coronary artery disease, peripheral artery disease, or cerebral-vascular disease), smoking, hypo-or hyperthyroidism, malabsorption, anticoagulant therapy, steroid therapy, anti-inflammatory drugs, statin and other hypolipidemic therapy, hypoglycemic agents, ARBs, TG > 400 mg/dL (for MetS subjects), oral contraceptives, use of antioxidant supplements in the past 6 months, pregnancy, abnormal complete blood count and alcohol consumption >1 oz/day, postmenopausal women on estrogen replacement therapy, recent surgery, inflammatory or malignant disease, leukocytosis and hsCRP >10 mg/l, chronic high intensity exerciser (exercise>100 min per week).

The Institutional Review Board at University of California, Davis approved the study. Fasting blood was collected after patient history and a physical examination was completed. Using standard laboratory techniques in the Clinical Pathology Laboratory, the following labs were completed: a complete blood count, plasma lipid and lipoprotein profile, urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, glucose and TSH. Insulin levels, IL-6, IL-8, LBP, nitrotyrosine and leptin were assayed by ELISA (Linco Biosystems) and HOMA-IR was calculated from glucose and insulin levels. Another screening was completed and a select number of subjects UC Davis CCRC for fasting blood draw of basic chemistries, HOMA and CRP and an early morning urine sample.

Early morning urine samples frozen at −70 °C were used from patients who meet the criteria for MetS and matched controls to evaluate the metabolites. All patients had normal hepatic and renal function and all metabolites are expressed as a ratio to urine creatinine to correct for urine concentration. The NIH West Coast Metabolomics Center quantified the samples by employing Gas chromatography time-of-flight mass spectrometry (GC-TOF MS) following standardized technique.[5] Samples were extracted by cold isopropanol/acetonitrile/water, dried and derivatized by trimethylsilylation to increase the volatility for GC-TOF MS data acquisition. Metabolites were identified by retention time to external standards and quantified by peak heights. Metabolites were normalized to urine creatinine levels. After metabolite quantification, we compared the following salient characteristics of MetS and inflammatory parameters to control subjects: sex, age, waist circumference, BMI, systolic and diastolic blood pressures, glucose, lipid profile, HOMA-IR, HbA1C hsCRP, IL-1b, IL-6, TNF, IL-8, monocyte TLR-2 & TLR-4, sTNFR1, sTNFR2, endotoxin, chemerin, leptin and adiponectin. The biomarkers of inflammation have been reported in previous communications on this cohort.[3–5] Thereafter we compared gamma-aminobutyric acid (GABA), D-pyroglutamic acid (PGA), and *N*-acetyl-D-tryptophan (NAT) levels in urine samples of nascent MetS compared to matched controls. We also analyzed correlations between these metabolites to inflammatory biomediators.

2.2. Statistical analysis

For the aforementioned variables, results are expressed as mean and standard deviation (SD) or as median and interquartile ranges for skewed variables. Log transformation was applied to variables with skewed distributions prior to parametric analyses. Wilcoxon Rank Sum test or two-sample *t*-tests were used to compare the control and metabolic syndrome groups. Analysis of covariance was used to adjust for age, BMI, and waist circumference. The association between continuous variables was assessed with Spearman rank correlation coefficients by combining the control and metabolic syndrome groups. General statistical analysis was performed using SAS statistical software (SAS Institute, Cary, NC). The level of significance for hypothesis testing was a two-sided alpha of 0.05 unless pre-specified differently. Adjustment for multiple testing was employed as appropriate using a Bonferroni method (e.g., Bonferroni-Holm) or adjustments via simulation to control the type I error rate. Data transformations or nonparametric tests were employed if needed to meet analysis assumptions.

3. Results

In our studied population, we saw a significant increase in waist circumference, BMI, systolic blood pressure, glucose, triglycerides, HOMA-IR, HbA1c and hsCRP levels in MetS patients when compared to controls; the only significant decreased levels in MetS patients was for HDL-cholesterol (Table 1). There was an increase in biomarkers of inflammation including IL-1b, IL-6, sTNFR1 ($p = 0.05$), sTNFR2 and endotoxin, monocyte-TLR-4, chemerin and leptin for the MetS patients (Table 2).

Fig. 1 depicts our 3 metabolites. GABA and PGA levels were significantly increased in MetS patients compared to controls: 2.8-fold and 2.9-fold median increases respectively with $p < 0.0001$ and $p = 0.004$. NAT was significantly decreased by 90% in MetS patients compared to controls, $p < 0.001$. While there were no significant differences in L-tryptophan levels between groups ($p = 0.41$), Glutamate levels were significantly decreased in patients with MetS compared to controls; median and interquartile ranges of 0.4(0.3–0.6) versus 2.3(1.1–3.6) respectively, $p = 0.0001$.

GABA had a significant positive correlation with the CM features of waist circumference, and systolic blood pressure (Table 3). GABA also correlated significantly with the following inflammatory markers and adipokines: endotoxin, chemerin, leptin and SAT secreted Fetuin-A. PGA correlated positively with IL-6, leptin, SAT-secreted Fetuin-A, Nitrotyrosine.

NAT correlated inversely with CM features of waist circumference, BMI, BP-systolic, BP-diastolic, glucose, triglycerides, and HbA1c.

Table 1
Baseline characteristics for MetS and control patients.

Variable	Controls (n = 20)	MetS (n = 30)	p-Value controls vs MetS*
Sex, F/M	17/3	22/7	0.5
Age (yrs)	48 ± 13	53 ± 9	0.16
Waist (cm)	92 ± 14	109 ± 14	**0.0002**
BMI (kg/m^2)	30.2 ± 5.6	35.1 ± 6.3	**0.01**
BP-systolic (mm Hg)	117 ± 12	132 ± 11	**0.0006**
BP-diastolic (mm Hg)	74 ± 9	80 ± 9	0.1
Glucose (mg/dL)	86 ± 8	98 ± 13	**0.003**
Total cholesterol (mg/dL)	193 ± 25	195 ± 31	0.85
Triglycerides (mg/dL)	63 (48–92)	147 (103–177)	**0.0002**
HDL-cholesterol (mg/dL)	52 ± 12	40 ± 11	**0.002**
LDL cholesterol (mg/dL)	126 ± 20	123 ± 23	0.85
HOMA-IR	1.6 (1.1, 2.8)	2.8 (1.9, 5.8)	**0.003**
HbA1C (%)	5.2 ± 0.4	5.7 ± 0.4	**0.007**
hsCRP (mg/L)	1.3 (0.4, 3.2)	4.0 (2.2, 5.8)	**0.005**

Results are presented as mean ± standard deviation or median (25th percentile, 75th percentile).

* p-Value for sex from Fisher's Exact test, p-values for continuous variable from the Wilcoxon Rank Sum test.

Table 2
Biomarkers of inflammation and adipokines for MetS and control patients.[3–5]

Variable	Controls (n = 20)	MetS (n = 30)	p-Value controls vs MetS*
IL-1b (pg/mL)	599 (412, 845)	879 (728, 1056)	**0.02**
IL-6 (pg/mL)	991 ± 595	1896 ± 421	**0.0001**
TNF (pg/mL)	366 ± 172	495 ± 421	0.92
IL-8 (pg/mL)	746 (524, 1256)	945 (746, 1975)	0.06
TLR-2 (MFI/106 cells)	26 ± 12	33 ± 18	0.28
TLR-4 (MFI/106 cells)	24 ± 12	41 ± 23	**0.002**
sTNFR1 (pg/mL)	930 ± 210	1163 ± 218	**0.05**
sTNFR2 (pg/mL)	1847 ± 345	2314 ± 415	**0.02**
Endotoxin (EU/mL)	4.0 ± 0.6	12.3 ± 2.9	**0.0003**
Chemerin (ng/mL)	277 ± 66	374 ± 61	**0.002**
Leptin (ng/mL)	37.5 (31, 55)	80 (51, 106)	**0.01**
Adiponectin (μg/mL)	6.3 (4.5, 12)	5.4 (3.3, 8.4)	0.31

Results are presented as mean ± standard deviation or median (25th percentile, 75th percentile).
* p-Value for sex from Fisher's Exact test, p-values for continuous variable from the Wilcoxon Rank Sum test.

NAT also correlated inversely with hsCRP, IL-6 ($p = 0.05$), monocyte-TLR-4, chemerin, RBP4 and LBP.

4. Discussion

In this report we document further abnormalities in the metabolome of nascent MetS. We show a profound decrease in NAT and increases in both GABA and PGA. Furthermore we show important correlations with biomarkers of inflammation and adipose tissue dysregulation.

NAT was first reported in humans by Pavlova et al in 2017 when they quantified 8 metabolites of tryptophan in urine.[6] They showed that NAT was a product of the host-microbial metabolism and was detected in the urine of pregnant women at micro-molar levels. Additionally, NAT was shown to be a possible biomarker to assess the status of the gut microbiota.[7] This study showed that treatment with piperacillin/tazobactam caused a disruption in colonization resistance, illustrated by, a 10-fold increase in NAT in the urine samples of mice during recovery phase of the microbiota. They concluded that NAT is useful in indicating an intact colonization resistance. From this study we can understand the decreased levels of NAT as a marker of compromised gut colonization resistance during the pathophysiology of MetS. There is a confirmed link between the state of the gut microbiome and the pro-inflammatory state of MetS as reviewed recently.[8] This inflammatory process upsets the balance of the gut microbiota causing endotoxemia, as well as altered levels of bacteria such as *Bacteriodes*, *Clostridia*, and Proteobacteria, etc. We documented in MetS patients that there are higher levels of endotoxin/LBP, which suggest greater gut microbiota disruption with potential increased gut permeability compared to controls. Our negative correlations of LBP and NAT further underscores that NAT is a potentially useful marker of gut microbiota disruption and endotoxemia in MetS patients.[4]

Recent studies have shown NAT to have anti-inflammatory as well as antioxidant properties in-vitro. *N*-acetyl kynureneine (NAK) is a major degradation product of NAT, was found in low molecular weight fraction of 5% human serum albumin (LMWF5A).[9] LMWF5A has recently been shown to have anti-inflammatory effects such as decreasing TNF release in LPS stimulated human peripheral blood mononuclear cells (PMBC) and increasing prostaglandin release to resolve inflammation with the PMBC model.[9] This paper highlights that NAK inhibits the

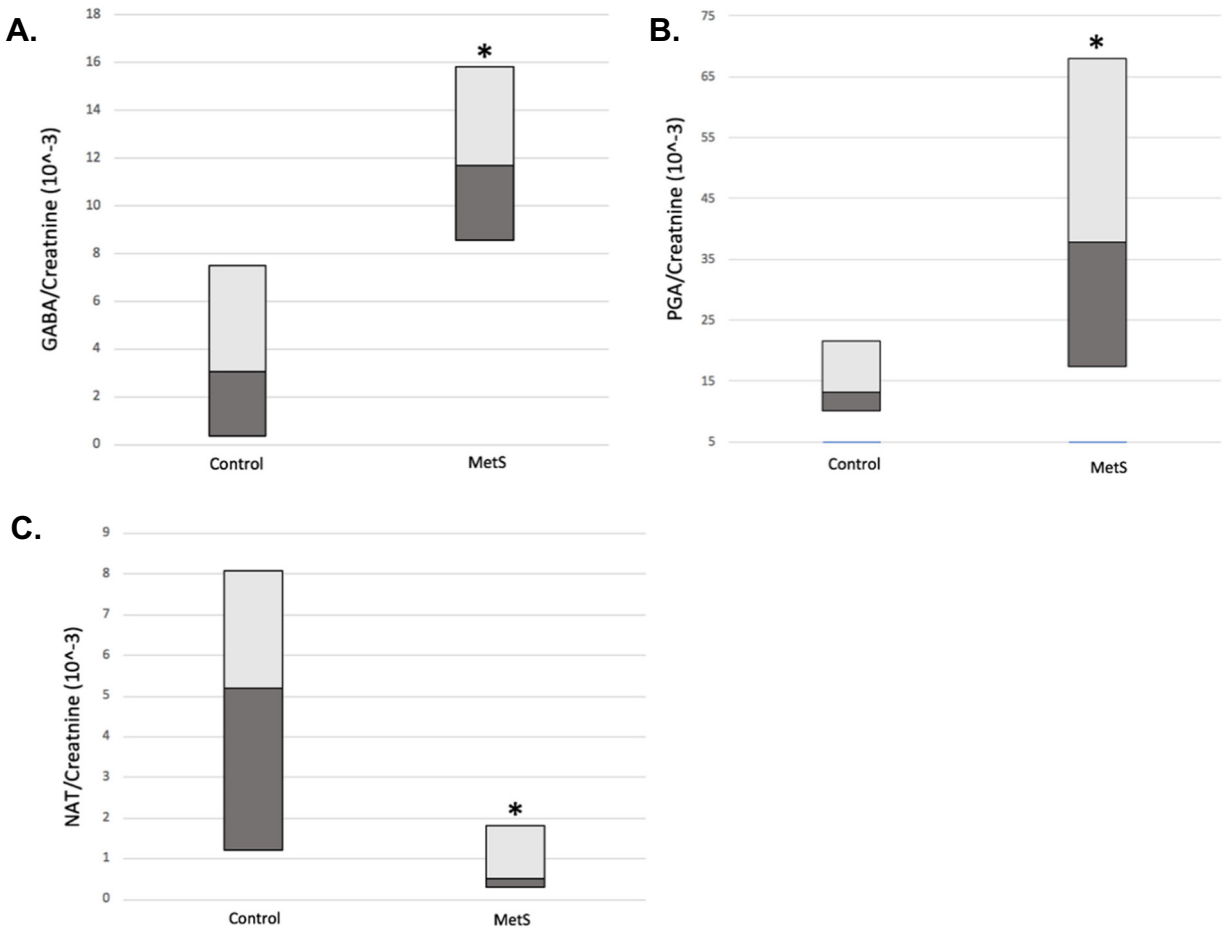

Fig. 1. This figure depicts the 3 metabolites (A) GABA, (B) PGA (C) NAT. The comparisons between controls and MetS patients are shown. Asterisks denote significance between groups.

Table 3
Relevant correlations of metabolites.[3–5]

Variable	GABA (r value, p value)	D pyroglutamic acid (r value, p value)	*N*-acetyl D_tryptophan (r value, p value)
Waist circumference (cm)	**(0.40, 0.005)**	(0.27, 0.06)	**(−0.42, 0.003)**[***]
BMI (kg/m^2)	(0.21, 0.19)	(0.23, 0.13)	**(−0.32, 0.04)**
BP-systolic (mm Hg)	**(0.34, 0.02)**	(0.24, 0.10)	**(−0.49, 0.0004)**
BP-diastolic (mm Hg)	(0.21, 0.17)	(0.26, 0.09)	**(−0.32, 0.04)**
Glucose (mg/dL)	(0.19, 0.19)	(0.05, 0.74)	**(−0.32, 0.03)**
Total cholesterol (mg/dL)	(−0.15, 0.32)	**(−0.31, 0.03)**	(−0.11, 0.47)
Triglycerides (mg/dL)	(0.29, 0.06)	(0.04, 0.81)	**(−0.45, 0.002)**
HDL cholesterol (mg/dL)	(−0.26, 0.084)	(−0.12, 0.44)	(0.28, 0.06)
LDL cholesterol (mg/dL)	(−0.22, 0.13)	**(−0.32, 0.03)**	(−0.04, 0.81)
HOMA-IR	(0.26, 0.090)	(0.02, 0.90)	(−0.20, 0.20)
HbA1C (%)	(0.18, 0.3)	(0.16, 0.38)	**(−0.46, 0.01)**
hsCRP (mg/dL)	(0.24, 0.10)	(0.09, 0.56)	**(−0.33, 0.02)**
IL-1b (pg/mL)	(0.23, 0.13)	(0.13, 0.41)	(−0.09, 0.55)
IL-6 (pg/mL)	(0.26, 0.091)	**(0.32, 0.04)**	**(−0.30, 0.05)**
TNF (pg/mL)	(−0.22, 0.14)	(−0.06, 0.71)	(0.01, 0.95)
IL-8 (pg/mL)	(0.18, 0.24)	(0.03, 0.83)	(−0.07, 0.63)
TLR-2 (MFI/106 cells)	(0.01, 0.95)	(−0.01, 0.94)	(−0.21, 0.18)
TLR-4 (MFI/106 cells)	(0.19, 0.23)	(−0.18, 0.27)	**(−0.33, 0.03)**
sTNFR1 (pg/mL)	(0.22, 0.35)	(−0.07, 0.75)	(−0.07, 0.75)
sTNFR2 (pg/mL)	(0.18, 0.44)	(−0.06, 0.79)	(−0.15, 0.52)
Endotoxin (EU/mL)	**(0.47, 0.02)**	(0.35, 0.08)	(−0.22, 0.29)
LBP	(0.32, 0.060)	(0.18, 0.31)	**(−0.44, 0.01)**
Chemerin (ng/mL)	**(0.39, 0.02)**	(0.26, 0.14)	**(−0.37, 0.03)**
Leptin (ng/mL)	**(0.45, 0.01)**	**(0.36, 0.03)**	(−0.22, 0.20)
Adiponectin (μg/mL)	(−0.13, 0.45)	(0.00052, 0.10)	(0.21, 0.23)
FetuinAng_gFat	**(0.39, 0.03)**	**(0.50, 0.004)**	(−0.18, 0.33)
RBP4	(0.09, 0.58)	(0.04, 0.84)	**(−0.45, 0.01)**
Nitrotyro_Hep	(0.20, 0.29)	**(0.44, 0.01)**	(−0.21, 0.25)

[***] Bolded values indicate p-values <0.05.

pro-inflammatory cytokine IL-6 and pro-inflammatory chemokine CXCL-10 release from the activated macrophages. Since LMWF5A inhibits certain TLRs and we showed an inverse correlation between NAT and TLR4 this could be advanced as the potential mechanism. NAT has also shown to have antioxidant properties in an in-vitro study by Dion et al[10] Using 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) as the generator of oxidative stress, the authors demonstrated the antioxidant effect of NAT.[10]

N-acetyl tryptophan (NAT) was found in decreased quantity in the MetS patients compared to control subjects and had negative correlations with 4 out of the 5 criteria of MetS: TG, Glucose, WC, BP as well as a trend with the last criterion, HDL-cholesterol ($r = 0.28$, $p = 0.06$). Such decreased levels of NAT with anti-inflammatory and antioxidant properties, makes it an attractive biomarker in the genesis of the MetS given the crucial role of inflammation in MetS. Furthermore, we can speculate, given the surging interest in ant-inflammatory therapies in cardio-metabolic disorders that if further studies confirm that indeed NAT is anti-inflammatory in-vivo, it could offer another welcome therapeutic strategy.

The two metabolites that were found in increased quantities in the MetS patients GABA and PGA, had positive correlations with both cardio-metabolic features and inflammatory biomarkers (Table 3).

We make the novel discovery that GABA was increased and shown to have pro-inflammatory effects that can promote the pathogenesis of MetS. In the published literature GABA was shown to have both pro and anti-inflammatory properties. It was found to regulate the secretion of cytokines from human CD4 T-Cells by regulating levels of both TH1 and Th2 responses. Depending on the concentration of GABA it was observed that at levels of <100 nM GABA had pro-inflammatory effects which increased the release of cytokines, while at GABA levels >500 nM there were anti-inflammatory effects that inhibited the release of cytokines.[11] GABA works by preventing B cells apoptosis. GABA also has been shown to stimulate the release of insulin and c-peptide.[12] In another study it was observed that GABA could improve insulin resistance in streptozotocin-induced diabetic rats that were given a high fat diet. GABA did this by increasing GLUT4 and by decreasing the gluconeogenesis pathway. There are many studies on the importance of GABA in pancreatic cells and its protective effect in diabetes. Our study is novel in that it shows a positive correlation between GABA and inflammatory markers suggesting that it could have pro-inflammatory effects in humans, outside of the pancreatic cells.[13]

Recently, in an elegant study Ikegama et al showed that increased GABA in brown adipose tissue (BAT) of obese mice promotes BAT dysregulation.[14] They showed that GABA feeding resulted in an increase GABA in BAT, whitening of BAT, decreased Uncoupling Protein 1 (UCP1) expression in BAT and worsened glucose intolerance. Also using a knockout mice model of GABA receptor signaling they could abrogate all these effects. Finally in 19 patients with BMI ranging from 18 to 31 they showed that in supraclavicular BAT, higher GABA levels was associated with lower levels of UCP-1. Overall increased GABA, per this study, was shown to have a deleterious effect on the protective effect of brown adipose tissue against MetS and obesity.[14] Our data in human MetS showed that GABA was increased and has pro inflammatory effects adding a translational advance to their study. Collectively these 2 studies suggest that GABA should be further investigated as a metabolite that could be crucial pivot in obesity and metabolic syndrome.

Data on the metabolite, D-pyroglutamic acid (PGA), is scanty. Notably, there was a study that aimed to examine key metabolites related to weight reduction by studying individuals who participated in a dietary intervention with black soybean peptides for 12 weeks.[15] The participants had a reduction in BMI, body fat, triglyceride levels and an increase in HDL-cholesterol levels. A study of their sera showed a significant increase of multiple metabolites, including PGA. In our study, there was a significant increase in PGA in the urine of MetS patients when compared to controls which appeared to be pro-inflammatory. Given the paucity of data, much further work is need to draw any form conclusions with regards to PGA.

Given the significantly lower levels of glutamate in patients with MetS we can speculate that conversion to both GABA and PGA (both increased) could explain in part this decrease in glutamate.[16,17]

5. Conclusion and study limitations

In conclusion, in this exploratory study of the metabolome, we make the novel observation of increased GABA and PGA and decreased NAT in patients with MetS. While GABA and PGA correlates positively with CM features and biomediators of inflammation, the metabolite NAT correlated inversely. Thus, GABA and PGA could contribute to the pro-inflammatory state of MetS and NAT could mitigate the inflammatory response. However our exciting findings in a well-defined cohort need to be validated in studies with larger sample sizes.

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