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ORIGINAL ARTICLE

Free-amino acid metabolic profiling of visceral adipose tissue from obese subjects

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Abstract

Interest in adipose tissue pathophysiology and biochemistry have expanded considerably in the past two decades due to the ever increasing and alarming rates of global obesity and its critical outcome defined as metabolic syndrome (MS). This obesity-linked systemic dysfunction generates high risk factors of developing perilous diseases like type 2 diabetes, cardiovascular disease or cancer. Amino acids could play a crucial role in the pathophysiology of the MS onset. Focus of this study was to fully characterize amino acids metabolome modulations in visceral adipose tissues (VAT) from three adult cohorts: (i) obese patients (BMI 43–48) with metabolic syndrome (PO), (ii) obese subjects metabolically well (O), and (iii) non obese individuals (H). 128 metabolites identified as 20 protein amino acids, 85 related compounds and 13 dipeptides were measured by ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) and gas chromatography-/mass spectrometry GC/MS, in visceral fat samples from a total of 53 patients. Our analysis indicates a probable enhanced BCAA (leucine, isoleucine, valine) degradation in both VAT from O and PO subjects, while levels of their oxidation products are increased. Also PO and O VAT samples were characterized by: elevated levels of kynurenine, a catabolic product of tryptophan and precursor of diabetogenic substances, a significant increase of cysteine sulfinic acid levels, a decrease of 1-methylhistidine, and an up regulating trend of 3-methylhistidine levels. We hope this profiling can aid in novel clinical strategies development against the progression from obesity to metabolic syndrome.

Keywords Obesity · Metabolomics · Body mass index · Metabolic syndrome · Branched chain amino acids · Histidine · Tryptophan · Adipose tissue

Abbreviations

BMI	Body mass index
HDL	High-density lipoprotein
GC/MS	Gas chromatography/mass spectrometry
LC/MS/MS	Liquid chromatography/mass spectrometry
RF	Random forest analysis
H	Healthy
O	Obese
PO	Pathological obese
BKCDH	Branched-chain alpha-keto acid dehydrogenase complex
IR	Insulin resistant
TCA	Tricarboxylic acid cycle
BCAA	Branched chain amino acids
MS	Metabolic syndrome
KYN	Kynurenine
KP	Kynurenine pathway
KYNA	Kynurenic acid
XA	Xanturenic acid
CSAD	Cysteine sulfinic acid decarboxylase
T2D	Type 2 diabetes
TDO	Tryptophan 2,3 dioxigenase
IDO	Indoleamine 2,3 dioxigenase
KMO	Kynurenine-3-monooxigenase
VAT	Visceral adipose tissue

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GAD-65 Glutamic acid dehydrogenase-65/GAD2
3-HKYN 3-Hydroxykynurenine

Introduction

Obesity is a multifactorial disease involving, with overweight, over a third of the world population. It has been estimated that, in 2015, a high body mass index (BMI) caused 4 million deaths worldwide due to the associated co-morbidities such as type 2 diabetes mellitus, cardiovascular disease, cancer, hypertension, and chronic kidney disease (Ng et al. 2014; Afshin et al. 2017). Some of these disorders define also the metabolic syndrome (MS), a mild-pathological condition characterized by three of following criteria: central/visceral obesity, hypertriglyceridemia, high fasting glucose levels, low levels of serum high-density lipoprotein (HDL) and hypertension. People with metabolic syndrome are considered at high risk of developing type 2 diabetes and/or cardiovascular disease. However, despite over nutrition and the presence of visceral fat, a subpopulation of obese individuals does not show symptoms of MS and indeed is metabolically healthy. Patients with obesity and type 2 diabetes have been the subject of a large number of plasma metabolic profiling with the purpose to further explore the pathophysiology of excess body fat, fostering the discovery of useful biomarkers predictive of obesity associated metabolic disorders and thus attain more effective therapies (Libert et al. 2018). In this context amino acids are interesting molecules to investigate because they act either as substrates in protein synthesis and also as metabolic modulators. A number of observational works have revealed positive association between variations in plasma amino acid concentration (including BCAA) and systemic pathological condition like obesity, insulin resistance (IR) and diabetes (Felig et al. 1969; Fiehn et al. 2010; Wurtz et al. 2013). Additionally a large body of literature indicates that various lipid molecules (including fatty acids) are involved in the onset and development of metabolic syndrome and its associated comorbidities. (Lopategi et al. 2016; Candi et al. 2018). Taken together these findings suggest that lipids and amino acids may act synergistically in promoting poor glycemic control and insulin resistance (Newgard et al. 2009) and prompted us to investigate the free-amino acid metabolic profiling of human visceral adipose tissue taken from healthy subjects and an obese cohort stratified following clinical diagnosis of metabolic syndrome. We have previously profiled the metabolomic composition of visceral adipose tissues (VAT) from an obese cohort (Candi et al. 2018) where we focused on markers of oxidative stress, elevated glucose levels and lipid pathway involved into insulin resistance and inflammation. As a follow up of our previous work, here in we investigated the principal amino acid variations detected in visceral fat samples from three cohorts: healthy obese (O), pathological obese (PO), and healthy (H). We aimed to identify altered amino acid profile reflecting the specific adipose tissue metabolism differences in the context of obesity and insulin resistance states.

Materials and methods

Study population

In our research 53 patients, hospitalized at the surgical Unit of the University of Rome Tor Vergata for bariatric or general surgery, were considered (Table S1). The project was approved by the Medical Ethics Committee of the Institution and before the study all participants signed a written and informed consent. Patients were divided into three study groups as indicated in Table S2. Group 1: 17 healthy (H) subjects, BMI = 25.31 ± 0.91, normal waist circumference, matched to the obese groups for approximate age and sex. Group 2: 18 obese patients without metabolic syndrome, indicated as obese (O). Group 3: 18 patients with obesity-related metabolic syndrome indicated as pathologically obese, PO. Pathological obese subject were defined according to the National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) (Grundy et al. 2004). Inclusion criteria of patients with Metabolic Syndrome (PO) were: waist circumference over 40 inches (men) or 35 inches (women), blood pressure over 130/85 mmHg, fasting triglyceride level over 150 mg/dl, fasting high-density lipoprotein (HDL) cholesterol level less than 40 mg/dl (men) or 50 mg/dl (women) and fasting blood sugar over 100 mg/dl. Subjects were between 21 and 73 years old while mean ages were 55.1 years for H, 43 years for O and 51.8 years for PO patients.

Preliminary tests like physical examination, ECG, chest X-ray and routine chemical analyses were performed on all subjects. Healthy subjects did not show any present or past history of chronic systemic condition, diabetes, hyperlipidemia and hypertension. Patients did not follow particular diet prior to surgery. Subjects with history of drug or alcohol dependence, chronic infection, autoimmune inflammatory disease and were not included in the study. Additional demographic and clinical characteristics of population study are reported in Tables S2 and S3.

Sample preparation

Samples preparation was conducted as previously described (Candi et al. 2018). Briefly, adipose tissue was collected during surgery. A flap of adipose tissue of about 2 cm, in the periphery of the greater omentum, was lifted, divided and delivered to the assistant researcher, present in the operating

room during the surgical procedure. The specimens were collected in falcon tubes, inventoried and immediately stored at – 80 °C. Metabolon LIMS system was used to uniquely identify and track each sample throughout processing and analysis. Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes.

Metabolomic analysis

The extracted samples were divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. For further details on data quality and process variability, pathway enrichment analysis see the "Supplementary Materials and Methods" section previously described (Candi et al. 2018).

Pathway enrichment analysis

For each individual pair-wise comparison, pathway enrichment displays the number of experimentally regulated compounds relative to all detected compounds in a pathway, compared with the total number of experimentally regulated compounds relative to all detected compounds in the study. Enrichment: [# of significant metabolites in pathway (*k*)/total # of detected metabolites in pathway (*m*))/(total # of significant metabolites (*n*)/total # of detected metabolites (*N*)] (k/m)/(n/N).

Results

Clinical parameters and global metabolic analysis

53 adult patients (Table S1) were classified into three groups according to clinical parameters indicated in Table S2: Group 1: 17 healthy (H), Group 2: 18 healthy obese (O) and Group 3: 18 pathologically obese (PO). Healthy (H) subjects were used as controls, matched to the obese groups for comparable age and sex. Patients with and without metabolic syndrome were indicated as obese (O) and pathologically obese (PO) respectively. PO patients showed increased waist circumference 18/18, lipid abnormalities 17/18, hypertension 7/18 and impaired glucose tolerance 12/18 (Table S3).

128 metabolites identified as 20 protein amino acids, 85 related compounds and 13 dipeptides were measured by ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) and gas chromatography/mass spectrometry GC/MS, (Candi et al. 2018). A selection of the free-metabolites obtained, is shown in Table 1 (see Table S4, for complete data analysis). Random forest analysis was performed in order to display segregation between the three groups and resulted in 71% and 81% predictive accuracy in discriminating the (O) and (PO) VAT samples respectively from (H) VAT samples from healthy controls (H) (Supplementary Fig. S1). Predictive accuracy of RF classification between (O) and (PO) was 69% and reflected a more limited segregation. Change in metabolites was evaluated as PEVs obtained via the differential enrichment pathways among groups (Table 1 and Supplementary Table S4).

BCAA catabolism is increased in pathologically obese samples

Our results show a significant down regulation of valine, isoleucine ($0.05 > p > 0.10$) and leucine ($p \leq 0.05$) in visceral fat of PO subjects when compared with their H counterparts suggesting increased BCAA catabolism in PO samples. Similar decrease in BCAA has been observed in O vs. H samples ($p \leq 0.05$) (Table 1, Fig. 1a–c). We noted that BCAA concentration is slightly higher in PO vs. O samples but still significantly lower than H subjects. Notably, consistent this hypothesis, the reduction in BCAA levels is accompanied by an up regulation of 2-methylbutyrylcarnitine (C5) ($0.05 > p > 0.10$) in PO vs. H subjects and also in PO vs. O subjects ($p \leq 0.05$) (Fig. 1g). The same up regulating trend in PO vs. H and in PO vs. O subjects is observed for isovalerylcarnitine (C5) and isobutyrylcarnitine (C4) although not significantly (Table 1). Levels of ethylmalonate also, a by-product of isoleucine catabolism (Linster et al. 2011), were higher in PO vs. H subjects even if not significantly (Table 1). These BCAA oxidation catabolites were rather down regulated in O vs. H samples, although modulation did not reach statistical significance (fold change 0.695–0.894). A significant decrease of the three BCKDH substrates, 4-methyl-2-oxopentanoate (from Leu), 3 methyl-2-oxovalerate (from Ile), 3-methyl-2-oxobutyrate (from Val) was also observed in PO and O samples ($p \leq 0.05$) (Fig. 1d–f). These results seem to indicate an upregulated catabolism of BCAA in PO samples.

Tryptophan metabolism is altered in the adipose tissue of pathological obese subjects

Here we detected differential levels of tryptophan metabolites originating from the Kynurenine pathway (KP), the main tryptophan oxidation route (Fig. 2a). Higher levels of KYN are significantly detected in PO tissue samples when compared to H samples (fold change 1.72; $p \leq 0.05$) (Table 1, Fig. 2c). VAT from O patients showed higher levels of KYN compared to H samples albeit without

Table 1 Fold of change values from selected aminoacid pathways

		Fold of change		
Sub pathway	**Biochemical name**	**O/H**	**PO/H**	**PO/O**
Histidine metabolism	Histidine	0.7062	0.7551	1.0692
	1-Methylhistidine	0.5112	0.787	1.5395
	3-Methylhistidine	0.5376	1.6279	3.0279
	N-acetylhistidine	0.4828	0.5344	1.107
	Trans-urocanate	0.4751	0.5733	1.2067
	Imidazole propionate	0.2156	0.2953	1.3699
	Imidazole lactate	0.7729	0.796	1.03
	Carnosine	0.6305	0.8878	1.4081
	Histamine	0.3986	0.4691	1.1768
	1-Methylhistamine	0.709	0.829	1.1692
	1-Methylimidazoleacetate	0.8125	0.87	1.0707
	4-Imidazoleacetate	0.4971	0.5865	1.18
Tryptophan metabolism	Tryptophan	0.5275	0.5753	1.0906
	3-Indoxyl sulfate	0.4444	0.5112	1.1504
	N-Acetyltryptophan	1	1	1
	Indolelactate	0.6208	1.0285	1.6567
	Indoleacetate	0.2327	0.3191	1.3712
	Kynurenine	1.2821	1.7243	1.345
	Tryptophan betaine	0.8434	0.4657	0.5522
	C-glycosyltryptophan	0.5425	0.8094	1.4919
Leucine, isoleucine and valine metabolism	Leucine	0.575	0.6958	1.2101
	4-Methyl-2-oxopentanoate	0.5342	0.5159	0.9657
	Isovalerylcarnitine (C5)	0.8974	1.3325	1.4848
	Beta-hydroxyisovalerate	0.6913	0.7674	1.1101
	Isoleucine	0.6965	0.8213	1.1792
	3-Methyl-2-oxovalerate	0.5354	0.6516	1.2171
	Alpha-hydroxyisovalerate	0.9214	1.0579	1.1481
	2-Methylbutyrylcarnitine (C5)	0.8822	1.6346	1.8529
	Tiglylcarnitine (C5:1-DC)	0.797	0.8357	1.0486
	Ethylmalonate	0.6952	1.3389	1.926
	Valine	0.5599	0.7374	1.3171
	3-Methyl-2-oxobutyrate	0.4338	0.4894	1.1282
	Isobutyrylcarnitine (C4)	0.8666	1.2876	1.4858
Methionine, cysteine, SAM and taurine metabolism	Methionine	0.5946	0.6702	1.1271
	N-acetylmethionine	0.5919	0.9071	1.5326
	N-formylmethionine	0.5385	0.5958	1.1065
	Methionine sulfone	0.4	1.0846	2.7116
	N-acetylmethionine sulfoxide	0.59	0.9439	1.5998
	S-adenosylhomocysteine (SAH)	0.8635	0.9169	1.0618
	Cysteine	0.8414	1.1103	1.3195
	S-methylcysteine	0.5285	0.5003	0.9466
	Cystine	0.6051	1.2316	2.0352
	Cysteine sulfinic acid	1.5687	1.5083	0.9615
	Hypotaurine	0.6916	0.6004	0.8681
	Taurine	0.9549	1.0796	1.1306
	N-acetyltaurine	0.714	0.7469	1.046

Values in the table indicate ratio

Green: ($p \leq 0.05$) and light green ($0.05 > p > 0.10$) indicate negative significant differences between groups shown, metabolite ratio < 1.00. Red: ($p \leq 0.05$) and pink: ($0.05 > p > 0.10$) indicate positive significant differences between groups shown, metabolite ratio ≥ 1.00. Non-colored: mean values are not significantly different for that comparison. See Supplementary Fig. S3 for further details

statistical significance. Furthermore, KYN metabolite is present in higher concentration in PO vs. O samples, highlighting the implication of the KP in the pathogenesis of T2 diabetes and thus in the metabolic syndrome which

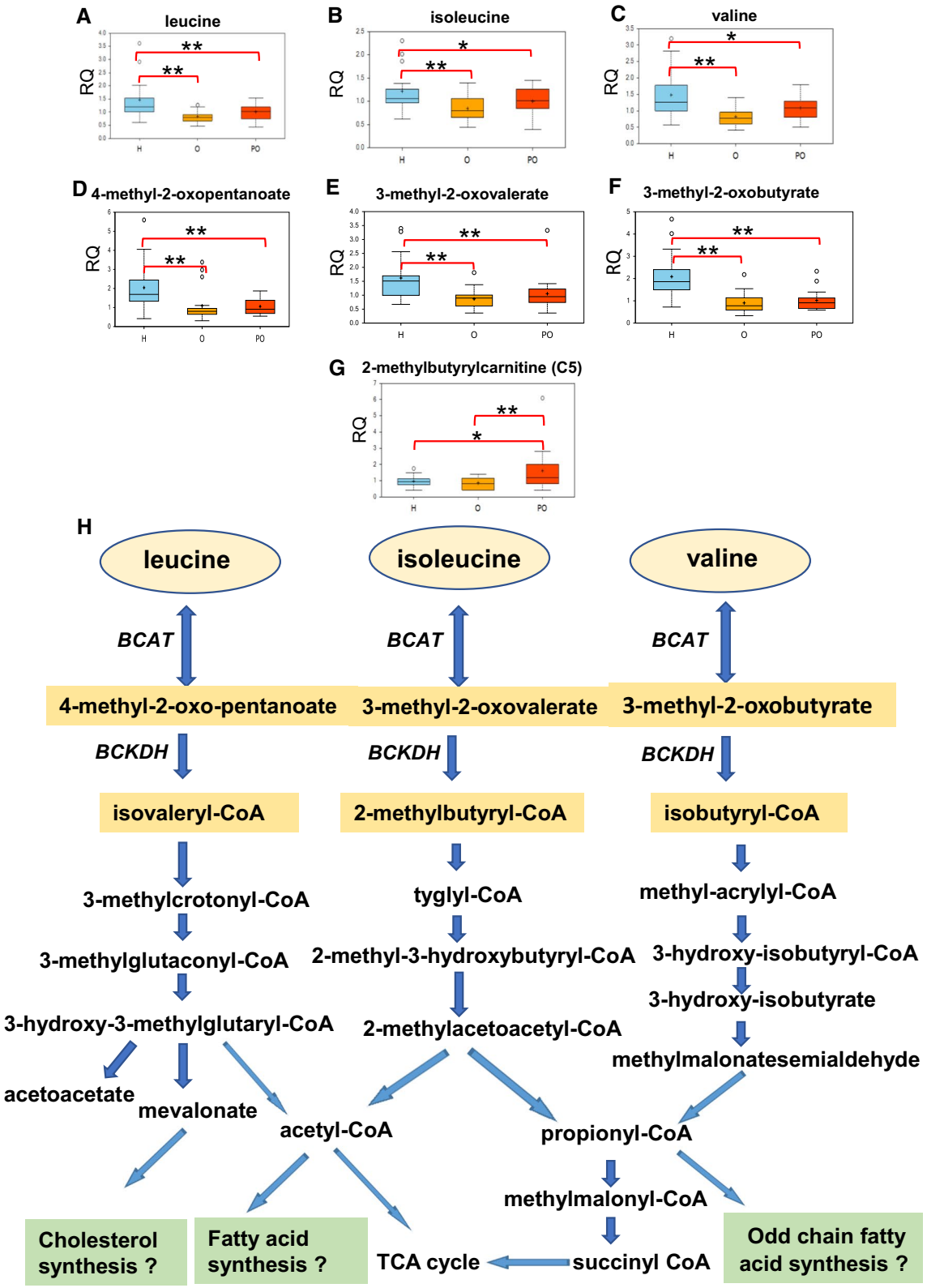


Fig. 1 BCAA catabolism is increased in patologically obese samples. Box-Whisker plots of visceral adipose tissue BCAA and their catabolic products levels show significant modulations between groups. **a**–**c** BCAA levels are decreased in PO and O vs. H; **d**–**f** levels of BCKDH substrates are decreased in PO and O vs. H; **g** one BCKDH product is increased in PO vs. H samples; **h** BCAA catabolic pathway, yellow shaded compounds are connected to the cited metabolites in the text. *Indicates ANOVA contrast p-value $= 0.05 > p > 0.10$. **Indicates ANOVA contrast p-value $= p \leq 0.05$

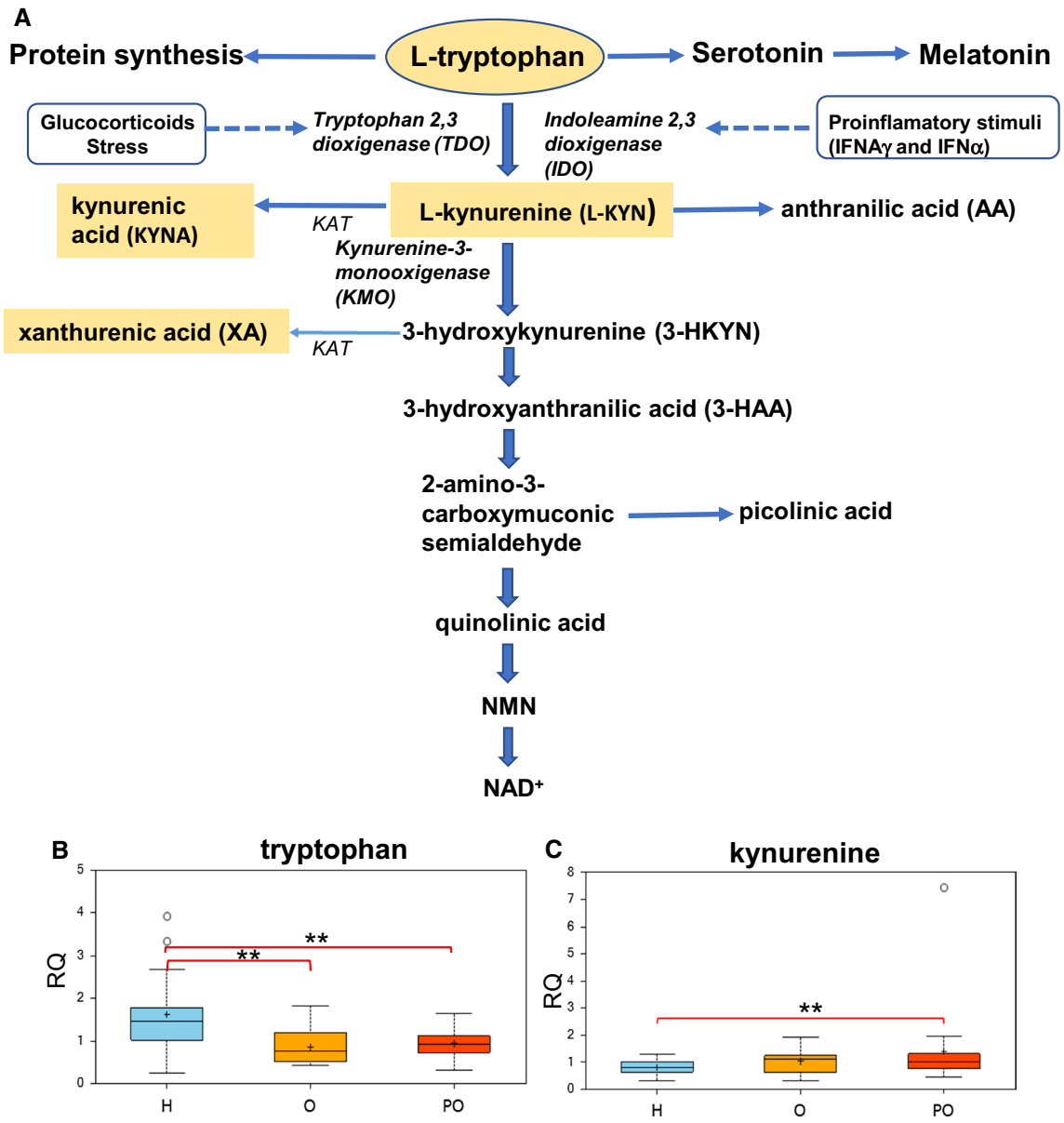


Fig. 2 Tryptophan catabolism in increased in obese and in pathologically obese samples. Box-Whisker plots of visceral adipose tissue tryptophan and of kynurenine levels show significant modulations between groups. **a** Tryptophan catabolic pathway, yellow shaded compounds are connected to the cited metabolites in the text; **b** Tryptophan levels are decreased and **c** kynurenine levels are increased in O and PO compared to H subjects. *Indicates ANOVA contrast p-value $= 0.05 > p > 0.10$. **Indicates ANOVA contrast p-value $= p \leq 0.05$

identifies PO subjects. Tryptophan levels are found significantly reduced in O and PO samples when compared to H samples (Table 1, Fig. 2b), (fold change 0.5275–0.5753; $p \leq 0.05$). This result is not unexpected since one of the KP function is to clear excess of plasma TRP (Kanai et al. 2009). In this analysis, adipose tissue from O and even more from PO subjects shows to be a production site of the tryptophan related diabetogenic precursor KYN and perhaps also a contributor to the homeostasis of plasma TRP concentration.

Methionine, cysteine, SAM, and taurine metabolism are altered in the adipose tissue of obese subjects

Cysteine sulfinic acid is an intermediate product in the synthesis of taurine from cysteine (Sörbo and Ewetz 1965) (Fig. 3a). Its levels are significantly increased both in PO and O vs. H samples ($p \leq 0.05$) (Table 1, Fig. 3d) and this may suggest a lower activity of the cysteine sulfinic acid decarboxylase (CSAD) (Fig. 3a). According to this hypothesis, levels of the CSAD product hypotaurine, are significantly

decreased in PO and O samples vs. H ($p \leq 0.05$, fold change 0.6–0.69) (Fig. 3f, Table 1). We may thus speculate that CSAD activity and by consequence, levels of cysteine sulfinic acid might be related with obesity and type 2 diabetes although we cannot make any significant assumption regarding the molecular nature of such relationship.

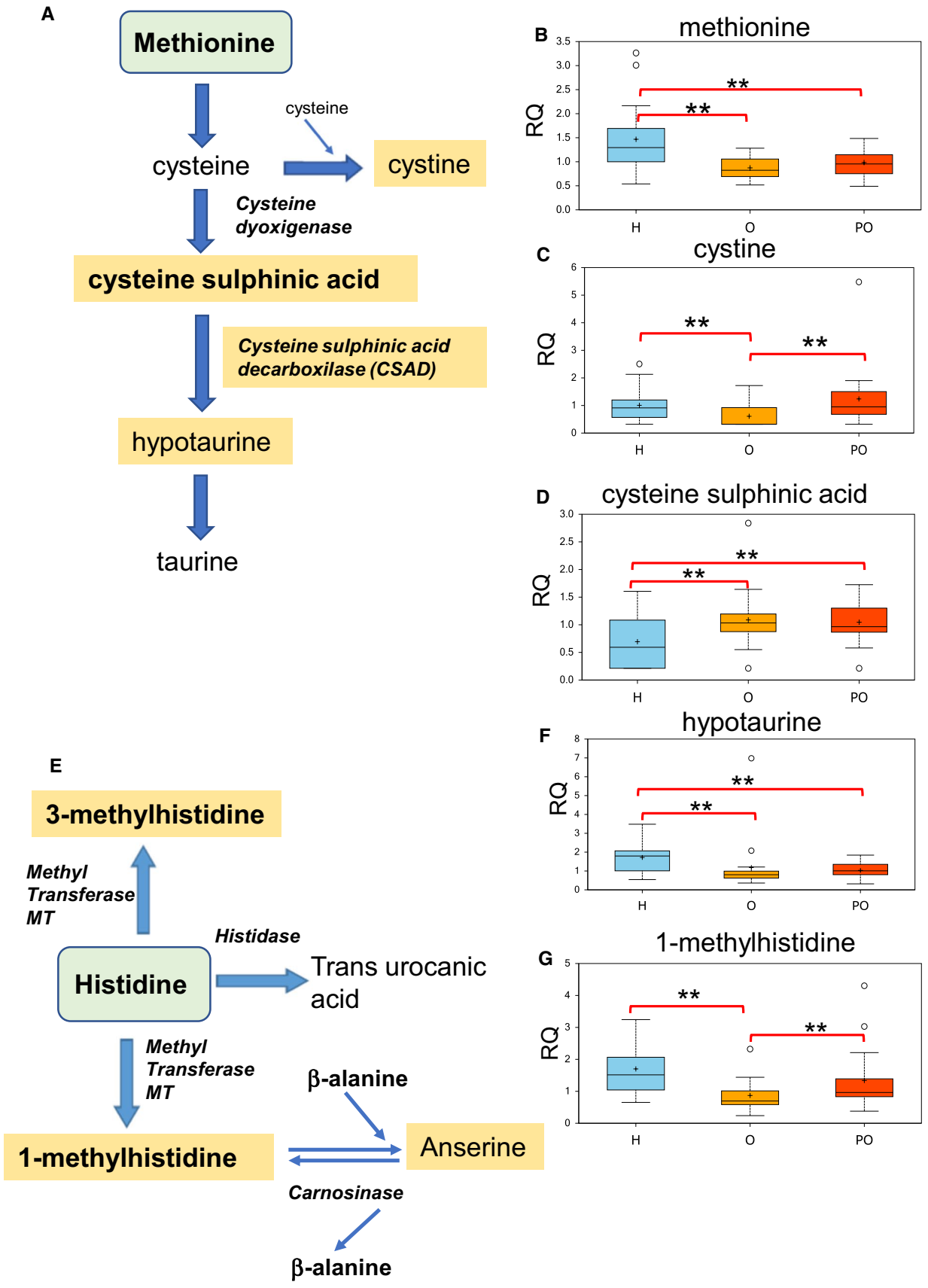
Cystine levels are also slightly elevated in PO vs. O samples ($p \leq 0.05$), and in PO vs. H albeit not significantly (Fig. 3a, c) (Table 1). High levels of cystine might reflect high levels oxidative stress (Samiec et al. 1998).

Histidine metabolism variation in the adipose tissue of obese subjects

1-methyl-histidine (1-MH), is a metabolite produced only in animals and not in humans and it is present in lower concentration in O and PO samples respect to H subjects ($p \leq 0.05$ and $0.05 > p > 0.10$) (Fig. 3e, g; Table 1). 3-methylhistidine (3-MH) instead is present in muscle proteins and its levels are up regulated in adipose tissue of PO vs. H samples, although without statistical significance. The metabolic evaluation of these two compounds might evidence elevated muscle protein turnover (Kochlik et al. 2018) or sarcopenia (Elia et al. 1981; Kim et al. 2010).

Discussion

Obese people can be classified into two weight independent categories: those without metabolic dysregulation (O) and those characterized by metabolic syndrome and higher level of inflammatory markers (PO). This distinction underlines the importance of investigating the body's differential metabolic response to prolonged excess calories intake leading to the development of chronic low-grade inflammation, T2D and IR. Plasma amino acid levels are often evaluated for this purpose (Libert et al. 2018). We also believe that investigating the amino acid metabolome in the context of the dysfunctional adipose tissue would provide valuable insights in the detection of altered biochemical pathways involved in obesity and metabolic unwellness. Thus, herein we report free amino acids metabolic profiling of visceral adipose tissue from obese subjects with and without metabolic syndrome and from the healthy relative counterpart. Increased plasma levels of amino acids and specifically of BCAAs, seems to be a characteristic of insulin resistance state in adult obese humans (Newgard et al. 2009). In longitudinal studies, elevated plasma BCAA levels were able to predict the onset of diabetes with 12 year-advance (Wang et al. 2011). The biochemical reason for plasma BCAA increase in insulin resistant states has not yet been clarified, and it is still a matter of debate whether BCAA are a contributing factor in insulin resistance and T2DM or just a marker of the suboptimal insulin action. Conversely to the data obtained in the literature with plasma samples, we observe a down-regulation of the three BCAA levels in the visceral adipose tissue of PO and O vs. H patients and an up regulation of their metabolic derivatives, the short chain acyl-carnitines, in PO vs. H patients. At tissue level, the concentrations of BCAA are determined by the difference between the rates of events that cause their accumulation and those that facilitate their disappearance. Thus dietary intake, tissue proteolysis, protein synthesis and BCAA catabolism, all contribute to tissue BCAA concentration which by consequence affects also plasma BCAA levels. It is likely, however, that white adipose tissue, regulating BCAA catabolism, may collaborate in maintaining physiological concentrations of BCAA in plasma (Herman et al. 2010; Zimmerman et al. 2013). The elevated acyl-carnitines that we report on PO subjects highlight an up regulation of BCAA catabolism in dysregulated VAT, at least through the BCKDH step included. BCAA catabolism starts with the mitochondrial isoform of BCATm (branched-chain amino transferase) that generates branched chain ketoacids (BCKAs). These molecules are then the substrates of the BCKA dehydrogenase (BCKDH) complex, the rate limiting enzyme of BCAA catabolism which produces acyl-CoA esters (Harris et al. 2005). Defective expression/quantity of BCAA degradation enzymes in liver and adipose tissue of rodents models have been considered as partially responsible of elevated plasma BCAA levels usually associated with obesity, diabetes and insulin resistance (She et al. 2007; Herman et al. 2010). In adipose tissue BCAT2 transcript levels did not differ between adipocytes from lean and obese Pima Indians (Lackey et al. 2013). At protein level, obesity related changes of BCAT2 found in fed state, disappeared in adipose tissue from fasted obesity model ob/ob and Zucker rodents (She et al. 2007). Thus BCAT2 does not seem to be consistently downregulated in obese rodents and humans. On the opposite, activities of the BCAA rate limiting step BCKDH have been found to be reduced in several tissues of obese compared to lean Zucker rats (She et al. 2013), and BCKDH expression was downregulated in adipose tissue of ob/ob mice (Zhou et al. 2019). Thus, our results seems in contradiction with previous studies where, a lower expression of BCAA catabolic enzymes has been reported in adipose tissue of obese humans (Pietiläinen et al. 2008). However, the relationship between BCAA catabolic enzymes levels and global rate of BCAA oxidation is still a point of discussion. In male obese Zucker rats, the rate of whole body leucine oxidation was 60% higher when compared to lean animals, (She et al. 2013) although important declines of BCKDH activity were observed in kidney, heart, and muscle, but not in liver. Also, in some circumstances, food intake might be also a contributing factor to elevated plasma level of BCAA. In fact, in Zucker rats obesity model, the absolute plasma BCAA increase is modest and tends


A
Methionine
cysteine
cysteine
cystine
Cysteine dyoxigenase
cysteine sulphinic acid
Cysteine sulphinic acid decarboxilase (CSAD)
hypotaurine
taurine
B
methionine
RQ
H
O
PO
C
cystine
D
cysteine sulphinic acid
E
3-methylhistidine
Methyl Transferase MT
Histidase
Histidine
Trans urocanic acid
Methyl Transferase MT
β-alanine
1-methylhistidine
Anserine
Carnosinase
β-alanine
F
hypotaurine
G
1-methylhistidine

◂**Fig. 3** Methionine and Histidine metabolisms are altered in obese and in pathologically obese samples. Box-Whisker plots of visceral adipose tissue methionine and histidine metabolites show significant modulations between groups. Yellow shaded compounds are connected to the cited metabolites in the text. **a** Pathway for the synthesis of taurine from methionine; **b** methionine levels are decreased in both in O and PO samples vs. H; **c**, **d** cystine and cysteine sulphinic acid levels are increased in PO vs. O and H samples; **e** Histidine dipeptide metabolism; **f**, **g** levels of hypotaurine and 1-methylhistidine are decreased both in O and PO samples compared to H; *Indicates ANOVA contrast p-value $= 0.05 > p > 0.10$. **Indicates ANOVA contrast p-value $= p \leq 0.05$

to decrease or even disappear under fasting conditions. In adipose tissue of ob/ob mice and Zucker rats, BCKDH protein levels were reduced in obese compared to their lean controls, but these changes decreased after an overnight fast. Levels of BCKDH inactivating kinase BCKD were obesity stimulated in ob/ob mice but not in fasted Zucker rats (She et al. 2007). On the other hand, most of the studies analysing BCAA plasma levels, have considered human or rodents in the overnight fasted state, suggesting that a more complex system regulates plasma concentrations of BCAA. However, despite the great deal of data evidencing a lower global expression of some of the BCAA catabolic enzymes in adipose tissue from various obese and insulin-resistant rodent models, it has not been defined if said changes correspond to lower BCAA utilization and catabolism in VAT from humans. In (visceral) omental adipose tissue of obese women with metabolic syndrome, the mRNA levels of BCKDA and BCKDB (two enzymes of the BCKDH complex) were lower than those of healthy obese women of equal weight (men were not examined), although no information was provided on mRNA levels of lean healthy women counterpart (Lackey et al. 2013). It is worth noting that the mechanism that causes these alterations in the BCAA catabolizing genes remains unknown. Most of these studies have been conducted using excellent animal models of obesity and although they provide an in-depth view of the metabolism of BCAA in adipose tissue, they may not conclusively demonstrate that the observed mechanisms are also applicable to human subjects at the population level.

In our analysis, in case of BCKDH down regulation in PO samples, we would expect substrate build up and thus elevated levels of the three BCKAs. On the contrary we observed a significant decrease of the three BCKDH substrates, 4-methyl-2-oxopentanoate (from Leu), 3 methyl-2-oxovalerate (from Ile), 3-methyl-2-oxobutyrate (from Val) in PO and O samples (Fig. 1d–f; Table 1), and a concomitant upregulation in PO samples of the respective BCKDH products, isovaleryl-carnitine (C5), 2-methylbutyryl-carnitine (C5) and isobutyryl-carnitine (C4). Catabolism of Ile and Val produces also propionyl-CoA that is converted to a C3 acyl-carnitine (Fig. 1h), Table 1). Acyl-carnitines allow the excess of Acyl-CoAs to cross the mitochondrial membrane and be transported out of the mitochondria and cells. Elevated muscle and serum levels of acyl-carnitines which derive from incomplete fatty acid oxidation process have been consistently associated with insulin resistance and obesity (Koves et al. 2008; She et al. 2013). Indeed, in skeletal muscle, over nutrition and high fat feeding have been proposed to promote an increase of β–oxidation of fatty acids that is not matched to a parallel upregulation in TCA cycle enzymes (Muoio and Newgard 2008; Koves et al. 2008). As a consequence, in mitochondria, accumulation of incomplete oxidized lipid by-products like acyl-carnitine is observed. These activated lipid species might cause dysfunctional insulin signalling through mitochondrial stress and impaired GLUT4 translocation (Muoio and Newgard 2008). Specifically, in adipocytes BCAA derived Acetyl-CoA and succinyl-CoA may fuel the TCA cycle o be utilized as substrates for the synthesis of other amino acids or lipids including odd chain fatty acids and cholesterol (Fig. 1h) (Roberts et al. 2009; Halama et al. 2016; Green et al. 2016). Moreover, the specific accumulation of C4 and C5 acyl-carnitines that we detect in PO samples, has been reported also in muscle and plasma from insulin resistant rodents as a combined effect of BCAA addition in the context of HF diet, and in plasma of insulin resistant humans (Newgard et al. 2009). Thus we can propose that, in obesity pathological setting, the likely increased flux of BCAA catabolism in VAT might contribute to the mitochondrial stress leading to global insulin resistance, through the production of an excess of C4 and C5 acyl-carnitines. The mechanism through which an overproduction of these metabolic intermediates can interfere with the oxidation of fatty acids and insulin resistance has yet to be unveiled. It also is likely that our data possibly reflects the peculiar metabolism of adipocytes in PO samples where increased production of BCAA catabolites may supply precursors for the synthesis of fatty acids and cholesterol which could act as inflammation/stress-inducing molecules in VAT (Candi et al. 2018).

Alternatively, such increase in BCAA derived acyl-carnitines found in our PO subjects might also be due a reduced activity of an enzyme located downstream of the BCKDH step in the catabolic BCAA pathway. However this is a rather unlikely hypothesis since after the decarboxylation step by BCKDH, catabolism of the three BCAA is divergent, at least through the formation of the final short chain CoA-esters that can enter the TCA cycle or follow anabolic metabolism. Unfortunately, our targeted metabolomic analysis does not allow us to evaluate such downstream products, dissect the catabolic pathway and draw conclusions. We cannot exclude, however, that the increase in C4 and C5 acyl-carnitine detected in adipose tissue of PO subjects could be of ectopic origin. In this case, the blunted BCAA catabolism generally observed in adipose tissue in insulin resistant states might be associated with an increased BCAA

catabolic flux in other tissues as it has recently detected by Neinast et al. (2019). Therefore, an inter-tissue exchange of C4, C5 acyl-carnitines might justify the rise in these metabolites levels in our PO vs. H samples.

One of the most prominent hypotheses on the molecular origin/consequence of T2D implies metabolites from the catabolic pathway of tryptophan (TRP). Kynurenine (KYN) is a pivotal compound of this pathway and, in the present metabolomic analysis is up regulated in O and more clearly in PO samples vs. H samples. This may potentially be an effect of the obesity-linked inflammation state, typical of PO subjects. In fact stress and/or inflammatory factors seem to play a crucial role in up regulating the first two enzymes of the KP: the hepatic TRP-2,3 dioxygenase (TDO) or extra hepatic indoleamine 2,3 dioxygenase (IDO1), and KYN-monooxygenase (KMO) with consequent increased levels of their products, KYN and 3-hydroxykynurenine (3-HKYN) respectively (Fig. 2a) (Connick and Stone 1985; Alberati-Giani et al. 2002). KYN and 3-HKYN generate, in turn, KYNA and XA, two diabetogenic metabolites. KYNA, KYN and XA exert their diabetogenic action throughout several mechanisms, like impairment of insulin biosynthesis or activity (Oxenkrug 2015). Elevated levels of XA have been found in the urines of T2D patients, compared to healthy people (Hattori et al. 1984). Oxenkrug recently confirmed these data by detecting higher concentration of KYN, XA, and KYNA in plasma of 30 T2D patients compared to 24 healthy controls (Oxenkrug 2015). Similar results were obtained in a larger cohort of T2D patients and controls. Moreover, up regulation of several KP enzymes in visceral adipose tissue of obese women well correlated with their BMI (Favennec et al. 2015). We might speculate that if the dysfunctional adipose tissue proves to be a circulating kynurenine production site, it may eventually establish a vicious cycle where the expanding adipose tissue promotes insulin resistance and diabetes which in turn support hyperphagia and obesity.

Activation of KP increases plasma KP-metabolites/TRP ratio and it is important also for plasma TRP clearance and homeostasis. The relevant reduction of TRP levels in both O and PO subject vs. H samples that we observed is in total agreement with an increase of KYN levels in O and PO vs. H samples (Fig. 2b, c). Indeed, when KP was impaired in KO mice, plasma and hepatic TRP concentration raised sharply up to 20 fold and 15 fold, compared to WT mice (Kanai et al. 2009). Up regulation of TDO and/or IDO1 provokes a sharp decrease in plasma TRP levels which is likely to subtract Trp bio-availability to the brain for serotonin synthesis, whose low levels in CNS are suspected to be involved in major depressive disorder (MDD), a pathological condition often associated to metabolic syndrome (Badawy 2013; Opel et al. 2015). IDO1 activation may act as a double edged-sword since elicits both diabetogenic metabolites synthesis as well as protective mechanism against over-activation of the immune system by stimulating the differentiation of regulatory T cell (Treg) (Mellor and Munn 2004).

The up regulation of cysteine sulfinic acid, an intermediate of taurine synthesis, that we noted in PO and O samples (Fig. 3a, b) could potentially unveil a deficiency in the activity of CSAD, the enzyme that transform cysteine sulfinic acid in hypotaurine that is then oxidized to taurine.

Curiously, CSAD enzyme shows 50% amino acid sequence identity with GAD-65 (Glutamic acid dehydrogenase-65/GAD2). Anti CSAD antibodies cross reacts with GAD-65, a target of auto antibodies in T1D (Baekkeskov et al. 1990), and have also been found in sera of patients with autoimmune polyendocrine syndrome type 1 (APS1) (Sköldberg et al. 2004). A recent GWAS screening in Japanese population found a primary association between a polymorphic site within one intronic region of CSAD gene and susceptibility to fulminant type 1 diabetes (Kawabata et al. 2019). It is important to note, however, that at present it is not possible to infer any significant association and/or a biochemical link between T2D and cysteine sulfinic acid and its decarboxylase.

Cystine derives from the oxidation of two cysteine residues and its increased levels may uncover oxidative stress or very early renal dysfunction in PO patients (Samiec et al. 1998; Pastore et al. 2015). These data are in agreement with our previous study where increased levels of oxidative stress markers characterized VAT from PO samples (Supplementary Fig. S2) (Candi et al. 2018).

1-methylhistidine (1-MH) and 3-methylhistidine (3-MH) are two compounds produced by methylation of histidine in muscles protein. 1-MH derives from the degradation of the dipeptide anserine and is synthesized in animals but not in humans (Fig. 3e, g) (Butt and Fleshler 1965; Elia et al. 1981; Kochlik et al. 2018). Thus 1-MH concentration is independent of muscle proteins turnover and mainly reflects meat intake (Hiroki et al. 1993). In our analysis 1-MH is significantly present in lower concentration in O samples when compared to H subjects and also in PO vs. H subjects (although not significantly). These values may be due to a diet based on lower meat consumption in O and PO subjects if compared to H. The metabolite 3-MH is found in fibrillar proteins actin and myosin and is considered a plasma and urine biomarker of muscle breakdown, although it may derives also from meat and fish intake. Moreover, by evaluating both 1-MH and 3-MH concentration in urine and/or blood samples it is possible to extrapolate the endo/exogenous origin of 3-MH. Here, we found that 3-MH levels are up regulated in adipose tissue of PO vs. H samples, although values did not reach statistical significance. This results might suggests that 3-MH levels in PO patients are probably due to inter-tissue exchange due to elevated muscle protein turnover (She et al. 2013; Kochlik et al. 2018) or to

sarcopenia, an age associated decline of muscle and strength that is often reported to be associated with type 2 diabetes (Smith et al. 1989; Kim et al. 2010, 2013; Zhou et al. 2013). We might speculate that increased protein turnover sustains elevated plasma BCAA levels in obesity states, while increased BCAA oxidation in some tissues, might lower those elevations. However, most studies are observational only and a thorough investigation is needed to clarify the relationship between insulin resistance, obesity and protein turnover.

Conclusions

Our analysis indicates that amino acid metabolism is altered in dysfunctional adipose tissue**.** In contrast with the up regulating trend of BCAA levels often observed in plasma samples from patients with obesity and metabolic syndrome, BCAA are significantly decreased in visceral adipose tissue of O and PO patients. Upregulation of C4 and C5 acylcarnitines observed in PO subjects, suggest an increased BCAA catabolic flux under pathological conditions. Tryptophan oxidation in adipose tissue of PO subjects produces elevated levels of kynurenine, a precursor of diabetogenic substances frequently associated to T2D metabolic setting. A marked decrease of 1-methylhistidine characterized obesity. Levels of cysteine sulfinic acid are increased in both PO and O vs. H samples, while cystine is up regulated in metabolic unwellness only. These alterations suggest that amino acid metabolism in deranged adipose tissue should be further investigated in humans and strengthens the concept of fat tissue as a site of direct observation of the metabolic variations implicated in obesity and its associated co morbidities. We believe that future research and insights on the adipose tissue metabolites and their related pathways that in our analysis are associated to poor metabolic health, such as kynurenine or cysteine sulfinic acid, could potentially unveil new specific roles of these compounds during adipogenesis and adipose tissue progression towards a state of unwellness. This valuable information might support the discovery of more effective therapies, biomarkers and diet prevention strategies to fight obesity and its progression towards metabolic syndrome.

Strengths and limitations

The main strength of this study is the evaluation of levels of protein amino acids, related compounds and dipeptides in VAT from three different metabolic settings, through the absolute and relative values of 128 compounds. Unlike most studies of similar design, our metabolomic profiling has been conducted on human VAT deriving from naturally healthy/pathological obese or lean subjects, as opposite to VAT extracted from rodent models of obesity and T2D, which might not always recapitulate human pathological conditions. Furthermore, in this study we used metabolic syndrome as inclusion criteria thus allowing comparison with data from other similar studies.

Several limitations have also to be taken into consideration. First, the targeted nature of the metabolomics did not include the detection of metabolites that could have been important to dissect the causes and the consequences of the observed changes and instead were not considered in the profiling. Second, our research did not take into consideration lifestyle factors such as patients' diet or exercise, which could have had an important impact on the profile of the fat metabolome. Third, our study was not supported by crucial information resulting from the transcriptomic and proteomic analysis, as well as from the plasma metabolome of the patients themselves, which would have helped us to further deepen the physiological origin of the altered pathways in the deranged adipose tissue.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards approved by the institutional "Comitato Etico Indipendente" (Independent Ethics Committee) reference number 159/2016 and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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