Original Article

MOLECULAR METABOLISM

Metabolomic profiling identifies potential pathways involved in the interaction of iron homeostasis with glucose metabolism

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ABSTRACT

Objective: Elevated serum ferritin has been linked to type 2 diabetes (T2D) and adverse health outcomes in subjects with the Metabolic Syndrome (MetS). As the mechanisms underlying the negative impact of excess iron have so far remained elusive, we aimed to identify potential links between iron homeostasis and metabolic pathways.

Methods: In a cross-sectional study, data were obtained from 163 patients, allocated to one of three groups: (1) lean, healthy controls ($n = 53$), (2) MetS without hyperferritinemia ($n = 54$) and (3) MetS with hyperferritinemia ($n = 56$). An additional phlebotomy study included 29 patients with biopsy-proven iron overload before and after iron removal. A detailed clinical and biochemical characterization was obtained and metabolomic profiling was performed via a targeted metabolomics approach.

Results: Subjects with MetS and elevated ferritin had higher fasting glucose ($p < 0.001$), HbA1c ($p = 0.035$) and 1 h glucose in oral glucose tolerance test ($p = 0.002$) compared to MetS subjects without iron overload, whereas other clinical and biochemical features of the MetS were not different. The metabolomic study revealed significant differences between MetS with high and low ferritin in the serum concentrations of sarcosine, citrulline and particularly long-chain phosphatidylcholines. Methionine, glutamate, and long-chain phosphatidylcholines were significantly different before and after phlebotomy ($p < 0.05$ for all metabolites).

Conclusions: Our data suggest that high serum ferritin concentrations are linked to impaired glucose homeostasis in subjects with the MetS. Iron excess is associated to distinct changes in the serum concentrations of phosphatidylcholine subsets. A pathway involving sarcosine and citrulline also may be involved in iron-induced impairment of glucose metabolism.

Keywords Metabolomics; Hyperferritinemia; Iron overload; Metabolic syndrome; Glucose

1. INTRODUCTION

Obesity is associated with type 2 diabetes (T2D) [1] and non-alcoholic fatty liver disease (NAFLD) [2]. NAFLD has been linked to insulin resistance (IR) and the Metabolic Syndrome (MetS) and is commonly regarded as the hepatic manifestation of the MetS [3–5]. Different iron phenotypes such as obesity-related iron deficiency as well as iron overload have been observed in association with obesity [6]. Iron overload linked to NAFLD is referred to as dysmetabolic iron overload syndrome (DIOS) [6].

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Abbreviations: T2D, type 2 diabetes mellitus; MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease; IR, insulin resistance; DIOS, dysmetabolic iron overload syndrome; BMI, body mass index; PCOS, polycystic ovary syndrome; oGTT, oral glucose tolerance test; WHO, World Health Organization; HOMA-IR, homeostatic model assessment-insulin resistance; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HDL, high density lipoproteins; LDL, low density lipoproteins; CRP, C-reactive protein; IL, interleukin; TNF, tumor necrosis factor; RBC, red blood count; +Fe, with iron overload; −Fe, without iron overload; WHR, waist hip ratio; MRI, magnet resonance imaging; FoxO1, forkhead transcription factor O1; Akt/PKB, Akt/protein kinase B; GSK3β, glycogen synthase kinase 3β; GLUT1, glucose transporter 1; HIF1α, hypoxia-inducible factor 1α; CDP, Cytidinediphosphat; PEMT, phosphatidylethanolamine N-methyltransferase; VLDL, very low-densitylipoproteins; PC, phosphatidylcholine; PC_E, plasmalogens; GNMT, glycine N-methyltransferase

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Serum ferritin concentrations are commonly used in the clinical routine as an indicator of body iron stores [7]. The MetS results in a complex dysregulation of iron homeostasis [8]. Serum ferritin concentrations increase with the number of features of the MetS [9] and several studies have confirmed the association between ferritin concentrations and IR [10], body mass index (BMI) [11], visceral fat mass [12], blood pressure [13], MetS [9,14], and polycystic ovary syndrome (PCOS) [15]. In line with this, a high serum ferritin concentration has been identified as a risk factor for the development of T2D and gestational diabetes [16,17]. In morbidly obese subjects, ferritin strongly correlated with waist circumference and IR. One year after gastric banding, ferritin did not change significantly despite weight loss and glucose tolerance improvement. However, ferritin concentrations were still correlated with IR at follow-up [18]. According to these observations, ferritin concentrations may indicate pronounced IR in overweight or obesity independent from other components of the MetS. Additionally, the incidence of the MetS after 6 years was more than four-fold higher in subjects with ferritin and transferrin in the highest tertile compared with participants in the lowest tertile, suggesting prognostic relevance [19]. In particular, serum ferritin concentrations may indicate more severe hepatic IR and a higher risk for progression to relevant clinical endpoints [20].

Since the role of iron overload in the pathogenesis of altered glucose metabolism has so far been incompletely studied, we aimed to identify potential metabolic pathways using a two-phased metabolomics approach. First, subjects with MetS and iron excess were compared to MetS subjects without iron excess and to control subjects; second, the metabolomic changes in response to phlebotomy treatment were investigated.

2. MATERIALS AND METHODS

2.1. Clinical and laboratory assessment

In a cross-sectional study, data were obtained from 163 patients, allocated to one of three groups: (1) lean, healthy controls (n = 53), (2) MetS without iron overload (n = 54) and (3) MetS with iron overload (n = 56). The second phlebotomy study included 29 patients with biopsy-proven iron overload before and after iron removal. Subjects were recruited among patients of the First Department of Internal Medicine, Paracelsus Medical University, Salzburg, Austria, and the Department of Internal Medicine, Hospital Oberndorf, Austria. In all subjects, an oral glucose tolerance test (oGTT) with 75 g of glucose in 300 ml of water according to WHO recommendations was performed [21]. The homeostasis model assessment (HOMA-IR; fasting insulin [μU/L] × fasting glucose [mmol/dL]/22.5) was used to calculate IR. T2D was classified as use of diabetes medication or HbA1c ≥ 6.5% (48 mmol/mol) or oGTT > 11.1 mmol/L (200 mg/dl) after 2 h or fasting glucose > 7.0 mmol/L (126 mg/dl). Following an overnight fast, venous blood was drawn. Full blood counts were obtained in all subjects by standard laboratory methods. In addition, blood was centrifuged, and plasma was analyzed for ferritin, transferrin and serum iron content, serum transaminases (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), gamma-glutamyl transpeptidase (GGT), serum lipid profile (including triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL)), glucose, insulin and C-reactive protein (CRP), adiponectin, interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and leptin. HbA1c was quantified by HPLC using Adamts H-8160 (Menarini, Florence, Italy). A standard physical examination was performed that included blood pressure readings, BMI, and waist and hip circumference. Blood pressure was taken by a nurse and determined after a 5-min rest in a sitting position; the average of two readings was used as the measurement of blood pressure. Waist circumference was measured at the highest point of the iliac crest with subjects standing in an upright position. BMI was calculated as weight/height squared (kg/m^2). Performance of liver biopsy and assessment of iron deposition by Pearl's stain was described previously [22].

2.2. Definition of study groups

Subjects for the cross-sectional study were recruited from approximately 2563 participants of the population-based Salzburg Colon Cancer Prevention Initiative (SAKKOPI study). The study design and details of the clinical and biochemical work-up of included subjects have been reported previously [23]. Subjects were allocated to one of three groups: (1) lean, healthy control group (n = 53), (2) MetS without iron overload (n = 54) and (3) MetS with iron overload (n = 56). According to the "National Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults" (NCEP-ATP-III) subjects were considered to have MetS if at least 3 of the following 5 criteria are present: waist circumference of more than 102 cm in men or more than 88 cm in women, serum triglycerides of more than 150 mg/dL, HDL-cholesterol below 40 mg/dL in men or below 50 mg/dL in women, blood pressure of 130/85 mmHg or above, fasting plasma glucose of more than 110 mg/dL (or a previous diagnosis of T2D) [24]. Iron overload was defined as serum ferritin values of 400–1100 ng/ml, those subjects considered to have MetS without iron overload had ferritin values from 30 to 120 ng/ml in men and women. Clinically relevant active inflammation was excluded as a confounder of elevated serum ferritin. Exclusion criteria were laboratory or clinical evidence of autoimmune, viral (viral hepatitis, HIV), or hereditary causes (Wilson disease, hereditary hemochromatosis, alpha-1 antitrypsin deficiency) of liver disease, malignancy, and clinically relevant alcohol consumption (>20 g per day for men and >10 g per day for women, a limit considered to be below the traditional level for alcohol-induced liver disease [25]).

Lean healthy control subjects had normal liver tests (ALT <35 IU/L for men and <19 IU/L for women), no components of the MetS according to NCEP-ATP-III criteria and normal biochemical iron parameters. The control group was matched with the MetS groups according to gender and age.

Throughout the study period, medication, especially lipid-lowering and antihypertensive drugs, remained unchanged. None of the patients was on insulin or oral antidiabetic therapy.

The study was approved by the local ethics committee (Ethikkommission des Landes Salzburg, approval no. 415-E/1675/6-2013), and informed consent was obtained from all participants.

2.3. Phlebotomies

Phlebotomies were performed biweekly until serum ferritin concentrations were between 50 and 100 μg/L. At every visit, serum ferritin and hemoglobin concentrations were checked to assess therapeutic efficacy and to avoid anemia. In case of anemia or fatigue, the interval was extended up to four weeks. Patients underwent 7.2 (5–12) phlebotomies resulting in 3.7 ± 2.8 g of removed iron per patient. Iron removal was calculated by the amount of red blood count (RBC) collected (1 ml of RBC = 1 mg of iron).

2.4. Metabolomics

We performed a metabolomic profiling in all subjects. In the intervention cohort, serum for the metabolomic analysis was drawn before

the first and 10–14 days after the final phlebotomy. We used a targeted metabolomics approach of combined direct flow injection and liquid chromatography MS/MS using the AbsolutelDQTM p180 kit (BIOCRATES life Sciences AG, Innsbruck, Austria) according to manufacturers' instructions. The methodological details are provided as supplementary material and have been reported previously [26].

2.5. Statistical analysis

In the cross-sectional study, both the MetS cohorts (i.e. with and without iron overload) was compared to the lean healthy control group. Data were compared before and after phlebotomy. Analyses of clinical and metabolic characteristics as well as graphic visualizations were performed using the R statistics environment (version 3.2.1, R Foundation for Statistical Computing, www.r-project.org) [27].

Metabolomics data was explored via principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) provided by the MetaboAnalyst webserver [28–30] to evaluate overall and group-specific variances.

Concerning the cross-sectional study, group differences in demographic, clinical, anthropometric, and laboratory characteristics were assessed by Analysis of Variance (ANOVA) with Tukey's HSD-post hoc test for normally distributed data, Kruskal–Wallis test and Dunn's post-hoc analysis for non-normally distributed data, and chi-squared test for qualitative variables. The corresponding metabolomics data were logarithmized and analyzed using ANOVA with Tukey's HSD-post hoc test.

In the phlebotomy study, laboratory characteristics after phlebotomy were compared to baseline values by Student's t-test for normally distributed and Wilcoxon rank-sum test for non-normally distributed data, respectively. Metabolomics data of these subjects were logarithmized and analyzed using paired Student's t-test.

All p-values were adjusted for multiple testing using the Benjamini–Hochberg principle [31] provided by the 'metabolomics'-package [32] for R. An adjusted p-value below 0.05 was considered statistically significant.

Multivariate linear regression analysis was performed to assess associations of metabolomic parameters with hyperferritinemia adjusting for age, BMI, sex, ALT, oGTT 1 h and platelets.

3. RESULTS

3.1. Cross-sectional study — population 1

3.1.1. Clinical characteristics

Subjects with MetS with and without iron overload were studied in order to identify clinical and metabolic differences related to iron; additionally, healthy control subjects were assessed. The groups were similar in age and sex distribution. According to the group definitions, the control group and the groups with MetS differed in blood pressure, lipids, liver enzymes, glucose homeostasis, and iron profile. Subjects with MetS were matched for their MetS components and only differed in their iron profiles. Ferritin levels in the MetS group without iron overload were significantly and clinically relevant lower than in the MetS group with iron overload (74.9 $\pm$ 27.9 vs. 434.9 $\pm$ 210.4 ng/ml, $p < 0.001$). Differences between the two MetS groups were observed regarding glucose parameters and liver enzymes. Subjects with MetS and high ferritin had higher indices of impaired glucose homeostasis as assessed by fasting glucose (97.6 $\pm$ 17.2 vs. 107.3 $\pm$ 15.1 mg/dl, $p < 0.001$) and glucose concentration at 1 h in the oGTT (152.9 $\pm$ 41.1 vs. 184.0 $\pm$ 46.1 mg/dl, $p = 0.002$) compared to subjects without iron overload. Likewise, patients with iron overload had higher ALT (27.4 $\pm$ 11.6 vs. 33.5 $\pm$ 16.4 U/l, $p = 0.077$) and GGT (37.8 $\pm$ 32.3 vs. 53.9 $\pm$ 68.5 U/l, $p = 0.024$) levels. However, the two groups were similar in their lipid profile, CRP, blood pressure, and BMI. There were significant differences in levels of adiponectin, IL-6, TNFα, and leptin between the control group and those patients with MetS, but no further differences between the subjects with and without iron overload were noted. The clinical and biochemical characteristics are summarized in Table 1.

3.1.2. Serum metabolomic analysis

Several results between MetS and healthy controls confirmed previously reported differences. Thus, metabolite concentrations of sarcosine, alanine, glutamate, glycine, kynurenine, leucine, valine, and a number of phosphatidylcholines (PC) were different between the healthy control group and those with the MetS.

Comparison of subjects with high and normal iron parameters revealed significant differences in the serum concentrations of sarcosine, citrulline, methioninsulfoxide, and, particularly, several long-chain PCs. The detailed results of the metabolomic analysis in cross sectional study comparing subjects with MetS with high and low ferritin are shown in Table 2 and Figure 1.

3.2. Phlebotomy study — population 2

3.2.1. Clinical characteristics

In order to compare clinical and metabolic characteristics before and after iron removal therapy, a phlebotomy intervention was performed in a separate cohort. The population baseline characteristics were comparable with the MetS group with iron overload (shown in Table 3). Patients were overweight, most of them diagnosed with MetS, and had pathological glucose homeostasis; all had high ferritin concentrations and hepatic steatosis with mild to moderate iron deposition as assessed by Pearl's stain on liver biopsy examination. We observed a trend toward significance for the reductions of ALT and GGT as previously reported in response to phlebotomy but no significant differences were observed with regard to HOMA index, fasting glucose and insulin, or lipid profile.

3.2.2. Serum metabolomic analysis

Metabolomics profiling before and after phlebotomy identified a number of significant differences including methionine, glutamate, and long-chain PCs. These changes are summarized in Table 4 and Figure 2. In order to identify metabolites related to iron status, we looked for metabolites, which were both different between the two MetS groups and also changed in response to iron removal in the intervention study. Metabolites that fulfilled both criteria were long-chain PCs including PC 40:2, PC 40:3, PC 40:4, and PC 42:1, suggesting an interaction of iron status with the homeostasis of these compounds. Univariate Spearman-rank correlation analysis among subjects with the MetS revealed significant correlations for the above mentioned metabolomic analytes with high ferritin concentrations and the clinical variables ALT, platelets, oGTT 1 h. We then performed a multivariate regression analysis that included these variables and found that after adjustment for age, sex, ALT, platelets, and oGTT 1 h, only sarcosine and PC42_6, but not citrulline, PC32_2, PC38_4, PC40_2, PC40_3, PC40_4, PC42_5, PC_E40_3, PC_E40_4, PC_E42_1 remained associated with high ferritin concentrations. None of these variables was independently associated with clinical parameters before and after phlebotomy in the multivariate model in the phlebotomy group (details not shown).

Table 1 — Cross-sectional study. Demographic, clinical, anthropometrical, and laboratory characteristics. Data are expressed as means and standard deviations. p-values were assessed by Analysis of Variance (ANOVA) with Tukey's HSD-post hoc test, Kruskal—Wallis test, and Dunn's post-hoc analysis or chi-squared test and adjusted for multiple comparison. Abbreviations: MetS — Metabolic Syndrome; −Fe — without iron overload; +Fe — with iron overload; BMI — body mass index; WHR — waist hip ratio; GGT — gamma glutamyl transpeptidase; AST — aspartate aminotransferase; ALT — alanine aminotransferase; oGGT — oral glucose tolerance test; HOMA — Homeostasis model assessment; Tchol — total cholesterol, HDL-C — high density lipoproteins, LDL-C — low density lipoproteins, TG — triglyceride, CRP — C-reactive protein; IL — interleukin; TNF — tumor necrosis factor, y — years, kg — kilogram, m — meter, ng — nanogram, ml — milliliter, mg — milligram, dl — deciliter, µg — microgram, g — gram, U — unit, l — liter.

	Lean healthy n = 53	MetS−Fe n = 54	MetS+Fe n = 56	Adjusted P-value	p 1 vs 2	p 1 vs 3	p 2 vs 3
Sex (male/female)	26/27	27/27	28/28				
Age (y)	54.7 ± 7.9	56.4 ± 6.4	58.3 ± 9.2	0.065			
Systolic blood pressure (mmHg)	115.2 ± 7,3	137.1 ± 14.6	140.2 ± 14.9	<0.001	<0.001	<0.001	0.446
Diastolic blood pressure (mmHg)	74.3 ± 6.5	84.2 ± 7.9	85.0 ± 10.2	<0.001	<0.001	<0.001	0.783
BMI (kg/m^2)	24.0 ± 2.5	29.9 ± 3.5	31.1 ± 5.0	<0.001	<0.001	<0.001	0.497
WHR	0.91 ± 0.07	0.96 ± 0.06	0.99 ± 0.07	<0.001	0.002	<0.001	0.039
Ferritin (ng/ml)	53.5 ± 20.4	74.9 ± 27.9	434.9 ± 210.4	<0.001	0.008	<0.001	<0.001
Transferrin (mg/dl)	282.8 ± 36.8	276.2 ± 38.8	253.4 ± 35.0	<0.001	0.638	<0.001	0.004
Transferrin saturation (%)	27.5 ± 9.2	26.1 ± 9.0	32.5 ± 9.1	0.001	0.459	0.009	0.001
Serum iron (µg/dl)	108.3 ± 34.5	99.8 ± 32.2	115.6 ± 33.7	0.058			
Hemoglobin (g/dl)	14.5 ± 1.2	14.7 ± 1.7	15.2 ± 1.0	0.008	0.131	0.005	0.131
GGT (U/l)	20.7 ± 12.7	37.8 ± 32.3	53.9 ± 68.5	<0.001	<0.001	<0.001	0.024
AST (U/l)	20.9 ± 6.4	23.2 ± 7.0	26.6 ± 10.9	0.005	0.100	0.003	0.144
ALT (U/l)	19.1 ± 7.4	27.4 ± 11.6	33.5 ± 16.4	<0.001	<0.001	<0.001	0.077
Fasting plasma glucose (mg/dl)	92.8 ± 7.7	97.6 ± 17.2	107.3 ± 15.1	<0.001	0.095	<0.001	<0.001
Fasting insulin (µU/ml)	5.6 ± 3.0	13.0 ± 15.1	13.5 ± 7.1	<0.001	<0.001	<0.001	0.218
oGGT 1 h (mg/dl)	139.8 ± 32.5	152.9 ± 41.1	184.0 ± 46.1	<0.001	0.295	<0.001	0.002
HbA1c (%)	5.4 ± 0.4	5.5 ± 0.3	5.7 ± 0.4	0.001	0.441	<0.001	0.035
HOMA-IR	1.30 ± 0.75	3.66 ± 7.37	3.59 ± 2.19	<0.001	<0.001	<0.001	0.106
Tchol (mg/dl)	219.5 ± 37.6	230.7 ± 47.0	238.6 ± 53.0	0.103			
HDL-C (mg/dl)	72.1 ± 17.6	47.1 ± 9.9	47.8 ± 12.9	<0.001	<0.001	<0.001	0.962
LDL-C (mg/dl)	132.9 ± 34.1	155.5 ± 39.7	155.7 ± 41.2	0.005	0.008	0.008	0.886
TG (mg/dl)	86.5 ± 25.8	192.7 ± 74.2	224.7 ± 189.1	<0.001	<0.001	<0.001	0.702
CRP (mg/dl)	0.13 ± 0.13	0.37 ± 0.38	0.70 ± 2.1	<0.001	<0.001	<0.001	0.319
Adiponectin (µg/ml)	13.6 ± 4.2	10.4 ± 4.1	10.2 ± 3.7	<0.001	<0.001	<0.001	0.834
IL-6 (pg/ml)	2.9 ± 1.2	4.1 ± 2.2	5.9 ± 10.6	<0.001	<0.001	<0.001	0.096
TNFα (pg/ml)	6.1 ± 2.1	7.2 ± 1.6	7.6 ± 2.4	<0.001	0.003	0.001	0.737
Leptin (ng/ml)	5.9 ± 4.2	19.9 ± 16.9	20.3 ± 12.8	<0.001	<0.001	<0.001	0.398

Table 2 — Cross-sectional study. Serum metabolome analysis. All concentrations are µM (micromole/L). Data are expressed as means and standard deviations unless otherwise indicated. p-values were assessed by ANOVA with Tukey's HSD-post hoc test and adjusted for multiple comparison. Abbreviations: MetS — Metabolic Syndrome; MetS−Fe — Metabolic Syndrome without iron overload; MetS+Fe — Metabolic Syndrome with iron overload; Met_So — Methioninsulfoxide; PC — phosphatidylcholines, PC_E — plasmalogens.

	Control	MetS−Fe	MetS+Fe	Adjusted p-value	1 vs 2	1 vs 3	2 vs 3
Sarcosine	5.71 ± 1.72	9.06 ± 4.55	18.74 ± 16.3	<0.001	<0.001	<0.001	<0.001
Citrulline	36.03 ± 7.14	33.55 ± 7.05	29.39 ± 7.51	<0.001	0.125	<0.001	0.001
Glutamate	60.04 ± 31.59	83.61 ± 30.39	96.25 ± 34.43	<0.001	<0.001	<0.001	0.066
Met_So	1.07 ± 0.57	1.20 ± 0.97	1.84 ± 1.77	0.014	0.656	0.002	0.007
Alanine	342.31 ± 68.75	413.49 ± 80.24	442.54 ± 84.74	<0.001	<0.001	<0.001	0.062
Glycine	273.54 ± 63.70	240.35 ± 49.36	225.77 ± 45.91	<0.001	0.003	<0.001	0.101
Kynurenine	2.57 ± 0.56	3.35 ± 0.75	3.60 ± 0.97	<0.001	<0.001	<0.001	0.183
Leucine	141.24 ± 31.32	167.87 ± 28.86	168.53 ± 41.52	<0.001	<0.001	<0.001	0.776
Valine	190.27 ± 27.09	216.87 ± 30.82	217.29 ± 34.12	<0.001	<0.001	<0.001	0.995
PC_38_3	42.50 ± 8.71	54.37 ± 12.70	57.97 ± 19.25	<0.001	<0.001	<0.001	0.335
PC_38_4	96.08 ± 20.39	117.13 ± 27.84	132.49 ± 42.22	<0.001	<0.001	<0.001	0.052
PC_40_2	0.35 ± 0.29	0.389 ± 0.40	0.71 ± 0.80	0.045	0.956	0.013	0.010
PC_40_3	0.72 ± 0.31	0.81 ± 0.46	1.02 ± 0.61	0.015	0.325	0.001	0.020
PC_40_4	3.50 ± 0.77	4.30 ± 1.20	5.41 ± 2.88	<0.001	0.001	<0.001	0.003
PC_42_5	0.40 ± 0.11	0.41 ± 0.14	0.53 ± 0.21	0.001	0.681	<0.001	<0.001
PC_E30_0	0.53 ± 0.14	0.46 ± 0.15	0.42 ± 0.10	0.001	0.002	<0.001	0.381
PC_E42_1	0.46 ± 0.20	0.50 ± 0.26	0.74 ± 0.52	0.001	0.419	<0.001	0.002

4. DISCUSSION

In this two-phased study, we aimed to identify molecular links between iron status and metabolic changes in subjects with the MetS. Clinically, subjects matched for age, sex and the presence of the MetS were different with regard to indicators of impaired glucose homeostasis. Although published data have linked higher iron stores to other components of the MetS, our results suggest that iron may be linked predominantly to impaired glucose homeostasis if subjects with the MetS are compared. Epidemiologically, iron stores have

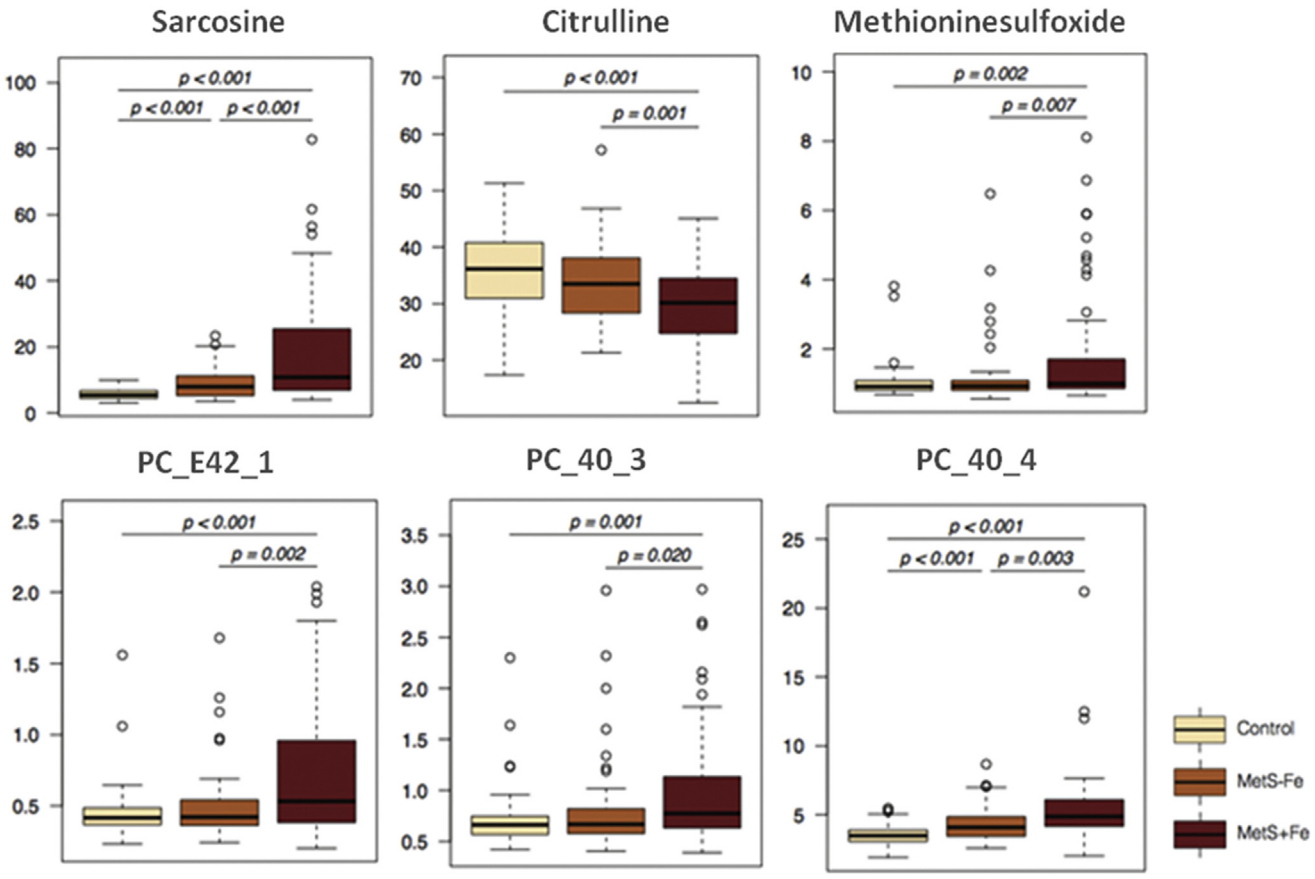

Figure 1: Cross-sectional study. Serum concentrations of selected metabolites. All concentrations are μM (micromole/L). Each horizontal line denotes the p-value comparing the respective groups by ANOVA. Abbreviations: MetS — Metabolic Syndrome; MetS−Fe — Metabolic Syndrome without iron overload; MetS+Fe — Metabolic Syndrome with iron overload, PC — phosphatidylcholine, PC_E — plasmalogens.

been positively associated with risk of T2D [33] and this finding was later confirmed in meta-analyses [34]. Liver iron, as measured by magnetic resonance imaging (MRI), is positively associated with T2D and IR [35]. Iron removal by phlebotomy has been associated with a lower risk of T2D in previously non-diabetic individuals [36] and with an improvement of IR in patients with MetS [37]. Unexpectedly, we were not able to reproduce the well-recognized improvement of glucose homeostasis in our phlebotomy study. Despite this fact, we found an improvement of liver enzymes as an indicator of phlebotomy effectiveness. We cannot exclude that some subjects did not adhere to the dietary request of the study protocol (i.e. fasting state at time of post-phlebotomy blood sample), which could limit the clinical interpretation of the glucose parameters. We would ideally recommend studying the effects of phlebotomy in the same subjects who were also analyzed for group comparisons, which was not possible in our study.

Excess iron in the liver causes hyperinsulinemia via decreased insulin degradation and impaired insulin signaling [38]. The ensuing hyperinsulinemic state may further augment the uptake of extracellular iron by inducing the redistribution of transferrin receptors to the cell surface [39] while downregulating hepcidin expression [40]. Additionally, cell culture experiments have demonstrated that iron accumulation led to IR and impaired glucose utilization in adipocytes [41]. Conversely, iron chelation re-established insulin receptor signaling by increasing the phosphorylation of forkhead transcription factor O1 (FoxO1), Akt/protein kinase B (Akt/PKB), and glycogen synthase kinase 3β (GSK3β), which constitute key pathways for insulin's effect on gluconeogenesis and glycogen synthesis. Similarly, molecules of glucose utilization such as glucose transporter 1 (GLUT1) or hypoxia-inducible factor 1α (HIF1α) were increased in cultured hepatoma cells in response to iron reduction resulting in glucose removal from the culture supernatant [42]. Although these observations suggest that iron and glucose or lipid homeostasis interact at the molecular level, data on the site of these interactions have remained scarce in humans. Profiling of the serum metabolome has been used as a valuable tool to identify subtle changes in various diseases such as cancer, cardiovascular diseases, or obesity [43—45].

We therefore reasoned that the association of metabolites with the presence of iron in MetS and their changes in response to phlebotomy may provide biochemical insight to pathways linked to the interaction of iron homeostasis with IR. We identified several metabolite concentrations to be associated with the MetS. Sarcosine, alanine, glutamate, glycine, kynurenine, leucine, valine and a number of PCs were significantly different between a healthy population and the two MetS groups combined. These observed differences between healthy subjects and the MetS widely confirm published results [46—48], which renders validity to our study results. The association of metabolomic patterns with impaired glucose homeostasis could serve to identify pathways involved in the development of T2D. Our data suggest that sarcosine, alanine, glutamate, glycine, kynurenine, branched-chain amino acids leucine and valine and a number of PCs may be involved in the pathophysiology of the MetS. A role for plasma

Table 3 — Phlebotomy study. Demographic, clinical, anthropometrical, and laboratory characteristics. Data are expressed as means and standard deviations unless otherwise indicated. p-values were assessed by Student's t-test or Wilcoxon rank-sum test and adjusted for multiple comparison. Abbreviations: T2DM — type 2 diabetes mellitus, BMI — body mass index; WHR — waist hip ratio; AST — aspartate aminotransferase; ALT — alanine aminotransferase; GGT — gamma glutamyl transpeptidase; HOMA — Homeostasis model assessment; Tchol — total cholesterol, HDL-C — high density lipoproteins, LDL-C — low density lipoproteins, TG — triglyceride, CRP — C-reactive protein; IL — interleukin; TNF — tumor necrosis factor, y — years, kg — kilogram, m — meter, ng — nanogram, ml — milliliter, mg — milligram, dl — deciliter, µg — microgram, g — gram, U — unit, l — liter.

	Baseline n = 30	After intervention	p-value before/after intervention
Sex (male/female)	28/2		
Age (y)	56.1 ± 7.4		
T2DM n (%)	8 (26.7)		
BMI (kg/m^2)	29.6 ± 2.5		
Ferritin (ng/ml)	677.8 ± 239.2	91.5 ± 84.9	<0.001
Transferrin saturation (%)	35.2 ± 10	20.8 ± 9.8	<0.001
Hemoglobin (g/dl)	15.5 ± 1	14.1 ± 1.2	<0.001
AST (U/l)	42.6 ± 14.3	43.3 ± 16.2	0.839
ALT (U/l)	51.4 ± 26.7	41.2 ± 20.3	0.112
GGT (U/l)	117 ± 87.1	111.5 ± 84.9	0.632
Fasting plasma glucose (mg/dl)	111.1 ± 19	107.7 ± 15.9	0.106
Fasting insulin (µU/ml)	23.2 ± 12.8	21.7 ± 10.7	0.536
HOMA-IR	6.8 ± 4,2	6.1 ± 3.6	0.615
Tchol (mg/dl)	212.4 ± 42	200.2 ± 39.8	0.097
HDL-C (mg/dl)	48.5 ± 11.4	51.3 ± 12.5	0.281
LDL-C (mg/dl)	129.1 ± 36.2	119.2 ± 32	0.228
TG (mg/dl)	222 ± 128.3	196.3 ± 97.7	0.318
IL-6 (pg/ml)	3.7 ± 2	6.2 ± 6.2	0.010
TNFα (pg/ml)	5.9 ± 1.2	5.7 ± 1	0.577
Leptin (ng/ml)	12.5 ± 6.9	12.6 ± 6.7	0.917

Table 4 — Phlebotomy study. Serum metabolome analysis before and after phlebotomy. All concentrations are µM (micromole/L). Data are expressed as means and standard deviations unless otherwise indicated. p-values were assessed by paired Student's t-test and adjusted for multiple comparison. Abbreviations: Met_So — Methioninsulfoxide; PC — phosphatidylcholine, PC_E plasmalogens.

	Baseline	After phlebotomy	Adjusted p-value
Sarcosine	5.98 ± 3.40	5.83 ± 2.90	0.997
Citrulline	35.62 ± 12.62	37.23 ± 10.67	0.559
Glutamate	298.38 ± 180.67	226.41 ± 127.63	0.006
Methionine	22.24 ± 9.84	28.90 ± 9.05	0.006
Met_So	6.30 ± 6.37	2.82 ± 1.74	0.733
PC_36_0	2.50 ± 1.17	1.79 ± 0.90	0.002
PC_38_0	3.36 ± 0.95	2.92 ± 0.91	0.009
PC_38_1	2.50 ± 1.27	1.95 ± 0.72	0.006
PC_38_3	50.02 22.96	49.18 16.91	0.997
PC_38_4	123.18 ± 56.31	120.46 ± 36.78	0.988
PC_40_2	1.34 ± 1.24	0.81 ± 0.64	0.027
PC_40_3	1.24 ± 0.90	0.83 ± 0.52	0.006
PC_40_4	5.43 ± 2.51	5.13 ± 1.88	0.633
PC_42_0	0.61 ± 0.25	0.50 ± 0.19	0.008
PC_42_1	0.43 ± 0.18	0.33 ± 0.12	0.006
PC_42_4	0.55 ± 0.42	0.39 ± 0.26	0.004
PC_42_5	0.70 ± 0.31	0.62 ± 0.23	0.223
PC_E30_0	0.35 ± 0.13	0.33 ± 0.11	0.671
PC_E36_1	11.90 ± 7.85	8.64 ± 4.78	0.001
PC_E38_1	4.81 ± 4.61	3.02 ± 2.89	0.002
PC_E38_2	6.32 ± 5.15	4.39 ± 3.30	0.006
PC_E38_3	12.12 ± 8.58	8.83 ± 5.84	0.001
PC_E40_1	2.10 ± 0.86	1.78 ± 0.59	0.040
PC_E40_2	2.83 ± 1.56	2.13 ± 1.13	0.002
PC_E40_3	5.23 ± 4.53	3.51 ± 2.84	0.002
PC_E40_4	4.37 ± 2.54	3.52 ± 1.85	0.004
PC_E40_5	6.36 ± 3.50	5.12 ± 2.41	0.002
PC_E42_1	0.90 ± 0.65	0.61 ± 0.35	0.004
PC_E44_3	0.28 ± 0.22	0.19 ± 0.15	0.002

branched-chain amino acids in T2D has been demonstrated previously which is also supported by our findings [47].

The key aim of the study was to identify metabolic indicators linked to higher iron status in subjects with the MetS. We found that the serum concentrations of sarcosine, citrulline, methioninsulfoxide, and a subset of long-chain PCs were significantly different between the MetS group with normal and high ferritin. For most metabolites in which differences were observed, the high-iron phenotype was linked to a further increase or decrease compared to the difference between control subjects and low-iron MetS. These observations confirm that iron excess is related to a pronounced adverse metabolic derangement in subjects with the MetS, which was particularly evident for sarcosine, methioninsulfoxide, and a subset of long-chain PCs.

Since a high number of metabolites analyzed in metabolomics studies increase the probability of identifying falsely positive results, we aimed to reduce the number of false positives by additionally analyzing changes in response to iron removal. We reasoned that this two-phased approach would help identify metabolites truly linked to iron status in the MetS, although potentially at the cost of missing metabolites biologically linked to iron. This was especially and surprisingly the case for several long-chain PCs, which so far have not been implicated in iron homeostasis and implicated only marginally in the pathophysiology of the MetS [49—52]. The interaction of ferritin and PCs level could result from a direct influence of the liver iron on the hepatic PCs production. Additionally, possible mechanisms include changes of oxidative stress and subclinical inflammation in the case of increased hepatic iron stores. In our study, levels of CRP and IL-6 were not significantly different between groups of MetS with and without iron overload. However, there is a trend toward increased inflammation with iron overload, and this might lead to changes in PCs levels. Biosynthesis of PCs occurs via cytidine diphosphate (CDP)-choline and phosphatidylethanolamine N-methyltransferase (PEMT) pathway, the major site for the synthesis of PCs is the liver. PCs play a key role in very low-density lipoproteins (VLDL) secretion, cell regeneration, and membrane integrity [53—55]. In animal models of impaired PCs biosynthesis, the impaired VLDL secretion resulted in an accumulation of hepatic triglycerides [56—61], inducing the histological hallmark of NAFLD. Additionally, PCs have an essential contribution to membrane integrity, suggesting that higher serum concentrations could reflect more advanced cell membrane damage. A pronounced decrease of hepatic PCs levels lead to membrane damage and triggered a pro-inflammatory cascade that further initiated progression to steatohepatitis [56,62].

Sarcosine is an intermediate of glycine biosynthesis and degradation. It came into research focus recently as a new marker of prostate cancer and its metastatic process [63—65]. Cleavage of glycine to sarcosine is facilitated by glycine N-methyltransferase (GNMT) that is further involved in gluconeogenesis [66,67]. Interestingly, sarcosine remained independently associated after adjustment for clinical variables, which suggests that its role in the MetS and DIOS should be studied further. There is evidence for citrulline as a key regulator in lipid metabolism, inflammation, and oxidative stress [68—71]. In addition, studies support its role in skeletal muscle protein metabolism and its trophic

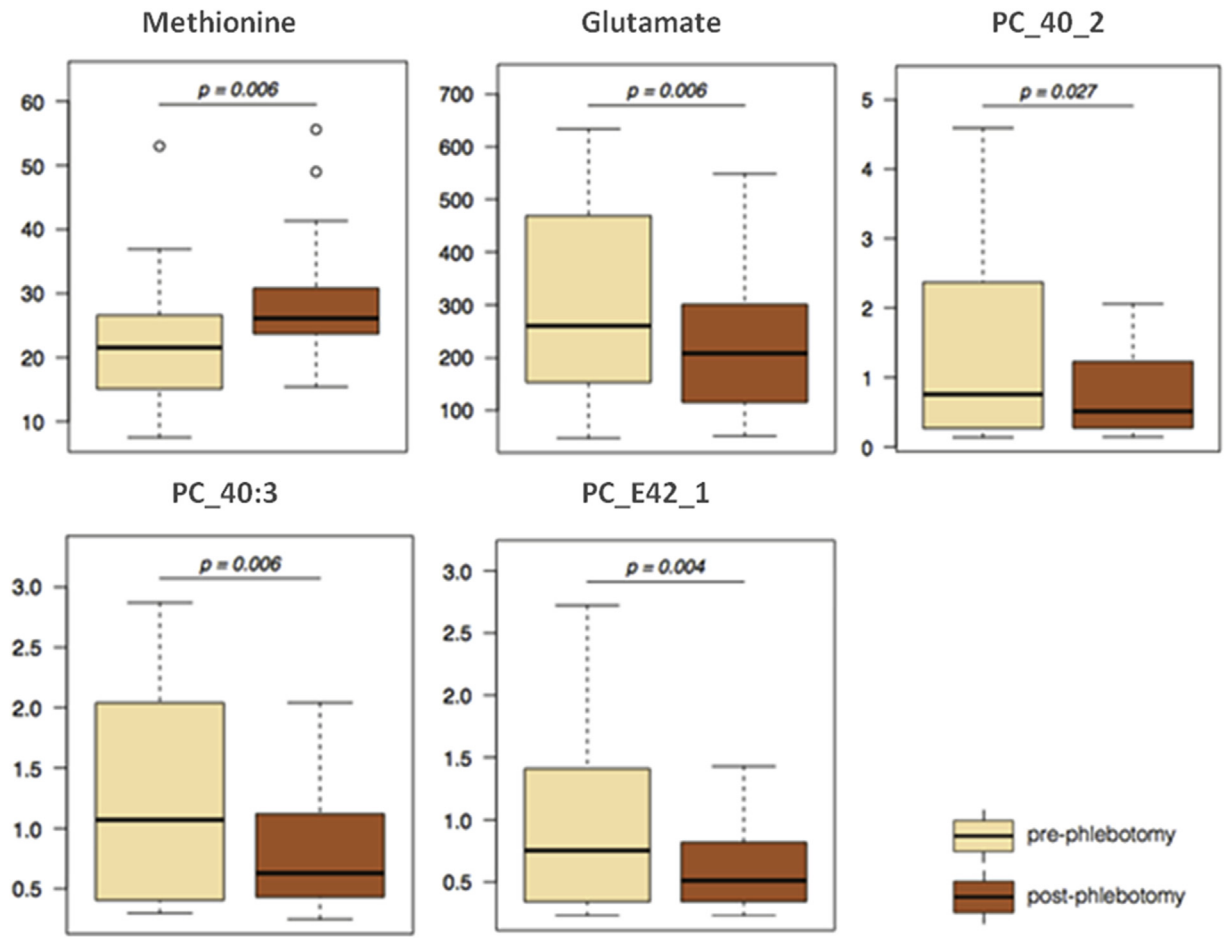

Figure 2: Phlebotomy study. Serum concentrations of selected metabolomics. All concentrations are μM (micromole/L). Horizontal line denotes the p-value comparing the groups by paired t-test. Abbreviations: PC — phosphatidylcholine, PC_E — plasmalogens.

effect on the gut [70,72]. Recent findings indicate that citrulline may offer a therapeutic strategy for NAFLD [68,69]. Citrulline is produced in the urea cycle mainly from ornithine and carbamoyl phosphate [73]. The urea and glutamate cycles are linked by aspartate. Despite this connection, it is unclear if serum citrulline concentrations are influenced by these pathways in healthy humans. Recent data on the effect of a glucagon-like peptide 2 analog give evidence that citrulline levels are mainly determined by enterocyte mass. Hence, higher citrulline concentrations may also indicate enhanced enterocyte mass and, therefore, may represent a non-causative association with higher ferritin concentrations [74].

Even if the population of the phlebotomy study is similar to the cohort with MetS and iron overload regarding the criteria of MetS, the baseline concentrations of metabolites differ. The reason for the difference is speculative but might be the higher baseline ferritin concentrations and the relevant higher insulin resistance in the cohort that underwent phlebotomies. As the precise biochemical pathways between metabolomics changes on the one side and iron overload and glucose metabolism on the other side is not clear, the ranked importance of PCs, citrulline, and sarcosine will require further research. Nevertheless, our data strongly suggest that iron may interact with human intermediary metabolism in multiple, so far unrecognized, biochemical pathways beyond the known interactions with insulin receptor signaling, gluconeogenesis, and adipokines. Our data, particularly on ferritin and PCs, citrulline, and sarcosine, may also indicate that some mechanisms play a role in certain subgroups of patients with the MetS. In addition, the metabolomics changes could reflect different pathophysiological pathways of hyperferritinemia and the altered glucose metabolism in these patients.

In summary, our data confirm that high serum ferritin concentrations are linked to impaired glucose homeostasis. Additionally, our study identifies novel associations of iron excess in MetS subjects with distinct subsets of PCs as well as a pathway involving sarcosine and citrulline. These metabolic pathways may be involved in iron-induced augmentation of IR.

AUTHOR CONTRIBUTIONS

LS — Data analysis, drafting, and writing of manuscript, SKE — data analysis, AW, WP, AF, MS, SA, DN, UHS, BP, SZ, SR, DW, EHB, CG, ER — patient recruitment, data acquisition and revision of manuscript for important intellectual content, CD, TKF, EA — study concept and design, analysis and interpretation of data, outlining, and revising the manuscript.

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CONFLICTS OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.10.006.

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