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

Lars Stechemesser 1, Sebastian K. Eder 1,2, Andrej Wagner 1, Wolfgang Patsch 3, Alexandra Feldman 1,2, Michael Strasser 1, Simon Auer 4, David Niederseer 5,6, Ursula Huber-Schönauer 4, Bernhard Paulweber 1, Stephan Zandanell 1, Sandra Ruhaltinger 1, Daniel Weghuber 3, Elisabeth Haschke-Becher 4, Christoph Grabner 7, Eva Rohde 7, Christian Datz 7,8, Thomas K. Felder 2,4,8, Elmar Aigner 1,2,8

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].

Received September 18, 2016 • Revision received October 17, 2016 • Accepted October 24, 2016 • Available online 31 October 2016

http://dx.doi.org/10.1016/j.molmet.2016.10.006

© 2016 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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/L]/22.5) was used to calculate IR. T2D was classified as serum ferritin values of 400–1100 ng/ml, ferritin concentrations may indicate pronounced IR in overweight or obesity independent from other components of the metabolic syndrome. Additionally, the incidence of the metabolic syndrome 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.

2.2. 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 (SACKOPI 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 AbsoluteIDQTM 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 logarthimized 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 metabolic 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 liver 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 ± 27.9 vs. 434.9 ± 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 ± 17.2 vs. 107.3 ± 15.1 mg/dl, p < 0.001) and glucose concentration at 1 h in the oGTT (152.9 ± 41.1 vs. 184.0 ± 46.1 mg/dl, p = 0.002) compared to subjects without iron overload. Likewise, patients with iron overload had higher ALT (27.4 ± 11.6 vs. 33.5 ± 16.4 U/l, p = 0.077) and GGT (37.8 ± 32.3 vs. 53.9 ± 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, TNF-α, 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 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, PC42_5, PC_E40_3, PC_E40_4, PC_E42_1 remained associated with high ferritin concentrations. None of these variables were independently associated with clinical parameters before and after phlebotomy in the multivariate model in the phlebotomy group (details not shown).
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...
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 1 (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

**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.
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 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 tripic
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.

**FINANCIAL SUPPORT**

Elmar Aigner is supported by PMU Research Fund PMU-FFF (E-13/17/086-A1G and Forschungsförderung der ÖGLMKC 2012). Support from
Spar Austria to Christian Datz is gratefully acknowledged. The funding sources had no involvement in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication.

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.

REFERENCES

[1] Haslam, D.W., James, W.P., 2005. Obesity. Lancet 366(9492):1197–1209.
[2] Haynes, P., Liangpunsakul, S., Chalasani, N., 2004. Nonalcoholic fatty liver disease in individuals with severe obesity. Clinics in Liver Disease 8:535–547.
[3] Marchesini, G., Bugianesi, E., Forlani, G., Cerrelli, F., Lenz, M., Manini, R., et al., 2003. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 37:917–923.
[4] Marceau, P., Biron, S., Hould, F.S., Marceau, S., Simard, S., Thung, S.N., et al., 1999. Liver pathology and the metabolic syndrome X in severe obesity. Journal of Clinical Endocrinology and Metabolism 84:1513–1517.
[5] Knobler, H., Schattner, A., Zornicki, T., Mainick, S.D., Keter, D., Sokolowska, N., et al., 1999. Fatty liver — an additional and treatable feature of the insulin resistance syndrome. QJM 92:73–79.
[6] Aigner, E., Feldman, A., Datz, C., 2014. Obesity as an emerging risk factor for iron deficiency. Nutrients 6:3587–3600.
[7] Ong, S.Y., Nicoll, A.J., Delatycki, M.B., 2016. How should hyperferritinemia be investigated and managed? European Journal of Internal Medicine 33:21–27.
[8] Datz, C., Felder, T.K., Niederseer, D., Aigner, E., 2013. Iron homeostasis in the metabolic syndrome. European Journal of Clinical Investigation 43(2):215–224.
[9] Wrede, C.E., Buettner, R., Bolheimer, L.C., Scholmerich, J., Pailitzsch, K.D., Hellerbrand, C., 2006. Association between serum ferritin and the insulin resistance syndrome in a representative population. European Journal of Endocrinology 154(2):333–340.
[10] Haap, M., Fritsche, A., Mensing, H.U., Häring, H.U., Stumvoll, M., 2003. Association of high serum ferritin concentration with glucose intolerance and insulin resistance in healthy people. Annals of Internal Medicine 139:869–871.
[11] Gillum, R.F., 2001. Association of serum ferritin and indices of body fat distribution and obesity in Mexican American men—the Third National Health and Nutrition Examination Survey. International Journal of Obesity and Related Metabolic Disorders 25(6):639–645.
[12] Iwasaki, T., Nakajima, A., Yoneda, M., Yamada, Y., Mukasa, K., Fujita, K., et al., 2005. Serum ferritin is associated with visceral fat area and subcutaneous fat area. Diabetes Care 28(10):2486–2491.
[13] Piperno, A., Trombini, P., Gelosa, M., Mauri, V., Pecchi, V., Vergani, A., et al., 2002. Increased serum ferritin is common in men with essential hypertension. Journal of Hypertension 20(8):1513–1518.
[14] John, N., Clark, J.M., Guillard, E., 2004. Serum ferritin and risk of the metabolic syndrome in U.S. adults. Diabetes Care 27(10):2422–2428.
[15] Martinez-Garcia, M.A., Luque-Ramírez, M., San-Millan, J.L., Escobar-Morreale, H.F., 2009. Body iron stores and glucose intolerance in premenopausal women: role of hyperandrogenism, insulin resistance, and genomic variants related to inflammation, oxidative stress, and iron metabolism. Diabetes Care 32(8):1525–1530.
[16] Forouhi, N.G., Harding, A.H., Allison, M., Sandhu, M.S., Welch, A., Luben, R., et al., 2007. Elevated serum ferritin levels predict new-onset type 2 diabetes: results from the EPIC-Norfolk prospective study. Diabetologia 50(5):949–956.
[17] Chen, X., Scholl, T.O., Stein, T.P., 2006. Association of elevated serum ferritin levels and the risk of gestational diabetes mellitus in pregnant women: the Camden study. Diabetes Care 29(5):1077–1082.
[18] Gastaldelli, A., Perego, L., Paganelli, M., Sesti, G., Hribal, M., Chavez, A.O., et al., 2009. Elevated concentrations of liver enzymes and ferritin identify a new phenotype of insulin resistance: effect of weight loss after gastric banding. Obesity Surgery 19:80–86.
[19] Vani, I.S., Balkau, B., Kettanee, A., André, P., Tichet, J., Fumeron, F., et al., 2007. Ferritin and transferrin are associated with metabolic syndrome abnormalities and their change over time in a general population: data from an Epidemiological Study on the Insulin Resistance Syndrome (DESI). Diabetes Care 30:1795–1801.
[20] Ellervik, C., Marott, J.L., Tykkäri-Hansen, A., Schnohr, P., Nordestgaard, B.G., 2014. Total and cause-specific mortality and markedly increased ferritin concentrations: general population study and metaanalysis. Clinical Chemistry 60(11):1419–1428.
[21] Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation, 2006. WHO.
[22] Aigner, E., Theurl, I., Haufe, H., Seifert, M., Hohlra, F., Scharinger, L., et al., 2008. Copper availability contributes to iron perturbations in human nonalcoholic fatty liver disease. Gastroenterology 135(2):680–688.
[23] Stadlmayr, A., Aigner, E., Steger, B., Scharinger, L., Lederer, D., Mayr, A., et al., 2011. Nonalcoholic fatty liver disease: an independent risk factor for colorectal neoplasia. Journal of Internal Medicine 270(1):41–49.
[24] Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). JAMA 285:2486–2497.
[25] Neuschwander-Tetri, B.A., Caldwell, S.H., 2003. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. Hepatology 37:1202–1219.
[26] Feldman, A., Eder, S.K., Felder, T.K., Kedenko, L., Paulweber, B., Stadlmayr, A., et al., 2016. Clinical and metabolic characterization of lean Caucasian subjects with non-alcoholic fatty liver. American Journal of Gastroenterology. http://dx.doi.org/10.1038/ajg.2016.318.
[27] R Core Team, 2015. R: a language and environment for statistical computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing. Available from: http://www.R-project.org.
[28] Xia, J., Sineijnokov, I.V., Han, B., Wishart, D.S., 2015. Metabo Analyst 3.0—making metabolomics more meaningful. Nucleic Acids Research 43(W1):W251–W257.
[29] Xia, J., Mandal, R., Sineijnokov, I.V., Broadhurst, D., Wishart, D.S., 2012. Metabo Analyst 2.0—a comprehensive server for metabolic data analysis. Nucleic Acids Research 40(Web Server issue):W127–W133.
[30] Xia, J., Psychogios, N., Young, N., Wishart, D.S., 2009. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Research 37(Suppl 2):W652–W660.
[31] Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of Royal Statistical Society: Series B Methodology 57(1):289–300.
[32] Livera, A.M.D., Bowne, J.B., 2014. Metabolomics: analysis of metabolomics data [Internet]. Available from: http://CRAN.R-project.org/package=metabolomics.
[33] Salonen, J.K., Tuomainen, T.P., Nyyssonen, K., Lakkma, H.M., Punnonen, K., 1998. Relation between iron stores and non-insulin dependent diabetes in men: case-control study. BMJ 317(7160):727.
Fernandez-Real, J.M., Manco, M., 2014. Effects of iron overload on chronic metabolic diseases. Lancet Diabetes Endocrinology 2:513–526.

Haap, M., Machann, J., von Friedeburg, C., Schick, F., Stefan, N., Schwenzer, N.F., et al., 2011. Insulin sensitivity and liver fat: role of iron overload. J Clin Endocrinol Metab 96(6):E958–E961.

Jiang, R., Ma, J., Ascherio, A., Stampfer, M.J., Willett, W.C., Hu, F.B., 2004. Dietary iron intake and blood donations in relation to risk of type 2 diabetes in men: a prospective cohort study. American Journal of Clinical Nutrition 79:70–75.

Houshyar, K.S., Lüdtke, R., Dobos, G.J., Kalus, U., Broecker-Preuss, M., Rampp, T., et al., 2012. Effects of phlebectomy-induced reduction of body iron stores on metabolic syndrome: results from a randomized clinical trial. BMC Medicine 10:54.

Niederer, C., Berger, M., Stremler, W., Starke, A., Stromeyer, G., Ebert, R., et al., 1984. Hyperinsulinemia in non-cirrhotic haemochromatosis: impaired hepatic insulin degradation? Diabetologia 26(6):441–444.

Davis, R.J., Corvera, S., Czech, M.P., 1986. Insulin stimulates cellular iron uptake and causes the redistribution of intracelluar transferrin receptors to the plasma membrane. Journal of Biological Chemistry 261:8708–8711.

Wang, H., Li, H., Jiang, X., Shen, Z., Li, M., 2014. Hepcidin is directly regulated by insulin and plays an important role in iron overload in streptozotocin-induced diabetic rats. Diabetes 63:1506–1518.

Dongiovanni, P., Ruscica, M., Rametta, R., Recalcati, S., Steffani, L., Gatti, S., et al., 2013. Dietary iron overload induces visceral adipose tissue insulin resistance. American Journal of Pathology 182(6):2254–2263.

Dongiovanni, P., Valenti, L., Ludovica Fracanzani, A., Gatti, S., Cairo, G., Fargion, S., 2008. Iron depletion by deferoxamine up-regulates glucose uptake and insulin signaling in hepatoma cells and in rat liver. American Journal of Pathology 172(3):738–747.

Gao, X., Zhang, W., Wang, Y., Pedram, P., Cahill, F., Zhai, G., et al., 2016. Serum metabolic biomarkers distinguish metabolically healthy peripherally obese from unhealthy centrally obese individuals. Nutrition Metabolism 13:33.

Wuertz, P., Havulinna, A.S., Soininen, P., Tynkkynen, T., Prieto-Merino, D., Hao, D., Sarfaraz, M.O., Farshidfar, F., Bebb, D.G., Lee, C.Y., Card, C.M., et al., 2016. Temporal characterization of serum metabolite signatures in lung cancer patients undergoing treatment. Metabolomics 12:58.

Lee, C.C., Watkins, S.M., Lorenzo, C., Wagenknecht, L.E., Il, C.A., et al., 2009. Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women. Diabetes Care 32:1678–1683.

Drogan, D., Dunn, W.B., Lin, W., Bujishe, B., Schulze, M.B., Langenberg, C., et al., 2015. Untargeted metabolic profiling identifies altered serum metabolites of type 2 diabetes mellitus in a prospective, nested case control study. Clinical Chemistry 61(3):487–497.

Floegel, A., Stefan, N., Yu, Z., Mühlenbruch, K., Drogan, D., Joost, H.G., et al., 2013. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes 62(2):639–648.

Wang-Sattler, R., Yu, Z., Herder, C., Messias, A.C., Floegel, A., He, Y., et al., 2012. Novel biomarkers for pre-diabetes identified by metabolomics. Molecular Systems Biology 8:615.

Rhee, E.P., Cheng, S., Larson, M.G., Wilford, G.A., Lewis, G.D., McCabe, E., et al., 2011. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. Journal of Clinical Investigation 121(4):1402–1411.

Ling, J., Chaba, T., Zhu, L.F., Jacobs, R.L., Vance, D.E., 2012. Hepatic ratio of phosphatidylcholine to phosphatidylethanolamine predicts survival after partial hepatectomy in mice. Hepatology 55(4):1094–1102.

Li, Z., Agellon, L.B., Allen, T.M., Umeda, M., Jewell, L., Mason, A., et al., 2006. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. Cell Metabolism 3(5):321–331.

Ikura, Y., Ohsawa, M., Suekane, T., Fukushima, H., Itabe, H., Jomura, H., et al., 2006. Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. Hepatology 43(3):506–514.

Verkade, H.J., Fast, D.G., Rusinol, A.E., Scabra, D.G., Vance, D.E., 1993. Impaired biosynthesis of phosphatidylcholine causes a decrease in the number of very low density lipoprotein particles in the Golgi but not in the endoplasmic reticulum of rat liver. Journal of Biological Chemistry 268(33):24990–249966.

Yao, Z.M., Vance, D.E., 1990. Reduction in VLDL, but not HDL, in plasma of rats deficient in choline. Biochemistry and Cell Biology 68(2):552–558.

Jacobs, R.L., Devlin, C., Tabas, I., Vance, D.E., 2004. Targeted deletion of hepatic CTP: phosphocholine cytidylyltransferase alpha in mice decreases plasma high density and very low density lipoproteins. Journal of Biological Chemistry 279(45):47402–47410.

Rinella, M.E., Elias, M.S., Smolak, R.R., Fu, T., Borenzajn, J., Green, R.M., 2008. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. Journal of Lipid Research 49(5):1066–1076.

Noga, A.A., Vance, D.E., 2003. A genderspecific role for phosphatidylethanolamine N-methyltransferase-derived phosphatidylcholine in the regulation of plasma high density and very low density lipoproteins in mice. Journal of Biological Chemistry 278(24):21851–21859.

Jacobs, R.L., Zhao, Y., Koonen, D.P., Sletten, T., Su, B., Lingrell, S., et al., 2010. Impaired de novo choline synthesis explains why phosphatidylethanolamine N-methyltransferase-deficient mice are protected from diet-induced obesity. Journal of Biological Chemistry 285(29):22403–22413.

Li, Z., Agellon, L.B., Vance, D.E., 2005. Phosphatidylcholine homeostasis and liver failure. Journal of Biological Chemistry 280(45):37798–37802.

Sreekumar, A., Poisson, L.M., Rajendiran, T.M., Khan, A.P., Cao, Q., Yu, J. et al., 2009. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature 457:910–914.

Issag, H.J., Waybright, T.J., Veenstra, T.D., 2011. Cancer biomarker discovery: opportunities and pitfalls in analytical methods. Electrophoresis 32:967–975.

Cavaliere, B., Maccioni, B., Monteleone, M., Naccarato, A., Sindona, G., Tagarelli, A., 2011. Sarcosine as a marker in prostate cancer progression: a rapid and simple method for its quantification in human urine by solide-phase microextraction-gas chromatography-triple quadruple mass spectrometry. Analytical and Bioanalytical Chemistry 400:2903–2912.

Mukherjee, S., Cruz-Rodriguez, O., Bolton, E., Irízug-Luí, J.A., 2012. The in vivo role of androgen receptor SUMOylation as revealed by androgen insensitivity syndrome and prostate cancer mutations targeting the proline/glycine residues of synergy control motifs. Journal of Biological Chemistry 287:31195–31206.

Cernei, N., Heger, Z., Gumulec, J., Zilka, 0., Masarik, M., Babula, P., et al., 2013. Sarcosine as a potential prostate cancer biomarker — a review. International Journal of Molecular Sciences 14:13893–13908.

Jegathesan, P., Beutheu, S., Ventura, G., Sarfati, G., Nubret, E., Kapel, N., et al., 2016. Effect of specific amino acids on hepatic lipid metabolism in fructose-induced non-alcoholic fatty liver disease. Clinical Nutrition 35(1):175–182.

Jegathesan, P., Beutheu, S., Ventura, G., Nubret, E., Berheim, I., al., 2015. Cirtulline and nonessential amino acids prevent fructose-induced nonalcoholic fatty liver disease in rats. Journal of Nutrition 145:2273–2279.

Moinard, C., Le Plenier, S., Norez, P., Morio, B., Bonnefont-Rousselot, D., Kharchi, C., et al., 2015. Cirtulline supplementation induces changes in body state.
composition and limits age-related metabolic changes in healthy male rats. Journal of Nutrition 145(7):1429–1437.

[71] Joffin, N., Jaubert, A.M., Durant, S., Bastin, J., De Bandt, J.P., Cynober, L., et al., 2014. Citrulline induces fatty acid release selectively in visceral adipose tissue from old rats. Molecular Nutrition and Food Research 58:1765–1775.

[72] Breuillard, C., Cynober, L., Moinard, C., 2015. Citrulline and nitrogen homeostasis: an overview. Amino Acids 47:685–691.

[73] Cox, M., Lehninger, A.L., Nelson, D.R., 2000. Lehninger principles of biochemistry, 3rd ed. New York: Worth Publishers. ISBN 1-57259-153-6.

[74] Seidner, D.L., Joly, F., Youssef, N.H., 2015. Effect of teduglutide, a glucagon-like peptide 2 analog, on citrulline levels in patients with short bowel syndrome in two phase III randomized trials. Clinical and Translational Gastroenterology 6:e93.