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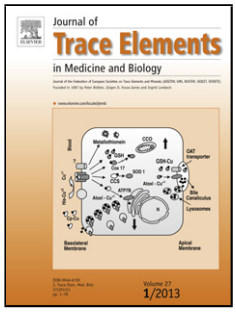

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<AT>Evaluation of tissue metal and trace element content in a rat model of non-alcoholic fatty liver disease using ICP-DRC-MS

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<ABS-Head><ABS-HEAD>Graphical abstract

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<*ABS-HEAD*>Highlights► NAFLD was characterized by hepatocyte steatosis and ballooning ► Insulin resistance, altered lipid profile and increased ALT activity was revealed ► NAFLD is associated with decreased total hepatic Co, Cu, I, Li, Mn, Se, Zn levels ► Hepatic Se and Mn levels remained decreased after adjustment for protein content ► Lower serum I, Li, Mn, and higher Co, Se, V, Sr levels were detected in NAFLD

<ABS-HEAD>Abstract

<ABS-P>The primary objective of the study was to assess the level of metals and trace elements in liver, serum, and hair of rats with diet-induced non-alcoholic fatty liver disease (NAFLD) using inductively coupled plasma dynamic reaction cell mass spectrometer (ICP-DRC-MS). 56 female 3-months-old Wistar rats divided into two equal groups were fed either standard (10% calories from fat) or high-fat high-carbohydrate diet (60% calories from fat in chow and 10% sucrose solution) for 6 weeks. Serum was examined for insulin resistance markers, lipid profile, and alanine aminotransferase (ALT) activity. Liver histology was assessed after hematoxylin and eosin staining. Metal and trace element concentrations were assessed by means of ICP-DRC-MS. Overfed animals were characterized by higher values of morphometric parameters. Liver examination revealed large and small droplet steatosis, hepatocyte ballooning and necrosis, being characteristic for NAFLD. Animals with NAFLD were characterized by insulin resistance, atherogenic changes of lipid profile and increased ALT activity. Significantly decreased hepatic Co, Cu, I, Li, Mn, Se, Zn levels were observed in rats with NAFLD. At the same time, only hepatic Mn and Se levels remained decreased after adjustment for total protein. Overfed animals were characterized by significantly lower I, Li, and Mn levels in blood serum, whereas concentration of Co, Se, V, and Sr exceeded the control values. In general, the results of the study demonstrate that NAFLD significantly affects metal and trace element status in experimental animals.

<KWD>Keywords: fatty liver; obesity; trace elements; minerals; insulin resistance.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by macrovesicular steatosis of more than 5% of hepatocytes in persons who do not consume alcohol. The prevalence of NAFLD is increasing worldwide reaching nearly 35% in some countries and being the most common liver disease [1]. NAFLD is often associated with obesity and adipokine dysregulation was supposed to play a key role in insulin resistance and NAFLD development [2]. Moreover, NAFLD is considered as a specific feature of metabolic syndrome with hepatic insulin resistance [3]. However, the causal relationship between NAFLD and metabolic syndrome is still questionable [4].

Like in the case of obesity [5] and metabolic syndrome [6], environmental pollution plays a significant role in NAFLD development [7]. It is supposed that heavy metals may play a significant role in NAFLD development. In particular, it has been demonstrated that cadmium [8], arsenic [9], and other heavy metal [10] exposure is significantly associated with NAFLD. A pathogenetic role of heavy metals in NAFLD development may be associated with their proinflammatory and prooxidant properties [11]. However, the existing data regarding the association between metal levels in the organism and NAFLD are inconsistent.

Essential trace elements also play a significant role in chronic liver diseases [12]. The most studied essential trace element in NAFLD is iron. In particular, it has been demonstrated that iron and copper dyshomeostasis may significantly contribute to NAFLD pathogenesis [13]. In particular, iron overload is tightly associated with NAFLD through activation of inflammation and oxidative stress [14]. However, certain contradictions exist. In particular, it has been demonstrated that both iron excess and deficiency may be associated with increased hepatic lipogenesis [15]. Experimental studies also revealed low hepatic iron in high fat fed animals [16]. Taking into account the role of insulin resistance [17] and oxidative stress [18] in NAFLD, other trace elements possessing insulin sensitizing (Cr, V, and Zn) and antioxidant properties (Se, Zn) [19] may also have a significant impact on the disease development. At the same time, data on the association between NAFLD and the level of these elements are still insufficient.

Nutritional strategies including trace element treatment are used for management of NAFLD [20]. Consequently, detailed data on mineral status of the organism in NAFLD are required for effective management of the disease.

Therefore, the primary objective of the study was to assess the level of metals and trace elements in liver, serum, and hair of rats with diet-induced NAFLD using inductively coupled plasma dynamic reaction cell mass spectrometer (ICP-DRC-MS).

Materials and methods

Experimental design

The protocol of the present investigation was approved by the Local Ethics Committee. All animal studies have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. A total of 56 female 3-months-old Wistar rats divided into two equal groups were used in the present study. The first group of animals (Control) obtained a standard diet (STD) containing 10% calories from fat (270 kcal/100 g, ``Orenburg food mixture factory", Orenburg, Russia) and pure drinking water with a total mineralization of not more than 250 mg/l. Animals from the second group (NAFLD) were maintained on a lard-based high-fat diet containing 60% calories from fat (429 kcal/100 g) and also obtained a 10% sucrose solution instead of drinking water (40 kcal/100 ml). No significant difference in trace elements content was detected between the STD and HFHCD (Table 1). The earlier data demonstrate that high-fat high-carbohydrate diet (HFHCD) is an effective dietary model to induce NAFLD in laboratory rodents [21]. The animals had free access to food and drinking solutions throughout the experiment. The mean chow consumption in STD and HFHCD groups were 23.5 ± 8.7 g and 24.5 ± 6.3 g, respectively ($p = 0.451$). The temperature in the animal room was 22 ± 2°C. The light and dark cycles in the animal room were 12 each (8.00 – 20.00). The total duration of dietary intervention was 6 weeks. At the end of the experiment liver was collected through a median laparotomic incision. Venous blood was collected from jugular vein with subsequent centrifugation to obtain serum. Hair samples were collected from the cranial part of the spine using ethanol-precleaned stainless scissors.

Morphometric and histological study

Body weight and naso-anal length (body length) were assessed in all animals. The values of thoracic circumference (TC) and abdominal circumference (AC) were used for the calculation of the AC/TC ratio.

The obtained liver samples (median lobe) were fixed in neutral buffered formalin. After embedding in paraffin, the blocks were sliced using microtome to obtain 5 μm-thick slices. The slices were stained with hematoxylin and eosin using standard techniques. The obtained sections were assessed and photographed using LOMO Micmed-6 (Lomo, Russia) microscope equipped with digital camera. Both periportal and centrilobular areas were assessed and photographed. Hepatocyte nucleus and lipid droplet areas were assessed using ImageJ software (NIH, Bethesda, MD, USA).

Blood biochemistry

The obtained serum was examined for glucose, total protein, albumin, triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C) concentrations and alanine aminotransferase (ALT) activity using the respective Olvex kits (St. Petersburg, Russia). Serum total lipid levels were assessed using Lachema kit (Lachema, Brno, Czech Republic) spectrophotometrically. Circulating insulin levels were estimated by means of enzyme-linked immunosorbent assay using Alpco kit (Alpco Diagnostics, Windham, NH). The values of serum glucose and insulin were used for calculation of insulin resistance index by the homeostasis model assessment (HOMA-IR) as follows: HOMA-IR = (glucose x insulin)/22.5 [22].

Oxidative stress biomarkers

Serum and liver samples were used for assessment of routine biomarkers of oxidative stress. Liver homogenization (1:10; w/v) for determination of total thiols (TSH) and thiobarbituric acid-reactive substances (TBARS) level was performed in an ice-cold 1/15 M phosphate buffer (pH = 7.4) with subsequent centrifugation (3,000 g, 10 min, 4°C). The obtained supernatant was used for analysis. Serum and supernatant levels of TSH [23] and TBARS [24] were assessed spectrophotometrically at PD-303UV spectrophotometer (Apel, Japan). The obtained levels were expressed

per mg of protein in a sample. The total protein concentration in liver homogenate was determined using Lowry-Folin method [25].

Metal and trace element analysis

The obtained samples were differentially prepared for trace elements analysis. Liver samples were rinsed with ice-cold distilled deionized water and separated from connective tissue using precleaned stainless instruments. The obtained hair samples were washed with acetone and rinsed thrice with distilled deionized water to remove possible external contamination and subsequently dried on air at 60°C. Serum samples were diluted with an acidified diluent (1:15 v/v) based on 1% 1-butanol (Merck KGaA, Darmstadt, Germany), 0.1% Triton X-100 (Sigma-Aldrich, Co., St. Louis, USA), and 0.07% HNO_3 (Sigma-Aldrich, Co., St. Louis, USA) in distilled deionized water. All studied samples in a quantity of 50 mg were introduced into Teflon tubes with concentrated HNO_3 for subsequent microwave digestion in Berghof speedwave (Berghof, Eningen, Germany) system for 20 minutes at 180°C. After cooling the system, the obtained solutions were used for chemical analysis.

Metal and trace element concentrations were assessed by means of inductively coupled plasma mass spectrometry at NexION 300D (PerkinElmer Inc., Shelton, CT 06484, USA) using Dynamic Reaction Cell (DRC) technology for the removal of major polyatomic interferences [26]. Sampling was performed with ESI SC-2 DX4 autosampler (Elemental Scientific Inc., Omaha, NE 68122, USA).

Calibration of the system prior the analysis was performed using solutions of metals with a final concentration of 0.5, 5, 10, and 50 μg/l prepared from Universal Data Acquisition Standards Kit (PerkinElmer Inc., Shelton, CT 06484, USA) by dilution of a stock solution with distilled deionized water and acidification with 1% HNO_3. Internal online standardization was performed using an internal standard containing 10 μg/l yttrium prepared from Yttrium (Y) Pure Single-Element Standard (PerkinElmer Inc., Shelton, CT 06484, USA) on a matrix containing 8% 1-butanol (Merck KGaA), 0.8% Triton X-100 (Sigma-Aldrich, Co.), 0.02% tetramethylammonium hydroxide (Alfa-Aesar, Ward Hill, MA 01835 USA), and 0.02% ethylenediaminetetraacetic acid (Sigma-Aldrich, Co). Laboratory quality control was performed using standard reference materials of hair (GBW09101, Shanghai Institute of Nuclear Research, Shanghai, China) and plasma (ClinCheck Plasma Control, lot 129, levels 1 and 2; RECIPE Chemicals + Instruments GmbH, Germany). The recovery rates for all analyzed metals and trace elements always exceeded 80%.

The obtained data on metal and trace elements content in liver, hair, and serum were expressed as μg/g, μg/g, μg/ml, respectively. Liver and serum metal and trace elements levels were also adjusted for total protein concentration and expressed as ng/mg liver protein and ng/g serum protein, respectively.

Statistical analysis

The obtained data were treated with Statistica 10 software (Statsoft, Tulsa, OK, US). Shapiro-Wilk test was used for data normality assessment. Data are expressed as mean and the respective standard deviation (± SD) for normally distributed values and median and 25 and 75 percentile boundaries for those non-normally distributed [27]. Group comparison of the parameters characterized by Gaussian distribution was performed using one-way ANOVA. Mann-Whitney U-test was used for group comparisons of non-normally distributed values. All differences were considered significant at $p < 0.05$.

Results

Morphometric parameters

High fat high carbohydrate feeding resulted in a significant increase in morphometric parameters (Fig 1). The most expressed changes were detected in weight gain (Fig 1B), being more than 2-fold higher in the NAFLD group. At the same time, the values of total body (Fig 1A) and liver weight (Fig 1F), and AC (Fig 1E) in overfed animals were 21, 20, and 16% higher than those in the control group. Despite a 4 and 8% increase in body length (Fig 1C) and TC (Fig 1D) values, the observed difference was also significant according to one-way ANOVA.

Liver histology

The obtained data demonstrate that STD-fed animals were characterized by normal liver structure in both periportal (Fig 2A) and centrilobular (Fig 2B) areas. The mean area of hepatocyte nuclei in centrilobular and periportal areas are 84 (72-98) and 88 (73-100) μm^2, respectively. Light microscopy of the obtained sections did not reveal any cytoplasmic inclusions. Oppositely, rats fed a high-fat high-carbohydrate diet had significantly altered hepatic histology being characterized by the appearance of large lipid droplets in hepatocyte cytoplasm especially in the periportal areas (Fig 2C). These areas were also characterized by the presence of hepatocyte ballooning and necrosis. Centrilobular hepatocytes are characterized by both small and large droplet steatosis (Fig 2D). However, hepatocyte necrosis is less frequent than that in the periportal area. The above mentioned changes were detected in 100% of HFHCD-fed animals. Mean cytoplasmic lipid droplet area in periportal and centrilobular hepatocytes was 101 (58-198) and 88 (54-143) μm^2. Mean nucleus area in centrilobular and periportal hepatocytes of high-fat high-carbohydrate fed animals was lower than that in the control group by 25 ($p < 0.001$) and 30% ($p < 0.001$), being 63 (54-76) and 62 (53-75) μm^2, respectively.

Blood biochemistry

NAFLD in rats was also associated with insulin resistance (Table 2). In particular, serum levels of glucose, insulin, and HOMA-IR values were 30, 76, and 150% higher as compared to the control values. Serum ALT activity in NAFLD group significantly exceeded the control values by a factor of 2. It is also notable that HFHC-fed animals were characterized by a significant 9% increase in total protein. However, no marked group difference was detected in serum albumin concentration. The activity of ALT in NAFLD rats was nearly 3-fold higher than that in the

healthy animals. Lipid spectrum was also significantly altered in animals with NAFLD. In particular, serum total lipid and TG levels exceeded the respective control values by a factor of more than 2 and 3. At the same time, total cholesterol in HFHC-fed animals was 32% higher as compared to that in STD rats. Despite a 16% decrease in serum HDL-C concentration in rats with NAFLD, the observed changes were not significant. In turn, high-fat high-carbohydrate feeding resulted in a significant more than 2-fold elevation of serum LDL-C levels as compared to the STD-fed animals.

Systemic and liver oxidative stress

The obtained data demonstrate that HFHC feeding did not significantly alter the levels of routine oxidative stress markers in serum (Table 3). Similarly, no significant difference in hepatic TBARS levels between the studied groups was detected. At the same time, rats with NAFLD were characterized by 31% lower levels of hepatic total thiol content as compared to the STD-fed controls.

Liver trace elements

NAFLD resulted in a significant alteration of essential metal and metalloid content in liver (Table 4). In particular, liver levels of Co, Cu, I, Li, Mn, Se, and Zn were 33, 14, 35, 30, 36, 44, and 22% lower than the respective control values. At the same time, no significant group difference in liver Cr and V content was detected. A 7% decrease in liver Fe content was nearly significant in agreement with Mann-Whitney U-test. Toxic metals were also affected by HFHC feeding with 18 and 37% lower hepatic concentration of As and Cd. At the same time, liver levels of Al, and Ni did not differ significantly between the study groups. Surprisingly, hepatic Sr levels were 37% higher than those in the control animals. However, the changes were nearly significant.

It is notable that after adjustment for total liver protein content the level of metals and trace elements was more stable. In particular, NAFLD was associated with a significant decrease of protein-adjusted liver Mn and Se content, being 25 and 35% lower than the respective control values. Oppositely, high-fat high-carbohydrate feeding resulted in a significant 60% increase in protein-adjusted hepatic Sr levels.

Serum trace elements

The character of alteration of serum trace elements profile in rats with NAFLD was quite different from that in liver (Table 5). In particular, HFHC-fed animals had 36 and 44% higher Co and Se levels in comparison to the control group values. Oppositely, serum levels of I, Li, and Mn in rats with NAFLD were 26, 44, and 12% lower than those in the STD group. In turn, no significant group difference in serum Cr, Cu, Fe, V, and Zn was detected. Serum toxic trace elements spectrum was characterized by a significant 92% increase in Sr levels, whereas the concentration of Al, As, and Ni was not affected. Cadmium was not detected in serum of rats from both groups.

Protein-adjusted levels of metals and trace elements were differentially affected by NAFLD than the total concentrations. In particular, protein-adjusted level of serum Co and V in HFHCD-fed animals did not differ significantly from the control values. Oppositely, a significant 19% decrease in protein-adjusted serum Zn levels was detected in the overfed animals. Similarly to the total serum concentration, NAFLD was associated with a 55, 49, and 28% decrease of protein-adjusted serum I, Li, and Se levels, as well as elevated Sr content in comparison to the respective control values.

Hair trace elements

The level of the studied metals in hair was more stable as compared to blood and liver (Table 6). At the same time, hair Cu, I, Li, and Zn content was 9, 36, 38, and 6% lower as compared to the respective control values. Oppositely, NAFLD was accompanied by a significant 24% increase in hair V level. No significant group difference in hair Co, Cr, Fe, Mn, and Se was detected. The level of toxic metals in hair of HFHC-fed rats was not significantly affected.

Discussion

The obtained data demonstrate that HFHCD consumption resulted in a significant increase in liver weight, as well as liver steatosis and hepatocellular ballooning, being indicative of the presence of NAFLD in experimental animals [28]. These data confirm earlier indications that high-fat high-carbohydrate feeding is a good model of NAFLD in experimental animals [29].

Serum examination also revealed a significant systemic insulin resistance and altered lipid profile in rats with NAFLD, being in agreement with the earlier data [30]. In particular, it has been demonstrated that inflammatory cytokines and adipokines affect insulin signaling in NAFLD leading to insulin resistance [2]. It is also notable that insulin resistance also provides a link between NAFLD and altered lipid profile resulting in proatherogenic state [30].

Hepatic total thiol content was affected by dietary manipulation in experimental animals being indicative of the presence of local oxidative stress in NAFLD [31]. Earlier studies demonstrated that oxidative stress plays a significant role in NAFLD progression [18]. It is also notable that oxidative stress is interrelated with insulin resistance in pathogenesis of fatty liver and NAFLD [32]. At the same time, the absence of systemic oxidative stress in the current model may be associated with the type of dietary fat. In particular, it has been demonstrated that saturated and monounsaturated fatty acids being present in lard are resistant to free radical oxidation [33]. Moreover, the results of TBARS assay may depend on the presence of various compounds limiting the informativeness of the analysis [34].

Therefore, the obtained animal model of NAFLD was characterized by liver steatosis, insulin resistance, altered lipid profile, and local hepatic oxidative stress.

It is demonstrated that NAFLD impaired hepatic and systemic (serum and hair) metal and metalloid levels. The most important finding is that the decreased hepatic levels of Se and Mn in NAFLD group were observed even after adjustment for liver protein content, being indicative of the profound alteration of these elements metabolism in NAFLD development.

Despite the presence of multiple indications of altered Se homeostasis in alcoholic liver disease [35,36], its metabolism in NAFLD is insufficiently studied. Taking into account the role of oxidative stress in NAFLD development and progression [18] it is supposed that decreased hepatic Se levels may be associated with increased Se requirement due to oxidative stress. At the same time, earlier data demonstrate that patients with non-alcoholic steatohepatitis have significantly lower hair Se levels, whereas no significant difference was detected between the control and liver steatosis group [37].

The obtained data demonstrate a significant decrease in liver and serum Mn content in NAFLD. However, previous experimental study failed to detect any significant alterations in liver Mn content in response to HFD [38]. In general, our findings are in agreement with the earlier indication of decreased serum Mn levels in diabetic obese women [39]. Experimental data also demonstrate that NAFLD in male mice is associated with a significant decrease in liver Mn-SOD activity [40], that is also used as a marker of Mn status [41].

We also observed a significant decrease in hepatic Co, Cu, I, Li, Mn, Se, and Zn levels in animals with NAFLD. However, these changes became insignificant after adjustment for hepatic protein content.

The observed decrease in hepatic Cu content in NAFLD rats corresponds to the earlier data demonstrating significantly lower liver Cu in NAFLD patients as compared to the control ones [42]. A detailed study by Church et al. (2015) demonstrated that liver Cu is significantly decreased in *ob/ob* mice even after liver fat extraction [43]. Experimental data also demonstrated that fructose-induced NAFLD is associated with copper deficiency due to altered duodenal Ctr1 activity [44]. Moreover, it has been noted that copper status is associated with impaired iron metabolism in NAFLD [45].

Experimental data demonstrate that dietary obesity in mice is associated with a significantly lower hepatic zinc levels [46], being in agreement with the obtained data. Hypothetically, decreased liver zinc may be associated with hepatic insulin resistance as Zn plays a significant role in insulin signaling [47]. Surprisingly, the level of other trace elements, Cr and V, also involved in insulin signal transduction [48, 49], was not affected by dietary manipulation.

The existing data on metabolism of Co, a structural component of B12 vitamin, in NAFLD are insufficient. It has been noted that feeding rats with high-fat folate-deficient diet significantly decreased liver B_{12} content as compared to standard folate-deficient diet [50]. At the same time, the examination of patients with biopsy-proven NAFLD failed to detect any significant difference in serum B_{12} levels in comparison to the control levels. Moreover, B_{12} levels did not correlate with the metabolic parameters [51].

The existing studies demonstrate that NAFLD is associated with thyroid dysfunction [52]. However, iodine status in NAFLD is poorly studied. We detected a significant decrease in hepatic I content in NAFLD animals that may be associated with decreased deiodination of thyroid hormones in liver [53]. Oppositely, excessive iodine has been shown to induce hepatic steatosis [54].

We have detected significantly lower hepatic Li levels in animals with NAFLD. Oppositely, the most recent study demonstrated that Li overexposure may increase liver fibrosis and fatty degeneration [55]. It is supposed that decreased liver Li content in rats with NAFLD may be associated with inhibition of Li absorption by high fat diet [56].

The present study failed to detect any significant alteration of hepatic iron levels in rats with NAFLD, being in contrast to the existing data. In particular, earlier data demonstrate that iron overload is tightly associated with NAFLD and insulin resistance through activation of inflammation and oxidative stress [14]. It has been also noted that systemic inflammation may significantly contribute to liver iron accumulation in NAFLD patients [57] and high fat diet-induced liver steatosis [58]. Oppositely, a growing body of data demonstrate that high-fat feeding results in decreased hepatic iron levels due to a proinflammatory state [16, 59]. It has been also noted that HFD decreases iron absorption and tissue accumulation without significant alteration of hepcidin expression [60]. Moreover, the authors state that the observed iron deficiency is not associated with iron sequestration in reticulo-endothelial system [60]. Therefore, the existing data demonstrate that inflammation in obesity and NAFLD may result in both increased and decreased hepatic iron levels. Moreover, it is stated that both iron deficiency and overload may result in altered lipid metabolism and increased intrahepatic lipid accumulation [15]. Finally, the results of the current study are confirmed by the presence of a model of high fat-induced steatohepatitis without increased liver iron stores [61].

Interesting data were obtained on toxic trace elements levels in NAFLD. It is supposed that heavy metals may play a significant role in NAFLD development [7]. In particular, a recent study demonstrated that low-dose Cd exposure induces metabolic and genetic alterations associated with fatty liver in mice [62]. Arsenic exposure has also been shown to interfere with NAFLD pathogenesis [10, 63]. At the same time, all above mentioned studies demonstrate the effect of heavy metal exposure, whereas the association between NAFLD and heavy metal levels in tissues of non-exposed organisms is insufficiently studied. The results of the present study showed a significant decrease of hepatic As and Cd content in NAFLD rats. Lower As content in rats' liver may be associated with altered arsenic distribution [64]. At the same time, the mechanism of decreased hepatic heavy metal content in a rat model of NAFLD is unknown.

It is notable that in general decreased hepatic metal and trace element content is associated with increased serum and hair levels. The observed situation may occur due to increased mobilization of metals from liver being the main

metal depot in the mammalian organism. The mechanism of such redistribution is unknown. It has been noted that certain infections and the accompanying inflammation are associated with a shift of trace elements between internal organs and blood [65,66]. However, it is unknown whether NAFLD-associated inflammation plays a key role in the observed changes.

Therefore, the obtained data demonstrate:

i) <LIST ><i)**1**>NAFLD is associated with significantly decreased total hepatic Co, Cu, I, Li, Mn, Se, Zn levels, whereas after adjustment for protein content only Se and Mn levels remained decreased;

<ii)**1**>Rats with NAFLD are characterized by significantly lower I, Li, and Mn levels in blood serum, while the concentration of Co, Se, V, and Sr exceeded the control values. The protein-adjusted levels of I, Li, Se, and Zn were also lower after high-fat high-carbohydrate feeding, whereas Sr content remained elevated.

ii) <iii)**1**>Hair analysis in animals with NAFLD revealed significantly decreased Cu, I, Li, and Zn content, and the level of V was elevated.</LIST>

In general, the results of the study demonstrate that NAFLD significantly affects metal and trace element status in experimental animals, being in agreement with our recent findings of altered liver trace element homeostasis even in initial stages of obesity [67]. Data on altered trace elements status in NAFLD may be considered during development of nutritional strategies for diseases management. In particular, special attention should be given to profound alteration Se and Mn metabolism in NAFLD. Hypothetically, modification of Se and Mn status may improve metabolic parameters in NAFLD. However, additional *in vitro* and *in vivo* studies are required to estimate the mechanisms of trace element dyshomeostasis in NAFLD and the causal relationship between these states. It is also notable that serum is not always a good indicator of metal and trace elements homeostasis in NAFLD and more parameters should be assessed prior the modulation of trace element status.

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<Figure>Fig. **1**. Morphometric parameters of control and NAFLD rats. A) Final body weight, B) Weight gain, C) Body length, D) Thoracic circumference; E) Abdominal circumference; F) Liver weight. Graph represents median (–), interquartile range ([]), and non-outlier range (I). The values are expressed as (Median (25 - 75). Individual p values are indicated in accordance with one-way ANOVA.

<Figure>Fig. **2**. Liver histology in experimental animals (hematoxylin and eosin staining, ×300). a – periportal area of liver in control animals characterized by normal hepatic structure; b – centrilobular area of liver in STD-fed animals characterized by normal hepatic histology; c – periportal area of liver fed a high fat high carbohydrate diet. Hepatocytes are characterized by the presence of large lipid droplets, signs of ballooning. Single necrotic hepatocytes are observed; d – centrilobular area of HFHCD-fed animals characterized by the presence of small and large droplet steatosis.

Tables

<Table>Table 1. Trace element content in diets (μg/g)

Element	STD	HFHCD
Co	1.0 ± 0.1	0.96 ± 0.15
Cr	0.65 ± 0.05	0.53 ± 0.09
Cu	16 ± 2	13 ± 3
Fe	210 ± 20	190 ± 38
I	1 ± 0.1	0.95 ± 0.2
Li	0.13 ± 0.01	0.12 ± 0.03
Mn	80 ± 10	75 ± 14
Se	0.20 ± 0.03	0.16 ± 0.04
V	0.40 ± 0.03	0.38 ± 0.05
Zn	30 ± 3	25 ± 3
Al	68 ± 4	59 ± 8
As	0.35 ± 0.01	0.33 ± 0.05
Cd	0.05 ± 0.01	0.04 ± 0.02
Ni	2.66 ± 0.30	2.31 ± 0.37
Sr	72.1 ± 1.0	68.2 ± 2.6
STD – standard diet; HFHCD – high fat high carbohydrate diet; Data expressed as mean ± SD.		

<Table>Table 2. Biochemical indicators of carbohydrate, protein and lipid metabolism in rats' serum

Parameter	Control	NAFLD	P values
Glucose, mmol/l	11.4 (8.6-13.4)	14.8 (11.5-17.5)	0.006 *
Insulin, IU/l	0.82 (0.59-1.19)	1.44 (1.02-2.40)	0.001 *
HOMA-IR	0.38 (0.22-0.64)	0.95 (0.74-1.46)	< 0.001 *
ALT, U/L	44.3 (31.4-53.8)	121.9 (88.2-221.5)	< 0.001 *
Total protein, g/l	92.2 (73.5-98.7)	99.9 (86.4-132.1)	0.044 *
Albumin, g/l	53.2 (50.4-60.7)	54.5 (48.4-61.4)	0.891
Total lipids, g/l	1.3 (1.2-1.6)	2.6 (1.7-3.9)	< 0.001 *
TG, mmol/l	1.5 (1.0-2.0)	4.6 (2.8-6.7)	< 0.001 *
TC, mmol/l	1.6 (1.3-2.0)	2.1 (1.4-3.4)	0.027 *
HDL-C, mmol/l	1.2 (1.0 – 1.4)	1.0 (0.9 – 1.3)	0.102
LDL-C, mmol/l	0.45 (0.41 – 0.68)	1.01 (0.59 – 2.02)	< 0.001 *

HOMA-IR – homeostatic model assessment insulin resistance index; ALT – alanine aminotransferase; TG – triglycerides; TC – total cholesterol; HDL-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol;
Data expressed as median (25 – 75);
* - significant difference between the group values at $p < 0.05$

<Table>Table 3. The level of TSH and TBARS in serum and liver of experimental animals

Parameter	Control	NAFLD	P values
Serum TBARS, nmol/mg protein	0.31 ± 0.17	0.25 ± 0.11	0.289
Serum TSH, mmol/mg protein	0.06 ± 0.04	0.08 ± 0.04	0.153
Liver TBARS, nmol/mg protein	0.94 ± 0.15	1.01 ± 0.47	0.876
Liver TSH, mmol/mg protein	0.84 ± 0.19	0.58 ± 0.27	< 0.001 *

TBARS – thiobarbituric acid reactive substances; TSH – total thiols;
Data expressed as median (25 – 75);
* - significant difference between the group values at $p < 0.05$

<Table>Table 4. Trace element content in liver of experimental animals

Element	µg/g liver tissue			ng/mg liver protein		
	Control	NAFLD	p-value	Control	NAFLD	p-value
Co	0.122 (0.099-0.148)	0.081 (0.0675-0.096)	< 0.001 *	0.578 (0.402 - 0.800)	0.410 (0.339 - 0.600)	0.132
Cr	0.211 (0.1-0.327)	0.196 (0.1105-0.2775)	0.695	0.711 (0.535 - 1.384)	1.14 (0.56 - 1.56)	0.624
Cu	5.12 (4.86-5.48)	4.37 (3.73-5.17)	0.003 *	24.62 (18.91 - 29.95)	23.86 (18.30 - 29.98	0.936
Fe	339 (311-376)	313 (262-349)	0.072	1551 (1127 - 2055)	1690 (1281 - 1916)	0.504
I	0.134 (0.081-0.154)	0.087 (0.024-0.1145)	0.016 *	0.474 (0.358 - 0.754)	0.460 (0.109 - 0.638)	0.322
Li	0.0040 (0.0021-0.0061)	0.0028 (0.0008-0.0036)	0.034 *	0.016 (0.010 - 0.023)	0.011 (0.006 - 0.021)	0.202
Mn	2.29 (1.99-2.60)	1.46 (1.38-1.57)	< 0.001 *	10.48 (8.06 - 12.29)	7.78 (5.82 - 10.51)	0.022 *
Se	1.24 (1.19-1.32)	0.697 (0.546-0.847)	< 0.001 *	5.54 (4.47 - 7.12)	3.59 (2.53 - 5.04)	0.002 *
V	0.0078 (0.006-0.011)	0.0069 (0.0054-0.0100)	0.301	0.040 (0.026 - 0.067)	0.036 (0.027 - 0.058)	0.964
Zn	35 (33.24-37.66)	26.99 (22.82-29.11)	< 0.001 *	163.98 (124.99 - 216.04)	148.53 (109.15 - 183.58)	0.250
Al	0.771 (0.548-1.23)	0.746 (0.5905-	0.901	3.58 (2.41 - 6.09)	4.17 (3.03 - 6.55)	0.281

		0.9755)				
As	0.091 (0.078-0.109)	0.074 (0.0615-0.088)	0.015 *	0.394 (0.320 - 0.625)	0.389 (0.296 - 0.541)	0.624
Cd	0.023 (0.019-0.026)	0.0145 (0.013-0.02)	< 0.001 *	0.109 (0.088 - 0.136)	0.093 (0.060 - 0.117)	0.127
Ni	0.014 (0.0098-0.019)	0.0145 (0.012-0.018)	0.775	0.060 (0.043 - 0.109)	0.076 (0.058 - 0.114)	0.215
Sr	0.026 (0.02-0.036)	0.0355 (0.026-0.045)	0.081	0.122 (0.094 - 0.251)	0.195 (0.140 - 0.266)	0.048 *
Data expressed as median (25 – 75); * - significant difference between the group values at $p < 0.05$						

<Table>Table 5. Trace element concentration in serum of experimental animals

Element	µg/ml serum			ng/g serum protein		
	Control	NAFLD	p-value	Control	NAFLD	p-value
Co	0.00265 (0.0023-0.003)	0.0036 (0.0029-0.0042)	0.001 *	0.031 (0.025 - 0.042)	0.037 (0.025 - 0.043)	0.662
Cr	0.0023 (0.002-0.0025)	0.0023 (0.002-0.0027)	0.609	0.027 (0.023 - 0.032)	0.023 (0.016 - 0.031)	0.114
Cu	1.4 (1.23-1.605)	1.44 (1.32-1.6)	0.447	16.46 (14.00 - 19.83)	15.04 (10.07 - 18.19)	0.081
Fe	3.19 (2.82-3.635)	3.43 (3.19-3.86)	0.153	39.56 (34.04 - 47.64)	34.03 (25.42 - 42.29)	0.106
I	0.0905 (0.0865-0.0995)	0.067 (0.057-0.074)	< 0.001 *	1.072 (0.935 - 1.340)	0.568 (0.476 - 0.788)	< 0.001 *
Li	0.0050 (0.0045-0.00565)	0.0028 (0.0025-0.0035)	< 0.001 *	0.055 (0.051 - 0.071)	0.028 (0.019 - 0.038)	< 0.001 *
Mn	0.409 (0.383-0.4275)	0.357 (0.347-0.39)	< 0.001 *	0.054 (0.041 - 0.068)	0.067 (0.047 - 0.111)	0.056
Se	0.0044 (0.0037-0.0058)	0.0073 (0.0055-0.01)	< 0.001 *	4.86 (4.05 - 5.78)	3.48 (2.43 - 4.10)	0.001 *
V	0.0009 (0.00075-0.0009)	0.0009 (0.0009-0.0009)	0.021 *	0.010 (0.008 - 0.011)	0.009 (0.007 - 0.011)	0.127
Zn	1.135 (1.02-1.215)	1.07 (0.971-1.15)	0.091	13.21 (11.54 - 16.03)	10.65 (7.71 - 12.34)	0.002 *
Al	0.025 (0.02-0.033)	0.023 (0.019-0.033)	0.950	0.269 (0.220 - 0.440)	0.278 (0.164 - 0.355)	0.296
As	0.00535 (0.00435-0.00715)	0.0058 (0.0052-0.0073)	0.268	0.072 (0.051 - 0.091)	0.062 (0.042 - 0.079)	0.228
Ni	0.0066 (0.0063-0.0102)	0.0073 (0.0055-0.0086)	0.979	0.078 (0.067 - 0.135)	0.064 (0.051 - 0.100)	0.055
Sr	0.0475 (0.041-0.052)	0.091 (0.075-0.098)	< 0.001 *	0.563 (0.452 - 0.723)	0.840 (0.524 - 1.245)	0.004 *
Data expressed as median (25 – 75); * - significant difference between the group values at $p < 0.05$						

<Table>Table 6. Trace element content in hair of experimental animals (µg/g dry hair)

	Control	NAFLD	p-value
Co	0.0275 (0.024-0.0315)	0.0255 (0.0235-0.0335)	0.949
Cr	0.1975 (0.1015-0.34)	0.1255 (0.073-0.2755)	0.322
Cu	12.975 (12.42-14.665)	11.69 (10.94-12.615)	< 0.001 *
Fe	20.04 (18.545-24.925)	20.745 (19.35-24.58)	0.595
I	5.915 (4.31-7.155)	3.77 (2.815-4.62)	< 0.001 *
Li	0.0755 (0.0575-0.095)	0.0465 (0.0405-0.0545)	< 0.001 *
Mn	0.832 (0.735-1.125)	1.0045 (0.851-1.445)	0.084
Se	0.4605 (0.4365-0.49)	0.4445 (0.4235-0.5015)	0.383
V	0.0565 (0.0275-0.0765)	0.07 (0.0515-0.099)	0.045 *
Zn	224.5 (214.5-244)	210.5 (204-227)	0.008 *

Al	1.635 (1.265-2.06)	1.87 (1.5-2.22)	0.102
As	0.0335 (0.0285-0.0385)	0.0305 (0.026-0.034)	0.067
Cd	0.00315 (0.0025-0.00455)	0.0038 (0.00305-0.0055)	0.225
Ni	0.1375 (0.1005-0.1795)	0.1385 (0.108-0.154)	0.927
Sr	0.832 (0.671-1.125)	0.804 (0.7325-0.9935)	0.515
Data expressed as median (25 – 75); * - significant difference between the group values at $p < 0.05$			

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