

A Role for Low Hepatic Copper Concentrations in Nonalcoholic Fatty Liver Disease

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- OBJECTIVES:** Copper has a role in antioxidant defense, lipid peroxidation, and mitochondrial function, and copper deficiency has been linked to atherogenic dyslipidemia. We aimed to investigate the potential role of copper availability in the pathogenesis of nonalcoholic fatty liver disease (NAFLD).
- METHODS:** Patients with NAFLD ($n = 124$) were compared to patients with chronic hepatitis C ($n = 50$), hemochromatosis ($n = 35$), alcoholic liver disease ($n = 13$), autoimmune hepatitis ($n = 11$), and control subjects ($n = 27$). We determined liver and serum copper concentrations with correlation to clinical, histological, and biochemical parameters in humans. The effect of dietary copper restriction on liver histology and intermediary metabolism in rats was investigated.
- RESULTS:** Hepatic copper concentrations in patients with NAFLD were lower than in control subjects (17.9 ± 8.4 vs. 31.4 ± 8.2 $\mu\text{g/g}$; $P < 0.001$) and in patients with other liver diseases ($P < 0.05$ for all liver diseases). In patients with NAFLD, lower liver copper was correlated with more pronounced hepatic steatosis ($R = -0.248$; $P = 0.010$), fasting glucose ($R = -0.245$; $P = 0.008$), and components of the metabolic syndrome (MetS; $R = 0.363$; $P < 0.001$). Patients with nonalcoholic steatohepatitis (NASH; $n = 31$) had lower hepatic copper concentrations than those with simple steatosis ($n = 93$; $P = 0.038$). Restriction of dietary copper in rats induced hepatic steatosis and insulin resistance (IR).
- CONCLUSIONS:** Reduced hepatic copper concentrations are found in human NAFLD and are associated with more pronounced hepatic steatosis, NASH, and components of the MetS. The development of hepatic steatosis and IR in response to dietary copper restriction in rats suggests that copper availability may be involved in the development of NAFLD.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/ajg>

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become the most frequent cause of elevated liver enzymes in Western societies (1). The histological and clinical spectrum of NAFLD ranges from benign steatosis to nonalcoholic steatohepatitis (NASH) with inflammation and scarring that may potentially progress to cirrhosis, end-stage liver disease, and hepatocellular carcinoma (2,3). NAFLD is closely linked to insulin resistance (IR) and has been firmly established as the hepatic manifestation of the metabolic syndrome (MetS) that comprises visceral obesity, arterial

hypertension, dyslipidemia, and impaired glucose tolerance or diabetes (4). The prevalence of NAFLD and other manifestations of IR are expected to increase further in the near future as a consequence of a sedentary lifestyle, high-caloric diets, and a predisposing genetic background (5).

An adequate supply of copper is essential for healthy life, as chronic copper deficiency can elicit anemia, leucopenia, myelopathy, or skin abnormalities. Data from rodent models provide evidence of an adverse effect of marginal copper deficiency on lipid metabolism (6,7). Increased oxidative stress is considered

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a key trigger in the pathogenesis of human NAFLD (8) and one of the enzymes counteracting oxidative stress, Cu/Zn superoxide dismutase depends on adequate copper availability (9), suggesting a link between copper availability and antioxidant defense in NAFLD. In addition, Sprague–Dawley rats exhibited an increased activity of the pro-inflammatory protein cyclooxygenase-2, when fed a diet with a low copper content (10). Moreover, IR and, in particular, NAFLD are frequently accompanied by perturbations of iron homeostasis (11,12) that are molecularly linked to low copper bioavailability and decreased levels of the copper containing ferroxidase ceruloplasmin (13,14). Systemic copper deficiency causes mitochondrial dysfunction in mice (15) and similar morphological and functional alterations have also been described in human NAFLD (16).

Given the multifaceted biological properties of copper, it appeared attractive to investigate the potential contribution of copper bioavailability to the development of NAFLD. In extension of a previous study (13), we aimed to compare hepatic copper concentrations (HCCs) of patients with NAFLD, chronic hepatitis C, alcoholic liver disease (ALD), hereditary hemochromatosis (HH), autoimmune hepatitis (AIH), and subjects with normal liver tissues and their relationships to clinical, histological, and biochemical parameters in a larger population. Furthermore, we aimed to examine the effect of dietary copper supply on liver histology and metabolic parameters in rats.

METHODS

Patient and control groups

We included 260 consecutively biopsied patients in our study. Among these, 124 patients were diagnosed with NAFLD. Patients with NAFLD had elevated liver enzymes, bright liver on ultrasound examination, and underwent liver biopsy after serological exclusion of infectious, immunological, or hereditary causes of liver disease and liver histology was compatible with NAFLD. As liver biopsies from “truly healthy” subjects are difficult to obtain, 27 subjects (20 women, 7 men) who underwent liver biopsy during the same period of time for unexplained elevation of liver enzymes. These patients had no clinical, serological (infectious, immunological, or hereditary causes of liver disease) evidence of liver disease and normal results of ultrasound examination and/or magnetic resonance tomography. When liver histology was reported as normal liver tissue, these patients were classified as control subjects for our investigation. Complete biochemical, clinical, and liver data were available from 59 NAFLD patients and 10 control subjects from a previous study and these were included in the present extended analysis (13). Furthermore, we compared patients with NAFLD to patients with other liver diseases as “disease control groups” that included patients with chronic hepatitis C, all infected with genotype 1 (hepatitis C virus infection (HCV), $n = 50$), AIH ($n = 11$), ALD ($n = 13$), and HH ($n = 35$).

Clinical evaluation

None of the study patients had signs of cardiac or renal insufficiency or suffered from cancer, systemic autoimmune diseases,

or infections. A diagnosis of diabetes was established if fasting glucose levels were above 126 mg/100 ml, glycosylated hemoglobin (HbA1c) was 6.0% or higher, or when patients were on oral antidiabetic medication. Elevated fasting glucose was defined with glucose concentrations between 110 and 126 mg/100 ml. As insulin is known to influence ceruloplasmin expression (17), diabetics on insulin therapy were not included in the study. To assess the prevalence of the MetS in the study groups, we used the WHO Clinical Criteria for Metabolic Syndrome (18). Because the waist-to-hip ratio and urinary albumin excretion were not available in our study population, patients were diagnosed as having MetS when they suffered from IR defined as type 2 diabetes, impaired fasting glucose (110–126 mg/100 ml), or impaired glucose tolerance (pathological glucose tolerance test) plus any two of the following conditions: (i) arterial hypertension (use of antihypertensive medication and/or high blood pressure >140 mm Hg systolic of >90 mm Hg diastolic); (ii) serum triglycerides >150 mg/100 ml; (iii) low high-density lipoprotein cholesterol <35 mg/100 ml in men or <39 mg/100 ml in women, or (iv) body mass index >30 kg/m² were present. Patients' charts were reviewed with regard to cigarette smoking and use of oral contraceptive medication as these factors are known to affect ceruloplasmin levels (19,20). Written informed consent was obtained from all study participants to use clinical data and histological results for scientific purposes, and the analysis was performed in accordance with the ethical standards set forth by the Helsinki Declaration of 1975 and revised in 1983. The study has been approved by the local ethics committee (Ethikkommission des Landes Salzburg).

Histological examination of liver biopsy samples

In humans, a minimum of 1.5 cm of the liver biopsy specimen was obtained for histopathological analysis. A small fraction of liver tissue was used for copper quantification as described below. Liver biopsy sets according to Menghini (Hepafix Luer Lock) were obtained from Braun (Melsungen, Germany) and are produced copper-free according to the manufacturer's data sheet. Plastic containers were used for transport and storage of liver tissue throughout the process to ensure copper-free handling. Liver biopsies were fixed in buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin and Masson's trichrome for morphological evaluation. All liver biopsies were first assessed independently by two pathologists unaware of clinical data and the study objective. In cases of discrepant results of the histological examination, samples were jointly re-evaluated by the two pathologists and the result agreed on was used for analysis. Histological examination of NAFLD liver biopsies was performed according to criteria proposed by Brunt *et al.* (21). Biopsy specimens were evaluated for the degree of steatosis 0–3 (0, <5%; 1, up to 33%; 2, 34–66%; and 3, >66% of hepatocytes affected), hepatocellular ballooning (0, no ballooning; 1, mild; and 2, marked ballooning). Inflammation was graded 0–3 (mild, moderate, and severe) according to criteria proposed and portal inflammation was graded 0–3 as described. Fibrosis was scored as fibrosis stage 1, zone 3

pericellular fibrosis; stage 2, pericellular and portal fibrosis; stage 3, bridging fibrosis; stage 4, cirrhosis. The diagnosis of NAFLD was established in liver biopsies showing steatosis with or without evidence of steatohepatitis (inflammation and hepatocyte ballooning, with or without Mallory's hyaline or fibrosis) associated with an increased body mass index and alcohol consumption of less than 20 g per day for both genders. Furthermore, biopsies with steatosis and mild lobular inflammation but without ballooning or perisinusoidal fibrosis were grouped with steatosis and not classified as NASH. Biopsies with steatosis and any stage of fibrosis other than above were classified as NASH. Also, biopsies with steatosis and lobular inflammation (1–3) and hepatocellular ballooning (1–2) were considered as NASH in the absence of fibrosis.

Inflammation and the stage of fibrosis in all other liver biopsies except for NAFLD (HCV, AIH, HH, ALD) were scored according to the METAVIR scoring system to allow for comparability of different liver diseases. Hence, inflammation was graded 0–3 and fibrosis was staged 0–4 (cirrhosis) as described (22). Steatosis was assessed as above in biopsies of disease control groups.

Laboratory evaluation

Venous blood was drawn after an overnight fast for determination of liver function tests, a full blood count, serum iron status including ferritin, transferrin, transferrin saturation, and serum iron, copper, ceruloplasmin, C-reactive protein, fasting glucose, lipids by standardized automated laboratory methods on the day the liver biopsy was performed. Insulin was measured by standard laboratory techniques, and IR was calculated using homeostasis model assessment for insulin resistance (HOMA-IR; fasting insulin ($\mu\text{IU/ml}$) \times fasting glucose (mmol/l)/22.5). HCCs were determined by standardized automated mass spectroscopy analysis in all study subjects and reported as $\mu\text{g/g}$ of dry weight.

Experimental copper deficiency *in vivo*

To study the effects of dietary copper supply on liver histology, development of NAFLD, and surrogate markers of the MetS, we prospectively studied rats that were fed with standard diets containing varying copper concentrations. We deliberately avoided an established NAFLD animal model to exclude overlapping dietary or genetic influences on the development of liver steatosis or IR and to restrict experimental variation purely to copper intake. Because oral feeding of Sprague–Dawley rats not requiring a very high-fat diet has recently been shown to serve as a useful model of NAFLD (23), 30 animals were fed Klība Petfood Purified Diet (Klība Petfood, Kaiseraugst, Switzerland), containing 15% casein, for 2 weeks. Thereafter, 10 rats each were grouped randomly to receive either a copper-depleted, copper-enriched, or a normal diet. Copper-depleted diet containing <2 p.p.m. copper (**Supplementary Table 1**). Additional copper supply was modified through drinking water with 0, 72, and 598 mg/l of copper, respectively. This resulted in daily copper intakes of 0.05, 2.5, and 12 mg, respectively. After 8 weeks of feeding, we killed rats and analyzed their livers and serums. Rat serum insulin concen-

tration was determined using the Rat Insulin ELISA kit (Linco Research, St. Charles, MI). Leptin receptor-deficient Zucker (fa/fa) rats ($n=6$), which spontaneously develop IR, obesity, and steatosis, and their lean (Fa/-) controls ($n=6$) were fed identical standard diets as detailed above for 8 weeks. Animal experiments were approved by the local animal use committee of the Kanton of Bern, Switzerland (Approval No. 96/08) and all animals received care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health.

Statistical analysis

Statistical analyses were carried out using SigmaStat 3.1 statistics package (Systat Software, Erkrath, Germany). All data are presented as mean \pm s.d. unless otherwise indicated. Differences between NAFLD and healthy or disease control groups were calculated by Student's *t*-test or Mann–Whitney *U*-test in case of non-Gaussian distribution of parameters. Proportions were compared using Fisher's exact and χ^2 methods. Associations among the various parameters in the different groups were calculated using Spearman's rank correlation coefficient. Differences in rats on diets with various copper concentrations were analyzed using analysis of variance.

RESULTS

Clinical and biochemical characteristics of the study cohort

To gain insight into the potential role of copper in NAFLD, we compared data from 124 patients with NAFLD to groups of patients with chronic liver diseases of different etiology and control subjects without evidence of liver disease. Clinical, biochemical, and histological details of the study groups are summarized in **Table 1**.

Liver and serum copper status of different liver diseases

When studying hepatic copper levels within the different patient groups, we found a significantly lower copper content in biopsies from patients with NAFLD (17.7 ± 8.3 $\mu\text{g/g}$ of dry weight; 95% CI 16.0–19.3) than in control biopsies (32.1 ± 9.5 $\mu\text{g/g}$ of dry weight; 95% CI 28.3–35.9; $P<0.001$) or in those from patients with other liver diseases ($P<0.05$; **Figure 1a**). Similarly, serum copper and ceruloplasmin levels were also lower in patients with NAFLD as compared to controls and patients with other liver diseases except for HH (**Figure 1b** and **c**). In patients with NAFLD, serum copper or ceruloplasmin levels were only marginally correlated to liver copper concentrations ($R=0.188$, $R=0.088$, and $R=0.225$; $P=0.057$, respectively). We found that patients who were diagnosed with the severe form of NAFLD, i.e. NASH ($n=31$), had lower HCCs compared to NAFLD patients without NASH ($n=93$; 15.1 ± 7.8 vs. 19.0 ± 8.9 $\mu\text{g/g}$; $P=0.038$) and, similarly, NAFLD patients diagnosed with MetS had lower HCCs compared to NAFLD patients without MetS (14.1 ± 7.5 vs. 20.5 ± 9.2 $\mu\text{g/g}$; $P<0.001$) as shown in **Figure 2**. The degrees of hepatic steatosis were not different between NASH patients (34.8 \pm 18.3%) and NAFLD patients with simple steatosis (36.3 \pm 17.4%).

Table 1. Clinical, biochemical, and histological details of patients and control subjects

	NAFLD	Control	HCV	AIH	ALD	HH	P
Number of Pts	124	27	50	11	13	35	
Female (%)	31 (25%)	20 (74.1%)	19 (38.0%)	7 (63.6%)	2 (15.4%)	9 (25.7%)	<0.001
Age (years)	51.8±13.8	47.7±10.6	53.1±13.1	45.0±16.3	47.6±5.7	55.7±13.1	0.153
AST (10–32 U/ml)	43.5±24.1	32.9±12.4	54.4±34.8	228.5±353.1*	88.9±54.0	43.0±29.5	0.039
ALT (10–32 U/ml)	67.3±40.3	52.7±28.8	70.4±47.1	388.4±681.4*	78.6±27.1	49.9±47.1	0.076
AP (40–129 U/ml)	77.9±25.6	111.3±39.0	68.6±18.5	110.8±26.2*	97.7±58.3	83.3±33.3	<0.001
GGT (10–71 U/ml)	89.7±83.5	142.8±85.6	78.9±73.8	198.6±121.6*	563.7±468.3	91.1±112.8	0.022
Cholesterol (mg/100ml)	213.5±48.1	225.8±46.8	174.3±37.5*	180.1±54.2*	244.9±53.8	207.9±43.0	0.256
LDL-cholesterol (mg/100ml)	142.9±42.5	141.3±43.3	108.5±31.7*	107.1±42.1*	152.4±47.1	132.2±43.3	0.928
HDL-cholesterol (mg/100ml)	52.6±15.3	71.8±14.3	59.5±20.5	63.3±26.3*	67.2±23.1	56.2±17.0	<0.001
Triglycerides (mg/100ml)	173.6±100.5	91.7±66.9	93.7±46.1*	96.0±33.4*	220.2±180.5	159.2±129.2	0.019
Ferritin (30–300 ng/ml)	536±392	133±118	252±221	357±377*	901±709	1866±1524	<0.001
Tf Sat (%)	35.0±11.4	27.3±8.3	33.8±14.2	36.4±17.6	43.9±13.0	71.5±17.3	0.003
Fasting glucose (mg/100ml)	107.1±18.5	91.7±7.2	96.0±10.6*	93.4±6.3*	105.2±36.3	103.9±16.8	<0.001
HOMA-IR ^a	5.1±5.0	1.4±0.6	3.2±2.7*	3.1±2.0*	3.8±2.1	4.1±2.2	<0.001
Pts with DM	46 (37.1%)	0	4 (8.0%)*	1 (9.1%)	4 (30.7%)	12 (34.3%)	0.001
BMI (kg/m ²)	28.8±2.9	25.5±2.3	25.4±2.8*	27.6±4.1	27.4±1.8	25.7±2.9	<0.001
Pts with BMI > 30	51 (41.1%)	0	10 (20.0%)*	3 (27.3%)	5 (38.5%)	8 (22.8%)	<0.001
Pts with triglycerides > 150	66 (53.2%)	4 (14.8%)	4 (8.0%)*	1 (9.1%)*	6 (46.2%)	13 (37.1%)	<0.001
Pts with low HDL	16 (12.9%)	0	1 (2.0%)*	1 (9.1%)	4 (30.7%)	5 (14.3%)	0.128
Pts with hypertension	49 (39.5%)	1 (3.7%)	15 (30%)	2 (18.2%)	3 (23.1%)	14 (40.0%)	0.007
Pts with MetS	44 (35.5)	0	3 (6%)*	0*	3 (23.1%)	9 (25.7%)	0.002
Pts with NASH (%)	31 (25.0%)	0	—	—	—	5 (14.3%)	0.003
Degree of steatosis (%)	34.9±17.6	0	6.1±10.0*	3.8±10.6*	25.0±16.7	13.8±11.7	<0.001
Pts with fibrosis (%)	29 (23.4%)	0	39 (78.0%)*	8 (72.7%)*	7 (53.8%)	17 (48.6%)	0.024
Degree of fibrosis	0.3±0.4	0	1.4±1.2*	1.7±1.5*	1.4±1.6	1.2±1.5	0.031
Hemoglobin (g/100ml)	15.8±1.2	14.6±1.3	15.4±1.2	15.0±1.2	15.5±0.7	15.0±1.2	<0.001
Smokers (%)	10 (8.1%)	3 (11.1%)	4 (8.0%)	1 (9.1%)	8 (61.5%)	2 (5.7%)	0.854

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; DM, type 2 diabetes mellitus; GGT, gamma glutamyl-transpeptidase; HCV, hepatitis C virus; HDL, high-density lipoprotein; HH, hereditary hemochromatosis; HIC, hepatic iron concentration; HOMA-IR, homeostasis model assessment for insulin resistance; LDL, low-density lipoprotein; MetS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; (%) refers to percentage of patients in the respective study group; Pts, patients.

Data are shown as mean ± s.d.

P denotes significance level between NAFLD patients and control subjects calculated as described above.

*denotes significant difference between NAFLD patients and respective disease control groups $P < 0.05$.

^aData obtained from 81 patients with NAFLD.

Correlations between hepatic copper and clinical, histological, and biochemical characteristics

To study putative functional interactions between liver copper concentrations and clinical, biochemical, or histological parameters, we performed Spearman's rank correlation analysis. In patients with NAFLD, HCCs were inversely correlated to the degree of hepatic steatosis ($R = -0.248$; $P = 0.010$), fasting glucose concentrations ($R = -0.245$; $P = 0.008$), the diagnosis of diabetes ($R = -0.271$; $P = 0.007$), IR (HOMA-IR; $R = -0.198$; $P = 0.032$), arterial hypertension ($R = -0.194$; $P = 0.052$), serum ferritin

($R = -0.201$; $P = 0.029$), and the sum of features of the MetS present ($R = -0.363$; $P < 0.001$). As IR reflects the common pathogenesis of various components of the MetS, we performed linear regression analysis to adjust for HOMA-IR and found that the relationship of liver copper concentrations with steatosis was weakened ($P = 0.069$). In patients with HH ($n = 35$), HCCs were inversely correlated with fasting glucose concentration ($R = -0.394$; $P = 0.020$) and with low-density lipoprotein cholesterol concentrations ($R = -0.351$; $P = 0.039$). A proportion of patients with HH ($n = 10$, 28.6%) had concomitant NAFLD. In patients with HCV

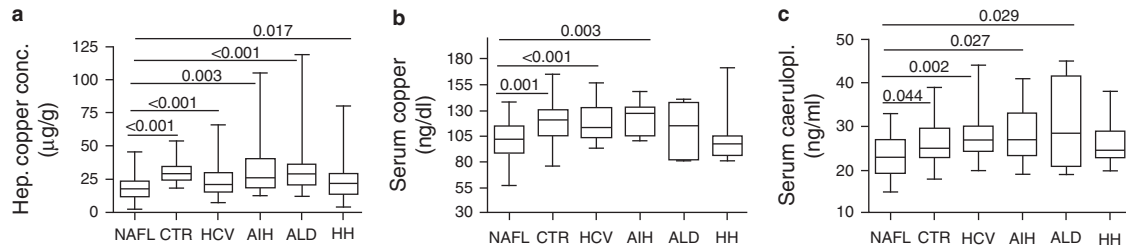


Figure 1. Hepatic (a) and serum copper parameters (b, c) in liver diseases and control subjects. Values are presented as means (horizontal lines), 25th and 75th percentiles (boxes), and minimum/maximum ranges. Calculations for statistically significant differences between NAFLD and healthy or respective disease control groups were performed by Student's *t*-test or Mann–Whitney *U*-test in case of non-Gaussian distribution of parameters. The figure above each horizontal line denotes the *P* value comparing the two groups at the end of the line. AIH, autoimmune hepatitis; ALD, alcoholic liver disease; HCV, hepatitis C virus infection; HH, hereditary hemochromatosis; NAFLD, nonalcoholic fatty liver disease.

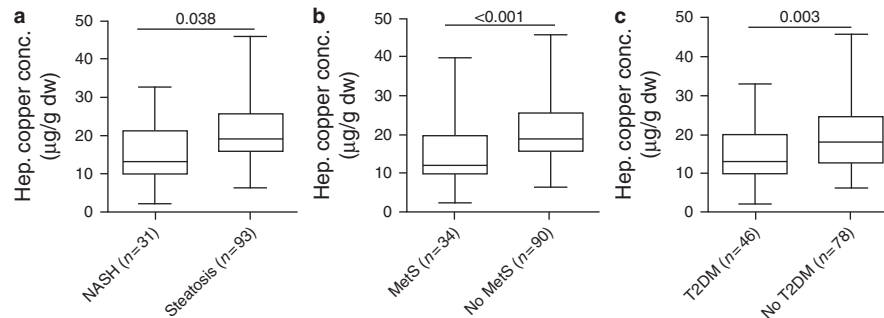


Figure 2. Hepatic copper concentrations in NAFLD patients with NASH (a), MetS (b), or type 2 diabetes (c) compared to patients without them. Values are presented as means (horizontal lines), 25th and 75th percentiles (boxes), and minimum/maximum ranges. Calculations for statistically significant differences between NAFLD and healthy or respective disease control groups were performed by Student's *t*-test or Mann–Whitney *U*-test in case of non-Gaussian distribution of parameters. The figure above the horizontal line denotes the *P* value comparing the two groups. Hep. copper conc., hepatic copper concentration; MetS, metabolic syndrome; NASH, nonalcoholic steatohepatitis; T2DM, type 2 diabetes mellitus.

($n=50$), HCCs were inversely correlated with high-density lipoprotein cholesterol concentrations ($R = -0.341$; $P = 0.029$). A total of four (8%) of patients with HCV also had liver steatosis on histological examination. No further correlations of copper parameters with other characteristics were detected in patients with HH and HCV, and these calculations were not performed in other liver diseases due to a low sample number.

Copper-restricted diet induces hepatic steatosis and IR in Sprague–Dawley rats

To study the putative causative role of copper deficiency for the development of liver steatosis *in vivo*, we subjected Sprague–Dawley rats to identical diets with varying amounts of copper as described in Methods section. After 8 weeks on variable copper supply, liver and serum copper concentrations were decreased in rats restricted for copper, whereas copper supplementation increased HCCs, but left serum copper concentrations unchanged. The latter effect was expected because serum copper levels are hardly affected by dietary copper. Thus, serum copper is not a reliable marker of copper status (24). Rats on a copper-deficient diet tended to have lower body weight with a relative increase of liver weight. Hemoglobin levels were decreased reflecting the effect of low copper availability on iron absorption and mobilization. We found that rats kept on a copper-deficient diet for 8 weeks presented with a marked periportal (zones 1 and 2)

macrovesicular steatosis without evidence of inflammation or fibrosis (Figure 3a) whereas rats kept on a normal or a copper-supplemented diet (Figure 3b and c) showed no steatosis indicating a direct effect of dietary copper supply on the development of steatosis in rats *in vivo*. Moreover, copper-deficient rats presented with surrogate markers of insulin resistance (HOMA-IR) and higher serum total cholesterol concentrations compared to rats on a normal or copper-enriched diet. However, serum triglyceride levels were decreased in copper-deficient animals, which was different from results published (7). Metabolic and biochemical copper characteristics of rats on diets with different copper concentrations are summarized in Table 2. To assess whether there was an effect of liver fat content on the results of HCC measurements, we analyzed livers from leptin receptor-deficient Zucker rats for hepatic copper and found that both obese Zucker rats ($n=6$) and lean littermates ($n=6$) had similar copper concentrations (Zucker rats $3.3 \pm 0.54 \mu\text{g/g}$ dry weight; lean controls $3.9 \pm 1.06 \mu\text{g/g}$ dry weight; NS).

DISCUSSION

The prevalence of NAFLD is continuously rising and represents a growing clinical problem (25). Although NAFLD is widely recognized as the hepatic manifestation of IR, neither animal models nor investigations in humans have provided a complete under-

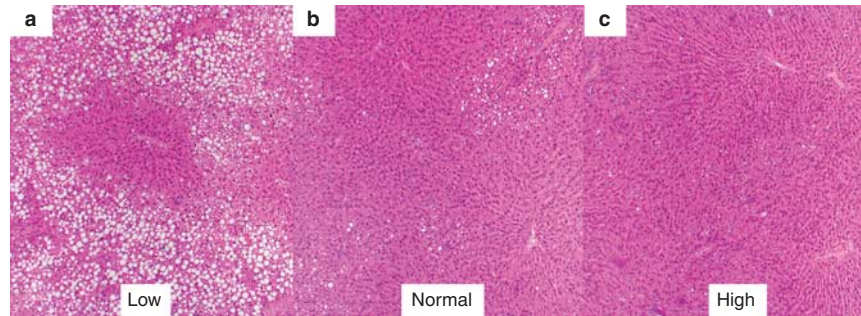


Figure 3. Liver histology of rats on different dietary copper concentrations. Sprague–Dawley rats were fed a copper-deficient ($n=10$; **a**), normal ($n=10$; **b**), or a copper-enriched ($n=9$; **c**) diet for 8 weeks. Copper-deficient rats developed marked macrovesicular steatosis as compared to rats on a normal or on a copper-enriched diet.

Table 2. General and metabolic characteristics of rats on varying copper supply

	Low, $n=10$	Normal, $n=10$	High, $n=9$	<i>P</i> (ANOVA)
<i>General characteristics</i>				
Body weight (g)	502.4±30.2	523.1±30.9	498.2±33.4	0.050
Liver weight (g)	14.9±1.2	15.1±2.0	12.3±1.5	0.003
Heart weight (g)	1.7±0.1	1.8±0.1	1.5±0.1	0.189
Hemoglobin (g/100ml)	13.3±1.2	14.5±0.4	14.7±0.5	<0.001
Hepatic copper (μg/g)	2.27±0.85	4.05±0.61	7.19±1.52	<0.001
Serum copper (μg/ml)	0.44±0.14	7.80±0.72	7.57±0.74	<0.001
<i>Intermediary metabolism</i>				
Serum glucose (mg/100ml)	247.7±77.8	227.1±28.3	169.0±12.1	0.001
Serum insulin (ng/ml)	3.4±1.9	2.2±1.1	1.3±1.1	0.012
HOMA-IR	2.1±1.2	1.3±0.7	0.5±0.5	0.003
Serum triglyceride (mg/100ml)	111.3±43.9	153.6±49.6	89.1±34.2	0.259
Serum cholesterol (mg/100ml)	80.9±16.4	66.2±9.8	60.4±10.0	0.004

ANOVA, analysis of variance; HOMA-IR, insulin resistance as assessed by homeostasis model assessment for insulin resistance.

P (ANOVA) is the difference between groups as calculated by ANOVA.

Data are presented as mean±s.e.m.

standing of its pathophysiology (1). Previous studies in humans suggested that availability of copper may be low in patients with NAFLD (13,26). In this study, we show that liver copper concentrations in patients with NAFLD are lower than in individuals with other chronic liver diseases and subjects with normal liver histology and that there is an inverse correlation between hepatic copper content and the degree of steatosis. In addition, we report that rats kept on a copper-deficient diet develop hepatic steatosis and IR. These findings suggest that low copper availability may contribute to the pathogenesis of NAFLD.

Because obesity, IR, and NAFLD are associated with Western dietary habits, observing a relative deficiency of specific nutrients in a condition of general overfeeding is unexpected. Evidence is mounting that in obesity-related conditions, nutrient composition is relevant to the development of IR even beyond net caloric intake (27,28). Currently, we are lacking information with regard to trace element composition of various diets (29) and whether specific

dietary components could affect copper bioavailability. Our data suggest that dietary copper availability may represent an additional disease-modifying factor relevant to the epidemic of NAFLD and IR. In our investigation, low liver copper concentrations were associated with higher degrees of hepatic steatosis among patients with NAFLD and rats on low dietary copper developed liver steatosis. These findings suggest that inadequate copper availability may increase lipid accumulation in the liver. Epidemiological studies have shown that copper deficiency is linked to atherogenic dyslipidemia (30,31) and investigations in rodent models found that copper deficiency induces hypertriglyceridemia, hypercholesterolemia, and also modifies low-density lipoprotein and very low-density lipoprotein composition (6,7,32). In contrast, low concentrations of serum and muscle cholesterol were detected after feeding supranormal copper concentrations in chicken (33). These studies provide evidence that low copper bioavailability can profoundly affect lipid metabolism and that it may therefore also be involved

in the development of NAFLD. However, due to the observational design of our study, we did not perform detailed examination of dietary habits in our patients and, therefore, cannot establish a link between specific nutrient intake and liver copper concentrations. Moreover, mitochondrial dysfunction accompanied by structural distortion has been implicated in the pathogenesis of NAFLD (15,34). Similarly, systemic copper deficiency leads to changes in mitochondrial morphology due to its key role in respiratory chain physiology, especially in cytochrome *c* oxidase activity (35), which resemble changes in respiratory chain function, and consecutively altered fatty acid β oxidation as described in human NAFLD (36). However, in our investigation we did not assess mitochondrial structure by electron microscopy in our patients. Hence, several metabolic pathways may be affected as a consequence of low liver copper concentrations and their further elucidation shall provide relevant information concerning the role of copper status as a potentially steatogenic factor in human NAFLD.

Liver fat accumulation is generally regarded benign in the absence of inflammation or fibrosis that distinguishes steatohepatitis from simple steatosis. Risk factors for the progression of NAFLD include—among others—increases in visceral adipose tissue and/or IR, or female sex (37). Among these, IR emerges as the key condition associated with progression from fatty liver to steatohepatitis (30,31). In our analysis, we observed lower HCCs among NAFLD patients with MetS ($P < 0.001$) and NASH ($P = 0.038$) compared to those without MetS and NASH, and we found an association between low copper concentrations in NAFLD patients with IR (HOMA-IR), elevated fasting glucose levels, diabetes, and hypertension. The consistent association of low copper concentrations with clinical, histological, and biochemical parameters of IR suggests a biologically relevant contribution of low copper availability to the development of NAFLD and also the progression to NASH. Similar to our findings in patients with NAFLD, we observed that copper-deficient rats were more insulin resistant than those on adequate copper supply, and we observed improved IR along with reduced serum cholesterol concentrations in rats receiving dietary copper fortification. In humans, copper deficiency has previously been linked to cardiovascular disease and features of the MetS including hypertension, atherogenic dyslipidemia, and high serum triglyceride levels (38). Our results suggest that this may also be the case in NAFLD. As NAFLD and obesity are characterized by increased levels of pro-inflammatory cytokines (39,40), which also induce ceruloplasmin gene expression, the absence of enhanced ceruloplasmin levels in patients with NAFLD is surprising. However, it needs to be determined if overweight and obese patients with NAFLD have different copper status as compared to obese subjects without NAFLD.

The liver is the central organ of copper storage and excretion, and quantification of HCCs is regarded to best reflect body copper status. However, this cannot be easily obtained in clinical routine due to biopsy-related risk (41). Determination of liver copper is routinely performed by measuring microgram of copper per gram dry weight of liver tissue through mass spectroscopy. It can be assumed that liver tissue composition, i.e. lipid content, may influence the measurement of HCCs and thus yield low copper

concentrations in NAFLD biopsies. However, this question has not been addressed so far. Assessment of liver copper concentrations in an established genetic model of fatty liver diseases and lean littermates, however, yielded identical concentrations of hepatic copper, supporting the notion that low copper concentrations in our investigation is not a consequence of lipid accumulation in the liver, but possibly, rather the cause. Of note, although we found decreased copper concentrations in liver tissue of patients with NAFLD, measurement of serum copper and ceruloplasmin levels yielded less pronounced differences, which suggests that the specific relevance and the cause of low liver copper concentrations to the various biological functions of copper will need to be clarified in future investigations. Nevertheless, we found that patients with NASH have lower liver copper concentrations as compared to patients with simple steatosis, although these two groups had a similar amount of fat within liver cells indicating that lipid content is not responsible for lower liver copper concentrations in patients with more pronounced steatosis or IR. In addition, the development of liver steatosis and IR in response to copper deficiency in rats supports the assumption of a causative role of low copper concentrations in the development of IR and NAFLD.

In summary, we report that liver copper concentrations in human NAFLD are decreased compared to control subjects and patients with other chronic liver diseases and that low copper concentrations are associated with steatosis and IR. Moreover, we provide evidence that dietary copper deficiency induces liver steatosis and IR in rats thus reporting a novel animal model that is potentially useful to study pathways for the development of human NAFLD and IR. This finding seems relevant with regard to disease progression because NAFLD patients with NASH had lower liver copper concentrations compared to NAFLD patients without NASH. The causative mechanisms underlying low copper bioavailability as well as the pathophysiological role of copper for hepatic and systemic IR deserve further investigation. Particularly, it remains to be determined whether IR is the cause or consequence of copper homeostasis and whether either condition perpetuates the other. Moreover, the effect of copper supply on key lipogenic pathways regulators of lipid turnover requires further study as well as the influence of copper depletion on established models of NAFLD to clarify the contribution of copper to the pathophysiology of NAFLD and IR.

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CONFLICT OF INTEREST

Guarantor of the article: Christian Datz, MD.

Specific author contributions: Design of the study: Elmar Aigner, Felix Stickel, and Christian Datz; clinical care of the patients, generation and analysis of the data: Elmar Aigner, Heike Haufe, Thomas Sonnweber, Florian Hohla, Andreas Stadlmayr, Marc Solioz, Herbert Tilg, Guenter Weiss, and Wolfgang Patsch; writing of the article: Elmar Aigner, Guenter Weiss, Felix Stickel, and Christian Datz; all authors read and approved of the final version of the article.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease and is regarded as the hepatic manifestation of insulin resistance.
- ✓ Its pathogenesis is multifactorial and incompletely understood.
- ✓ Copper has a role in antioxidant defense, lipid peroxidation, and mitochondrial function, and copper deficiency has been linked to atherogenic dyslipidemia.

WHAT IS NEW HERE

- ✓ Copper concentrations are low in liver biopsies from patients with NAFLD compared to control biopsies and biopsies from patients with other liver diseases, and are related to the severity of the disease and insulin resistance.
- ✓ Rats on dietary copper restriction developed insulin resistance and liver steatosis, suggesting that low copper availability may have a role in NAFLD.

REFERENCES

1. Neuschwander-Tetri BA. Fatty liver and the metabolic syndrome. *Curr Opin Gastroenterol* 2007;23:193–8.
2. Yeh MM, Brunt EM. Pathology of nonalcoholic fatty liver disease. *Am J Clin Pathol* 2007;128:837–47.
3. Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* (Baltimore, Md) 2006;43:S99–112.
4. Greenfield V, Cheung O, Sanyal AJ. Recent advances in nonalcoholic fatty liver disease. *Curr Opin Gastroenterol* 2008;24:320–7.
5. Cornier MA, Dabelea D, Hernandez TL *et al.* The metabolic syndrome. *Endocr Rev* 2008;29:777–822.
6. al-Othman AA, Rosenstein F, Lei KY. Copper deficiency alters plasma pool size, percent composition and concentration of lipoprotein components in rats. *J Nutr* 1992;122:1199–204.
7. al-Othman AA, Rosenstein F, Lei KY. Copper deficiency increases *in vivo* hepatic synthesis of fatty acids, triacylglycerols, and phospholipids in rats. *Proc Soc Exp Biol Med* 1993;204:97–103.
8. Sanyal AJ. Mechanisms of disease: pathogenesis of nonalcoholic fatty liver disease. *Nat* 2005;2:46–53.
9. Prohaska JR, Geissler J, Brokate B *et al.* Copper, zinc-superoxide dismutase protein but not mRNA is lower in copper-deficient mice and mice lacking the copper chaperone for superoxide dismutase. *Exp Biol Med* (Maywood) 2003;228:959–66.
10. Schuschke DA, Adeagbo AS, Patibandla PK *et al.* Cyclooxygenase-2 is upregulated in copper-deficient rats. *Inflammation* 2009;32:333–9.
11. Zelber-Sagi S, Nitzan-Kaluski D, Halpern Z *et al.* NAFLD and hyperinsulinemia are major determinants of serum ferritin levels. *J Hepatol* 2007;46:700–7.
12. Mandler MH, Turlin B, Moirand R *et al.* Insulin resistance-associated hepatic iron overload. *Gastroenterology* 1999;117:1155–63.
13. Aigner E, Theurl I, Haufe H *et al.* Copper availability contributes to iron perturbations in human nonalcoholic fatty liver disease. *Gastroenterology* 2008;135:680–8.
14. Aigner E, Theurl I, Theurl M *et al.* Pathways underlying iron accumulation in human nonalcoholic fatty liver disease. *Am J Clin Nutr* 2008;87:1374–83.
15. Nose Y, Kim BE, Thiele DJ. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. *Cell Metab* 2006;4:235–44.
16. Wei Y, Rector RS, Thyfault JP *et al.* Nonalcoholic fatty liver disease and mitochondrial dysfunction. *World J Gastroenterol* 2008;14:193–9.
17. Seshadri V, Fox PL, Mukhopadhyay CK. Dual role of insulin in transcriptional regulation of the acute phase reactant ceruloplasmin. *J Biol Chem* 2002;277:27903–11.
18. World Health Organization. Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. Technical Report 992. WHO: Geneva, Switzerland, 1999.
19. Galdston M, Levytska V, Schwartz MS *et al.* Ceruloplasmin. Increased serum concentration and impaired antioxidant activity in cigarette smokers, and ability to prevent suppression of elastase inhibitory capacity of alpha 1-proteinase inhibitor. *Am Rev Respir Dis* 1984;129:258–63.
20. Sobrio GA, Granata A, Granese D *et al.* Sex hormone binding globulin, cortisol binding globulin, thyroxine binding globulin, ceruloplasmin: changes in treatment with two oral contraceptives low in oestrogen. *Clin Exp Obstet Gynecol* 1991;18:43–5.
21. Brunt EM, Janney CG, Di Bisceglie AM *et al.* Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999;94:2467–74.
22. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* (Baltimore, Md) 1996;24:289–93.
23. Tipoe GL, Ho CT, Liong EC *et al.* Voluntary oral feeding of rats not requiring a very high fat diet is a clinically relevant animal model of non-alcoholic fatty liver disease (NAFLD). *Histol Histopathol* 2009;24:1161–9.
24. Danzeisen R, Araya M, Harrison B *et al.* How reliable and robust are current biomarkers for copper status? *Br J Nutr* 2007;98:676–83.
25. Torres DM, Harrison SA. Diagnosis and therapy of nonalcoholic steatohepatitis. *Gastroenterology* 2008;134:1682–98.
26. Ferenci P, Steindl-Munda P, Vogel W *et al.* Diagnostic value of quantitative hepatic copper determination in patients with Wilson's disease. *Clin Gastroenterol Hepatol* 2005;3:811–8.
27. Ouyang X, Cirillo P, Sautin Y *et al.* Fructose consumption as a risk factor for non-alcoholic fatty liver disease. *J Hepatol* 2008;48:993–9.
28. Zelber-Sagi S, Nitzan-Kaluski D, Goldsmith R *et al.* Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. *J Hepatol* 2007;47:711–7.
29. Moschen AR, Tilg H. Nutrition in pathophysiology and treatment of nonalcoholic fatty liver disease. *Curr Opin Clin Nutr Metab Care* 2008;11:620–5.
30. Klevay LM. Cardiovascular disease from copper deficiency—a history. *J Nutr* 2000;130:489S–92S.
31. Klevay LM. Dietary copper and risk of coronary heart disease. *Am J Clin Nutr* 2000;71:1213–4.
32. Bakalli RI, Pesti GM, Ragland WL *et al.* Dietary copper in excess of nutritional requirement reduces plasma and breast muscle cholesterol of chickens. *Poult Sci* 1995;74:360–5.
33. Pessayre D. Role of mitochondria in non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 2007;22 (Suppl 1): S20–7.
34. Zeng H, Saari JT, Johnson WT. Copper deficiency decreases complex IV but not complex I, II, III, or V in the mitochondrial respiratory chain in rat heart. *J Nutr* 2007;137:14–8.
35. Fromenty B, Robin MA, Igoudjil A *et al.* The ins and outs of mitochondrial dysfunction in NASH. *Diabetes Metab* 2004;30:121–38.
36. Chitturi S, Weltman M, Farrell GC *et al.* HFE mutations, hepatic iron, and fibrosis: ethnic-specific association of NASH with C282Y but not with fibrotic severity. *Hepatology* (Baltimore, Md) 2002;36:142–9.
37. Adams LA, Angulo P. Recent concepts in non-alcoholic fatty liver disease. *Diabet Med* 2005;22:1129–33.
38. Lalor PF, Faint J, Aarbodet Y *et al.* The role of cytokines and chemokines in the development of steatohepatitis. *Semin Liver Dis* 2007;27:173–93.
39. Engstrom G, Hedblad B, Stavenow L *et al.* Incidence of obesity-associated cardiovascular disease is related to inflammation-sensitive plasma proteins: a population-based cohort study. *Arterioscler Thromb Vasc Biol* 2004;24:1498–502.
40. Gitlin JD. Transcriptional regulation of ceruloplasmin gene expression during inflammation. *J Biol Chem* 1988;263:6281–7.
41. Olivares M, Mendez MA, Astudillo PA *et al.* Present situation of biomarkers for copper status. *Am J Clin Nutr* 2008;88:859S–62S.