A High-Calorie Diet Aggravates Mitochondrial Dysfunction and Triggers Severe Liver Damage in Wilson Disease Rats

Claudia Einer,¹,²,* Christin Leitzinger,¹,* Josef Lichtmannegger,¹ Carola Eberhagen,¹ Tamara Rieder,³ Sabine Borchard,¹ Ralf Wimmer,² Gerald Denk,² Bastian Popper,⁴,⁵ Frauke Neff,⁶ Elena V. Polishchuk,⁷ Roman S. Polishchuk,⁷ Stefanie M. Hauck,⁸ Christine von Toerne,⁸ Jennifer-Christin Müller,⁹ Uwe Karst,⁹ Bipin S. Baral,¹⁰ Alan A. DiSpirito,¹⁰ Andreas E. Kremer,¹¹ Jeremy Semrau,¹² Karl Heinz Weiss,¹³ Simon Hohenester,²,§ and Hans Zischka¹,³,§

¹Institute of Molecular Toxicology and Pharmacology, ²Institute of Pathology, ³Research Unit Protein Science, Helmholtz Center Munich, German Research Center for Environmental Health, Neuherberg, Germany; ⁴Department of Medicine II, University Hospital, Ludwig-Maximilians-University Munich, Munich, Germany; ⁵Institute of Toxicology and Environmental Hygiene, Technical University Munich, Munich, Germany; ⁶Department of Anatomy and Cell Biology, ⁷Core Facility Animal Models, Biomedical Center, Ludwig-Maximilians-University, Planegg-Martinsried, Germany; ⁸Telethon Institute of Genetics and Medicine, Pozzuoli (Naples), Italy; ⁹Institute of Inorganic and Analytical Chemistry, University of Münster, Münster, Germany; ¹⁰Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa; ¹¹Department of Medicine I, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany; ¹²Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, Michigan; ¹³Department of Gastroenterology, Internal Medicine IV, University Hospital Heidelberg, Heidelberg, Germany

SUMMARY
A high-calorie diet severely aggravates hepatic mitochondrial and hepatocellular damage in Wilson disease rats. A toxic triad of adenosine triphosphate depletion, increased reactive oxygen species, and increased bile salts lead to an earlier onset of the disease and to enhanced disease progression.

BACKGROUND & AIMS: In Wilson disease, ATP7B mutations impair copper excretion into bile. Hepatic copper accumulation may induce mild to moderate chronic liver damage or even acute liver failure. Etiologic factors for this heterogeneous phenotype remain enigmatic. Liver steatosis is a frequent finding in Wilson disease patients, suggesting that impaired copper homeostasis is linked with liver steatosis. Hepatic mitochondrial function is affected negatively both by copper overload and steatosis. Therefore, we addressed the question of whether a steatosis-promoting high-calorie diet aggravates liver damage in Wilson disease via amplified mitochondrial damage.

METHODS: Control Atp7b+/− and Wilson disease Atp7b−/− rats were fed either a high-calorie diet (HCD) or a normal diet. Copper chelation using the high-affinity peptide methanobactin was used in HCD-fed Atp7b−/− rats to test for therapeutic reversal of mitochondrial copper damage.

RESULTS: In comparison with a normal diet, HCD feeding of Atp7b−/− rats resulted in a markedly earlier onset of clinically apparent hepatic injury. Strongly increased mitochondrial copper accumulation was observed in HCD-fed Atp7b−/− rats, correlating with severe liver injury. Mitochondria presented
with massive structural damage, increased H₂O₂ emergence, and dysfunctional adenosine triphosphate production. Hepatocellular injury presumably was augmented as a result of oxidative stress. Reduction of mitochondrial copper by methanobactin significantly reduced mitochondrial impairment and ameliorated liver damage.

CONCLUSIONS: A high-calorie diet severely aggravates hepatic mitochondrial and hepatocellular damage in Wilson disease rats, causing an earlier onset of the disease and enhanced disease progression. (Cell Mol Gastroenterol Hepatol 2019;7:571–596; https://doi.org/10.1016/j.jcmgh.2018.12.005)

Keywords: Copper-Storage Disease; Steatosis; Steatohepatitis; Mitochondria; Methanobactin.

See editorial on page 684.

Wilson disease (WD) is an autosomal-recessively inherited disorder of copper metabolism caused by ATP7B gene mutations, resulting in impaired biliary copper excretion. Subsequent hepatic copper accumulation induces a heterogeneous phenotype that lacks a clear genotype correlation.⁵ Although some individuals remain rather unaffected, others develop mild to moderate chronic liver disease or even acute liver failure. The mechanisms underlying this heterogeneity are currently unknown. Pharmacologic therapies in WD aim to restore copper homeostasis.⁶ In Atp7b⁻/⁻ rats, an animal model mirroring the WD liver phenotype,⁷ hepatic copper accumulation causes a reduced mitochondrial adenosine triphosphate (ATP) production capacity, mitochondrial destruction, liver failure, and animal death.⁸,⁹ Heterozygous Atp7b⁺/⁻ rats do not accumulate copper and thus are highly stringent, nonaffected control animals.³,⁴ Copper-induced mitochondrial damage in Atp7b⁻/⁻ rats can be resolved efficiently by innovative treatments using the potent copper chelating agent methanobactin (MB), which has an extraordinarily high copper affinity.⁶,⁷ MB decreases mitochondrial copper within days, coinciding with liver tissue restoration and avoidance of liver failure and animal death.⁵

Besides mitochondrial impairments, fat accumulation (steatosis) is a frequently observed early characteristic in livers of WD patients.⁸,⁹ Indeed, WD often may be misdiagnosed as nonalcoholic fatty liver disease (NAFLD).⁹ The prevalence of NAFLD is increasing in Western societies, in many cases owing to high-calorie malnutrition (ie, excessive intake of fat and sugar), and the associated metabolic syndrome.¹⁰ Interestingly, in NAFLD patients, mitochondrial alterations similar to those found in WD patients have been reported (eg, altered cristae and reduced ATP production resulting from oxidative phosphorylation defects).¹¹,¹² Wild-type mice subjected to a high-fat, high-fructose–containing diet have functional deficits in their hepatic mitochondria, most prominently a reduced ATP production capacity.¹³,¹⁴ Thus, mitochondrial structural and functional impairments are hallmarks in both WD and NAFLD, suggesting a potential link between aberrant hepatic copper and lipid metabolism.

An obvious dietary recommendation for WD patients is to avoid copper-rich foods (eg, shellfish, nuts, or chocolate) to counteract an excessive hepatic copper accumulation.¹⁵ However, much less attention is given to other aspects of WD patient nutrition (eg, fat or sugar content in their diet). The potential influence of such environmental aspects on WD progression and severity came to our attention by a case report on monozygotic WD twins.¹⁶ One of the twins with nutritional disturbance (bulimia nervosa) had clinically apparent signs of liver failure (eg, ongoing hepatocyte necrosis), and had to undergo liver transplantation. Her twin sister, however, underwent a prolonged period of undernourishment, and presented with asymptomatic mild liver disease.¹⁷ This and further case reports suggests that lifestyle may impact WD progression, possibly contributing to differing WD phenotypes and to the conundrum of a lacking genotype–phenotype correlation in WD.¹⁸

Similar to the clinical situation, in WD research, treatments of relevant animal models have plausibly focused on the amelioration of copper-induced liver damage (eg, with the aim to avoid oxidative liver damage).¹⁹,²⁰ The opposite, that is, studies on diets that may aggravate disease progression, are virtually nonexistent. Only recently have reports suggested that misbalanced copper homeostasis participates in liver steatosis and may negatively influence not only lipid and cholesterol metabolism, but also the assembly and secretion of lipoproteins from intestinal enterocytes.²¹,²²

Driven by these findings and considerations, we asked whether malnutrition with a high-calorie diet (HCD), enriched in fat and sugar, would influence disease progression in Atp7b⁻/⁻ rats. We applied a variant of an HCD that particularly reflects the eating habits in Western society, causing the “American Lifestyle-induced Obesity Syndrome,”²³ and that represents a physiologically relevant, true-to-life model. The rationale of this study was that both enriched copper and fatty acids cause bioenergetic defects and therefore synergistically and detrimentally may coincide on hepatic mitochondria, which was found to be the case. We thus report here that an HCD accelerated and

Abbreviations used in this paper: Acetyl-CoA, acetyl coenzyme A; ADP, adenosine diphosphate; AST, aspartate aminotransferase; ATP, adenosine triphosphate; BSA, bovine serum albumin; Cs, cerulo-plasmin; CS, citrate synthase; EGTa, ethylene glycol-bis(-β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; F₄₅₀, adenosine triphosphate synthase; HAI, Histologic Activity Index; HCD, high-calorie diet; ICP, Inductively Coupled Plasma; MB, methanobactin; MS, mass spectrometry; NADH, reduced nicotinamide adenine dinucleotide; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; ND, normal diet; ROS, reactive oxygen species; TRIS, tris(hydroxymethyl)aminomethane; WD, Wilson disease.

*Authors share co-first authorship; †Authors share co-senior authorship.

© 2019 The Authors. Published by Elsevier Inc. on behalf of the AGA Institute. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

https://doi.org/10.1016/j.jcmgh.2018.12.005
aggravated liver damage in Atp7b⁻/⁻ rats. In comparison with Atp7b⁻/⁻ rats fed a normal diet (ND), profoundly increased mitochondrial copper accumulation caused severe bioenergetic defects in HCD-fed Atp7b⁻/⁻ rats. We conclude that lipid accumulation in copper-burdened hepatocytes may represent a second hit in WD, inducing liver damage, and suggest that further research should establish whether dietary counseling of WD patients may be of therapeutic benefit.

Results
An HCD Severely Aggravates and Strongly Accelerates Liver Damage in WD Rats

An HCD significantly increased the visceral fat mass (Figure 1A) and liver triglyceride levels (Figure 1B) within a few weeks of feeding in Atp7b⁻/⁻ control rats, compared with their ND-fed counterparts. This coincided with the
presence of abundant macrosteatosis in liver histology (Figure 1D) and an increased NAFLD Activity Score (NAS) of 3–5 (Figure 1C, Table 1). Despite steatosis, however, constant body and liver weights were encountered in the HCD-fed control Atp7b+/− rats (Figure 1E–G, Table 2). HCD- vs ND-fed Atp7b+/− rats (ie, WD rats) also had tendentiously increased visceral fat mass, significantly increased liver triglyceride levels, liver steatosis, and equal body but increased liver weight (Figures 1A–G, Table 2).

Importantly, clinically apparent liver injury (ie, serum aspartate aminotransferase [AST] level, >200 U/L) was present only in age-matched HCD-fed Atp7b+/− rats, but not in ND-fed Atp7b+/− or in HCD-fed Atp7b+/− rats (Figure 2A, Table 2). Gross liver damage was further detectable by histologic analyses only in HCD-fed Atp7b+/−, but not in the other groups (Figures 1D, 2C, and D, Table 3). Although in the other groups, single necrosis and apoptosis, low signs of inflammation or fibrosis, occasionally were observed, these features became severely apparent and increased significantly (vs ND-fed controls) only in HCD-fed Atp7b+/− rats (Figure 2C and D, Tables 1 and 3). Summation of these parameters resulted in low NAS and Histologic Activity Index (HAI) scores for the other rats, but showed strongly and significantly (vs both ND-fed controls and ND-fed Atp7b+/− rats) increased NAS and HAI scores for HCD-fed Atp7b+/− rats (Figures 1C and 2C). Because steatosis, hepatocyte ballooning, and inflammation are the hallmarks of nonalcoholic steatohepatitis,25 such nonalcoholic steatohepatitis was present in all 6 HCD-fed Atp7b+/− rats, but in only 2 of 6 HCD-fed Atp7b+/− rats (Table 1).

With respect to WD, we further tested for a difference in disease progression in HCD- vs ND-fed Atp7b+/− rats. As can be seen in Figure 2B, HCD feeding led to a earlier disease onset by approximately 20 days in HCD- vs ND-fed Atp7b+/− rats. This is a remarkable disease acceleration, because earlier we had determined a median survival of 106 days in ND-fed Atp7b+/− rats.4 In addition, a noticeable steeper slope of the trend curve for liver damage was observed in HCD- vs ND-fed Atp7b+/− rats (Figure 2B). Thus, HCD feeding causes severely aggravated liver damage that appears much earlier and progresses faster in HCD- vs ND-fed Atp7b+/− rats.

### An HCD Increases Serum and Mitochondrial Copper Load in Atp7b+/− Rats

In WD livers, copper-loading of ceruloplasmin (Cp) is impaired because of ATP7B mutations.2 Consequently, Cp oxidase activity and copper concentrations in peripheral blood typically are reduced.26 Accordingly, ND-fed Atp7b+/− rats presented almost no Cp oxidase activity and significantly decreased plasma copper levels compared with Atp7b+/− controls (Figure 3A and B). Of note, HCD feeding of Atp7b+/− rats resulted in significantly lower serum copper and Cp oxidase activity compared with ND-fed controls (Figure 3A and B). This is in line with observations in NAFLD patients, in whom reduced Cp oxidase activity was found to be associated strongly with hepatocyte ballooning or liver steatosis and therefore was suggested as a potential marker of liver dysfunction in fatty liver injury.27,28 In contrast to Atp7b+/− control rats, HCD feeding of Atp7b+/− rats still resulted in low Cp oxidase activity (Figure 3A), but in a significant increase of serum copper if compared with ND-fed Atp7b+/− rats (Figure 3B). Such increased non-CP bound serum copper is a hallmark of overt liver damage in WD that may arise from disintegrated hepatocytes, because

| Genotype | Chow | ND | ND | ND | ND | ND | ND | ND | HCD |
|----------|------|----|----|----|----|----|----|----|-----|
| Animal ID | Rat 3 | Rat 15 | Rat 1 | Rat 2 | Rat 14 | Rat 16 | Rat 17 | Rat 20 | Rat 5 |
| Steatosis grade | <5% | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | 5%–33% | 1 | | | | | | |
| | >33% to 66% | 2 | | | | | | |
| | >66% | 3 | | | | | | |
| Lobular inflammation | No foci | 0 | 0 | | | | | | |
| | <2 foci per 200× field | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 2–4 foci per 200× field | 2 | | | | | | |
| | >4 foci per 200× field | 3 | | | | | | |
| Ballooning | None | 0 | 0 | 0 | | | | | |
| | Few balloon cells | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | Many cells/ prominent ballooning | 2 | | | | | | |
| Diagnostic classification (NAS) | Not steatohepatitis | <3 | 2 | 2 | 1 | 2 | 1 | 2 | 2 |
| | Possible/ borderline | 3–4 | 3 | | | | | | |
| | Definite steatohepatitis | ≥5 | | | | | | | |
necrosis was largely present in the HCD-fed Atp7b⁻/⁻ rats (Figure 2D).

WD Atp7b⁻/⁻ rats, either ND- or HCD-fed, accumulated comparable copper amounts in whole liver homogenates and liver cytosol (Figure 3C and D). This finding shows a comparable copper intake via the ND/tap water diet vs the HCD/sugar water diet, respectively. In contrast, however, an excessive increase in copper was found in mitochondria isolated from HCD- vs ND-fed Atp7b⁻/⁻ rats (Figure 3E). Of note, a significant increase in mitochondrial copper also was determined in HCD- vs ND-fed control Atp7b⁺/⁻ rats. Importantly, such increased mitochondrial copper significantly correlated with a higher NAS and a progressive HAI score (Figure 3F).

### An HCD Strongly Amplifies Hepatic Mitochondrial Damage in Atp7b⁻/⁻ Rats

Structural and functional alterations in liver mitochondria are early key features in WD patients and related animal models. Mitochondrial alterations also are prominent features in NAFLD patients and related animal models. We therefore hypothesized that the combined effect of genetically driven copper accumulation owing to the Atp7b knockout and metabolic disturbance induced by the high-calorie nutrition may accelerate the deterioration of hepatic mitochondria.

In full agreement with this supposition, feeding Atp7b⁻/⁻ rats with an HCD severely affected their hepatic mitochondria (Figure 4). Compared with mitochondria from ND-fed Atp7b⁻/⁻ rats and from control (Atp7b⁺/⁻) rats, mitochondria from HCD-fed Atp7b⁻/⁻ rats appeared with detached inner and outer membranes, prominent matrix condensations, and ballooned cristae (Figure 4A and B, arrows). Such typical WD features were partly observed in mitochondria from age-matched ND-fed Atp7b⁻/⁻ rats, albeit to a significantly lower extent (Figure 4B, and quantification in 4C). In contrast to mitochondria from ND-fed Atp7b⁻/⁻ rats, HCD-fed Atp7b⁻/⁻ mitochondria had partly rounded vesicular cristae that also were abundantly present in HCD-fed Atp7b⁻/⁻ mitochondria (Figure 4A and B, asterisks). Thus, hepatic mitochondria are affected in structure by both copper deposition and HCD, and their combination resulted in a most severe mitochondrial phenotype.

These structural impairments were paralleled by remarkable mitochondrial functional deficits. The capacity to produce ATP was significantly lower in mitochondria from either HCD-fed Atp7b⁺/⁻ or ND-fed Atp7b⁻/⁻ rats in comparison with those from ND-fed Atp7b⁺/⁻ controls (Figure 5A). However, the strongest decrease in ATP production capacity was determined in mitochondria from HCD-fed Atp7b⁻/⁻ rats, in which ATP production capacity was significantly lower than in all other tested mitochondrial populations (Figure 5A). In contrast, HCD feeding hardly affected mitochondrial oxygen consumption (Figure 5C) and did not change respiratory control ratios (Figure 5D) because only nonsignificant tendencies for increased succinate-linked, leak, and maximum oxygen consumption rates were observed in the restricted number of investigated HCD- vs ND-fed animals. It thus remains for future studies to evaluate whether these alterations do or do not contribute to mitochondrial dysfunction in HCD-fed Atp7b⁻/⁻ rats.

| Table 1. Continued |
|---------------------|
| HCD | HCD | HCD | HCD | ND | ND | ND | ND | ND | ND | HCD | HCD | HCD | HCD | HCD | HCD |
| Rat 4 | Rat 6 | Rat 24 | Rat 23 | Rat 27 | Rat 31 | Rat 8 | Rat 7 | Rat 9 | Rat 29 | Rat 30 | Rat 11 | Rat 10 | Rat 12 | Rat 35 | Rat 36 | Rat 37 |
| 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 |
| 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 4 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

2019 High Calorie Diet and Wilson Disease 575
Importantly, mitochondria from HCD-fed Atp7b−/− rats appeared with 2 highly cell-toxic features that were changed significantly in comparison with the mitochondrial populations from all other rats. The first was a significantly lower ATP production (Figure 5A), and the second was that only mitochondria from HCD-fed Atp7b−/− rats showed significantly enhanced mitochondrial H2O2 emergence, whether tested with either respiratory complex II–linked succinate (Figure 5E) or with respiratory complex I–linked glutamate/malate (Figure 5F) as substrates. Appreciable mitochondrial reactive oxygen species (ROS) were neither emerging from mitochondria from (still) healthy ND-fed WD rats nor from HCD-fed control rats, but were exclusive features of severely damaged mitochondria in HCD-fed Atp7b−/− rats.

An HCD Increases Enzyme Abundancies of Hepatic Lipid and Bile Salt Synthesis in Atp7b−/− Rats

How does the combined challenge of decreased copper excretion (owing to Atp7b deletion) and increased fatty acid

### Table 2. An HCD Accelerates Disease Progression in Atp7b−/− Rats

| Animal ID | Genotype | Chow | Age, days | Sex | Body weight, g | AST, U/L | Bilirubin, mg/dL |
|-----------|----------|------|-----------|-----|----------------|----------|-----------------|
| Rat 1     | +/−      | ND   | 67        | M   | 206            | 181      | <0.5            |
| Rat 2     | +/−      | ND   | 73        | F   | 132            | 146      | <0.5            |
| Rat 3     | +/−      | ND   | 75        | F   | 155            | 137      | <0.5            |
| Rat 4     | +/−      | HCD  | 67        | F   | 148            | 108      | <0.5            |
| Rat 5     | +/−      | HCD  | 73        | F   | 140            | 125      | <0.5            |
| Rat 6     | +/−      | HCD  | 75        | F   | 167            | 127      | <0.5            |
| Rat 7     | −/−      | ND   | 67        | M   | 214            | 152      | <0.5            |
| Rat 8     | −/−      | ND   | 73        | F   | 145            | 130      | <0.5            |
| Rat 9     | −/−      | ND   | 75        | F   | 148            | 181      | <0.5            |
| Rat 10    | −/−      | HCD  | 67        | F   | 144            | 122      | <0.5            |
| Rat 11    | −/−      | HCD  | 73        | F   | 128            | 318<0.5  | <0.5            |
| Rat 12    | −/−      | HCD  | 75        | F   | 144            | 298<0.5  | <0.5            |
| Rat 13    | +/−      | ND   | 79        | M   | 259            | 93.5     | <0.5            |
| Rat 14    | +/−      | ND   | 79        | F   | 137            | 93.1     | <0.5            |
| Rat 15    | +/−      | ND   | 80        | F   | 139            | 95.8     | <0.5            |
| Rat 16    | +/−      | ND   | 80        | F   | 165            | 73.6     | <0.5            |
| Rat 17    | +/−      | ND   | 81        | M   | 272            | 78.5     | <0.5            |
| Rat 18    | +/−      | ND   | 81        | M   | 219            | 96.5     | <0.5            |
| Rat 19    | +/−      | ND   | 81        | M   | 248            | 222      | <0.5            |
| Rat 20    | +/−      | ND   | 82        | M   | 263            | 156      | <0.5            |
| Rat 21    | +/−      | ND   | 82        | M   | 226            | 123      | <0.5            |
| Rat 22    | +/−      | HCD  | 79        | M   | 285            | 90.4     | <0.5            |
| Rat 23    | +/−      | HCD  | 80        | F   | 168            | 65.2     | <0.5            |
| Rat 24    | +/−      | HCD  | 81        | M   | 281            | 69.9     | <0.5            |
| Rat 25    | +/−      | HCD  | 81        | M   | 272            | 77.6     | <0.5            |
| Rat 26    | +/−      | HCD  | 81        | M   | 244            | 123      | <0.5            |
| Rat 27    | +/−      | HCD  | 82        | M   | 275            | 139      | <0.5            |
| Rat 28    | +/−      | HCD  | 82        | M   | 243            | 125      | <0.5            |
| Rat 29    | −/−      | ND   | 80        | F   | 162            | 82.2     | <0.5            |
| Rat 30    | −/−      | ND   | 81        | M   | 271            | 80.5     | <0.5            |
| Rat 31    | −/−      | ND   | 82        | M   | 263            | 115      | <0.5            |
| Rat 32    | −/−      | ND   | 82        | M   | 246            | 141      | <0.5            |
| Rat 33    | −/−      | ND   | 82        | M   | 264            | 90.2     | <0.5            |
| Rat 34    | −/−      | ND   | 82        | M   | 262            | 117      | <0.5            |
| Rat 35    | −/−      | HCD  | 80        | F   | 167            | 460<0.5  | 4.4<0.5         |
| Rat 36    | −/−      | HCD  | 81        | M   | 250            | 486<0.5  | <0.5            |
| Rat 37    | −/−      | HCD  | 82        | M   | 282            | 610<0.5  | 1.8<0.5         |

F, female; M, male.

*aAnimals are considered as having clinically apparent WD if AST level is greater than 200 U/L and/or bilirubin level is greater than 0.5 mg/dL.
intake (via the HCD) alter hepatic lipid metabolism? To address this question, we subjected liver homogenates of Atp7b+/− and Atp7b−/− rats, either ND- or HCD-fed, to a quantitative proteomic comparison (Tables 4–7). This analysis provided evidence for a strongly increased mitochondrial β-oxidation in HCD-fed Atp7b−/− rats (Table 4). This finding agrees well with our earlier results observed in wild-type mice fed an HCD for a prolonged time, and may be an adaptive response to the increased nutritive fatty acid supply.13,14 In further agreement, we also observed higher levels of lipid biosynthesis enzymes, partly in HCD-fed Atp7b+/− control rats, but very prominently in HCD-fed Atp7b−/− rats (Table 5). In line with these observations, increased triglyceride levels were observed in livers from HCD- vs ND-fed rats (Figure 1B), but not in serum (Figure 6A), and only mildly nonsignificantly increased levels of serum nonesterified free fatty acids (Figure 6B). Thus, the highly increased supply of fatty acids via the HCD vs ND14 plausibly causes a 2-fold adaptation in hepatocytes: first, their increased degradation in mitochondria via β-oxidation, and, second, their esterification to triglycerides, which preferentially are stored in cytosol.

How would hepatocytes deal with an increasing acetyl coenzyme A (acetyl-CoA) amount resulting from increased mitochondrial β-oxidation of fatty acids? One response is increased lipid biosynthesis and storage. However, acetyl-CoA also is the precursor in hepatic ketogenesis and cholesterol biosynthesis.32 The proteomic comparison showed only slightly increased to doubled levels of the ketogenic mitochondrial enzymes (Table 6), and first analyses of serum levels indicated comparable 3-hydroxybutyrate levels in HCD-vs ND-fed Atp7b−/− rats (3.25 ± 0.07 vs 3.58 ± 1.23 mg/dL, respectively), indicating minor effects on ketogenesis. In contrast, approximately 4-fold increased enzyme levels (in comparison with ND-fed control rats) were found for nearly the whole cholesterol biosynthesis pathway (Table 6).

Figure 2. An HCD causes severe liver damage in Atp7b−/− rats. (A) Serum AST is increased specifically in HCD-fed Atp7b−/− rats (N = 6–12). (B) After disease progression, animals are considered as having clinically apparent WD if AST level is greater than 200 U/L (blue dashed line). HCD causes an earlier disease onset in Atp7b−/− rats compared with ND-fed animals (HCD, N = 6; ND, N = 18). (C) Total hepatic injury score (HAI, N = 6–8) as well as the (D) HAI score parameters of necrosis, apoptosis, liver cell injury, and fibrosis (N = 6–8) increase significantly only in HCD-fed Atp7b−/− rats. One-way analysis of variance with the (A) Tukey multiple comparisons test, (B) nonlinear curve fitting, or (C and D) nonparametric Kruskal–Wallis test. N, number of analyzed animals. *Significant to Atp7b+/− ND. †Significant to Atp7b+/− HCD. ‡Significant to Atp7b−/− ND. *#†P < .05; **#††P < .01; ***#†††P < .001; ****#††††P < .0001.
Moreover, in HCD-fed $Atp7b^{+/−}$ rats, strong increases were found in enzymes responsible for cholesterol excretion via bile acid biosynthesis and bile excretion (Table 7). These data indicated an increased synthesis of cholesterol and plausibly, consequently elevated bile salts in HCD-fed $Atp7b^{+/−}$ rats. In fact, although unchanged cholesterol levels were determined in serum (Figure 6C) of HCD- vs ND-fed rats, significantly increased bile salt levels were determined only in HCD-fed $Atp7b^{+/−}$ rat serum (Figure 6D).

### Table 3. Histologic Assessment of Hepatic Injury (HAI Score) in Livers of ND- and HCD-Fed $Atp7b^{+/−}$ and $Atp7b^{−/−}$ Rats

| Genotype | Chow | ND | ND | ND | ND | ND | ND | ND |
|----------|------|----|----|----|----|----|----|----|
| Animal ID | Rat 3 | Rat 15 | Rat 1 | Rat 2 | Rat 14 | Rat 16 | Rat 17 |
| Steatosis Location | Zone 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Zone 1 | 1 |
| Azonai | 2 |
| Panacinar | 3 |
| Microvesicular steatosis Absent | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Present | 1 |
| Inflammation Microgranulomas Absent | 0 |
| Present | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Large lipogranulomas Absent | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Present | 1 |
| Portal inflammation None to minimal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| More than minimal | 1 |
| Liver cell injury Acidophil bodies None to rare | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Many | 1 |
| Pigmented macrophages None to rare | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Many | 1 |
| Megamitochondria None to rare | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Many | 1 |
| Necrosis (periportal/ perisomial interface hepatitis) Piecemeal necrosis Absent | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mild Focal, few portal areas | 1 | 1 |
| Focal, most portal areas | 2 |
| Continuous, approximately | 3 |
| <50% tracts/septa |
| Severe Continuous, approximately | 4 |
| >50% tracts/septa |
| Confluent necrosis Absent | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Focal confluent necrosis | 1 |
| Zone 3 necrosis-some areas | 2 |
| Zone 3 necrosis-most areas | 3 |
| Zone 3 necrosis + occasional bridging (p-c) | 4 |
| Zone 3 necrosis + multiple bridging (portal-central) | 5 |
| Panacinar/multiacinar necrosis | 6 |
| Lytic necrosis/ apoptosis/ focal inflammation No foci | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ≤1 foci per 100× field | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2–4 foci per 100× field | 2 |
| 5–10 foci per 100× field | 3 |
| >10 foci per 200× field | 4 |
| Fibrosis Stage None Perisinusoidal or periportal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mild, zone 3 | 1 |
| Moderate, zone 3 | 1A |
| Portal/periportal | 1B |
| Perisinusoidal and periportal bridging fibrosis | 1C |
| Cirrhosis | 2 |
| Other findings Mallory’s hyaline None to rare | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Many | 1 |
| Total | 4 | 2 | 3 | 1 | 5 | 2 | 2 | 2 |

The bacteria-derived peptide MB efficiently reverses HCD-induced mitochondrial dysfunction and improves liver integrity in $Atp7b^{−/−}$ rats. The High-Affinity Copper Binding Peptide MB efficiently reverses HCD-induced mitochondrial dysfunction and improves liver integrity in $Atp7b^{−/−}$ rats. This therapeutic effect is largely owing to efficient hepatic de-coppering (Figure 7A), most prominently in the mitochondrial compartment restoring...
Because we aimed here to improve the mitochondrial status in HCD-fed Atp7b−/− rats as fast as possible, together with a low stress level due to therapy, we chose a once-daily treatment for 5 consecutive days only. We previously observed that such short-term treatments already resulted in mitochondrial improvement, whereas more intense treatments were needed to rescue diseased animals. Consequently, we assessed here whether the beneficial effect of MB treatment still would hold in HCD-fed Atp7b−/− rats. Animals were treated once daily for 5 consecutive days with MB, starting at day 75 (ie, at an age when HCD-fed Atp7b−/− rats presented with marked liver damage) (Figure 2A and B).

MB treatment clearly improved the mitochondrial structure, as evidenced either in situ or at the level of isolated mitochondria (Figure 7B). A significantly lower

| Table 3. Continued | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/
number of isolated mitochondria from MB-treated HCD-fed Atp7b−/− rats presented with cristae detachments and matrix condensations in comparison with mitochondria from untreated HCD-fed Atp7b−/− rats (Figure 7B, and quantification shown in 7C). This structural normalization was paralleled by a 50% reduction in mitochondrial copper load (Figure 7D, Table 8), highly comparable with our previous results for this treatment regimen (ie, 32%–62% mitochondrial copper depletion using 5 single consecutive MB injections). Moreover, strongly enforced mitochondrial ATP production capacity (Figure 7E) and significantly decreased mitochondrial H₂O₂ emergence was observed (Figure 7F). Thus, a 5-day-only MB treatment efficiently ameliorated copper-induced structural and functional deficits in liver mitochondria from HCD-fed Atp7b−/− rats.

Besides the mitochondrial status, we next investigated effects on overt liver damage upon treatment. All animals experienced a significant decrease in AST serum levels after the MB treatment vs pretreatment levels (Figure 8A, Table 8). One animal that additionally showed increased serum bilirubin levels before treatment returned to levels below the detection limit, and all animals regained weight, showing a positive therapeutic effect (Table 8). In addition, if compared with untreated HCD-fed Atp7b−/− rats, we found significantly lower serum copper levels and a massive decrease in total serum bile salts (Figure 8B and C). Thus,

Figure 3. An HCD increases the serum and mitochondrial copper load in Atp7b−/− rats. (A) Serum Cp activity is depleted in Atp7b−/− rats. (B) Serum copper is decreased in Atp7b−/− rats compared with Atp7b+/− rats, but increases upon HCD in Atp7b−/− rats. (C and D) Equally increased copper load in (C) whole-liver homogenate and (D) hepatic cytosol in either HCD- or ND-fed Atp7b−/− rats. (E) Massive mitochondrial copper load in HCD-fed Atp7b−/− rats. (F) The mitochondrial copper load significantly correlates with NAS and HAI score (N = 31). One-way analysis of variance with the (A–E) Tukey multiple comparisons test (N = 6–12), or (F) Spearman correlation. *Significant to Atp7b+/− ND. #Significant to Atp7b+/− HCD. †Significant to Atp7b−/− ND. *r.p < .05; **,##,††p < .01; ***,###,†††,****,####,††††p < .001; ***,##,†††,****,####,††††p < .0001. Cu, copper.
In WD, ATP7B malfunction impairs hepatic copper excretion. This leads to a progressive copper burden in mitochondria. Copper ultimately causes mitochondrial destruction, hepatocyte death, liver failure, and decease of WD Atp7b<sup>−/−</sup> rats. We previously reported that efficient depletion of mitochondrial copper load with the high-affinity copper chelator MB leads to full recovery from even severe states of liver damage. Upon pausing still, the rate of mitochondrial copper re-accumulation determines the rate of re-occurring liver damage. These findings show that hepatic mitochondria are exceptionally susceptible to liver copper overload, thereby being a key organelle in WD pathogenesis. However, mitochondria also readily respond to environmental changes other than increased copper by metabolic adaptations and can balance imposed challenges to different extents for a long time. We reasoned that this mitochondrial flexibility might contribute to the high variability of the clinical presentation of WD. The ATP7B genotype is not clearly predictive for the age of onset, the disease presentation or progression, or for the response to treatment. This absence of a genotype-phenotype correlation may be best exemplified by studies on genetically identical WD twins, whose clinical appearance ranged from presymptomatic phenotypes to liver failure. Consequently, it has been suggested that the WD phenotype may be highly attributable to environmental factors.

Steatosis is a frequently observed early characteristic in livers of WD patients, and mutations in the ε3 and ε4 isoforms of the apolipoprotein E gene are associated with WD onset. Moreover, mutations in the lipase gene PNALP3 are linked to the percentage of liver steatosis in patients with WD. Furthermore, lower serum cholesterol levels were observed in WD patients with hepatic symptoms, and WD animal models, such as Atp7b<sup>−/−</sup> mice, present a down-regulation of enzymes involved in cholesterol and lipid metabolism, whereas Long-Evans Cinnamon rats show lower serum but higher liver cholesterol and triglyceride levels. These studies clearly indicate a link between the lipid/cholesterol metabolism and WD pathophysiology. We therefore asked whether a steatosis-promoting diet would influence WD-related liver damage, and especially mitochondrial damage, in Atp7b<sup>−/−</sup> rats. The rationale was that both enriched copper and fatty acids cause bioenergetic defects and therefore synergistically and detrimentally may coincide on hepatic mitochondria.

An HCD caused strongly increased and accelerated liver damage, evidenced by serum markers of liver damage (Figure 2A and B) and histologic assessment (HAI score and NAS). In HCD- vs ND-fed Atp7b<sup>−/−</sup> rats, a drastically increased copper load was found in mitochondria, but equal cytosolic or overall hepatic copper contents. Compared with mitochondria from either HCD- or ND-fed control rats or from ND-fed Atp7b<sup>−/−</sup> rats, mitochondria from HCD-fed Atp7b<sup>−/−</sup> rats appeared to have the most severe mitochondrial structural alterations, a significantly lower ATP production, and a significantly enhanced mitochondrial ROS emergence.

**Discussion**

**Figure 4.** An HCD amplifies hepatic mitochondrial damage in WD Atp7b<sup>−/−</sup> rats. (A and B) Mitochondria either (A) in situ (scale bar: 250 nm) or (B) isolated (scale bar: 1 μm) from HCD-fed Atp7b<sup>−/−</sup> rats presenting with severe structural alterations, including detachments of the mitochondrial inner and outer membranes (arrows) or matrix condensations together with ballooned cristae (asterisk). (C) Quantification of structurally altered mitochondria from the 4 animal groups. One-way analysis of variance with the Tukey multiple comparisons test (N = 2–3, 350–750 mitochondria per group of animal). *Significant to Atp7b<sup>−/−</sup> ND. †Significant to Atp7b<sup>−/−</sup> HCD. ‡Significant to Atp7b<sup>−/−</sup> ND. *#P < .05; **#; ††P < .01; ***#; †††P < .001; ****##; ††††P < .0001.

Overt liver damage was rescued by this short-term MB treatment. At a histologic level, however, liver damage of HCD-fed Atp7b<sup>−/−</sup> rats only partially was resolved (Figures 8D–F), resulting in a mild but significant decrease in NAS (Figures 8F), while liver triglyceride levels were unchanged (Figure 8G).
Hence, the combination of an HCD with an increasing copper load caused severe structural and functional mitochondrial impairments, whereas mitochondrial copper overload strongly correlated with progressive liver damage (Figures 2–5).

Thus, a simple change in nutrition from a normal diet to an HCD severely aggravated and accelerated WD pathophysiology in HCD-fed $Atp7b^{+/−}$ rats. This could be the result of 2 not mutually exclusive reasons: enhanced copper uptake and/or an additional metabolic burden imposed on the hepatocytes and their mitochondria because the later organelles are both the prime site for cellular copper utilization (via complex IV of the respiratory chain) and for fatty acid degradation. Based on reports that ND-fed control animals consumed similar amounts of food and water as their HCD-fed counterparts, we adjusted for an equal copper supply by the 2 diets (see the Materials and Methods section). In subsequent testing, however, we found that the rats consumed less HCD than ND food, but more fructose water instead of water, respectively. Upon recalculating the supplied copper amounts, we found that the HCD/syrup diet provided approximately 18% more copper. Interestingly, this slightly increased copper supply, however, did not result in correspondingly increased liver copper levels (Figure 3) because we observed equal copper loads in total liver homogenates and in liver cytosol. To the contrary, mitochondria from HCD- vs ND-fed control animals had drastically and significantly increased copper levels (Figure 3). This may indicate that the distribution of hepatocyte copper changes upon HCD feeding. In the HCD-fed $Atp7b^{−/−}$ rat liver mitochondria this copper overload caused severe structural and functional mitochondrial

Figure 5. An HCD severely impairs mitochondrial function in $Atp7b^{−/−}$ rats. (A) HCD feeding significantly reduces mitochondrial ATP production capacity in $Atp7b^{−/−}$ rats ($N = 5–7$). (B) Tendentially lowest $F_{1}F_{0}$ activity (ATP synthase, normalized to CS activity) in mitochondria from HCD-fed $Atp7b^{−/−}$ rats ($N = 5$). HCD feeding (C) hardly affected mitochondrial oxygen consumption and (D) did not change respiratory control ratios (RCR; $N = 3$). (E and F) Strongly increased $H_{2}O_{2}$ emergence from mitochondria of HCD-fed $Atp7b^{−/−}$ rats using either (E) succinate/rotenone and ADP or (F) glutamate/malate as substrates ($N = 5$). One-way analysis of variance with the Tukey multiple comparisons test. *Significant to $Atp7b^{+/−}$ ND. †Significant to $Atp7b^{+/−}$ HCD. ‡Significant to $Atp7b^{+/−}$ ND. $*, **, ***, ###, †††, ††††P < .05, ***, ###, †††, ††††P < .01; ***, ###, †††, ††††P < .001; ***, ###, †††, ††††P < .0001. CII-linked_P, succinate-linked phosphorylation; LEAK, oxygen consumption upon oligomycin treatment; ETS, electron transfer system capacity in a noncoupled carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP)-treated state.
Table 4. Quantitative Proteome Comparisons of Liver Homogenate Proteins of the +/- ND, +/- HCD, and +/- HCD Groups Vs +/- ND Group: Fatty Acid Degradation (β-Oxidation)

| Symbol   | Description                                                                                   | +/- ND vs +/- ND | +/- HCD vs +/- ND | +/- HCD vs +/- ND | Cellular localization |
|----------|-----------------------------------------------------------------------------------------------|------------------|------------------|------------------|-----------------------|
| Acot13   | Acyl-CoA thioesterase 13                                                                      | 0.87             | 1.12             | 3.12             | Mitochondrion         |
| Echdc2   | Enoyl-CoA hydratase domain containing 2                                                        | 1.08             | 1.00             | 2.39             | Mitochondrion         |
| Echdc3   | Enoyl-CoA hydratase domain containing 3                                                          | 1.15             | 0.90             | 2.11             | Mitochondrion         |
| Eftdh    | Electron-transferring-flavoprotein dehydrogenase                                               | 1.16             | 0.84             | 2.09             | Mitochondrion         |
| Ivd      | Isovaleryl-CoA dehydrogenase                                                                  | 1.26             | 1.33             | 3.03             | Mitochondrion         |
| Mcee     | Methylmalonyl CoA epimerase                                                                    | 0.94             | 1.14             | 2.68             | Mitochondrion         |
| Acadsb   | Acyl-CoA dehydrogenase, short/branched chain                                                   | 0.93             | 1.73             | 3.09             | Mitochondrion         |
| Decr1    | 2,4-Dienoyl CoA reductase, 1, mitochondrial                                                    | 0.99             | 0.60             | 1.62             | Mitochondrion         |
| Acaa2    | Acetyl-CoA acyltransferase 2                                                                   | 0.99             | 0.93             | 1.65             | Mitochondrion         |
| Acads    | Acyl-CoA dehydrogenase, C-2 to C-3 short chain                                               | 0.99             | 0.95             | 1.56             | Mitochondrion         |
| Auh      | Adenosine-uridine RNA binding protein/enoyl-CoA hydratase                                     | 1.09             | 1.03             | 1.58             | Mitochondrion         |
| Echs1    | Enoyl-CoA hydratase, short chain, 1, mitochondrial                                           | 0.90             | 1.28             | 2.00             | Mitochondrion         |
| Efta     | Electron-transfer-flavoprotein, α polypeptide                                                 | 0.98             | 1.13             | 1.98             | Mitochondrion         |
| Eftb     | Electron-transfer-flavoprotein, β polypeptide                                                 | 1.08             | 1.21             | 1.79             | Mitochondrion         |
| Gcdh     | Glutaryl-CoA dehydrogenase                                                                    | 0.86             | 1.09             | 1.91             | Mitochondrion         |
| Hac1     | 1-Hydroxyacyl-CoA lyase 1                                                                     | 1.24             | 0.75             | 1.92             | Mitochondrion         |
| Hadh     | Hydroxacyl-CoA dehydrogenase                                                                  | 0.96             | 0.75             | 1.81             | Mitochondrion         |
| Mut      | Methylmalonyl CoA mutase                                                                      | 1.09             | 1.29             | 1.86             | Mitochondrion         |
| Pcca     | Propionyl-CoA carboxylase, α polypeptide                                                       | 1.08             | 1.19             | 1.81             | Mitochondrion         |
| Pccb     | Propionyl-CoA carboxylase, β polypeptide                                                       | 1.00             | 1.11             | 1.96             | Mitochondrion         |
| Acot2    | Acyl-CoA thioesterase 2                                                                       | 1.10             | 0.40             | 2.75             | Mitochondrion         |
| Acad9    | Acyl-CoA dehydrogenase family, member 9                                                       | 0.76             | 0.96             | 0.63             | Mitochondrion         |
| Mmaa     | Methylmalonic aciduria (cobalamin deficiency) cblA type                                       | 1.10             | 1.33             | 0.55             | Mitochondrion         |
| Eci1     | Enoyl-CoA delta isomerase 1                                                                   | 0.89             | 0.50             | 0.70             | Mitochondrion         |
| Ehhdh    | Enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase                                            | 1.20             | 0.65             | 0.99             | Mitochondrion         |
| Hadha    | Hydroxacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), α subunit | 0.86             | 0.62             | 1.07             | Mitochondrion         |
| Acad10   | Acyl-CoA dehydrogenase family, member 10                                                      | 0.86             | 0.90             | 1.30             | Mitochondrion         |
| Acad11   | Acyl-CoA dehydrogenase family, member 11                                                       | 0.91             | 0.90             | 0.75             | Mitochondrion         |
| Acad8    | Acyl-CoA dehydrogenase family, member 8                                                        | 0.97             | 1.26             | 1.02             | Mitochondrion         |
| Acadl    | Acyl-CoA dehydrogenase, long chain                                                            | 1.07             | 0.79             | 1.26             | Mitochondrion         |
| Acadm    | Acyl-CoA dehydrogenase, C-4 to C-12 straight chain                                            | 0.95             | 1.04             | 0.99             | Mitochondrion         |
| Acadv1   | Acyl-CoA dehydrogenase, very long chain                                                       | 0.87             | 0.72             | 0.98             | Mitochondrion         |
| Cpt1a    | Carnitine palmitoyltransferase 1a, liver                                                       | 1.10             | 0.68             | 0.94             | Mitochondrion         |
| Cpt2     | Carnitine palmitoyltransferase 2                                                               | 0.87             | 0.81             | 1.01             | Mitochondrion         |
| Mcat     | Malonyl-CoA-acyl carrier protein transacylase (mitochondrial)                                 | 0.71             | 0.74             | 1.12             | Mitochondrion         |
| Mecr     | Mitochondrial trans-2-enoyl-CoA reductase                                                      | 0.93             | 1.06             | 1.32             | Mitochondrion         |
| Acox2    | Acyl-CoA oxidase 2, branched chain                                                             | 1.08             | 0.83             | 2.54             | Peroxisome, mitochondrion |
| Phyh     | Phytanoyl-CoA 2-hydroxylase                                                                    | 1.20             | 1.48             | 2.39             | Peroxisome, mitochondrion |
| Abcd3    | ATP-binding cassette, subfamily D, member 3                                                   | 0.95             | 0.64             | 0.77             | Peroxisome, mitochondrion |
| Ech1     | Enoyl-CoA hydratase 1, peroxisomal                                                            | 1.01             | 0.66             | 1.06             | Peroxisome, mitochondrion |
| Acox3    | Acyl-CoA oxidase 3, pristanoyl                                                                 | 1.00             | 0.75             | 1.12             | Peroxisome, mitochondrion |
| Eci3     | Enoyl-CoA delta isomerase 3                                                                   | 0.91             | 0.91             | 1.34             | Peroxisome, mitochondrion |
deficits (Figures 4 and 5), paralleled by cell death (Figure 2). We recently reported that progressive mitochondrial copper accumulation causes a steady reduction of their capacity to produce ATP. This is owing to the direct impact of copper on the protein complexes involved in ATP production, but also ATP delivery to the cytosol.

Bioenergetic deficit matches the clinical presentation of liver damage in Atp7b−/− rats. A decrease in the ATP production capacity to 70% in comparison with mitochondria from control rats was found to be critical for the onset of clinically apparent liver damage (i.e., AST levels > 200 U/L). In the present study, a mitochondrial ATP production capacity of 80% was preserved in young ND-fed Atp7b−/− rats (Figure 5A). In agreement with our earlier study, these rats still were healthy (Figure 2). In a mouse study, we reported that the increased supply of fatty acids via an HCD causes lipidic alterations in the membranes of liver mitochondria that also reduce their ATP production capacity, along with only mild signs of liver impairment.

In agreement with this study, we determined a reduced mitochondrial ATP production capacity but comparatively mild signs of apparent liver damage in HCD-fed Atp7b−/− control vs Atp7b+/− rats (steatohepatitis was present in only 2 of 6 HCD-fed Atp7b+/− rats) (Figures 1C, 2, and 5A, Tables 1 and 3). This situation changed when an increasing copper load coincided with steatosis: a decreased ATP production capacity to less than 40% was paralleled by severe structural impairments and strongly increased ROS emergence in mitochondria from HCD-fed Atp7b−/− rats, in comparison with mitochondria from all other rats, whether HCD- or ND-fed controls or ND-fed Atp7b+/− rats (Figures 4 and 5). One interesting question for future experiments is why mitochondrial turnover and renewal via mitophagy was incapable to rescue the detrimental effects of copper and steatosis coinciding on mitochondria. Either such pathways were efficiently inactivated (possibly involving activated mechanistic target of rapamycin (mTOR) pathways), blocked by copper excess, negatively affected by bioenergetic deficits that were too strong, or simply overwhelmed. As a result, however, such mitochondrial damage strongly challenges hepatocytes, and cell death (both necrosis and apoptosis) was extensive (Figure 2D), paralleled by severe liver damage in HCD-fed Atp7b−/− rats (Figure 2, Tables 1–3). We therefore conclude that WD pathophysiology in HCD-fed Atp7b−/− rats is aggravated because of a highly detrimental combination of massive copper- and fatty acid–induced impacts on liver mitochondria.

Further support for the decisive role of mitochondrial damage in the pathophysiology of HCD-fed Atp7b−/− rats comes from the results of the applied short-term treatment with the copper chelator MB (Figures 7 and 8). We chose this drug, and applied it once daily for 5 consecutive days, to test for a fast mitochondrial recovery in HCD-fed Atp7b−/− rats, as a potential remedy against acute and overt liver damage. In comparison with our earlier reports, such treatments are relatively mild because we also had applied MB 2 or 3 times daily or for increased time periods of up to 1 month. Nevertheless, this short-term MB treatment significantly reduced mitochondrial structural damage and improved mitochondrial ATP production with a concomitant decrease in ROS emergence (Figure 7). This mitochondrial amelioration was paralleled by a rescue of overt/acute liver damage in all treated animals, as serum AST levels decreased, bilirubin levels were below detection and

---

**Table 4. Continued**

| Symbol | Description | /- ND vs +/ ND | +/- HCD vs +/ ND | +/- HCD vs +/ ND | Cellular localization |
|--------|-------------|----------------|-----------------|-----------------|----------------------|
| Eci3   | Enoyl-CoA delta isomerase 3 | 0.77 | 0.91 | 0.85 | Peroxisome, mitochondrion |
| Slc27a2 | Solute carrier family 27 (fatty acid transporter), member 2 | 1.03 | 0.82 | 1.07 | ER, mitochondrion |
| Hac1   | 2-Hydroxyacyl-CoA lyase 1 | 1.24 | 0.75 | 1.92 | Peroxisome |
| Aco4   | Acyl-CoA thioesterase 4 | 1.10 | 0.48 | 0.44 | Peroxisome |
| Aco8   | Acyl-CoA thioesterase 8 | 1.05 | 0.91 | 0.49 | Peroxisome, cytoplasm |
| Acox1  | Acyl-CoA oxidase 1, palmitoyl | 1.09 | 0.62 | 1.17 | Peroxisome |
| Acaa1a | Acetyl-CoA acyltransferase 1 | 1.01 | 1.00 | 0.82 | Peroxisome |
| Aco12  | Acyl-CoA thioesterase 12 | 1.04 | 0.86 | 0.99 | Cytoplasm |
| Acox1  | Acyl-CoA oxidase 1, palmitoyl | 0.87 | 0.81 | 1.49 | Peroxisome |
| Decr2  | 2,4-dienoyl CoA reductase 2, peroxisomal | 1.08 | 0.80 | 0.84 | Peroxisome |
| Hadhb  | Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), β subunit | 0.92 | 0.71 | 0.97 | Peroxisome |
| Hsd17b4 | Hydroxysteroid (17-β) dehydrogenase 4 | 1.04 | 0.85 | 1.01 | Peroxisome |

**NOTE.** Data are presented as mean ratios from 3 age-matched animals (age, 80–82 days). ER, endoplasmic reticulum.

*Fold-changes greater than 2 compared with +/−ND control.*

*Fold-changes between 1.5 and 2.0 compared with +/−ND control.*
animals regained body weight (Table 8). In addition, compared with untreated HCD-fed Atp7b−/− rats, serum copper and bile salt levels, plausibly increased upon hepatocyte disintegration, significantly decreased in MB-treated HCD-fed Atp7b−/− rats (Figure 8B and C).

Despite this highly beneficial effect of the applied short-term MB-treatment, mitochondrial and liver rescue was not complete. Although the mitochondrial copper content decreased to 50% (Figure 7D), in absolute terms, the remaining copper load still was a borderline burden.
Table 6. Quantitative Proteome Comparisons of Liver Homogenate Proteins of the -/- ND, +/- HCD, and +/- HCD Groups Vs +/- ND Group: Ketone Body and Cholesterol Synthesis

| Symbol       | Description                                                                 | -/- ND vs +/- ND | +/- HCD vs +/- ND | +/- HCD vs +/- ND | Cellular localization |
|--------------|-----------------------------------------------------------------------------|------------------|------------------|------------------|----------------------|
| Ketone body synthesis |                                                                              |                  |                  |                  |                      |
| Acss3        | Acyl-CoA synthetase short-chain family member 3                              | 0.90             | 1.26             | 2.39<sup>a</sup> | Mitochondrion        |
| Bdh1         | 3-Hydroxybutyrate dehydrogenase, type 1                                      | 0.95             | 0.82             | 3.20<sup>a</sup> | Mitochondrion        |
| Hmgcs2       | 3-Hydroxy-3-methylglutaryl-CoA synthase 2                                    | 1.04             | 0.58<sup>b</sup> | 1.95<sup>a</sup> | Mitochondrion        |
| Acat1        | Acetyl-CoA acetyltransferase 1                                               | 0.92             | 0.82             | 1.30             | Mitochondrion        |
| Hmgcl        | 3-Hydroxymethyl-3-methylglutaryl-CoA lyase                                   | 0.81             | 0.87             | 1.43             | Mitochondrion        |
| Cholesterol synthesis |                                                                              |                  |                  |                  |                      |
| Acat2        | Acetyl-CoA acetyltransferase 2                                               | 1.73<sup>b</sup> | 2.67<sup>a</sup> | 8.79<sup>a</sup> | Nucleus              |
| Hsd17b7      | Hydroxysteroid (17-β) dehydrogenase 7                                       | 1.57<sup>b</sup> | 2.03<sup>a</sup> | 6.52<sup>a</sup> | Cell membrane        |
| Idi1         | Isopentenyl-diphosphate delta isomerase 1                                    | 1.63<sup>b</sup> | 4.08<sup>a</sup> | 3.39<sup>a</sup> | Peroxisome           |
| Msmo1        | Methylsterol monoxygenase 1                                                  | 1.70<sup>b</sup> | 3.25<sup>a</sup> | 4.16<sup>a</sup> | ER                   |
| Cyp51        | Cytochrome P450, family 51, lanosterol 14-α demethylase                      | 1.31             | 2.60<sup>a</sup> | 4.75<sup>a</sup> | Unknown              |
| Hmgcs1       | 3-Hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)                          | 1.08             | 2.60<sup>a</sup> | 3.74<sup>a</sup> | Cytoplasm            |
| Mvk          | Mevalonate kinase                                                           | 1.21             | 2.21<sup>a</sup> | 4.12<sup>a</sup> | Peroxisome           |
| Sqle         | Squalene epoxidase                                                           | 1.47             | 3.62<sup>a</sup> | 4.05<sup>a</sup> | ER                   |
| Ebp          | Emopamil binding protein (sterol isomerase)                                 | 1.66<sup>b</sup> | 1.68<sup>b</sup> | 2.71<sup>a</sup> | ER                   |
| Dhcrt7       | 7-Dehydrocholesterol reductase                                              | 1.37             | 1.77<sup>b</sup> | 2.42<sup>a</sup> | ER                   |
| Lss          | Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)                   | 1.40             | 1.98<sup>b</sup> | 4.01<sup>a</sup> | ER                   |
| Pmvk         | Phosphomevalonate kinase                                                     | 1.22             | 1.73<sup>a</sup> | 4.47<sup>a</sup> | Peroxisome           |
| Tm7sf2       | Transmembrane 7 superfamily member 2                                         | 1.17             | 1.88<sup>a</sup> | 3.84<sup>a</sup> | ER                   |
| Fdps         | Farnesyl diphosphate synthase                                               | 1.49             | 1.30             | 3.99<sup>a</sup> | Cytoplasm            |
| Mvd          | Mevalonate (diphospho) decarboxylase                                         | 1.01             | 2.00<sup>a</sup> | 1.94<sup>a</sup> | Peroxisome           |
| Fdft1        | Farnesyl diphosphate farnesyl transferase 1                                 | 1.22             | 1.99<sup>b</sup> | 1.80<sup>a</sup> | ER                   |
| Hmgcs2       | 3-Hydroxy-3-methylglutaryl-CoA lyase                                         | 1.04             | 0.58<sup>b</sup> | 1.95<sup>a</sup> | Mitochondrion        |
| Acaa2        | Acetyl-CoA acetyltransferase 2                                               | 0.99             | 0.93             | 1.65<sup>a</sup> | Mitochondrion        |
| Dhcrt24      | 24-Dehydrocholesterol reductase                                             | 1.17             | 1.87<sup>a</sup> | 1.35             | ER, Golgi            |
| Acat1        | Acetyl-CoA acetyltransferase 1                                               | 0.92             | 0.82             | 1.30             | Mitochondrion        |
| Hmgcl        | 3-Hydroxymethyl-3-methylglutaryl-CoA lyase                                   | 0.81             | 0.87             | 1.43             | Mitochondrion        |
| Nsdhl        | NAD(P)-dependent steroid dehydrogenase-like                                  | 1.06             | 0.92             | 1.00             | ER                   |

NOTE. Data are presented as mean ratios from 3 age-matched animals (age, 80–82 days). ER, endoplasmic reticulum.

<sup>a</sup> Fold-changes greater than 2 compared with +/- ND control.

<sup>b</sup> Fold-changes between 1.5 and 2.0 compared with +/- ND control.

(Table 8) and comparable with the values of ND-fed Atp7b<sup>+/−</sup> rats (Figure 3E) that were about to develop hepatitis within days (Figure 2B). This borderline status may explain the noticeable but limited improvements in liver histology (Figure 8), and it remains for future studies to test whether prolonged/intensified MB treatments would result in a more complete reversal of mitochondrial and liver damage in HCD-fed Atp7b<sup>+/−</sup> rats.

HCD feeding caused steatosis in all HCD-fed animals. In contrast, steatohepatitis was present in only 2 of 6 HCD-fed Atp7b<sup>+/−</sup> rats, but in all 6 HCD-fed Atp7b<sup>−/−</sup> rats (Figure 1, Table 1). Accordingly, tendentious lower levels of visceral fat (Figure 1A) and lower levels of liver triglycerides (Figure 1B) were found in HCD-fed Atp7b<sup>−/−</sup> vs Atp7b<sup>+/−</sup> rats, indicating a comparatively higher energy turnover in Atp7b<sup>−/−</sup> livers. Indeed, mitochondrial enzymes involved in
fatty acid degradation were enriched in livers of HCD-fed Atp7b+/- rats, but especially in HCD-fed Atp7b-/- rat livers (Table 4). Interestingly, we also observed higher abundancies of lipid biosynthesis enzymes, partly in HCD-fed Atp7b+/- control rats, but very prominently in HCD-fed Atp7b-/- rats (Table 5). Although these increased enzyme abundancies do not necessarily mean an enhanced flux via these pathways, the highly increased supply of fatty acids via the HCD vs ND indicates a 2-fold adaptation in hepatocytes: first, their increased degradation in mitochondria via β-oxidation, and, second, their esterification to triglycerides that are preferentially stored in cytosol. Future measurements have to validate such enhanced metabolic fluxes and/or specific metabolites from these pathways. Furthermore, besides the mere levels of enzyme abundancies, metabolic enzyme activities may be modulated further via post-translational modifications such as acetylation and succinylation, which were not assessed in this study. Indeed, increased acetyl-CoA levels from augmented β-oxidation may result in increased acetylation of

Table 7. Quantitative Proteome Comparisons of Liver Homogenate Proteins of the +/- ND, +/- HCD, and +/- HCD Groups Vs +/- ND Group: Bile Acid Synthesis and Transport

| Symbol | Description | +/- ND vs +/- HCD vs +/- HCD | Cellular localization |
|--------|-------------|-----------------------------|----------------------|
| Acox2  | Acyl-CoA oxidase 2, branched chain | 1.08 0.83 2.54 | Mitochondrion, peroxisome |
| Amacr  | α-Methylacyl-CoA racemase | 1.07 0.99 2.34 | Mitochondrion, peroxisome |
| Cyp8b1 | Cytochrome P450, family 8, subfamily b, polypeptide 1 | 0.97 0.99 2.09 | ER |
| Acot8  | Acyl-CoA thioesterase 8 | 1.05 0.91 0.49 | Mitochondrion, peroxisome |

Bile transport and secretion

| Symbol | Description | +/- ND vs +/- HCD vs +/- HCD | Cellular localization |
|--------|-------------|-----------------------------|----------------------|
| Abcc2  | ATP-binding cassette, subfamily C, member 2 | 0.97 1.34 3.51 | Membrane |
| Slc10a1| Solute carrier family 10 (sodium/bile acid cotransporter), member 1 | 1.69 1.28 3.49 | Cell membrane |
| Slc22a7| Solute carrier family 22 (organic anion transporter), member 7 | 1.17 0.98 3.85 | Cell membrane |
| Slc22a1| Solute carrier family 22 (organic cation transporter), member 1 | 1.16 0.95 2.25 | Cell membrane |
| Slc27a5| Solute carrier family 27 (fatty acid transporter), member 5 | 1.36 0.98 4.37 | ER |
| Slc01a1| Solute carrier organic anion transporter family, member 1a1 | 0.94 0.86 6.44 | Cell membrane |
| Slc01a4| Solute carrier organic anion transporter family, member 1a4 | 1.30 2.45 10.96 | Cell membrane |
| Slc01b2| Solute carrier organic anion transporter family, member 1B2 | 1.35 0.88 2.41 | Cell membrane |
| Stard10| Steroidogenic acute regulatory protein-related lipid transfer domain containing 10 | 1.38 1.14 2.58 | Cell membrane |
| Sult2a1| Sulfotransferase family 2A, dehydroepiandrosterone-preferring, member 1 | 1.53 1.06 6.69 | Cytoplasm |
| Sult2a1| Sulfotransferase family 2A, dehydroepiandrosterone-preferring, member 2 | 2.09 1.00 4.40 | Cytoplasm |
| Abcb1a| ATP-binding cassette, subfamily B, member 1A | 0.55 1.34 0.34 | Cell membrane |
| Abcc3  | ATP-binding cassette, subfamily C, member 3 | 0.65 0.57 0.08 | Cell membrane |
| Ephx1  | Epoxide hydrolase 1, microsomal (xenobiotic) | 0.77 1.23 0.35 | ER |
| Ldlr   | Low-density lipoprotein receptor | 0.99 0.65 0.49 | Cell membrane, Golgi |
| Abcb11 | ATP-binding cassette, subfamily B, member 11 | 1.00 1.14 1.06 | Cell membrane |

NOTE. Data are presented as mean ratios from 3 age-matched animals (age, 80–82 days). ER, endoplasmic reticulum.

aFold-changes greater than 2 compared with +/- ND control.
bFold-changes between 1.5 and 2.0 compared with +/- ND control.
mitochondrial enzymes involved in the tricarboxylic acid cycle, fatty acid oxidation, amino acid and carbohydrate metabolism, ketone body synthesis, and the urea cycle. Moreover, an increasing acetyl-CoA amount would influence not only these pathways, but also possibly would cause increased cholesterol levels because acetyl-CoA is the precursor in hepatic cholesterol biosynthesis. Such hepatic cholesterol accumulation has been reported in NAFLD patients and rodents and correlated with histologic severity of the disease and thus seems to be associated with HCD malnutrition. In fact, increased enzyme abundancies for nearly the whole cholesterol biosynthesis pathway were found in HCD-fed Atp7b-/- rat livers (Table 6). Unexpectedly, however, we did not observe specifically increased cholesterol levels in these animals (Figure 6C). This may have been prevented by an increased routing of cholesterol into bile salts because we determined 2- to 11-fold increases in abundancies of enzymes involved in bile salt synthesis and bile excretion in HCD-fed Atp7b-/- rat livers (Table 7). In agreement, we found increased serum bile salts only in serum of HCD-fed Atp7b-/- rats (Figure 6D), which were reduced significantly upon MB treatment (Figure 8C). Clearly, such increased bile salt synthesis may be a further detrimental impact in HCD-fed Atp7b-/- rat livers because accumulating bile salts are hepatotoxic (especially to hepatic mitochondria).

In conclusion, the combination of accumulating copper with an HCD is highly detrimental to hepatic mitochondria. A toxic triad of ATP depletion, massively increased ROS, and bile salts seals the fate of affected hepatocytes. This indicates that a high- vs normal-calorie nutrition may have a tremendous impact on WD progression and severity and may contribute to the striking phenotype-genotype discrepancies encountered in WD patients, in agreement with a recent review article that indicated the importance of lifestyle modifications in WD. We therefore suggest monitoring such aspects of nutrition in much more detail in the future, to establish whether dietary counseling of WD patients may be of therapeutic benefit.

Materials and Methods

Animal Studies

Animals were maintained under the Guidelines for the Care and Use of Laboratory Animals of the Helmholtz Center Munich. Animal experiments were approved by the government authorities of the Regierung von Oberbayern, Munich, Germany.

Control Atp7b+/+ and WD Atp7b-/- rats of both sexes (Table 2; strain name, LPP crossbreed between Long Evans cinnamon rats and Piebald Virol Glaxo rats; bred in-house, provided by Borjigin) were used because we currently have no indication for a sex-dependent altered WD phenotype in these animals. Animals were fed ad libitum either on an ND (1314; Altromin Spezialfutter GmbH, Seelenkamp, Germany; copper content, 13.9 mg/kg; metabolic energy, 588 Einer et al Cellular and Molecular Gastroenterology and Hepatology Vol. 7, No. 3

![Figure 6. An HCD increases total serum bile salts in Atp7b-/- rats. (A) Serum triglycerides, (B) serum nonesterified fatty acids (NEFAs), and (C) total serum cholesterol do not differ between ND and HCD groups (N = 4-5). (D) Total serum bile salts increase in HCD-fed Atp7b-/- rats (N = 3-5). One-way analysis of variance with the Tukey multiple comparisons test. *Significant to Atp7b+/+ ND. †Significant to Atp7b-/- HCD. ‡Significant to Atp7b+/+ HCD. #{P < .05; **#{P < .01; ***#{P < .001; ****#{P < .0001.}
3301 kcal/kg; 14% kcal from fat) and tap water (copper content, <0.2 mg/L) or on an HCD (Altromin Spezialfutter GmbH; copper content, 9.3 mg/kg; metabolic energy, 4523 kcal/kg; 45% kcal from fat) and fructose syrup (metabolic energy, 722 kcal/L) in drinking water supplemented with 3.1 mg/L copper. Rats were fed an HCD starting at an age of 46–50 days until an age of 79–82 days. To additionally test for a difference in the age of onset and rate of liver damage progression on the 2 diets (HCD vs ND), a subset of animals was analyzed in parallel at an age of 67, 73, and 75 days (Table 2). Daily consumption values for rats were estimated from the literature to be approximately 20 g chow and 30 mL water, respectively. In subsequent control measurements in Atp7b+/− rats, we determined an average uptake of 16.1 ± 1.7 g ND food and 31 ± 3 mL water per day (ie, approximately 230 μg copper/day), and 12.5 ± 1.7 g HCD food and 50 ± 15 mL fructose syrup (ie, approximately 272 μg copper/day). Thus, the HCD/sugar-water diet supplied slightly more copper (approximately 18%) compared with the ND/tap water diet, but

**Figure 7.** MB rescues HCD-induced mitochondrial dysfunction in Atp7b+/− rats. (A) Laser ablation ICP-MS distribution maps (lower panels) show lower copper concentrations in liver samples from ND-fed, MB-treated Atp7b+/− rats compared with untreated ND-fed Atp7b+/− controls (copper concentration range, 0–500 μg/g; laser spot size, 25 μm; scan speed, 50 μm/s). (B) Electron micrographs of mitochondria either in situ (upper panel, scale bar: 500 nm) or isolated (lower panel, scale bar: 500 nm) show mitochondrial structure normalization upon MB treatment (right panels) vs untreated (left panels). (C) Quantification of isolated mitochondria with altered structure (N = 3, 700–750 mitochondria in each animal group). (D–F) Methanobactin treatment (D) decreases mitochondrial copper load (N = 5–6), (E) increases mitochondrial ATP production (N = 5), and (F) decreases mitochondrial H2O2 emergence (substrates, glutamate/malate; N = 5). Unpaired t test, significant if **P < .01; ****P < .0001.
nevertheless resulted in almost equal liver homogenate copper contents in both Atp7b+/− and Atp7b−/− rats (Figure 3C). MB treatment of HCD-fed Atp7b−/− rats was performed once daily for 5 consecutive days starting at an age of 74–75 days, as recently described (150 mg/kg body weight intraperitoneally).5

**Chemicals**

Chemicals were mostly obtained from Sigma–Aldrich (Taufkirchen, Germany). Nitric acid, K2HPO4, KCl, malate, iodacetamide, multi-element standard IV, copper (II) sulfate pentahydrate, ethanol, and xylene were purchased from Merck (Darmstadt, Germany). Acetyl-CoA, reduced nicotinamide adenine dinucleotide (NADH), phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase were obtained from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Carl-Roth (Karlsruhe, Germany). Tris-(hydroxymethyl)aminomethane (TRIS) was obtained from VWR International GmbH (Ismaning, Germany). Gelatin was purchased from Grüssing (Filsum, Germany). Rhodium Inductively Coupled Plasma (ICP) standard solution was purchased from SCP Science (Baie D’Urfé, Canada). Osmium tetroxide and uranyl-less contrasting agent were obtained from Science Services GmbH (Munich, Germany). Propylene oxide and epoxy resin were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Lead citrate was purchased from Leica Biosystems (Wetzlar, Germany).

**Liver Examination**

Serum AST and bilirubin levels were measured with the Reflotron system (Roche Diagnostics, Penzberg, Germany) and liver damage in animals was considered clinically apparent if the serum AST level was greater than 200 U/L and/or the bilirubin level was greater than 0.5 mg/dL.5 Serum cholesterol nonesterified fatty acids and serum triglycerides were analyzed with Respons910 (Diasys Greiner GmbH, Flacht, Germany) according to the manufacturer’s guidelines. Serum ceruloplasmin activity was measured as described elsewhere.49,50 Total serum bile salt concentrations were quantified in serum samples using the Diazyme total bile salt kit (Diazyme Laboratories, Poway, CA) according to the manufacturer’s instructions. Histologic evaluation was performed on formalin-fixed, paraffin-embedded H&E-stained liver samples. Morphologic features were summarized as an activity score as recommended for the diagnosis of steatohepatitis in NAFLD (NAS)25 as well as for hepatitis (HAI score).51 For quantification of liver triglycerides, 100 mg/mL liver tissue was sonicated within 5% NP40 solution.

---

### Table 8. MB Treatment (Intraperitoneally, Once Daily for 5 Days) Reduces Liver Damage and Copper Overload in Serum, Kidney, and Livers of Female HCD-Fed Atp7b−/− Rats

| Animal ID | Rat 38 | Rat 39 | Rat 40 | Rat 41 | Rat 42 | −/- HCD + MB, mean | −/- HCD, mean |
|-----------|--------|--------|--------|--------|--------|-------------------|---------------|
| Sex       | Female | Female | Female | Female | Female |                   |               |
| Age, day  | 79     | 79     | 80     | 80     | 80     | 80a               | 74a           |
| AST, U/L  |        |        |        |        |        |                   |               |
| Before    | 227    | 330    | 419    | 368    | 545    | 378               | 300           |
| After     | 172    | 123    | 196    | 136    | 136    | 191               | nd            |
| Bilirubin, mg/dL |       |        |        |        |        |                   |               |
| Before    | <0.5   | <0.5   | <0.5   | <0.5   | 1.2    | <0.5a             | <0.5a         |
| After     | <0.5   | <0.5   | <0.5   | <0.5   | <0.5   | <0.5a             | nd            |
| Body weight, g |       |        |        |        |        |                   |               |
| Before    | 121    | 141    | 128    | 122    | 102    | 123               | 146           |
| After     | 126    | 149    | 134    | 132    | 113    | 131               | nd            |
| Spleen weight, g |     |        |        |        |        |                   |               |
| Before    | 0.23   | 0.15   | 0.25   | 0.23   | 0.28   | 0.23              | 0.28          |
| After     | 1.93   | 2.39   | 2.66   | 1.99   | 2.04   | 2.20              | 2.53          |
| Visceral fat, g |     |        |        |        |        |                   |               |
| Serum Cu, ng/mL | n.d. | 455    | 296    | 224    | 525    | 375               | 640           |
| Kidney Cu, µg/g wet weight | 36   | 40     | 62     | 44     | 100    | 56                | 99            |
| Liver homogenate Cu, µg/g wet weight | 318 | 237    | 247    | 296    | 284    | 276               | 392           |
| Mitochondrial Cu, ng/mg protein | 538 | 384    | 386    | 445    | 467    | 444               | 893b          |
| Mitochondrial Cu depletion, %b | 39.8 | 57.0   | 56.7   | 50.1   | 47.7   | 50                | 0             |
| NAS       | 4      | 5      | 4      | 5      | 5      | 5a                | 6a            |
| HAI       | 8      | 10     | 8      | 12     | 12     | 10a               | 11a           |

**NOTE.** Data on AST, bilirubin, and body weight are present before and after 5 days of MB treatment. NAFLD activity score was as follows: ≤2, no nonalcoholic steatohepatitis; 3–4, borderline nonalcoholic steatohepatitis; ≥5 definite nonalcoholic steatohepatitis. Cu, copper; nd, not determined. Values are shown as medians. Mitochondrial copper depletion relative to the mean mitochondrial copper value of female untreated Atp7b−/− rats.
heated for 5 minutes at 96°C, and cooled on ice. Homogenates were cleared for 2 minutes at 20,000×g, and supernatants (diluted in 5% NP40 solution 1:1–1:10 as required) were analyzed with Respons910 (Diasys Greiner GmbH).13

**Metal Content Determination**

Copper in serum, liver homogenate, cytosol, and mitochondria, as well as kidney homogenate, were analyzed by ICP Optical Emission Spectrometry (Ciros Vision, SPECTRO...
Analytical Instruments GmbH, Kleve, Germany) after wet ashing of samples with 65% nitric acid.  

**Preparation of Rat Liver Cytosol and Mitochondria**

Freshly removed liver tissue was homogenized with a Teflon-glass homogenizer (B. Braun Biotech, Melsungen, Germany) in isolation buffer (pH 7.2) containing 300 mmol/L sucrose, 5 mmol/L EGTA, 0.2 mmol/L ethylenediaminetetraacetic acid (EGTA), and 0.1% BSA. Approximately 1 mL of the homogenate was centrifuged at 100,000×g (1 h, 4°C), and the supernatant (liver cytosol) was collected and stored at −80°C. For mitochondrial isolation, the remaining homogenate was clarified from debris and nuclei by an 800×g centrifugation step, and a crude mitochondrial fraction was pelleted at 18,900×g (20 min at 4°C). To purify mitochondria, the pellet was suspended in isolation buffer, loaded on a Nycodenz gradient (Axis-Shield PoC, Oslo, Norway; gradient composition was as follows: 2 mL of 40%, 1 mL of 33%, 3 mL of 28%, 2 mL of 27%, 2 mL of 24% Nycodenz solution; diluted in 10 mmol/L TRIS, pH 7.4), and centrifuged at 74,100×g (1 h, 4°C). The mitochondrial fraction (layer at the 28% gradient phase) was collected and suspended in isolation buffer without BSA and washed 2 times at 18,900×g (10 min at 4°C).  

**Mitochondrial ATP Production**

ATP production was determined using the ATP Bioluminescence Assay Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s guideline. In detail, 10 μg mitochondria were incubated with 160 μmol/L adenosine diphosphate (ADP) and 5 mmol/L succinate for 30 minutes at room temperature in a buffer containing 0.2 mol/L sucrose, 10 mmol/L 3-(N-morpholino)propanesulfonic acid-TRIS, 1 mmol/L inorganic phosphate, 10 μmol/L EGTA and 2 μmol/L rotenone. As background control, potassium cyanide (2 mmol/L, respiratory chain complex IV inhibitor) was added. Mitochondrial ATP production was calculated in pmol/min/mg protein based on background-corrected luminescence signals and ATP standard curves (Roche Diagnostics, Mannheim, Germany).  

**Mitochondrial Respiration**

Mitochondrial respiration was measured with an Oxygraph-2k instrument and processed via DatLab 6.2 software (Oroboros Instruments, Innsbruck, Austria). Per each chamber, 100 μg mitochondria was supplied in a buffer containing 0.25 mol/L sucrose, 1 mmol/L EGTA, 30 mmol/L K2HPO4, 15 mmol/L KCl, 5 mmol/L MgCl2, 25 mmol/L succinate, 1 μmol/L rotenone, and 0.5 mmol/L ADP, and oxygen consumption rates were measured as succinate-linked phosphorylation. To determine leak respiration, 2.5 mmol/L oligomycin was added (final, 2.5 μmol/L) to block ATP synthase. Subsequently, the protonophore carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone was titrated (2 μL steps from a 20 μmol/L stock solution) to induce maximum oxygen consumption (electron transfer system capacity). The respiratory control ratio was calculated by dividing the succinate-linked phosphorylation oxygen consumption by the oxygen consumption upon oligomycin addition.  

**Mitochondrial H2O2 Production**

Mitochondrial H2O2 production was analyzed after resorufin fluorescence (converted from Amplex Red; Molecular Probes, Invitrogen, Karlsruhe, Germany) at λex 540/20 nm and λem 620/40 nm. The assay was performed with 75 μg mitochondria in 150 μL buffer (pH 7.4) containing 125 mmol/L KCl, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 mmol/L MgCl2, 2 mmol/L K2HPO4, 5 μmol/L MnCl2, and as substrates either 10 mmol/L succinate/2 μmol/L rotenone and 3.2 mmol/L ADP, or 5 mmol/L glutamate/5 mmol/L malate. To start the reaction, 50 μL of a solution containing 320 μmol/L Amplex Red, 2 U/mL horseradish peroxidase, and 60 U/mL superoxide dismutase was added. Resorufin fluorescence was followed up in a plate reader (Synergy 2; BioTek Instruments, Inc, Bad Friedrichshall, Germany) and the Resorufin slope was converted into the rate of H2O2 production in pmol/min/mg with a H2O2 standard curve.  

**F1F0-Activity and Citrate Synthase Activity**

F1F0 activity was assessed as described before. In detail, 20 μg mitochondria were incubated in buffer containing either 50 mmol/L TRIS (pH 8.0) or an additional 3 μmol/L oligomycin (F1F0 inhibitor, negative control) for 5 minutes at 37°C in a plate reader (Synergy 2). To start the reaction, a final mixture of 0.5 mmol/L ATP, 3 μmol/L carbonyl cyanide 3-chlorophenylhydrazone, 1 mg/mL BSA, 1 μmol/L antimycin A, 10 mmol/L KCl, 4 mmol/L MgCl2, 0.2 mmol/L NADH, 2 mmol/L phosphoenolpyruvate, 4 U lactate dehydrogenase, and pyruvate kinase were added. The F1F0 activity was determined in the reverse direction after ATP hydrolysis and NADH oxidation in parallel to the conversion of pyruvate to lactate. The decrease in NADH absorbance at 340 nm was proportional to the ATPase activity and was calculated in nmol/min/mg protein. The F1F0 activity was normalized to the activity of the housekeeping enzyme citrate synthase (CS). The activity of mitochondrial CS was determined according to earlier reports. In brief, 280 μL of a solution containing 2.5% (wt/vol) Triton X-100, 100 μmol/L 5,5’-dithio-bis-(2-nitrobenzoic acid), 75 μg acetyl-CoA, and 500 μmol/L oxaloacetate was incubated at 37°C. The reaction was started by adding 20 μg mitochondria and followed at 412 nm for 5 minutes. CS activities were calculated from the linear slopes of the initial rates.  

**Electron Microscopy**

Animal livers and isolated mitochondria were fixed with 2.5% glutaraldehyde (Science Services GmbH), postfixed with 1% osmium tetroxide, dehydrated with ethanol and propylene oxide, and were embedded in epoxy resin. Sixty-
nanometer sections were cut using the Leica EM UC7 microtome (Leica Biosystems) or the Reichert-Jung Ultracut E microtome (now Leica Biosystems). Ultrathin sections were negative-stained with uranyl acetate (Uranyless) and lead citrate. Images were acquired using either a FEI Tecnai-12 electron microscope equipped with a VELETTA CCD digital camera (FEI, Eindhoven, The Netherlands) or a JEOL 1200 EXII electron microscope (Akishima, Tokyo, Japan) equipped with a KeanViewII digital camera (Olympus, Hamburg, Germany) and processed with the iTEM software package (anlySISFive; Olympus).

For structural analyses, mitochondria were grouped in normally structured mitochondria of the “condensed type” or in altered mitochondria with marked membrane detachments, matrix condensations, and ballooned cristae. A total of 350–750 mitochondria were included per group of animals.

**Proteome Analysis**

**Mass spectrometry (MS) sample preparation.** Liver homogenates were lysed in urea buffer (9 mol/L urea, 6 mol/L thiourea, 65 mmol/L dithiothreitol). A total of 10 µg protein per replicate was proteolytically cleaved by applying a modified filter-aided sample preparation procedure, including a quenching step using 1 mol/L dithiothreitol to bind unreacted iodacetamide. After elution of peptides, samples were acidified with 0.5% trifluoroacetic acid and analyzed on the Orbitrap XL (Thermo Fisher Scientific, Dreieich, Germany) as described.

**Mass spectrometry.** Liquid chromatography tandem-mass spectrometry analysis was performed on a LTQ-Orbitrap XL operated on a nano-high-performance liquid chromatography (UltiMate 3000 RSLCnano System; Thermo Fisher Scientific) as described elsewhere, with the modification of using a nonlinear 30-minute liquid chromatography gradient.

**Protein identification and label-free relative quantification.** Acquired spectra were analyzed using Progenesis QI for proteomics (v2.0; Nonlinear Dynamics, Newcastle upon Tyne, UK), as described previously, with the following adaptations: spectra were searched using the search engine Mascot (version 2.5.1; Matrix Science, London, UK) against the Ensembl rat database (release 80; 28,609 sequences). The Mascot-integrated decoy database search using the Percolator algorithm was set to a peptide false discovery rate of less than 1.5%. Peptide assignments were imported into Progenesis QI. Normalized abundances of peptides were summed up and allocated to the respective protein.

**Laser Ablation ICP-Mass Spectrometry**

For laser ablation ICP-MS analysis, tissue sections of rat liver samples embedded in paraffin were prepared with a thickness of 5 µm using a microtome HM 355S (Thermo Scientific, Bremen, Germany). To quantify the copper concentration in the tissue samples, matrix-matched standards based on 10% gelatin in aqueous solutions of copper (II) sulfate pentahydrate were prepared as described before. To validate the standard concentrations, bulk analysis after digestion with nitric acid was used as described before. A laser ablation system (LSX-213 G2+; Teledyne CETAC Technologies, Omaha, NE) was used. ICP-MS detection was performed with a quadrupole-based iCAP TQ (Thermo Fisher Scientific). The laser ablation and ICP-MS were connected with Tygon tubing Saint-Gobain (Courbevoie, France). The following ICP-MS parameters were applied for all measurements: forward power, 1550 W; cool gas flow, 14 L/min; and auxiliary gas flow, 0.8 L/min. In-house-developed software was used to convert the laser ablation ICP-MS data into 2-dimensional images. The copper concentration was calculated using a linear calibration function derived from the average signal intensities for each standard using Microsoft Excel 2016 (Microsoft Corp, Redmond, WA).

**Miscellaneous**

MB was isolated from the spent media of *Methylosinus trichosporium* OB3b as previously described. Protein quantification was performed by the Bradford or Biuret assay (T1949; Sigma-Aldrich).

**Statistics**

Throughout this study, N refers to the number of analyzed animals. Data are presented as means ± SD. Statistical significance was analyzed using 1-way analysis of variance with the Tukey multiple comparisons test, or the nonparametric Kruskal–Wallis test when comparing 3 or more sample sets (GraphPad Prism 7, GraphPad Software, Inc, San Diego, CA). For 2 group comparisons, the unpaired 2-tailed Student t test was used for parametric data and the Mann–Whitney test was used for nonparametric data, respectively (GraphPad Prism 7).

All authors had access to the study data and reviewed and approved the final manuscript.

**References**

1. Ferenci P. Phenotype-genotype correlations in patients with Wilson’s disease. Ann N Y Acad Sci 2014;1315:1–5.
2. Ferenci PCA, Stremmel W, Houwen R, Rosenberg W, Schilsky M, Jansen P, Moradpour D. EASL clinical practice guidelines: Wilson’s disease. J Hepatol 2012; 56:671–685.
3. Ahmed S, Deng J, Borjigin J. A new strain of rat for functional analysis of PINA. Brain Res Mol Brain Res 2005;137:63–69.
4. Zischka H, Lichtmannegger J, Schmitt S, Schulz S, Jagemann N, Schultz S, Wartini D, Jennen L, Rust C, Larchette N, Galluzzi L, Chajes V, Bandow N, Gilles VS, DiSpirito AA, Esposito I, Goettlicher M, Summer KH, Kroemer G. Liver mitochondrial membrane crosslinking and destruction in a rat model of Wilson disease. J Clin Invest 2011; 121:1508–1518.
5. Lichtmannegger J, Leitzinger C, Wimmer R, Schmitt S, Schulz S, Kabiri Y, Eberhagen C, Rieder T, Janik D, Neff F, Straub BK, Schirmacher P, DiSpirito AA, Bandow N,
Baral BS, Flatley A, Kremmer E, Denk G, Reiter FP, Hohenester S, Eckardt-Schupp F, Dencher NA, Adamski J, Sauer V, Niemietz C, Schmidt HH, Merle U, Gotthardt DN, Kroemer G, Weiss KH, Zischka H. Methanobactin reverses acute liver failure in a rat model of Wilson disease. J Clin Invest 2016;126:2721–2735.

6. Choi DW, Zea CJ, Do YS, Semrau JD, Antholine WE, Hargrove MS, Pohl NL, Boyd ES, Geesey GG, Hartsel SC, Shafe PH, McEllistrem MT, Kisting CJ, Campbell D, Rao V, de la Mora AM, Dispirito AA. Spectral, kinetic, and thermodynamic properties of Cu(I) and Cu(II) binding by methanobactin from Methylosinus trichosporium OB3b. Biochemistry 2006;45:1442–1453.

El Ghazouani A, Basle A, Firbank SJ, Knapp CW, Gray J, Graham DW, Dennison C. Copper-binding properties and structures of methanobactins from Methylosinus trichosporium OB3b. Inorg Chem 2011;50:1378–1391.

8. Sternlieb I. Mitochondrial and fatty changes in hepatocytes of patients with Wilson’s disease. Gastroenterology 1968;55:354–367.

9. Stattermayer AF, Traussnigg S, Dienes HP, Aigner E, Stauber R, Lackner K, Hofer H, Stift J, Jrba F, Stadlmayr A, Datz C, Strasser M, Maieron A, Trauner M, Ferenci P. Hepatic steatosis in Wilson disease–role of copper and PNPLA3 mutations. J Hepatol 2015;63:156–163.

10. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34:274–285.

Cortez-Pinto H, Chatham J, Chacko VP, Arnold C, Rashid A, Diehl AM. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. JAMA 1999;282:1659–1664.

12. Caldwell SH, Swerdlow RH, Khan EM, lezzeni JC, Hespenheide EE, Parks JK, Parker WD Jr. Mitochondrial abnormalities in non-alcoholic steatohepatitis. J Hepatol 1999;31:430–434.

13. Einer C, Hohenester S, Wottke L, Artmann R, Schulz S, Goisman C, Simmons A, Leitzinger C, Eberhagen C, Borchart S, Schmitt S, Hauck SM, von Toerne C, Jastroch M, Walheim E, Rust C, Gerbes AL, Popper B, Mayr D, Schnurr M, Vollmar AM, Denk G, Zischka H. Mitochondrial adaptation in steatotic mice. Mitochondrion 2018;40:1–12.

14. Einer C, Hohenester S, Wottke L, Artmann R, Schulz S, Goisman C, Simmons A, Leitzinger C, Eberhagen C, Borchart S, Schmitt S, Hauck SM, von Toerne C, Jastroch M, Walheim E, Rust C, Gerbes AL, Popper B, Mayr D, Schnurr M, Vollmar AM, Denk G, Zischka H. Data on chow, liver tissue and mitochondrial fatty acid compositions as well as mitochondrial proteome changes after feeding mice a western diet for 6-24 weeks. Data Brief 2017;15:163–169.

15. Pfeiffer RF. Wilson’s disease. Handb Clin Neurol 2011;100:681–709.

16. Brewer GJ, Yuzbasiyan-Gurkan V, Dick R, Wang Y, Johnson V. Does a vegetarian diet control Wilson’s disease? J Am Coll Nutr 1993;12:527–530.

17. Kegley KM, Sellers MA, Ferber MJ, Johnson MW, Joelson DW, Shrestha R. Fulminant Wilson’s disease requiring liver transplantation in one monozygotic twin despite identical genetic mutation. Am J Transplant 2010;10:1325–1329.

18. Kieffer DA, Medici V. Wilson disease: at the crossroads between genetics and epigenetics—a review of the evidence. Liver Res 2017;1:121–130.

19. Yamazaki K, Ohyama H, Kurata K, Wakabayashi T. Effects of dietary vitamin E on clinical course and plasma glutamic oxaloacetic transaminase and glutamic pyruvic transaminase activities in hereditary hepatitis of LEC rats. Lab Anim Sci 1993;43:61–67.

20. Kitamura Y, Nishikawa A, Nakamura H, Furukawa F, Imazawa T, Umemura T, Uchida K, Hirose M. Effects of N-acetylcycteine, quercetin, and phytic acid on spontaneous hepatic and renal lesions in LEC rats. Toxicol Pathol 2005;33:584–592.

21. Levy E, Brunet S, Alvarez F, Seidman E, Bouchard G, Escobar E, Martin S. Abnormal hepatobiliary and circulating lipid metabolism in the Long-Evans Cinnamon rat model of Wilson’s disease. Life Sci 2007;80:1472–1483.

22. Huster D, Purnat TD, Burkhead JL, Ralle M, Fiehn O, Stickert F, Olson NE, Teupser D, Lutsenko S. High copper selectively alters lipid metabolism and cell cycle machinery in the mouse model of Wilson disease. J Biol Chem 2007;282:8343–8355.

23. Pierson H, Muchenditsi A, Kim BE, Ralle M, Zachos N, Huster D, Lutsenko S. The function of ATPase copper transporter ATP7B in intestine. Gastroenterology 2018;154:168–180.e5.

24. Tetri LH, Basaranoglu M, Brunet EM, Yerian LM, Neuenschwander-Tetri BA. Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent. Am J Physiol Gastrointest Liver Physiol 2008;295:G987–G995.

25. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313–1321.

26. Tao TY, Gitlin JD. Hepatic copper metabolism: insights from genetic disease. Hepatology 2003;37:1241–1247.

27. Nobili V, Sirotto M, Bedogni F, Rava L, Pietrobattista A, Panera N, Alisi A, Squitti R. Levels of serum ceruloplasmin associate with pediatric nonalcoholic fatty liver disease. J Pediatr Gastroenterol Nutr 2013;56:370–375.

28. Aigner E, Strasser M, Haufe H, Sonnweber T, Hohla F, Stadlmayr A, Solioz M, Tlig H, Patsch W, Weiss G, Stickel F, Datz C. A role for low hepatic copper concentrations in nonalcoholic fatty liver disease. Am J Gastroenterol 2010;105:1978–1985.
milk (tx-j) mouse, a model for Wilson disease. Mol Genet Metab 2008;93:54–65.

31. Servidio G, Bellanti F, Tamborra R, Rollo T, Romano AD, Giudetti AM, Capitanio N, Petrella A, Vendemiale G, Altomare E. Alterations of hepatic ATP homeostasis and respiratory chain during development of non-alcoholic steatohepatitis in a rodent model. Eur J Clin Invest 2008;38:245–252.

32. McGarry JD, Foster DW. Ketogenesis and cholesterol synthesis in normal and neoplastic tissues of the rat. J Biol Chem 1969;244:4251–4256.

33. Koliaki C, Szendroedi J, Kaul K, Jelenik T, Nowotny P, McGarry JD, Foster DW. Ketogenesis and cholesterol synthesis in normal and neoplastic tissues of the rat. J Biol Chem 1969;244:4251–4256.

34. Bandmann O, Weiss KH, Kaler SG. Wilson disease: impact on extrahepatic tissues in the rat. J Biol Chem 1969;244:4251–4256.

35. Czlonkowska A, Gromadzka G, Chabik G. Monozygotic female twins discordant for phenotype of Wilson’s disease. Mov Disord 2009;24:1066–1069.

36. Medici V, Weiss KH. Genetic and environmental modifiers of Wilson disease. Handb Clin Neurol 2017;142:35–41.

37. Schiefermeier M, Kollegger H, Madl C, Polli C, Oder W, Kuhn H, Berr F, Ferenci P. The impact of apolipoprotein E genotypes on age at onset of symptoms and phenotypic expression in Wilson’s disease. Brain 2000;123:585–590.

38. Litwin T, Gromadzka G, Czlonkowska A. Apolipoprotein E gene (APOE) genotype in Wilson’s disease: impact on clinical presentation. Parkinsonism Relat Disord 2012;18:367–369.

39. Rodo M, Czonkowska A, Pulawska M, Swiderska M, Tarnacka B, Wehr H. The level of serum lipids, vitamin E and low density lipoprotein oxidation in Wilson’s disease patients. Eur J Neurol 2000;7:491–494.

40. Seesle J, Gohdes A, Gotthardt DN, Pfeiffer J, Eckert N, Stremlw W, Reuner U, Weiss KH. Alterations of lipid metabolism in Wilson disease. Lipids Health Dis 2011;10:83.

41. Ralle M, Huster D, Vogt S, Schirmeister W, Burkhead JL, Capps TR, Gray L, Lai B, Maryon E, Lutsenko S. Wilson disease at a single cell level: intracellular copper trafficking activates compartment-specific responses in hepatocytes. J Biol Chem 2010;285:30875–30883.

42. Newman JC, He W, Verdin E. Mitochondrial protein acylation and intermediary metabolism: regulation by sirtuins and implications for metabolic disease. J Biol Chem 2012;287:42436–42443.

43. Menzies KJ, Zhang H, Katsuya E, Auwerx J. Protein acetylation in metabolism - metabolites and cofactors. Nat Rev Endocrinol 2016;12:43–60.

44. Hirschey MD, Shimazu T, Jing E, Grueter CA, Collins AM, Aouizerat B, Stancakova A, Goetzman E, Lam MM, Schwer B, Stevens RD, Muehlabauer MJ, Kakar S, Bass NM, Kuusisto J, Laakso M, Alt FW, Newgard CB, Farese RV Jr, Kahn CR, Verdin E. SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. Mol Cell 2011;44:177–190.

45. Arguello G, Balboa A, Arrese M, Zanluongo S. Recent insights on the role of cholesterol in non-alcoholic fatty liver disease. Biochim Biophys Acta 2015;1852:1765–1778.

46. Schulz S, Schmitt S, Wimmer R, Aichler M, Eisenhofer S, Lichtmannegger J, Eberhagen C, Artmann R, Tookos F, Walch A, Krappmann D, Brenner C, Rust C, Zischka H. Progressive stages of mitochondrial destruction caused by cell toxic bile salts. Biochim Biophys Acta 2013;1828:2121–2133.

47. Hohenester S, Gates A, Wimmer R, Beuers U, Anwer MS, Rust C, Webster CRL. Phosphatidylinositol-3-kinase p110δ contributes to bile salt-induced apoptosis in primary rat hepatocytes and human hepatoma cells. J Hepatol 2010;53:918–926.

48. Weiss J. Haus- und versuchstierpflege: 80 Tabellen: Enke, Stuttgart, Germany 2003.

49. Schosinsky KH, Lehmann HP, Beeler MF. Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride. Clin Chem 1974;20:1556–1563.

50. Erel O. Automated measurement of serum ferroxidase activity. Clin Chem 1998;44:2313–2319.

51. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995;22:696–699.

52. Zischka H, Larochette N, Hoffmann F, Hamoller D, Jagemann N, Lichtmannegger J, Jennen L, Muller-Hocker J, Rogg F, Gottlicher M, Vollmar AM, Kroemer G. Electrophoretic analysis of the mitochondrial outer membrane rupture induced by permeability transition. Anal Chem 2008;80:5051–5058.

53. Schmitt S, Saathoff F, Meissner L, Schropp EM, Lichtmannegger J, Schulz S, Eberhagen C, Borchard S, Aichler M, Adamski J, Plesnila N, Rothenfusser S, Kroemer G, Zischka H. A semi-automated method for isolating functionally intact mitochondria from cultured cells and tissue biopsies. Anal Biochem 2013;443:66–74.

54. Schulz S, Lichtmannegger J, Schmitt S, Leitzinger C, Eberhagen C, Einer C, Kerth J, Aichler M, Zischka H. A protocol for the parallel isolation of intact mitochondria from rat liver, kidney, heart, and brain. Methods Mol Biol 2015;1295:75–86.

55. Muller FL, Liu Y, Abdul-Ghani MA, Lustgarten MS, Bhattacharya A, Jang YC, Van Remmen H. High rates of superoxide production in skeletal-muscle mitochondria respiring on both complex I- and complex II-linked substrates. Biochem J 2008;409:491–499.
Inhibition by DL-2-bromopalmitoyl-CoA and effect of hypothyroidism. Biochem J 1986;236:137–141.

58. Williams AJ, Coakley J, Christodoulou J. Automated analysis of mitochondrial enzymes in cultured skin fibroblasts. Anal Biochem 1998;259:176–180.

59. Hackenbrock CR. Ultrastructural bases for metabolically linked mechanical activity in mitochondria. II. Electron transport-linked ultrastructural transformations in mitochondria. J Cell Biol 1968;37:345–369.

60. von Toerne C, Kahle M, Schafer A, Ispiryan R, Blindert M, Hrabe De Angelis M, Neschen S, Ueffing M, Hauck SM. Apoe, Mbl2, and Psp plasma protein levels correlate with diabetic phenotype in NZO mice—an optimized rapid workflow for SRM-based quantification. J Proteome Res 2013;12:1331–1343.

61. Obermann J, Priglinger CS, Merl-Pham J, Geerlof A, Priglinger S, Gotz M, Hauck SM. Proteome-wide identification of glycosylation-dependent interactors of galectin-1 and galectin-3 on mesenchymal retinal pigment epithelial (RPE) cells. Mol Cell Proteomics 2017;16:1528–1546.

62. Hachmoller O, Aichler M, Schwamborn K, Lutz L, Werner M, Sperling M, Walch A, Karst U. Element bio-imaging of liver needle biopsy specimens from patients with Wilson’s disease by laser ablation-inductively coupled plasma-mass spectrometry. J Trace Elem Med Biol 2016;35:97–102.

63. Bandow NL, Gallagher WH, Behling L, Choi DW, Semrau JD, Hartsel SC, Gilles VS, Dispirito AA. Isolation of methanobactin from the spent media of methane-oxidizing bacteria. Methods Enzymol 2011;495:259–269.

64. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.

Acknowledgments
The authors would like to thank Dr E.E. Rojo for critical reading of the manuscript.

Author contributions
Claudia Einer and Christin Leitzinger performed experiments, analyzed data, and wrote the paper; Josef Lichtmannegger, Tamara Rieder, Sabine Borchard, Ralf Wimmer, and Andreas E. Kremer performed experiments; Frauke Neff, Bastian Popper, Carola Eberhagen, Elena V. Polishchuk, and Roman S. Polishchuk performed histochemical and transmission electron microscope analyses; Christine von Toerne and Stefanie M. Hauck performed the proteome analysis; Uwe Karst and Jennifer-Christin Müller performed laser ablation inductively coupled plasma-mass spectrometry; Alan A. Dispirito, Bipin S. Baral, and Jeremy Semrau produced the methanobactin samples; Gerald Denk reviewed the data and the manuscript; Karl Heinz Weiss analyzed the data and designed experiments; Simon Hohenester analyzed the data, designed experiments, guided data compilation, and wrote the paper; and Hans Zischka designed experiments, analyzed the data, wrote the paper, and directed this study.

Conflicts of interest
The authors disclose no conflicts.

Funding
This study was supported in part by Deutsche Forschungsgemeinschaft grants ZI1386/2-1 (H.Z.) and HO4460/3-1 (S.H.), and by Verein zur Förderung von Wissenschaft und Forschung at the Faculty of Medicine, LMU Munich, grant 7/16 (S.H.).