A high caloric diet augments mitochondrial dysfunction and triggers severe liver damage in Wilson disease rats

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**Abbreviations:** Aspartate aminotransferase (AST), body weight (bw), ceruloplasmin (Cp), citrate synthase (CS), copper (Cu), methanobactin (MB), non-alcoholic fatty liver disease (NAFLD), normal diet (ND), oxygen consumption rate (OCR), respiratory control ratio (RCR), triglyceride (TG), high caloric western diet (HCD), wet weight (w.w.), Wilson disease (WD).

**Abstract**

**Background and aim:** 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 associates with liver steatosis. Hepatic mitochondrial function is impaired by both copper overload and steatosis. Therefore, we addressed the question whether a steatosis-promoting high caloric diet aggravates liver damage in Wilson disease via amplified mitochondrial damage.

**Methods:** Control *Atp7b+/-* and Wilson disease *Atp7b-/-* rats were either fed a high caloric western diet (HCD) or a normal diet (ND). Copper chelation using the high-affinity peptide Methanobactin was employed in HCD-fed *Atp7b-/-* rats to test for therapeutic applicability.

**Results:** In comparison to ND, HCD feeding of *Atp7b-/-* rats resulted in a pronounced earlier onset of clinically apparent hepatic injury. Strongly elevated mitochondrial copper accumulation was observed in HCD-fed *Atp7b-/-* rats, correlating with liver injury. Mitochondria presented with severe structural damage, massive H2O2 production and dysfunctional ATP production. Hepatocellular injury was likely augmented due to oxidative stress. Reduction of mitochondrial copper by chelation reduced mitochondrial reactive oxygen species, ameliorated hepatitis and cured overt liver disease.

**Conclusion:** A high caloric diet severely aggravates hepatic mitochondrial and liver damage in Wilson disease rats. Effective mitochondrial copper depletion strongly ameliorates such damage together with improved liver steatosis, demonstrating a close relationship of hepatic copper and lipid metabolism.

**Introduction**

Wilson disease (WD) is an autosomal recessively inherited disorder of copper metabolism, due to *ATP7B* gene mutations, resulting in impaired biliary copper excretion. Subsequent hepatic copper accumulation induces a heterogeneous phenotype that lacks a clear genotype correlation [1](#_ENREF_1). While some individuals remain unaffected, others develop mild to moderate chronic liver disease or even acute liver failure [2](#_ENREF_2). The mechanisms underlying this heterogeneity are currently unknown. Pharmacological therapies in WD aim at the restoration of copper homeostasis [2](#_ENREF_2). In *Atp7b-/-* rats, an animal model mirroring the WD liver phenotype [3](#_ENREF_3), hepatic copper accumulation causes a reduced mitochondrial ATP production capacity, mitochondrial destruction, liver failure and animal death [4](#_ENREF_4), [5](#_ENREF_5). Heterozygous *Atp7b+/-* rats do not accumulate copper and are thus highly stringent, non-affected control animals [3](#_ENREF_3), [4](#_ENREF_4). Copper induced mitochondrial damage in *Atp7b-/-* rats can be efficiently resolved by innovative treatments using the potent copper chelating agent Methanobactin [5](#_ENREF_5), which has an extraordinarily high copper affinity [6](#_ENREF_6), [7](#_ENREF_7). Methanobactin decreases mitochondrial copper within days, coinciding with liver tissue restoration and avoidance of liver failure and animal death [5](#_ENREF_5).

Besides mitochondrial impairments, fat accumulation (steatosis) is a frequently observed early characteristic in livers of WD patients [8](#_ENREF_8), [9](#_ENREF_9). Indeed, WD may frequently be misdiagnosed as non-alcoholic fatty liver disease (NAFLD) [9](#_ENREF_9). Prevalence of NAFLD is on the rise in Western societies, frequently due to high caloric malnutrition and the associated metabolic syndrome [10](#_ENREF_10). Interestingly, in NAFLD patients, mitochondrial alterations similar to those found in WD patients have been reported, e.g. altered cristae and reduced ATP production due to oxidative phosphorylation defects [11](#_ENREF_11), [12](#_ENREF_12). Wild type mice subjected to a high-fat, high-fructose containing diet present functional deficits in hepatic mitochondria, most prominently a reduced ATP production capacity [13](#_ENREF_13), [14](#_ENREF_14). This variant of a high caloric diet particularly reflects the eating habits in Western society, causing the “American-Lifestyle-induced-Obesity-Syndrome” [15](#_ENREF_15), and represents a physiologically relevant, true-to-life-model. 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 (*e.g*. shellfish, nuts or chocolate) to prevent excessive hepatic copper accumulation [16](#_ENREF_16). However, much less attention is given to other aspects of WD patients nutrition [17](#_ENREF_17), *e.g*. 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 from monozygotic WD twins [18](#_ENREF_18). One of the twins with nutritional disturbance (bulimia nervosa) had clinically apparent signs of liver failure, *e.g*. 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 [18](#_ENREF_18). This (and further case reports) suggests a massive impact of lifestyle on WD progression, which could solve the conundrum of a lacking genotype-phenotype correlation in WD.

Similar to the clinical situation, in WD research, treatments of relevant animal models have focused on the amelioration of copper-induced liver damage, *e.g*. aiming at the avoidance of oxidative liver damage [19](#_ENREF_19), [20](#_ENREF_20). The opposite - studies on diets that may aggravate disease progression - are virtually non-existent. 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 [21-23](#_ENREF_21).

Driven by these findings and considerations, we asked whether malnutrition with a high caloric diet (HCD), enriched in fat and sugar, would influence disease progression in *Atp7b-/-* rats. The rationale was that both, enriched copper and fatty acids, cause bioenergetic defects and may therefore synergistically and detrimentally impact on hepatic mitochondria. We report here that a HCD accelerated and aggravated liver damage in *Atp7b-/-* rats. In comparison to *Atp7b-/-* rats fed a normal diet (ND), profoundly increased mitochondrial copper accumulation caused severe bioenergetic defects in HCD-fed *Atp7b-/-* rats. We conclude that (i) dietary conditions that may negatively impact on copper burdened mitochondria should be carefully monitored in WD patients, in order to avoid increased rates of liver damage and (ii) copper accumulation in steatotic hepatocytes may represent a “second-hit” inducing the progression to steatohepatitis.

**Materials and Methods**

***Animal studies***

Animals were maintained under the guidelines for the care and use of laboratory animals of the Helmholtz Center Munich and animal experiments were approved by the government authorities of the “Regierung von Oberbayern”, Munich, Germany.

Control *Atp7b+/-* and WD *Atp7b-/-* rats (strain name: LPP,  bred in-house, provided by Jimo Borjigin) [3](#_ENREF_3) were fed ad libitum either on normal caloric diet (ND, 1314, Altromin Spezialfutter GmbH, Seelenkamp, Germany, copper content: 13.9 mg/kg; 14% kcal from fat) and tap water or, starting at animal day 46 for 21–36 days, on high caloric diet (HCD, 45% kcal from fat, Altromin, Seelenkamp, Germany, copper content: 9.3 mg/kg) and fructose-syrup in drinking water supplemented with 3.1 mg/l copper [14](#_ENREF_14), [15](#_ENREF_15). Daily consumption values for rats are approx. 20 g chow and 30 ml water, respectively. Thus, both the ND/tap water diet or the HCD/sugar water diet supplied around 278 µg copper/day.

Methanobactin (MB) treatment of HCD-fed *Atp7b-/-* rats was done once daily for five consecutive days starting at animal day 74–75, as recently described (i.p. 150 mg/kg bw) [5](#_ENREF_5).

***Liver examination***

Serum AST and bilirubin were measured with Reflotron system (Roche Diagnostics, Penzberg, Germany) and liver damage in animals was considered clinically apparent if serum aspartate aminotransferase (AST) was > 200 U/l and/or bilirubin was > 0.5 mg/dl [5](#_ENREF_5). Serum cholesterol, non-esterified fatty acids (NEFAs), triglycerides and alkaline phosphatase (AP) were analyzed with Respons® 910 (Diasys Greiner GmbH, Flacht, Germany) according to manufactural guidelines. Serum ceruloplasmin activity was measured as described elsewhere [24](#_ENREF_24), [25](#_ENREF_25). Total serum bile salt (TBS) concentrations were quantified in serum samples using the Diazyme total bile salt kit (Diazyme Laboratrories, Poway, CA, USA), according to the manufacturer’s instructions. Histological evaluation was done on formalin-fixed, paraffin-embedded HE-stained liver samples. Morphological features were summarized as activity score as recommended for diagnosis of steatohepatitis in NAFLD (NAS) [26](#_ENREF_26) as well as for hepatitis (HAI-Score) [27](#_ENREF_27). Triglycerides in liver tissue were quantified as described elsewhere [13](#_ENREF_13).

***Metal content determination***

Copper in serum, liver homogenate, cytosol and mitochondria, as well as kidney homogenate were analyzed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Ciros Vision, SPECTRO Analytical Instruments GmbH, Kleve, Germany) after wet ashing of samples with 65% nitric acid (Merck KGaA, Darmstadt, Germany) [4](#_ENREF_4).

***Mitochondrial analyses***

Rat liver mitochondria were freshly isolated as described earlier [28-30](#_ENREF_28) and purified by a Nycodenz® step gradient(Axis-Shield PoC, Oslo, Norway). Mitochondria were directly used for measurements of ATP synthesis (ATP Bioluminescence Assay Kit, Roche, Germany), respiration (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), and H2O2 production using AmplexTM Red at λEx. 540/20 nm and λEm. 620/40 nm (Molecular Probes, Eugene, USA; Substrates for H2O2 measurements were either succinate (10 mM) / rotenone (2 µM) and ADP (3.2 mM), or glutamate (5 mM) / malate (5 mM) [31](#_ENREF_31), or were fixed with glutaraldehyde (2.5%, Science Services GmbH, Munich, Germany) for transmission electron microscopy [28](#_ENREF_28) (Jeol 1200 EXII, Akishima, Tokyo, Japan). Images were taken using a digital camera (KeenViewII, Olympus, Hamburg, Germany) and processed with the iTEM software package (anlySISFive, Olympus, Hamburg, Germany). For structural analyses, mitochondria were grouped in “normally structured” mitochondria of the “condensed type” [32](#_ENREF_32) or in “altered” mitochondria with marked membrane detachments, matrix condensations and ballooned cristae. Per group of animals, 350–750 mitochondria were included. Frozen mitochondria were used for F1FO-, citrate synthase-activity and metal analyses [13](#_ENREF_13), [33](#_ENREF_33).

***Electron microscopy***

Animal livers were fixed with 2.5% glutaraldehyde and embedded in epoxy resin. Sixty nm sections were cut at the Leica EM UC7 microtome (Leica Biosystems, Wetzlar, Germany) and images acquired using a FEI Tecnai-12 electron microscope equipped with a VELETTA CCD digital camera (FEI, Eindhoven, The Netherlands).

***Miscellaneous***

Methanobactin was isolated from the spent media of *Methylosinus trichosporium* OB3b as previously described [34](#_ENREF_34). Protein quantification was done by the Bradford- [35](#_ENREF_35) or Biuret assay (T1949, Sigma-Aldrich, Taufkirchen, Germany).

***Statistics***

Throughout this study “N” is the number of analyzed animals and “n” equals the number of technical replicates. Data are presented as mean ± standard deviation (SD). Statistical significance was analyzed using One-way ANOVA with Tukey’s multiple comparisons test when comparing 3 or more sample sets (GraphPad Prism 7, GraphPad Software, Inc.; California, USA). For 2 group comparisons, the unpaired two-tailed Student’s t-test was used for parametric data and the Mann-Whitney test for non-parametric data, respectively (GraphPad Prism 7). Statistically significant p-values are: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

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

**Results**

**A high caloric diet severely aggravates and strongly accelerates liver damage in Wilson disease rats.**

In contrast to a normal diet (ND), a significant increase of visceral fat mass (Figure 1A), liver weight (Figure 1B), and enhanced liver triglyceride levels (TG, Figure 1C) were detected in HCD-fed control *Atp7b+/-* and WD *Atp7b-/-* rats, coinciding with abundant macrosteatosis (Figure 1G). However, clinically apparent liver injury (serum AST > 200 U/l) was only present in HCD-fed *Atp7b-/-* rats, but not in age-matched HCD-fed *Atp7b+/-* or in ND-fed *Atp7b-/-* rats (Figure 1D). Histology confirmed marked liver damage in HCD-fed *Atp7b-/-* rats (Figure 1E-G). Ballooned hepatocytes, inflammatory infiltrations and cell death were abundantly present, resulting in a significantly raised HAI-Score (evaluating periportal ± bridging necrosis, intralobular degeneration/focal necrosis, portal inflammation, and fibrosis) (Figures 1F, G). Thus, HCD feeding of *Atp7b-/-* rats severely aggravated liver damage in comparison to ND-fed *Atp7b-/-* rats.

Furthermore, in order to test for a difference in the age-dependency of liver damage, we observed accelerated disease onset in HCD- vs. ND-fed *Atp7b-/-* rats (Supplementary Figure 1). Whereas HCD feeding caused a disease onset at an animal age of around 70 days, such signs of liver damage were not seen in ND-fed *Atp7b-/-* rats before an age of 87–90 days (Supplementary Figure 1), in agreement with our earlier report [5](#_ENREF_5). In addition, a strongly steeper slope of the trend curve for liver damage was observed in HCD- vs. ND-fed *Atp7b-/-* rats (Supplementary Figure 1) Thus, HCD-feeding did not only aggravate liver damage but also strongly accelerated WD progression in comparison to ND-fed *Atp7b-/-* rats.

**A high caloric diet increases serum and mitochondrial copper load in *Atp7b-/-* rats.**

In WD livers, copper-loading of ceruloplasmin (Cp) is impaired because of *ATP7B* mutations [2](#_ENREF_2). Consequently, Cp-oxidase activity and copper concentrations in peripheral blood are typically reduced [36](#_ENREF_36). Accordingly, ND-fed *Atp7b-/-* rats presented almost no Cp-oxidase activity and markedly decreased plasma copper levels compared to *Atp7b+/-* controls (Figure 2A, B). HCD-feeding of *Atp7b-/-* rats resulted in a significant elevation of serum copper (Figure 2B), despite a still low Cp-oxidase activity (Figure 2A), indicative of non-Cp bound copper in serum.

Expectedly, copper accumulated in livers of *Atp7b-/-* rats. Comparable copper levels were found in whole liver homogenates and liver cytosol of *Atp7b-/-* rats either ND- or HCD-fed (Figure 2C, D). This finding validated a highly comparable copper supply via the ND/tap water diet *vs*. the HCD/sugar water diet, respectively. In contrast, however, a massive rise in copper was found in mitochondria isolated from HCD- *vs*. ND-fed *Atp7b-/-* rats (Figure 2E). Of note, some elevation in mitochondrial copper was also determined in HCD- *vs*. ND-fed control *Atp7b+/-* rats. Thus, HCD feeding elevates mitochondrial copper, most prominently in *Atp7b-/-* rats, but also to some extent in *Atp7b+/-* rats. This elevation significantly correlated with accelerated WD progression and enhanced fatty liver injury (HAI-Score and NAS, Figure 2F).

**A high caloric diet strongly amplifies hepatic mitochondrial damage in *Atp7b-/-* rats.**

Structural and functional alterations in liver mitochondria are early key features in WD patients [5](#_ENREF_5), [8](#_ENREF_8), [37](#_ENREF_37) and related animal models [5](#_ENREF_5), [38](#_ENREF_38). Similarly, mitochondrial alterations are also prominent features in NAFLD patients [12](#_ENREF_12) [11](#_ENREF_11) and related animal models [13](#_ENREF_13), [39](#_ENREF_39). We therefore hypothesized that the combined effect of genetically driven copper accumulation owing to the *Atp7b* knockout and metabolic disturbance induced by the high caloric nutrition would accelerate the deterioration of hepatic mitochondria.

In full agreement with this supposition, feeding *Atp7b-/-* rats with a HCD massively affected their hepatic mitochondria (Figure 3). When compared to mitochondria from HCD-fed control (*Atp7b+/-*) rats, the former appeared with detached inner and outer membranes, prominent matrix condensations, and ballooned cristae (arrows in Figure 3A, B). Such typical WD features were partly observed in mitochondria from age-matched ND-fed *Atp7b-/-* rats albeit to a significantly lower extent (Figure 3B, quantification 3C). In contrast to mitochondria from ND-fed *Atp7b+/-* rats, HCD-fed *Atp7b+/-* mitochondria partly had rounded vesicular cristae that were also abundantly present in HCD-fed *Atp7b-/-* mitochondria (asterisks in Figure 3A, B). 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 strong 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 to those from ND-fed *Atp7b+/-* controls (Figure 4A). However, the strongest drop in ATP production capacity was determined in mitochondria from HCD-fed *Atp7b-/-* rats, significantly lower than in all other tested mitochondrial populations (Figure 4A). In addition, the lowest ATP synthase (F1FO) activity was found in mitochondria from HCD-fed *Atp7b-/-* rats (Figure 4B). If directly compared to mitochondria from ND-fed *Atp7b-/-* rats, HCD-fed *Atp7b-/-* mitochondria demonstrated elevated oxygen consumption (Figure 4C), indicative for partial inner membrane damage that was further substantiated by a lower respiratory control ratio (RCR) (Figure 4D).

Importantly, only mitochondria from HCD-fed *Atp7b-/-* rats demonstrated significantly enhanced mitochondrial H2O2 production, whether tested with either respiratory complex II-linked succinate (Figure 4E) or with respiratory complex I-linked glutamate/malate (Figure 4F) as substrates. This finding demonstrated that appreciable mitochondrial reactive oxygen species (ROS) are neither emerging from mitochondria from (still) healthy ND-fed Wilson disease rats nor from HCD-fed control rats, but are features of severely damaged mitochondria in HCD-fed *Atp7b-/-* rats.

**A high caloric diet increases hepatic lipid and bile salt synthesis in *Atp7b-/-* rats.**

How does the combined challenge of decreased copper excretion (due to *Atp7b* deletion) and increased fatty acid 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 (Supplementary Table 1A–D). This analysis provided first evidence for a strongly elevated mitochondrial ß-oxidation in HCD-fed *Atp7b-/-* rats (Supplementary Table 1A). This finding agrees well with our earlier results observed in wild type mice fed a HCD for prolonged time, and may be an adaptive response to the elevated nutritive fatty acid supply [13](#_ENREF_13), [14](#_ENREF_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 (Supplementary Table 1B). In line, elevated triglyceride levels were observed in livers from HCD- *vs*. ND-fed rats (Figure 1C), but not in serum (Figure 5A) and only mildly elevated serum levels of non-esterified free fatty acids (NEFAs, Figure 5B). Thus, the highly elevated supply of fatty acids via the HCD *vs*. ND [14](#_ENREF_14) plausibly causes a two-fold adaptation in hepatocytes, first, their increased degradation in mitochondria via ß-oxidation, and second, their esterification to triglycerides that are preferentially stored in cytosol.

How would hepatocytes deal with an increasing Acetyl-CoA amount resulting from elevated mitochondrial ß-oxidation of fatty acids? One response is an increased lipid biosynthesis and storage. However, Acetyl-CoA also is the precursor in hepatic ketogenesis and cholesterol biosynthesis [40](#_ENREF_40). The proteomic comparison demonstrated only slightly elevated to doubled levels of the ketogenic mitochondrial enzymes (Supplementary Table 1C), indicating a minor elevation in ketogenesis. In contrast, around four-fold elevated enzyme levels (in comparison to ND-fed control rats) were found for nearly the whole cholesterol biosynthesis pathway (Supplementary Table 1C). Moreover, in HCD-fed *Atp7b-/-* rats, strong elevations were found in enzymes responsible for cholesterol excretion via bile acid biosynthesis and bile excretion (Supplementary Table 1D). These data indicated an elevated synthesis of cholesterol and bile salts therefrom in HCD-fed *Atp7b-/-* rats. In fact, while unchanged cholesterol levels were determined in serum (Figure 5C) of HCD vs. ND-fedrats, significantly elevated bile salt levels were determined in HCD-fed *Atp7b-/-* rat serum (Figure 5D).

**The high affinity copper binding peptide Methanobactin rescues high caloric diet induced mitochondrial dysfunction and liver damage in *Atp7b-/-* rats.**

The bacteria-derived peptide Methanobactin (MB) prevents disease progression in *Atp7b-/-* rats [4](#_ENREF_4), [5](#_ENREF_5). This therapeutic effect is largely due to efficient hepatic de-coppering (Supplementary Figure 2) that we have reported to specifically restore mitochondrial structure and function [4](#_ENREF_4), [5](#_ENREF_5).

Consequently, we assessed here whether the beneficial effect of MB treatment would still hold in HCD-fed *Atp7b-/-* rats. Animals were treated once daily for five consecutive days with MB, starting at day 75, *i.e*., at an age when HCD-fed *Atp7b-/-* rats presented marked liver damage (Figure 1D, Supplementary Figure 1).

MB-treatment clearly improved the mitochondrial structure, as evidenced either *in situ* or at the level of isolated mitochondria (Figure 6A). A significantly lower number of isolated mitochondria from MB-treated HCD-fed *Atp7b-/-* rats presented with cristae detachments and matrix condensations in comparison to mitochondria from untreated HCD-fed *Atp7b-/-* rats (Figure 6A, quantification in 6B). This structural normalization was paralleled by a 50% reduction in mitochondrial copper load (Figure 6C, Supplementary Table 2), a strongly enforced mitochondrial ATP production capacity (Figure 6D) and a significantly decreased mitochondrial H2O2 emergence (Figure 6E). Thus, a 5-day only MB treatment efficiently ameliorated mitochondrial copper overload in HCD-fed *Atp7b-/-* rats, reduced mitochondrial damage and restored mitochondrial function.

This mitochondrial rescue was paralleled by restored liver integrity, as serum AST levels returned to normal (Figure 7A) and serum copper significantly decreased (Figure 7B) despite only moderately lower copper values in liver homogenates (Supplementary Table 2) in MB-treated *vs*. untreated HCD-fed *Atp7b-/-* rats. In line with restored Acetyl-CoA utilization by mitochondria, total serum bile salts significantly decreased (Figure 7C). Furthermore, a reduced presence of lobular inflammation and hepatic injury was apparent in HE-stained liver samples in treated *vs*. untreated HCD-fed *Atp7b-/-* rats (Figure 7D), resulting in beneficial changes in the hepatic injury score (Figure 7E, F).

**Discussion**

In Wilson disease (WD), ATP7B malfunction impairs hepatic copper excretion. This leads to a progressive copper burden in mitochondria [4](#_ENREF_4), [5](#_ENREF_5), [8](#_ENREF_8), [38](#_ENREF_38). Copper ultimately causes mitochondrial destruction, hepatocyte death, liver failure and decease of WD *Atp7b-/-* rats [5](#_ENREF_5). Efficient depletion of mitochondrial copper load with the high-affinity copper-chelator Methanobactin leads to full recovery from even severe states of liver damage [5](#_ENREF_5). If the treatment is paused, the rate of mitochondrial copper re-accumulation determines the rate of re-occurring liver damage [5](#_ENREF_5).

These findings define hepatic mitochondria as central integrators of liver copper overload and disease stage in WD. However, mitochondria readily respond to environmental changes by metabolic adaptations and can balance imposed challenges, also copper, to different extents quite longstanding [13](#_ENREF_13) [41](#_ENREF_41). This flexibility might contribute to the high variability of the clinical presentation of WD [1](#_ENREF_1). Neither is the *ATP7B* genotype anyhow predictive for the age of onset, the disease presentation or progression, nor for the response to treatment [1](#_ENREF_1), [42](#_ENREF_42). This absence of a genotype-phenotype correlation may be best exemplified by studies on genetically identical WD twins, whose clinical appearance ranged from pre-symptomatic phenotypes to liver failure [18](#_ENREF_18), [43](#_ENREF_43). Consequently, it has been suggested that the WD phenotype may be highly attributable to environmental factors [43](#_ENREF_43) [44](#_ENREF_44). However, no such factors have been conclusively identified to date.

We evaluated here the impact of a high caloric diet (HCD) on Wilson disease onset, progression and severity in *Atp7b-/-* rats. A HCD caused strongly increased and accelerated liver damage, evidenced by serum markers of liver damage and histological assessment (HAI Score), together with mitochondrial (but equal cytosolic or overall hepatic) copper overload and massive mitochondrial dysfunction in HCD-fed *Atp7b-/-* rats in comparison to (still) healthy ND-fed *Atp7b-/-* rats (Figures 1–3, Supplementary Figure 1). Liver damage correlated strongly with mitochondrial copper load (Figure 2F). Thus, a “simple” change in nutrition from a normal to a high caloric diet (providing equal copper supply) aggravated WD.

*Why is the Wilson disease liver phenotype severely aggravated in HCD- vs. ND-fed Atp7b-/- rats?*

From our data, we conclude that this is because of a highly detrimental combination of copper- and fatty acid-induced impacts on liver mitochondria. We have recently reported that progressive mitochondrial copper accumulation causes a steady reduction of their capacity to produce ATP [5](#_ENREF_5). This is due to a direct impact of copper on the protein complexes involved in ATP production, but also ATP delivery to the cytosol [4](#_ENREF_4). This bio-energetic deficit matches the clinical presentation of liver damage in *Atp7b-/-* rats. A drop in the ATP production capacity to 70% in comparison to mitochondria from *Atp7b+/-* control rats was found to be critical for the onset of clinically apparent liver damage (i.e., AST levels > 200 U/l) [5](#_ENREF_5). In the present study, a mitochondrial ATP production capacity of 80% was preserved in young ND-fed *Atp7b-/-* rats. In agreement with our earlier study, these rats were still clinically healthy (Figure 1D–F, Supplementary Figure 1). In a further study, we have reported that the increased supply of fatty acids via a HCD causes lipidomic alterations in the membranes of liver mitochondria that also reduce their ATP production capacity [13](#_ENREF_13). However, despite these mitochondrial alterations, only mild signs of liver impairment were observed [13](#_ENREF_13). In agreement with this further study, we determined here a reduced mitochondrial ATP production capacity but only mild signs of apparent liver damage in HCD-fed *Atp7b+/-* control rats (Figure 1, 4A). Thus, lipidomic membrane alterations in mitochondria induced by a HCD are less connected with liver damage in contrast to a direct copper association on essential mitochondrial protein complexes. This situation changes if both impacts coincide. A massive drop in the ATP synthase activity caused a decrease in the ATP production capacity to below 40%, severe structural impairments and strongly elevated ROS emergence in mitochondria from HCD-fed *Atp7b-/-* rats in comparison to control mitochondria (Figures 3, 4).Such mitochondrial damage is incompatible with hepatocyte survival, and cell death is extensive, causing severe liver damage (Figure 1D). Of note, short-term treatment with the copper chelator Methanobactin significantly reduced all of these features (Figures 6, 7), most prominently the mitochondrial copper load. This clearly argues for copper load to be the main driver of mitochondrial destruction, as treated animals remained on the HCD throughout.

HCD-feeding caused steatosis in control *Atp7b+/-* but steatohepatitis in *Atp7b-/-* rats (Figure 1). However, tendentious lower levels of visceral fat (Figure 1A) and lower levels of liver triglycerides (Figure 1C) were found in HCD-fed *Atp7b-/-* vs. *Atp7b+/-* rats, indicating a comparatively higher energy turnover in *Atp7b-/-* 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 (Supplementary Table 1A). Interestingly, we also observed higher levels of lipid biosynthesis enzymes, partly in HCD-fed *Atp7b+/-* control rats, but very prominently in HCD-fed *Atp7b-/-* rats (Supplementary Table 1B). Thus, the highly elevated supply of fatty acids via the HCD vs. ND [14](#_ENREF_14) causes a two-fold adaptation in hepatocytes, first, their increased degradation in mitochondria via ß-oxidation, and second, their esterification to triglycerides that are preferentially stored in cytosol. However, an increasing Acetyl-CoA amount, resulting from elevated mitochondrial ß-oxidation of fatty acids, could not only result in increasing lipid biosynthesis but also in elevated cholesterol levels, as Acetyl-CoA is also the precursor in hepatic cholesterol biosynthesis [40](#_ENREF_40). Such hepatic cholesterol accumulation has been reported in NAFLD patients and rodents [45](#_ENREF_45) and correlated with histological severity of the disease and thus seems to be associated with HCD malnutrition. In fact, elevated enzyme abundances for nearly the whole cholesterol biosynthesis pathway were found in HCD-fed *Atp7b-/-* rat livers (Supplementary Table 1C). Unexpectedly, however, we did not observe specifically elevated cholesterol levels in these animals (Figure 5C). This may have been prevented by an increased routing of cholesterol into bile salts, as we have determined two- to eleven-fold abundance increases in bile synthesis and bile excretion enzymes in HCD-fed *Atp7b-/-* rat livers (Supplementary Table 1D). In fact, we found massively elevated bile salts only in serum of HCD-fed *Atp7b-/-* rats (Figure 5D), which were significantly reduced upon Methanobactin treatment (Figure 7C). Clearly, such elevated bile salt synthesis may be a further detrimental impact in HCD-fed *Atp7b-/-* rat livers, as accumulating bile salts are hepatotoxic (especially to hepatic mitochondria) [46](#_ENREF_46), [47](#_ENREF_47).

In conclusion, the combination of accumulating copper with a HCD is highly detrimental to hepatic mitochondria. A toxic triad of ATP depletion, massively elevated ROS and bile salts seals the fate of affected hepatocytes. This indicates that a high *vs*. normal caloric nutrition may have a tremendous impact on WD progression and severity and may explain the striking phenotype-genotype discrepancies encountered in WD patients.

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**Figure Legends**

**Figure 1: A high caloric diet causes severe liver damage in *Atp7b-/-* rats.**

 (A-C) A high caloric diet (HCD) increases visceral fat mass (A, N=6–8), liver weight (B, N=5–6) and liver triglyceride content (C, N=5) in *Atp7b+/-* and *Atp7b-/-* rats. (D-F) Serum AST (D, N=6–8), NAFLD activity (NAS, E, N=6–8) and hepatic injury score (HAI, F, N=6–8) only increases significantly in HCD-fed *Atp7b-/-* rats. Statistics: one-way ANOVA with Tukey’s multiple comparisons test (A-D), non-parametric Kruskal-Wallis test (E, F), \*significant to *Atp7b*+/**-**ND, #significant to *Atp7b*+/**-** HCD, †significant to *Atp7b***-/-** ND. (G) Liver histology (HE-stain, scale bar: 100 µm) from HCD-fed *Atp7b+/-* and *Atp7b-/-* rats present macrosteatosis (open asterisk), ballooned hepatocytes (open arrow), inflammatory infiltrations (black arrow) and some signs of fibrosis (black asterisk), apoptosis (open arrowhead) or necrosis (black arrowhead).

**Figure 2: A high caloric diet increases serum and mitochondrial copper load in *Atp7b-/-* rats.**

(A) Serum ceruloplasmin oxidase (Cp) activity is depleted in *Atp7b-/-* rats. (B) Serum copper is decreased in *Atp7b-/-* rats compared to *Atp7b+/-* rats but increases upon HCD in *Atp7b-/-* rats. (C-D) Equally elevated copper load in whole liver homogenate (C) and hepatic cytosol (D) in either HCD- or ND-fed *Atp7b-/-* rats. (E) Massive mitochondrial copper load in HCD-fed *Atp7b-/-* rats. (F) Mitochondrial copper load significantly correlates with NAS and HAI-score. Statistics: one-way ANOVA with Tukey’s multiple comparisons test (A-E, N=6–8), (F) Spearman correlation, \*significant to *Atp7b*+/**-**ND, #significant to *Atp7b*+/**-** HCD, †significant to *Atp7b***-/-** ND.

**Figure 3: A high caloric diet amplifies hepatic mitochondrial damage in WD *Atp7b-/-* rats.**

(A-B) Mitochondria either *in situ* (A, scale bar: 250 nm) or isolated (B, scale bar: 1 µm) from HCD-fed *Atp7b-/-* rats present 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 four animal groups. Statistics: one-way ANOVA with Tukey’s multiple comparisons test (N=2-3, 350–750 mitochondria per group of animal), \*significant to *Atp7b*+/**-**ND, #significant to *Atp7b*+/**-** HCD, †significant to *Atp7b***-/-** ND.

**Figure 4: A high caloric diet severely impairs mitochondrial function in *Atp7b-/-* rats.**

(A) HCD feeding causes significantly lower mitochondrial ATP production capacity in *Atp7b+/-* and especially in *Atp7b-/-* rats (N=5–7, n=10–14). (B) Lowest F1FO-activity (ATP synthase, normalized to citrate synthase (CS) activity) in mitochondria from HCD-fed *Atp7b-/-* rats (N=5). (C-D) Increased oxygen consumption and decreased respiratory control ratio (RCR) in mitochondria from HCD- *vs*. ND-fed *Atp7b-/-* rats (N=3, CII-linked\_P: succinate-linked phosphorylation, LEAK: O2-consumption upon oligomycin treatment, ETS: electron transfer system capacity in a non-coupled FCCP-treated state). (E-F) Massively elevated H2O2 emergence from mitochondria of HCD-fed *Atp7b-/-* rats using either succinate/rotenone and ADP (E) or glutamate/malate (F) as substrates (N=5). Statistics: one-way ANOVA with Tukey’s multiple comparisons test, \*significant to *Atp7b*+/**-**ND, #significant to *Atp7b*+/**-** HCD, †significant to *Atp7b***-/-** ND.

**Figure 5: A high caloric diet increases total serum bile salts in *Atp7b-/-* rats.**

Serum triglycerides (A), serum non-esterified fatty acids (B, NEFAs) and total serum cholesterol (C) does not differ between ND and HCD groups (N=4-5). (D) Total serum bile salts increase only in HCD-fed *Atp7b-/-*rats (N=3-5). Statistics: one-way ANOVA with Tukey’s multiple comparisons test, \*significant to *Atp7b*+/**-**ND, #significant to *Atp7b*+/**-** HCD, †significant to *Atp7b***-/-** ND.

**Figure 6: Methanobactin rescues HCD-induced mitochondrial dysfunction in *Atp7b-/-* rats.**

(A) Electron micrographs of mitochondria either *in situ* (A, upper panel, scale bar: 500 nm) or isolated (A, lower panel, scale bar: 500 nm) demonstrate mitochondrial structure normalization upon Methanobactin treatment (MB, right panels) *vs*. untreated (left panels). (B) Quantification of isolated mitochondria with altered structure (N=3, n=18, “n” number of analyzed pictures including a total of 700–750 mitochondria in each animal group). (C-E) Methanobactin treatment decreases mitochondrial copper load (C, N=5–6), increases mitochondrial ATP production (D, N=5, n=10) and lowers mitochondrial H2O2 emergence (E, substrates: glutamate/malate, N=5). Statistics: unpaired t-test, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

**Figure 7: Methanobactin reduces liver damage and ameliorates steatosis in HCD-fed *Atp7b-/-* rats.**

(A-C) A short-term MB treatment markedly reduces the liver damage marker AST (A), and significantly decreases serum copper (B) and total serum bile salts (C). (D) Liver sections (scale bar: 100 µm) from MB-treated (right panel) *vs*. untreated HCD-fed *Atp7b-/-* rats present with a lower grade of steatosis (open asterisk), lobular inflammation (black arrow), apoptosis (open arrowhead) and necrosis (black arrowhead), but similar amounts of ballooned hepatocytes (open arrow) and signs of fibrosis (black asterisk), as also evidenced by a significantly lower NAS (E) and a slightly reduced HAI-Score (F). Statistics: (A-C, N=3–6) unpaired t-test, significant if \*p < 0.05, mean values ± SD; (E-F, N=5–6) non-parametric Mann-Whitney test, significant if \*p < 0.05, median values ± range.