Original article

MOLECULAR METABOLISM

Mitochondrial gene polymorphisms alter hepatic cellular energy metabolism and aggravate diet-induced non-alcoholic steatohepatitis

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ABSTRACT

Objective: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and is associated with an enhanced risk for liver and cardiovascular diseases and mortality. NAFLD can progress from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH). However, the mechanisms predisposing to this progression remain undefined. Notably, hepatic mitochondrial dysfunction is a common finding in patients with NASH. Due to a lack of appropriate experimental animal models, it has not been evaluated whether this mitochondrial dysfunction plays a causative role for the development of NASH.

Methods: To determine the effect of a well-defined mitochondrial dysfunction on liver physiology at baseline and during dietary challenge, C57BL/6J-mt$^{FVB/N}$ mice were employed. This conplastic inbred strain has been previously reported to exhibit decreased mitochondrial respiration likely linked to a non-synonymous gene variation (nt7778 G/T) of the mitochondrial ATP synthase protein 8 (mt-ATP8).

Results: At baseline conditions, C57BL/6J-mt$^{FVB/N}$ mice displayed hepatic mitochondrial dysfunction characterized by decreased ATP production and increased formation of reactive oxygen species (ROS). Moreover, genes affecting lipid metabolism were differentially expressed, hepatic triglyceride and cholesterol levels were changed in these animals, and various acyl-carnitines were altered, pointing towards an impaired mitochondrial carnitine shuttle. However, over a period of twelve months, no spontaneous hepatic steatosis or inflammation was observed. On the other hand, upon dietary challenge with either a methionine and choline deficient diet or a western-style diet, C57BL/6J-mt$^{FVB/N}$ mice developed aggravated steatohepatitis as characterized by lipid accumulation, ballooning of hepatocytes and infiltration of immune cells.

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List of abbreviations: ALT, alanine aminotransferase; Arg, arginine; AMP, adenosine monophosphate; AMPK, AMP-activated proteinkinase; pAMPK, phosphorylated AMP-activated proteinkinase; Asp, aspartic acid; ATP, adenosine triphosphate; ATP8, ATP synthase protein 8; CPT I, carnitine-palmitoyltransferase I; C0, free DL-carnitine; C16, hexadecanoyl-L-carntine; C18, octadecanoyl-L-carnitine; B6-mt^{B6}, C57BL/6; B6-mt^{FVB}, C57BL/6-mt$^{FVB/N}$; CD, control diet; CD3, cluster of differentiation receptor 3; CYP51A1, cytochrome P450, family 51, subfamily A, polypeptide 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Gr1, granulocyte differentiation antigen 1; H&E, hematoxylin–eosin staining; H_2O_2, hydrogen peroxide; Hsd17b7, 17-beta-hydroxysteroid dehydrogenase type 7; IDI1, isopentenyl-diphosphate delta isomerase 1; IPA, ingenuity pathway analysis; IL, interleukin; Ly6G, lymphocyte antigen 6 complex, locus G; mt, mitochondrial; Met, methionine; MCDD, methionine and choline deficient diet; MSMO1, methylsterol monooxygenase 1; ND3, NADH dehydrogenase subunit 3; NAFL, non-alcoholic liver steatosis; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation system; ROS, reactive oxygen species; PBS, phosphate buffered saline; SNPs, single nucleotide polymorphisms; SOD2, superoxide dismutase 2; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; KEGG, Kyoto Encyclopedia of Genes and Genomes; TNFα, tumor necrosis factor alpha; Tyr, tyrosine; WD, western-style diet

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Conclusions: We observed distinct metabolic alterations in mice with a mitochondrial polymorphism associated hepatic mitochondrial dysfunction. However, a second hit, such as dietary stress, was required to cause hepatic steatosis and inflammation. This study suggests a causative role of hepatic mitochondrial dysfunction in the development of experimental NASH.

Keywords NAFLD; Steatohepatitis; Mitochondrial gene polymorphism; Mitochondrial dysfunction; Lipid metabolism; TNFα

1. INTRODUCTION

Along with the worldwide obesity pandemic, the prevalence of non-alcoholic fatty liver disease (NAFLD) is constantly rising [1,2]. The NAFLD disease spectrum encompasses simple steatosis (NAFL), steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma [3]. NAFL is characterized by an accumulation of hepatocellular lipids — mainly triglycerides [4,5], whereas NASH is identified by the addition of inflammation [6]. In contrast to simple hepatic steatosis, the incidence of NASH substantially increases the risk for hepatic diseases as well as cardiovascular complications and overall mortality [7—12]. It is estimated that 10—20% of NAFL patients progresses to NASH, but the risk factors and mechanisms involved in this progression are still poorly understood [6].

An increasing body of literature supports the observation that mitochondrial dysfunction is involved in the development of NASH. This is based on the frequent detection of the abundant formation of hepatic reactive oxygen species (ROS), lipid peroxidation, cellular energy depletion, increased expression of pro-inflammatory cytokines and ultrastructural lesions of mitochondria [13,14]. Additionally, it was recently demonstrated that patients with NASH, but not with simple hepatic steatosis, exhibit reduced mitochondrial respiration [15]. However, whether hepatic mitochondrial dysfunction plays a causative role in the progression to NASH or is a consequence of hepatic inflammation remains uncertain due to a lack of appropriate experimental animal models [16,17].

Mitochondrial function is affected by both exogenous and endogenous factors. Exogenous factors include drugs, nutritional residues, aging, and lack of exercise, all of which are frequently present in modern societies [14]. With regard to endogenous factors, mitochondrial dysfunction is frequently induced by single nucleotide polymorphisms (SNPs) of the mitochondrial genome and insufficient mitochondrial DNA repair mechanisms [18]. In fact, a variation of the mitochondrial genome has already been linked to advanced stages of NAFLD in a case—control association study [19].

To further evaluate the association between mitochondrial gene polymorphisms and NASH, we used an established mouse model of mitochondrial dysfunction linked to a non-synonymous gene variation (nt7778 G/T) of the mitochondrially encoded ATP8, a complex V protein of the mitochondrial respiratory chain [20]. This gene polymorphism has been previously demonstrated to contribute to experimental diseases such as autoimmunity, neurodegeneration and β-cell dysfunction [21—23]. Here, we demonstrate initial evidence for the possible impact of this mitochondrial gene polymorphism on experimental NASH.

2. MATERIAL AND METHODS

2.1. Animal treatment and induction of experimental non-alcoholic steatohepatitis

Mice of the conplastic inbred strain C57BL/6J-mt$^{FVB/N}$ (B6-mt^{FVB}) exhibit a non-synonymous variation of the mitochondrial encoded *mt-ATP8* of the complex V of the mitochondrial respiratory chain as well as synonymous variations of *mt-ND3* (complex I) and *mt-tRNA^{Arg}* (Table 1). Next generation sequencing technology was used to exclude other mutations as well as heteroplasmy. The nuclear genome was identical between B6-mt^{FVB} and C57BL/6J (B6-mt^{B6}), which were used as control strain [20,21]. Mice were maintained in a regular 12-h light—dark cycle under standard conditions and were provided with food and water ad libitum. After weaning, at the age of 4 weeks, mice of both sexes were offered free access to control diet (CD; Altromin 1324) or methionine and choline deficient diet (MCDD; Ssniff MCD TD.90262) for 8 weeks. For a second dietary intervention, B6-mt^{FVB} and B6-mt^{B6} mice of both sexes were fed either the CD as above or a western-style diet for a period of 12 weeks (WD; Ssniff EF R/M acc. TD88137 mod.), which contained 40% of calories derived from butter fat and cholesterol and which was enriched by fructose-sweetened water (30% fructose, Sigma Aldrich, Germany). All experiments were performed in accordance with the animal care guidelines of the University of Lübeck, Germany (acceptance no.: 73-5/12). Procedures involving animals and their care were conducted in accordance with national and international laws and policies.

2.2. ROS and ATP measurement

For detection of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) levels in liver tissue were determined using a commercially available kit (Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, Invitrogen). ATP production was assessed as oxygen consumption rate (OCR) used to drive mitochondrial ATP production. For this, hepatocytes (2×10^4/100 μl) were isolated as described previously [24] and cultivated overnight in hepatocyte growth medium (DMEM, Gibco # 11966-025, 1% penicillin/streptomycin, 10% FCS). The XF Cell Mito Stress Test assay and the Seahorse Bioscience XF^e Extracellular Flux analyzer were used following the manufacturer's instructions.

2.3. Lipid peroxidation measurements

Lipid peroxidation products were determined utilizing a lipid peroxidation assay that measured malondialdehyde (MDA) in liver tissue according to the manufacturer's protocol (Eagle Biosciences, LIP39-K01).

2.4. Western blot

Western blot analysis was performed according to standard protocols as described previously [25], see Supporting information for more details.

2.5. Metabolomics

Metabolic analysis was performed at the genome analysis center of the Helmholtz Center Munich, Germany as reported previously [26,27]. In brief, liver and plasma samples from CD fed B6-mt^{FVB} and B6-mt^{B6} mice at the age of 12 weeks, 5 males and 5 females each, were analyzed. The spectrum of metabolites included acyl-carnitines,

Table 1 — Genetic difference between C57BL/6J-mt$^{FVB/N}$ and C57BL/6J.

Position	Variation	Gene	Amino acid change
nt7778	G/T	*mt-ATP8* (complex V)	Asp → Tyr
9821	8A/9A	*mt-tRNA^{Arg}*	
9461	T/C	*mt-ND3* (complex I)	syn.

The C57BL/6J-mt$^{FVB/N}$ mice are conplastic inbred mice and genetically differ only in the mitochondrial genome. Variations concern the *mt-ATP8* of complex V (nt7778, G/T) and *mt-ND3* of complex I (nt9461, T/C) of the mitochondrial respiratory chain as well as *mt-tRNA^{Arg}* (9821, A repeat, 8A/9A), whereas only the variation of the mt-ATP8 gene induces an amino acid change.

glycerophospholipids, sphingolipids, amino acids, and sugars. See Supporting information for more details.

2.6. Whole genome expression analysis

Total RNA was isolated from liver samples of 4 male mice per strain (12 weeks old, CD feeding) and used for genome wide expression analysis. These samples were hybridized on Affymetrix GeneChip Mouse Gene1.0 ST arrays in cooperation with the core facility for microarray analysis of the Rostock University Medical Center. Hybridization, washing, and scanning of the arrays were performed according to Affymetrix protocols using the WT Labeling procedure. After analysis of mRNA levels, all mRNAs were filtered based on expression and variance to exclude genes with a high variability of expression. As a result, 4347 transcripts out of a total of 35556 measured transcripts could be used for further clustering and were analyzed for statistical significance between B6-mt^{FVB} and B6-mt^{B6} mice using the Mann–Whitney U test (p-value < 0.05). Following this, a KEGG pathway analysis was performed with 327 significantly regulated transcripts utilizing the STRING database (http://string-db.org/). See Supporting information for further details on statistical analysis.

2.7. Lipid content of liver tissues

Liver tissue (100 mg) was homogenized, lysed using lysis buffer (50 mM Tris; 2 mM $CaCl_2$; 80 mM NaCl; 1% Triton X-100; pH 8.0) and centrifuged to remove remaining cell debris. The supernatant was collected for further analysis. The protein content was determined by SDS-Lowry assay. Liver cell lipids were extracted with chloroform/methanol (8:5). Total concentration of liver cholesterol concentration (free cholesterol and cholesteryl esters) was measured using the enzymatic Amplex Red Cholesterol Assay Kit (A12216 Invitrogen) according to the manufacturer's recommendations. Total triglyceride concentration was determined using commercially available enzymatic kits (Roche 11488872 and 11285874122) and the Amersham Biotrak2 plate reader (540 nm).

2.8. Histopathological and immunohistochemical analysis of mouse liver tissue

Preparation of liver sections and histopathological techniques was performed according to standard protocols. Two independent observers performed all histological assessments in a blinded fashion. NASH was determined using the established NAFLD activity score (NAS) on H&E stained random liver sections as described elsewhere [28]. Briefly, a score of 0–2 excludes NASH, a score of 3–4 defines "borderline NASH", and a score of 5 or higher warrants histological diagnosis of NASH. Furthermore, frozen sections of liver were stained using the lipid-specific Oil red O (Sigma Aldrich, Germany) or stained with the secondary antibody anti-rat Alexa 555 after incubating with anti-Ly6G or CD3 primary antibodies. Ly6G- and CD3-positive cells were counted per high-power field (magnification 200×) in 10 different areas chosen by chance on each slide (3 per mouse).

2.9. Quantitative real-time PCR

RNA extraction, reverse transcription and PCR using SYBR green were performed according to standard protocols and as described previously [25]. See Supporting information for details on procedures and primer sequences.

2.10. Statistical analysis

Results are expressed as mean with SEM. If not stated otherwise, p-values were calculated for two groups by a 2-tailed, non-parametric Mann–Whitney U test and for more than two groups by a 2-way ANOVA with Bonferroni post-test, both using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. p-values <0.05 were considered statistically significant and marked with *, p-values <0.01 were considered highly statistically significant and marked with ** and in case of $p < 0.001$ with ***.

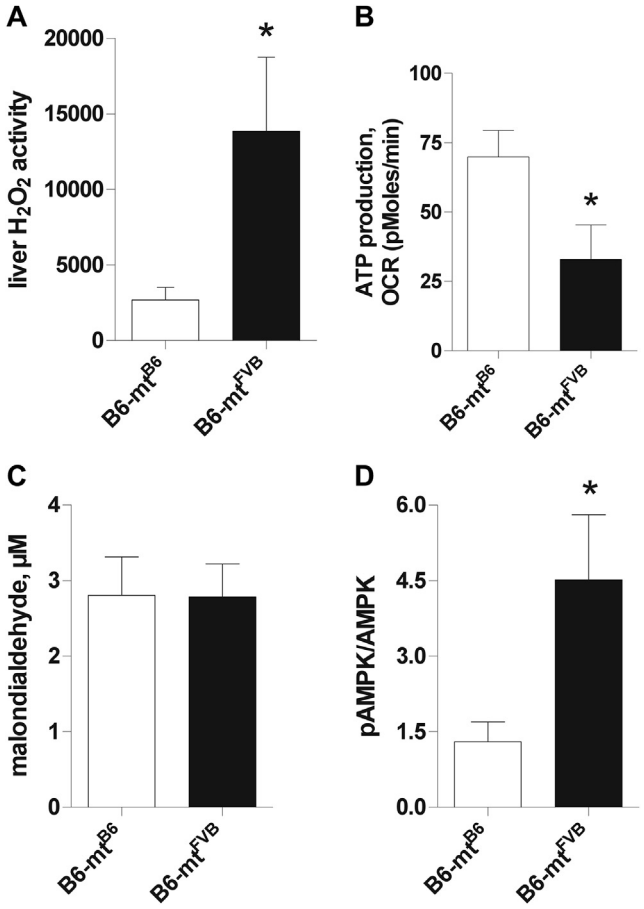

Figure 1: *B6-mt^{FVB} mice show hepatic mitochondrial dysfunction and hepatocellular energy deficiency.* Liver samples of B6-mt^{B6} and B6-mt^{FVB} mice at the age of 12 weeks were analyzed under baseline conditions (control diet feeding); data is shown from 2 independent experiments. (A) Levels of reactive oxygen species (ROS), n = 12. (B) ATP production determined by measurement of mitochondrial oxygen consumption, n = 10. (C) Malondialdehyde was determined as the representative lipid peroxidation products, n = 9. (D) Relative protein amount (in relation to GABDH) and respective ratio of phosphorylated to not phosphorylated AMPK, n = 9.

3. RESULTS

3.1. B6-mt^{FVB} mice exhibit hepatic mitochondrial dysfunction and hepatocellular energy deficiency

Previous reports have demonstrated that B6-mt^{FVB} mice exhibit reduced mitochondrial function in the brain, spleen and pancreatic β-cells [21–23]. Thus, we assessed whether these B6-mt^{FVB} mice exhibit hepatic mitochondrial dysfunction and whether this dysfunction plays a causal role in liver steatosis and inflammation.

Comparable levels of basal metabolic parameters, such as weight and serum levels of fasting glucose and lipids, were observed between B6-mt^{FVB} and B6-mt^{B6} mice at baseline (Figure S1A–B). To confirm mitochondrial dysfunction in the liver of B6-mt^{FVB}, we assessed hepatic ROS formation and ATP production [29]. It was determined that

ROS formation was increased more than 5-fold in B6-mt^{FVB} as compared to control B6-mt^{B6} mice. Further, ATP production was reduced by more than 2-fold in B6-mt^{FVB} mice (Figure 1A–B).
Generation of oxidative radicals, such as ROS, typically leads to lipid peroxidation, which has been reported to be involved in the progression of NAFLD [30]. We found that levels of malondialdehyde, the representative cytotoxic lipid peroxide, were similar between B6-mt^{FVB} and B6-mt^{B6} mice (Figure 1C). Additionally, hepatic mRNA expression of SOD2, a ROS clearing agent, was increased nearly 2-fold in the liver tissue of B6-mt^{FVB} mice (Figure S1C). Thus, no oxidative stress appeared to be functionally present in liver tissue under the baseline condition. Decreased ATP production indicates low energy supply and is known to influence the key energy sensor AMP-activated proteinkinase (AMPK), which genuinely plays a crucial role in the pathogenesis of NAFLD [31,32]. Interestingly, we observed a 3.5-fold increase in the phosphorylated, and thereby activated, pAMPK in liver of B6-mt^{FVB} versus B6-mt^{B6} mice (Figure 1D). While a combination of low ATP production and an increase of pAMPK implies a defective hepatic energy supply, the unchanged basal metabolic parameters suggest no eminent impact of the hepatic mitochondrial dysfunction on systemic metabolism as measured by body weight, serum glucose, and lipid levels.

3.2. B6-mt^{FVB} mice display unaffected hepatic morphology but alterations in metabolic profiling

The presence of hepatic mitochondrial dysfunction in B6-mt^{FVB} mice suggested susceptibility to spontaneous hepatic steatosis or inflammation. However, histological analysis revealed normal liver tissue architecture in mice at 3 months of age. No differences were detected regarding the shape of hepatocytes, infiltration of immune cells or lipid accumulation (Figure 2A). Monitoring the liver tissue architecture of B6-mt^{FVB} and B6-mt^{B6} control mice until the age of 12 months generated similar histological results (Figure 2B).
B6-mt^{FVB} mice presented features of hepatic energy deficiency, although no basal effect on liver morphology could be detected. To investigate for further consequences of mitochondrial dysfunction on energy homeostasis, we performed both hepatic and plasma metabolic profiling [33], which allows for a snapshot of *in vivo* energy metabolism. The wide spectrum of

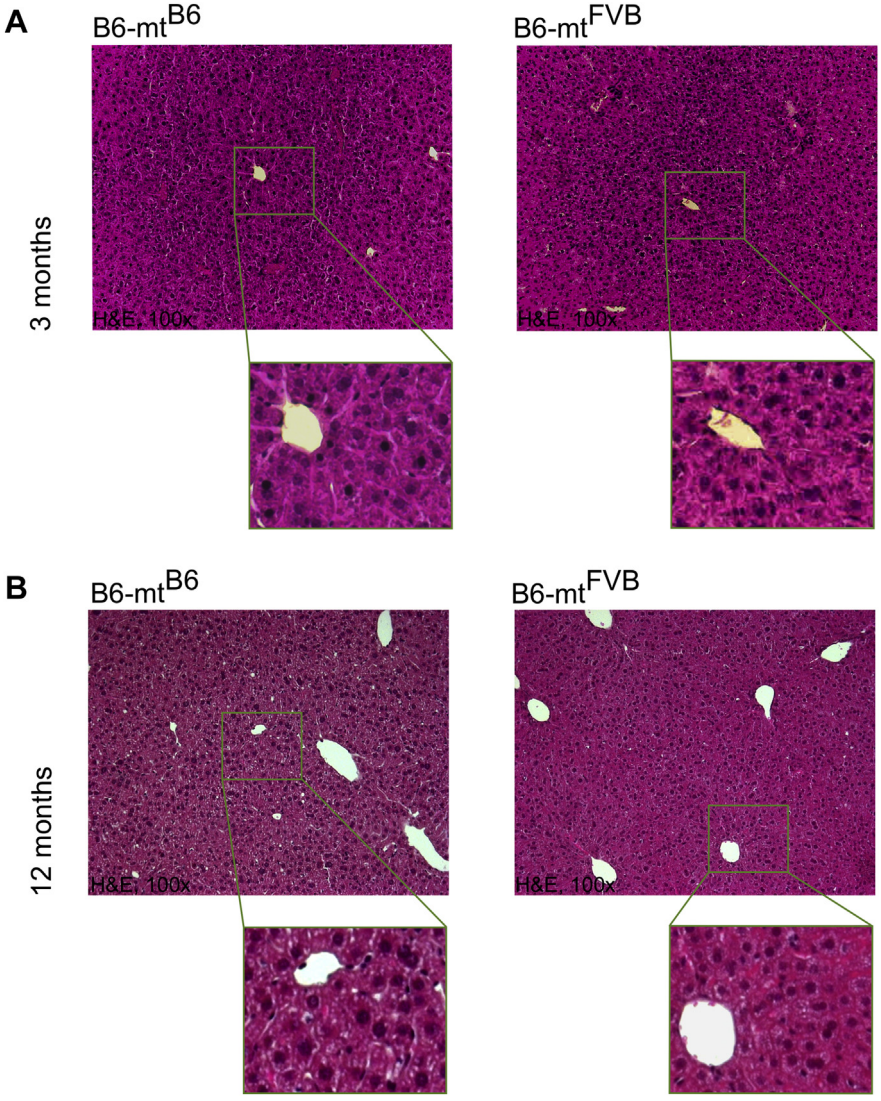

Figure 2: *Baseline liver morphology is not affected by hepatic mitochondrial dysfunction.* Exemplary pictures of H&E staining of liver tissue. (A) Staining of 3 months old B6-mt^{B6} (left) and B6-mt^{FVB} mice (right), amplification 100× plus magnified section. (B) Staining of 12 months old B6-mt^{B6} (left) and B6-mt^{FVB} (right), amplification 100× plus magnified section.

screened metabolites included acyl-carnitines, glycerophospho- and sphingolipids, amino acids, and sugars. We found various acyl-carnitines were altered in liver and plasma. Specifically, C6 (C4:1-DC), C3-OH, C3, C18:2, C18:1, and C16 were significantly changed in liver tissue, and C3-DC (C4-OH) and C18:2 were significantly changed in plasma of B6-mt^{FVB} mice compared to B6-mt^{B6} control mice (Figure 3A). Applying the ratio of free carnitine and the sum of the long-chain acyl-carnitines C16 and C18 (C0/[C16 + C18]) reflects function of carnitine-palmitoyltransferase I (CPT I), the enzyme necessary for free fatty acid transport into the mitochondria for β-oxidation [34,35]. Interestingly, the carnitine ratio was increased nearly 4-fold in the liver tissue but not in plasma of B6-mt^{FVB} mice compared to control B6-mt^{B6} mice (Figure 3B), suggesting hepatic CPT I deficiency in B6-mt^{FVB} mice. In addition to the altered acyl-carnitines, 5 out of 92 screened glycerophospholipids, 2 out of 15 hepatic sphingolipids, and the amino acids serine and methionine were altered in B6-mt^{FVB} livers, but no sugars were altered (Figures S2–S3). In summary, the observed changes point towards an impact of the mitochondrial genome on hepatocellular energy metabolism in B6-mt^{FVB} mice.

3.3. Alterations of metabolic pathways and the hepatic lipid content in liver tissue of B6-mt^{FVB} mice under baseline conditions

To further determine the mechanisms and specific genes that may be altered by hepatic mitochondrial dysfunction, we performed a genome-wide mRNA expression analysis of liver samples from control diet (CD) fed B6-mt^{FVB} and B6-mt^{B6} mice. This analysis revealed that 327 out of the initially screened 35556 transcripts were expressed differentially. KEGG pathway analysis using the STRING database revealed 56 transcripts to be jointly involved in 15 significantly changed pathways in B6-mt^{FVB} versus B6-mt^{B6} mice (Figure 4A). Remarkably, 24 of these 56 transcripts are involved in metabolic pathways. Considering that Cyp51, Idi1 and Hsd17b7 are involved in lipid metabolism and lipid accumulation is one central feature of the NAFLD/NASH pathology, we wanted to assess the functional relevance of these findings and therefore analyzed the lipid content of liver samples collected from CD fed B6-mt^{FVB} and B6-mt^{B6} control mice. Quantification of triglyceride levels in the livers of B6-mt^{FVB} mice revealed a significant increase (1.4 times) compared to

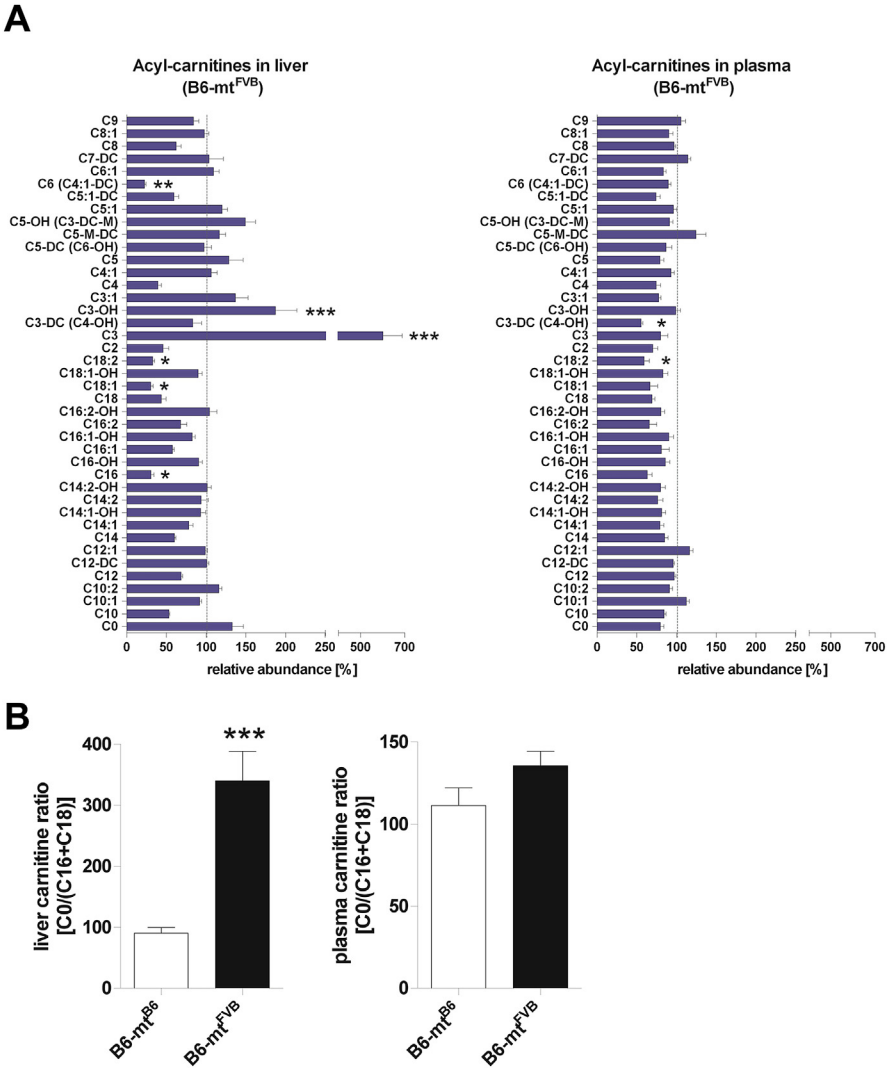

Figure 3: *Altered levels of acyl-carnitines in liver and plasma of B6-mt^{FVB} versus B6-mt^{B6}.* (A) List of all acyl-carnitines in liver tissue and plasma of B6-mt^{FVB} in relation to the value in B6-mt^{B6} mice (normalization to 100% is marked with a line). Data were acquired for metabolomics profiling at the genome analysis center Helmholtz Center Munich, Germany. See Supplement for list of all metabolites and according abbreviations. (B) Liver and plasma carnitine ratio of free carnitine (C0) to the sum of the long-chain acyl-carnitines C16 and C18 as an indicator of CPT I function.

B6-mt^{B6} mice (Figure 4B). Additionally, liver cholesterol was 1.4 times decreased in these mice (Figure 4B). Taken together, these findings point towards a functional impact of the mitochondrial genome on hepatocellular lipid metabolism and lipid composition in B6-mt^{FVB} mice at baseline, although it is effectively compensated under baseline conditions.

3.4. MCD diet-induced steatohepatitis was aggravated in B6-mt^{FVB} mice

To determine whether hepatic mitochondrial dysfunction and subsequent energy deficiency influences the development of NASH, B6-mt^{FVB} and B6-mt^{B6} mice were fed the NASH-inducing methionine and choline deficient diet (MCDD) [36]. After 8 weeks of feeding NASH progression was quantified based on histological analysis determining lipid accumulation, infiltration of immune cells and hepatocellular ballooning. Each of these criteria contributes to the NAFLD activity score (NAS) [28]. We observed that liver tissue from B6-mt^{FVB} mice fed MCDD displayed increased hepatic lipid deposition and increased numbers of immune cells and ballooned hepatocytes, resulting in a significantly higher NAS as compared to B6-mt^{B6} mice (Figure 5A). To quantify levels of hepatic lipid deposition, we stained liver sections with Oil red O. Lipid content was found to be 1.5 times higher in liver sections of B6-mt^{FVB} as compared to control mice (Figure 5B).

Hepatic inflammation is known to drive hepatocellular damage. In fact, serum levels of alanine transaminase (ALT), a clinical marker of hepatocellular damage, were increased (1.4 times) in B6-mt^{FVB} as compared to B6-mt^{B6} mice (Figure 5C). Considering the changes in liver content of cholesterol and triglycerides under baseline conditions, we also quantified cholesterol and triglyceride levels in the liver after MCDD. Interestingly, both cholesterol and triglyceride levels were significantly increased in B6-mt^{FVB} liver tissue samples as compared to those from either MCDD fed B6-mt^{B6} mice (Figure 5D**)** or CD fed B6-mt^{FVB} mice (Figure 4B).

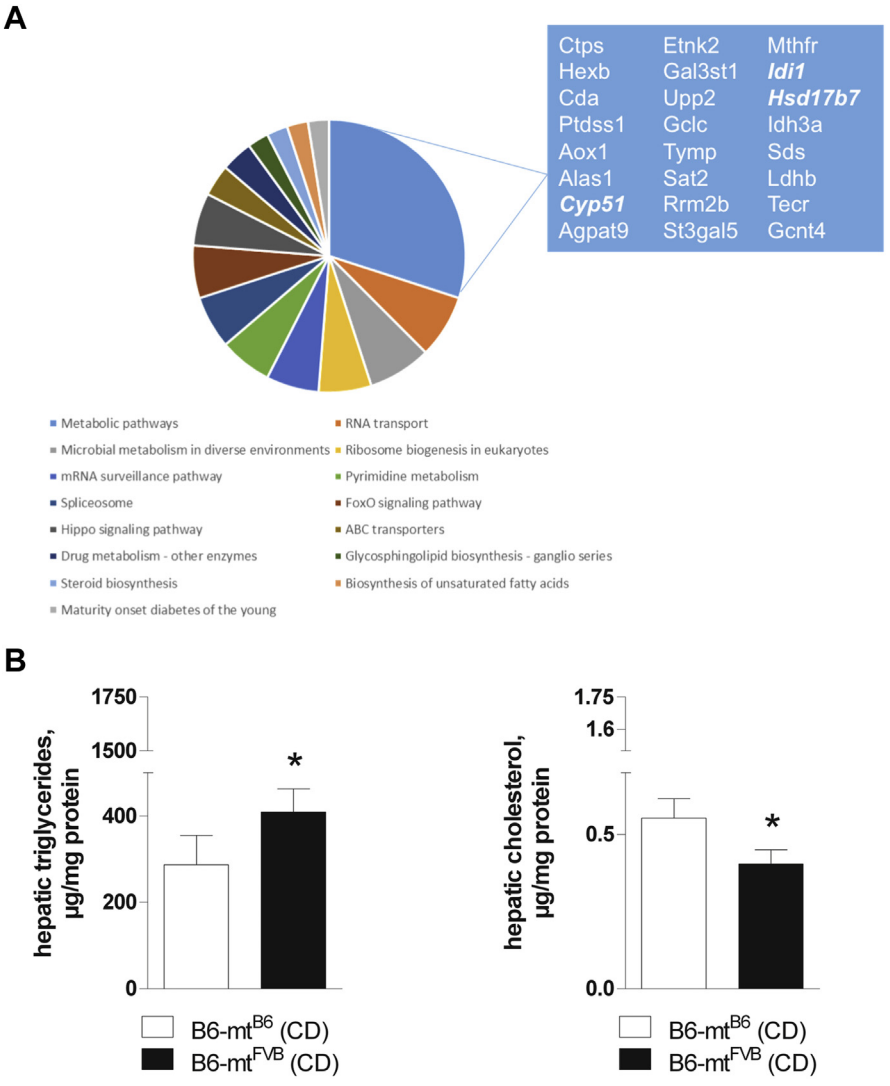

Figure 4: *Gene expression analysis of hepatic lipid content under baseline conditions in B6-mt^{FVB} mice.* (A) Genome-wide mRNA expression analysis of liver samples revealed 327 transcripts to be expressed significantly different in B6-mt^{FVB} mice compared to B6-mt^{B6} control mice (12 weeks old, control diet feeding). KEGG pathway analysis using the STRING database revealed 56 transcripts to be jointly involved in 15 significantly changed pathways in B6-mt^{FVB} versus B6-mt^{B6} mice. The list (blue box) shows 24 genes involved in metabolic pathways, of which Cyp51, Idi1, and Hsd17b7 (in bold) affect lipid metabolism. (B) Hepatic content of triglycerides (left) and cholesterol (right) measured under baseline conditions in 12 weeks old mice, n = 23 (B6-mt^{B6}), n = 29 (B6-mt^{FVB}), data from 2 independent experiments.

We further explored immune cell infiltration by incubating liver sections with antibodies against immune cell surface markers (Ly6G, marker for neutrophils; CD3, marker for T cells). A 1.5-times higher infiltration of Ly6G- or CD3-positive cells in B6-mt^{FVB} versus B6-mt^{B6} mice indicated increased hepatic infiltration of neutrophils and T cells (Figures S4A). In addition, we analyzed the expression of pro-inflammatory cytokines. Of note, under baseline conditions, the expression of TNFα was increased 3-fold in B6-mt^{FVB} mice at the

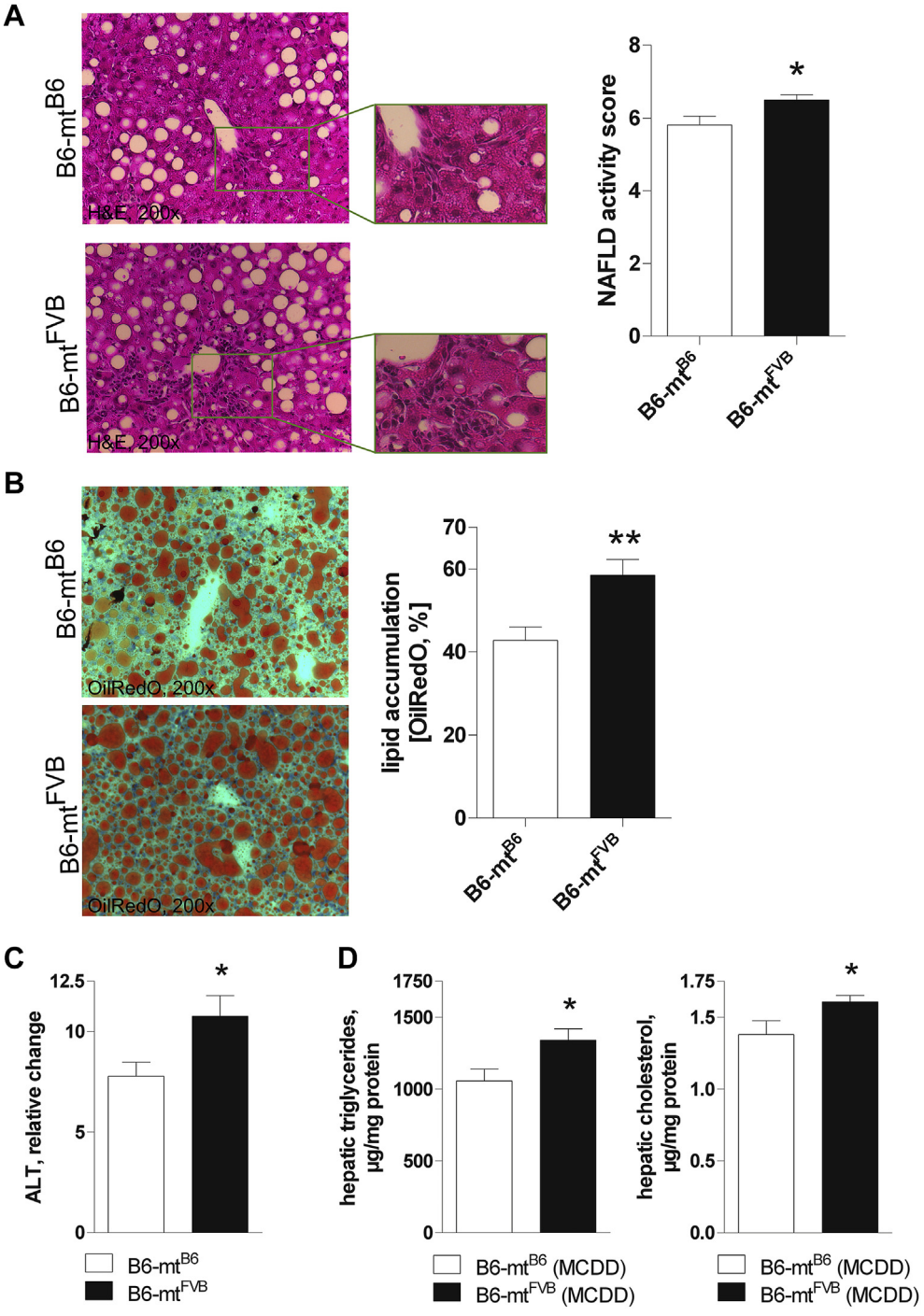

Figure 5: *MCDD-induced steatohepatitis is aggravated in B6-mt^{FVB} mice.* (A) Exemplary pictures of H&E stained liver section of B6-mt^{B6} (upper row) and B6-mt^{FVB} (lower row) mice after 8 weeks MCDD (age 12 weeks), amplification 200× plus magnified section. On the right, NAFLD activity score, scoring by two observers in a blinded fashion, n = 12 (B6-mt^{B6}), n = 16 (B6-mt^{FVB}), 2 independent experiments. (B) Exemplary pictures of Oil-Red-O stained liver section of B6-mt^{B6} (upper row) and B6-mt^{FVB} (lower row) mice after MCD diet for a period of 8 week (age 12 weeks), amplification 200×. On the right, quantification of hepatic lipid accumulation by two observers in a blinded fashion, n = 12 (B6-mt^{B6}), n = 16 (B6-mt^{FVB}). (C) MCDD induced a change of serum levels of ALT in relation to basal enzyme levels under CD, n = 16 (B6-mt^{B6}), n = 23 (B6-mt^{FVB}), 2 independent experiments. (D) Hepatic content of triglycerides (left) and cholesterol (right) measured in 12 weeks old mice after 8 weeks MCD diet, n = 12 (B6-mt^{B6}), n = 16 (B6-mt^{FVB}), data from 2 independent experiments.

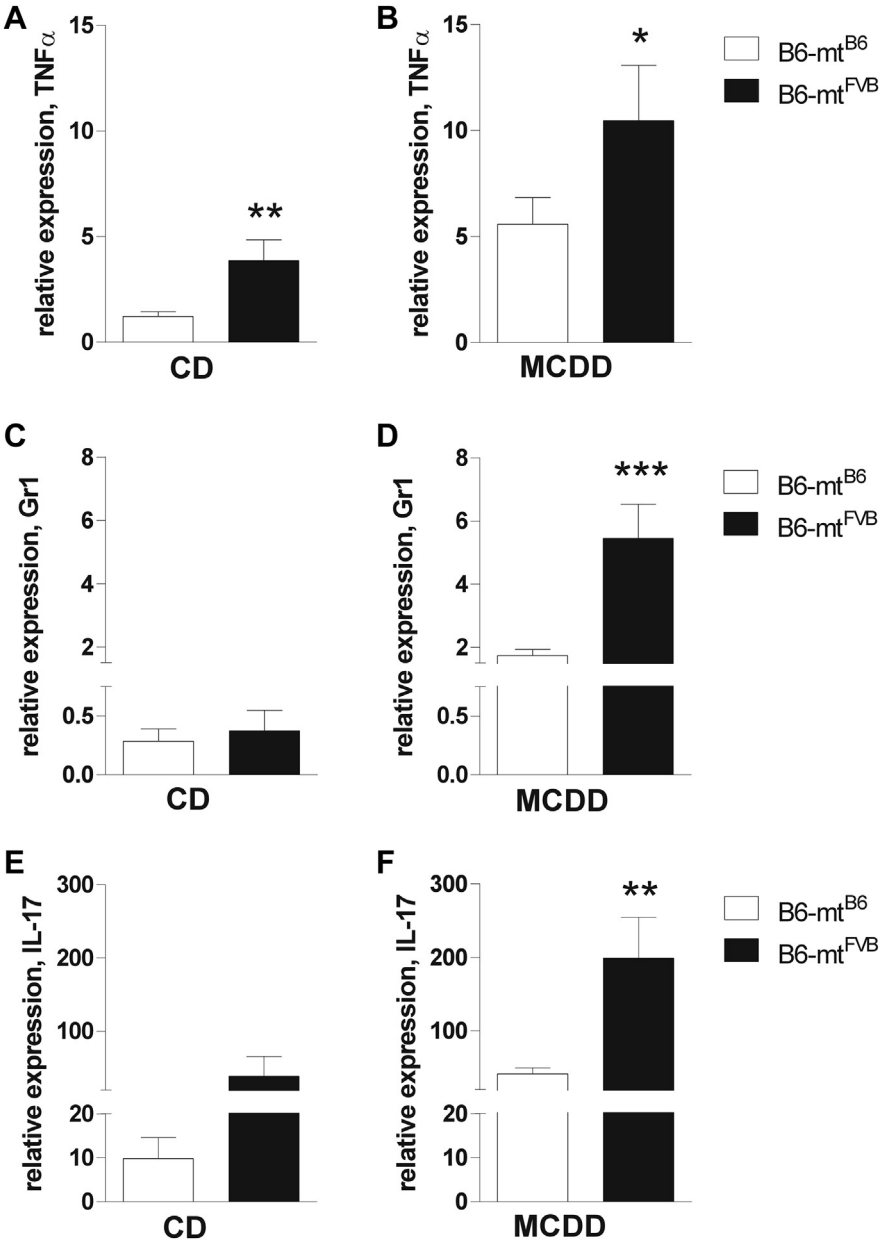

Figure 6: *Pro-inflammatory cytokine expression in B6-mt^{FVB} at baseline and upon MCDD feeding.* Relative mRNA abundance of TNFα after 8 weeks CD feeding (A) and 8 weeks MCDD feeding (B), Gr1 after 8 weeks CD feeding (C) and MCDD feeding (D), and IL-17 after 8 weeks control diet (E) and MCDD feeding (F); each in relation to beta-2 microglobulin, n = 12 (B6-mt^{B6}), n = 16 (B6-mt^{FVB}), 2 independent experiments.

age of 12 weeks (Figure 6A), while similar mRNA expression levels were detected for Gr1, IL-17, IL-6, IL-1β, and IL-18 (Figures 6C, E, S4B). However, in line with findings from histological analyses of MCDD-induced steatohepatitis, the expression of the pro-inflammatory cytokines such as TNFα, Gr1, and IL-17 were significantly increased in liver tissue of B6-mt^{FVB} mice compared to B6-mt^{B6} upon MCDD feeding (Figure 6B, D, F).

3.5. B6-mt^{FVB} mice are more prone to the metabolic challenge of a western-style diet

Considering that the well-established MCDD model directly affects hepatocellular function but leads to decreased body weight and malnutrition, we next evaluated whether the observed phenotype persisted during a calorie-enriched dietary feeding. To test this, we used a fructose-enriched high-fat western-style diet (WD) for a short metabolic intervention to detect early changes in liver physiology [37]. WD feeding resulted in higher body and liver weights in female B6-mt^{FVB} mice compared to female B6-mt^{B6} control mice, but no changes in body or organ weight of male B6-mt^{FVB} mice were observed (Figure S5A). Fasting glucose levels of both male and female B6-mt^{FVB} mice were increased compared to B6-mt^{B6} control mice, indicating a disturbed systemic glucose tolerance (Figure 7A). The expression levels of TNFα mRNA transcripts as well as the serum levels of ALT in B6-mt^{FVB} mice were significantly increased in both males and females, indicating exacerbated hepatic inflammation (Figure 7B–C). However, levels of hepatic Gr1

and IL-17 mRNA expression as well as the amount of liver lipids were similar in WD fed B6-mt^{FVB} and B6-mt^{B6} mice of both sexes (Figures 7D–E, S5B). Of note, the levels of hepatic lipids in B6-mt^{FVB} mice after WD feeding were as high as the levels after MCDD feeding (male and female). Further, histological analysis was employed to determine NAS in male and female B6-mt^{FVB} versus B6-mt^{B6} control mice (Figure 7E). We detected stronger lipid accumulation and immune cell infiltration in liver sections of B6-mt^{FVB} mice compared to B6-mt^{B6} control mice. Despite the short period of feeding, we found sporadic signs of hepatic ballooning that were accompanied by an increased (1.9 times) NAS in B6-mt^{FVB} versus B6-mt^{B6} mice (Figure 7E). In summary, B6-mt^{FVB} mice show a higher susceptibility to the metabolic challenge of a WD feeding.

4. DISCUSSION

Due to the frequent detection of indicators of mitochondrial dysfunction, such as ROS and low ATP levels, in patients or mouse models with NASH [13,16,38–40], mitochondrial dysfunction is widely thought to be a key promoter in the pathology of NASH. However, considering that NASH represents an inflammatory disease, which itself might induce these indicators of mitochondrial dysfunction, it is still a matter of debate whether hepatic mitochondrial dysfunction is a cause or consequence of the development of NASH [16]. Currently, the leading hypothesis concerning the development of NASH is the two-hit or multiple-hit hypothesis, which states that multiple adverse events jointly contribute to the full pathogenesis of NAFLD [41,42]. It is widely accepted that hepatic accumulation of triglycerides represents the first hit; the second hit is thought to potentially be a variety of environmental

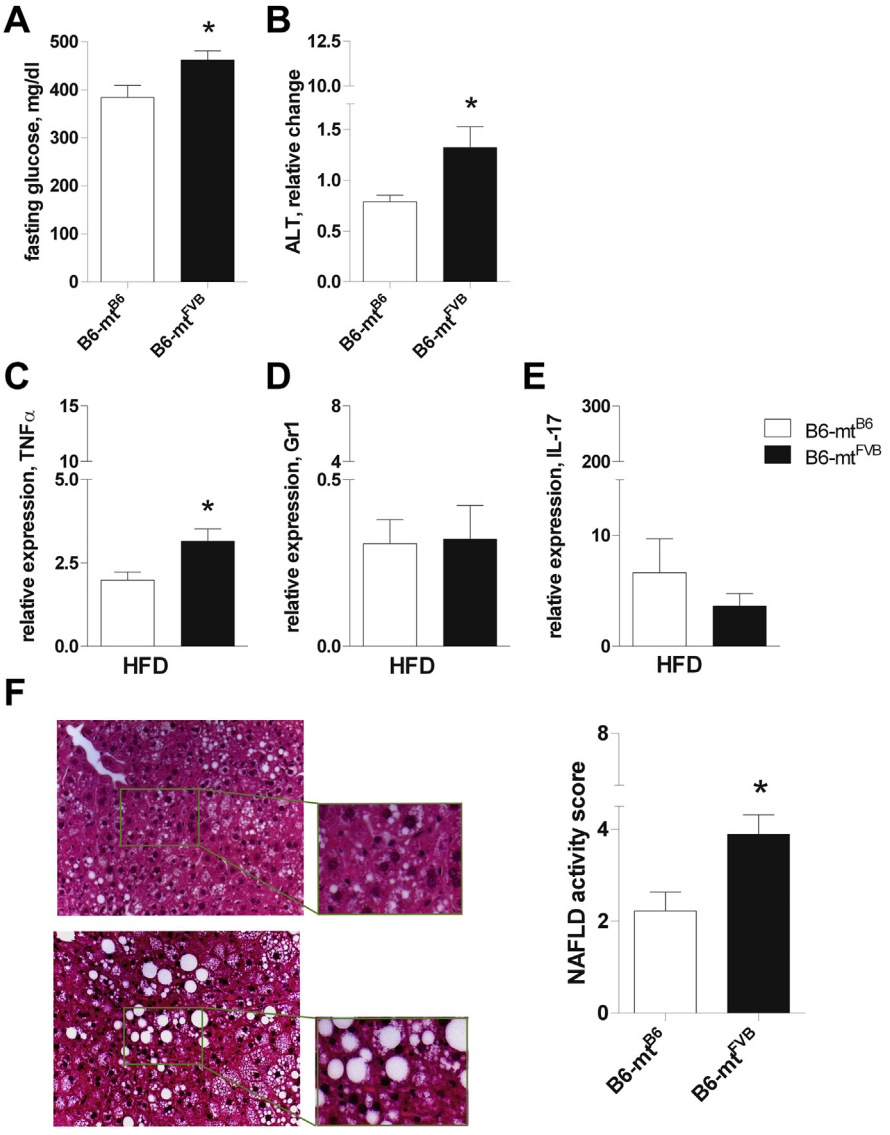

Figure 7: *B6-mt^{FVB} mice are more prone to the metabolic challenge of a western-style diet.* (A) Serum levels of fasting glucose after 12 weeks of western-style diet, n = 9 (B6-mt^{B6}), n = 10 (B6-mt^{FVB}). (B) Change of serum levels of ALT in relation to basal enzyme levels, n = 5. Relative mRNA expression of hepatic TNFα (C), Gr1 (D) and IL17 (E) in relation to beta-2 microglobulin, n = 9 (B6-mt^{B6}), n = 10 (B6-mt^{FVB}). (F) Exemplary pictures of H&E stained liver section of B6-mt^{B6} (upper row) and B6-mt^{FVB} (lower row), amplification 200× plus magnified section. On the right, NAFLD activity score, scoring by two observers in a blinded fashion after western-style feeding for 12 weeks (final age 16 weeks), n = 9 (B6-mt^{B6}), n = 10 (B6-mt^{FVB}).

stressors, such as dietary residues, hyperglycemia or hyperlipidemia. Of note, all of these are associated with the induction of mitochondrial dysfunction [43].

Mitochondrial gene polymorphisms frequently occur and can lead to mitochondrial dysfunction, whose impact is observed in various human diseases [18,19,44–46]. Due to the lack of recombination of mitochondrial DNA, mitochondrial gene variations are difficult to be mapped in experimental animal models [20]. Thus, conplastic inbred strains uniquely allow to analyze the impact of mitochondrial gene variations on mitochondrial function and disease outcome.

The B6-mt^{FVB} mice carry a non-synonymous variation of the *mt-ATP8* gene as well as synonymous gene variations of *mt-ND3* and *mt-tRNA^{Arg}* (Table 1). The mutation in the *mt-ATP8* gene is the only one involving an amino-acid change, so it is the most likely cause of the observed alterations. However, an impact of the other variations certainly cannot be ruled out. In agreement with previous reports in other tissues [21–23], we could verify that B6-mt^{FVB} mice display hepatic mitochondrial dysfunction. In addition, at baseline we detected increases in TNFα as a key feature of NASH reflecting the first hit [14,47]. However, we did not observe spontaneous lipid accumulation or signs of hepatic inflammation at baseline. Hence, this finding supports the idea that mitochondrial dysfunction alone is not sufficient to induce NASH. Indeed, upon dietary challenge, as a second hit, B6-mt^{FVB} mice displayed an aggravated shape of NAFLD/NASH. Most strikingly, we already observed a NASH-like phenotype after a short 12 weeks period of WD feeding. This represents approximately half of the time principally needed to evoke the full picture of NASH in wild-type mice [37]. Thus, as the most salient finding of this study we could show that hepatic mitochondrial dysfunction due to naturally occurring mitochondrial gene polymorphisms in combination with a dietary stimulus is sufficient to jointly induce the whole range of NASH.

Notably, during the short WD feeding we observed that female B6-mt^{FVB} mice had a higher body and liver weight compared to female B6-mt^{B6} mice, whereas the weight of the males was similar between the two groups. Nonetheless, the NAS, ALT and glucose levels were similarly increased in male and female B6-mt^{FVB} mice compared to B6-mt^{B6} control mice. The gender effect with respect to weight development remains to be elucidated, but, interestingly, similar gender-specific differences have already been described in humans in terms of NAFLD [48].

Oxidative stress has been shown to be involved in NASH pathology and is usually linked with mitochondrial dysfunction [38,49]. However, malondialdehyde, the major lipid peroxidation product, was unaltered in livers from B6-mt^{FVB} mice. Thus, we hypothesized that oxidative stress might not be involved in promoting the exacerbated NASH phenotype in B6-mt^{FVB} mice, most likely due to successful compensation. However, future experiments involving ROS clearing procedures are needed to finally dissect the impact of the higher ROS formation in this model. Considering that perturbed hepatic energy metabolism is described as part of the pathophysiology in NASH and that B6-mt^{FVB} mice displayed low hepatic levels of ATP and increased levels of pAMPK, we propose that changes in energy metabolism may be present in B6-mt^{FVB} mice [50]. In particular, metabolomics analysis indicated that various acylcarnitines were altered [33]. Since these metabolites are crucially involved in different energy pathways, this finding suggests a disturbance in energy homeostasis [51]. Moreover, the considerably increased hepatic carnitine ratio suggests a reduced CPT I activity in liver of B6-mt^{FVB} mice, in accordance with the literature [34,35]. This may account for increased hepatic, but not serum, triglyceride levels at baseline and upon diet, most likely resulting from defective free fatty acids transport into the mitochondria by the CPT I dependent carnitine shuttle [35]. With respect to the glucose-fatty acid cycle described by Randle, it remains to be elucidated why the observed increased levels of activated AMPK do not account for an increased CPT I activity in B6-mt^{FVB} mice [52]. Interestingly, propionyl-L-carnitine (C3) was previously demonstrated to improve liver respiratory chain activity [53]. In fact, levels of hepatic propionyl-L-carnitine were increased in B6-mt^{FVB} mice indicating a potential compensatory mechanism at baseline.

A recent study suggested that the nuclear genome is the major determinant of disease outcome in a mouse model of NAFLD [54]. In agreement, we show that the expression of various nuclear genes was changed in B6-mt^{FVB} mice. These findings highlight the likely importance of mitochondria–nucleus crosstalk [18]. We also

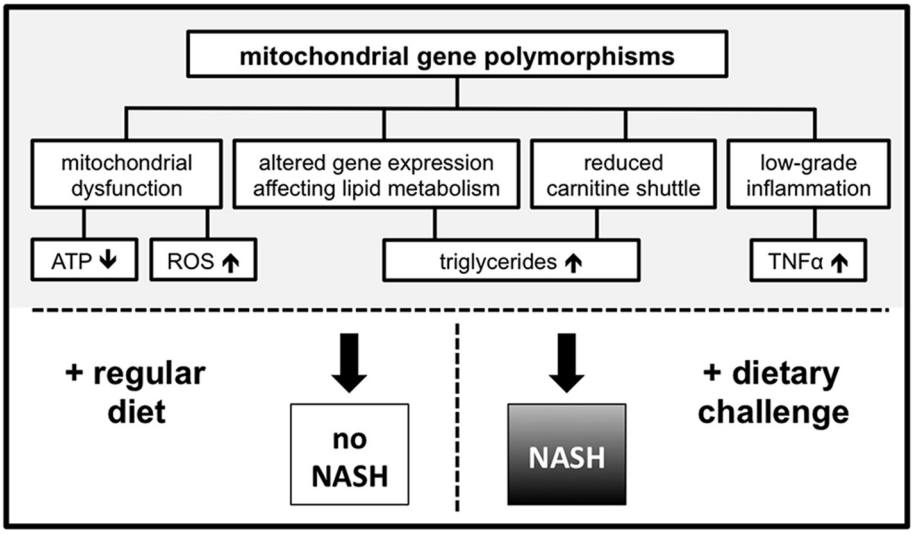

Figure 8: *Proposed explanatory scheme.* The figure shows the hepatic alterations due to the mitochondrial gene polymorphisms at baseline. These prerequisite alterations remain silent under regular diet; however, environmental stress (e.g. dietary challenge) leads to the NAFLD sequelae.

found that the changed gene expressions affect pathways predominantly involved in energy, especially lipid metabolism. This might be an additional reason why an altered liver lipid profile at baseline was observed. Upon diet-induced NASH, triglycerides and cholesterol were markedly increased in B6-mt^{FVB} mice. As growing evidence points towards a critical influence of lipid metabolism on the development of NASH, we propose that hepatic mitochondrial dysfunction may be a determinant of the relationship between altered lipid metabolism and NAFLD/NASH [55]. However, it should be mentioned that other factors might be involved in the increased susceptibility of B6-mt^{FVB} mice to dietary stress. A prominent candidate is the gut—liver axis determined by the intestinal microbiota involving IL-17 signaling, which is increasingly reported to play an important role for the development of NAFLD/NASH [56—59].

High caloric intake is frequent in affluent societies and usually accompanied by obesity and NAFL. The combination of obesity and NAFL alone does not lead to NASH in the majority of cases, although genotoxic stress, which impairs mitochondrial function, is ubiquitously present in obesity [45]. Recently, Koliaki et al. showed that during NAFL, the mitochondria can adapt to metabolic challenges, but, with the progression to NASH, these adaptive mechanisms fail, and mitochondrial dysfunction occurs [15]. Our current data support this notion and underline that the fragile balance of steady state compensation can be disturbed upon dietary challenge.

Taken all together, we hypothesize the following as a possible explanatory scheme (Figure 8). Mitochondrial gene polymorphisms lead to altered mitochondrial function, characterized by decreased hepatic energy supply, increases in ROS, and low-grade inflammation reflected by increased TNFα levels. This alters the lipid profile, and the carnitine ratio suggests a reduced CPT I function. Thus, the carnitine shuttle, which transports free fatty acids into the mitochondria for further metabolism, works inappropriately. This, in turn favors the hepatic accumulation of triglycerides, although this is not apparent. The combined alterations in liver tissue remain silent and without clinical consequence under regular diet, but, with an environmental stressor, such as dietary challenge, compensatory mechanisms collapse and increases in triglycerides and cholesterol may fuel the NAFLD sequelae.

In conclusion, we provide evidence that mitochondrial genetics impact hepatic mitochondrial function and that following alterations, such as generation of ROS and induction of TNFα signaling, act as a first hit within the pathology of NASH. Further, we demonstrated that environmental stress is required, as a second hit, to cause the full picture of NASH. The next important step will be the translation of these findings into the human NAFLD/NASH pathology. Consequently, this study may help to identify which patients with liver steatosis are at risk for NASH and its complications and may lead to a pharmacological approach preventing the emergence of NAFLD.

AUTHORSHIP

TS, DKu, FB, RP, SDe, STJ, KF, HL, SI, and CS participated in generation, analysis and interpretation of data. AS, AKB, MH, SM, AK, JB, IW, JH, DKo, JK, SDi, DG, SMS, and JA provided materials, technical support and participated in critical review of the manuscript. TS and CS participated in the conception and design of the study and wrote the manuscript.

All authors have reviewed the manuscript and approved the final version.

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CONFLICT OF INTEREST

The authors have no actual or potential conflicting interests.

APPENDIX A. SUPPORTING INFORMATION

Supporting information related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.01.010.

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