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Spontaneous cholelithiasis in C57BL/6 mice predisposes to liver cancer in NASH.

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Main text

C57BL/6 mice are widely used for metabolic, cancer, and immunological studies. High-caloric diets induce a heterogeneous phenotype in C57BL/6 mice, with the majority developing obesity and metabolic syndrome whilst others remaining lean and metabolically healthy\(^1\). Western diets (WD), methionine or choline deficient-based diets are used to study non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), important risk factors for hepatocellular carcinoma (HCC) development\(^2\)-\(^4\).

When conducting metabolic studies with NASH-inducing diets, we found several mice remained lean but developed liver cancer at time points where previously only NASH had been reported\(^5\)-\(^6\). These mice displayed elevated markers of biliary damage (serum total bile acids [TBA] and alkaline phosphatase [ALP]). To understand the link between these serum parameters and accelerated liver cancer development, we segregated mice prior to NASH-diet feeding and followed them prospectively (Figure 1a,b).

Screening male C57BL/6J littermates from Janvier at 6 weeks of age identified a heterogeneous serum TBA pattern (Figure 1c). Mice displaying serum TBA above 45 \(\mu\)M (9.1\%) were classified as cholemic/high-TBA (H-TBA) (Figure 1c), as complications caused by elevated serum TBA only occur above 40-45 \(\mu\)M\(^7\)-\(^8\). Cholemic was corroborated in C57BL/6J mice from Jackson Laboratories (14.6\%) and Charles River (23.8\%) (Supplementary Figure 1a,b). Importantly, cholemic was not observed in either B6-FVB/N-129 or DBA/2 mice (Supplementary Figure 1c,d).

Next, mice were fed either standard chow or WD (Figure 1d). Chow-feeding caused no accelerated liver cancer phenotype in either low-TBA (L-TBA) or H-TBA mice (data not shown). H-TBA mice lacked any differences in body weight prior to WD-feeding compared to L-TBA mice (Figure 1e). H-TBA mice showed accelerated and elevated liver damage (serum alanine aminotransferase [ALT]; ALP) upon WD-feeding (Figure 1f,g). H-TBA WD-fed mice showed
significantly lower levels of serum cholesterol compared to WD L-TBA mice, likely due to the requirement of cholesterol for bile acid synthesis (Figure 1h). H-TBA WD-fed mice remained lean throughout the experiment and displayed improved glucose homeostasis and serum triglyceride levels (Figure 1i-j, Supplementary Figure 1e-k).

At 32-week post-diet start, H-TBA WD-fed mice retained higher liver damage (serum ALT; ALP) and displayed liver cancer with 100% penetrance (Figure 1k-p, Supplementary Figure 1l). Thus, H-TBA/cholemic mice show metabolic improvements at the expense of accelerated liver cancer. Histological analyses revealed tumor nodules of different sizes in WD-fed H-TBA mice, with loss of collagen IV and increased hepatocyte proliferation, indicative of HCC (Figure 2a, Supplementary Figure 2a). H-TBA mice displayed profound biliary expansion, higher fibrosis, yellow coloration of the serum and elevated serum total bilirubin (Figure 2b-f). Although livers of H-TBA mice displayed biliary expansion, none of the tumors were of biliary origin (CK19), whilst showing positivity for the hepatocyte marker HNF4A (Supplementary Figure 2a). In addition, H-TBA mice fed a WD displayed significantly higher immune cell infiltrates (Supplementary Figure 2b-f). Notably, the exacerbated liver damage in H-TBA mice was evident as early as 5-weeks of WD feeding (Supplementary Figure 3a,b).

We observed no consistent differences in expression of genes involved in bile acid synthesis or transport between H-TBA and L-TBA mice fed a WD (Supplementary Figure 3c). TBA levels remained strongly elevated in H-TBA WD-fed mice at 32 weeks post-diet start (Figure 2g), primarily due to taurine-conjugates (taurocholic acid – TCA and tauro-beta-muricholic acid - T-β-MCA) and unconjugated cholic acid (Supplementary Figure 3d). Previous reports demonstrated that TCA is not only a predictive biomarker of cirrhosis but also drives liver cirrhosis⁹. Here, serum TBA and ALP strongly correlated with increased liver fibrosis (Figure 2h,i).

RNA-sequencing of liver homogenates from L-TBA and H-TBA mice fed a WD revealed a clear separation between groups (Figure 2j). Gene ontology (GO) and gene-set enrichment
analysis (GSEA) indicated increased fibrosis and proliferation as well as activation of the Notch and MAPK pathways (Figure 2k,l; Supplementary Figure 3e). GSEA also revealed enrichment for the Yes-associated protein (YAP) signature in H-TBA mice (Figure 2l), supporting previous findings showing that bile acids activate YAP to promote carcinogenesis.

The accelerated liver cancer phenotype in H-TBA cholemic mice was corroborated using an alternate Western diet with high trans-saturated fats or a choline-deficient HFD (Supplementary Figure 4a-d). Additionally, cholemic female mice also succumbed to accelerated liver cancer upon WD-feeding (data not shown). Nevertheless, cholemic mice did not show exacerbated liver damage to the well-characterized CCl₄-induced model of liver fibrosis (Supplementary Figure 4e-g). This suggests that cholema-induced accelerated liver damage and cancer may be specific to high-caloric feeding.

Overall, a subset (5-25%) of all C57BL/6 mice, obtained from different commercial breeders, develop spontaneous cholema, predisposing them to liver cancer upon high-caloric feeding. The molecular and genetic basis for development of spontaneous cholema in C57BL/6 mice remains to be investigated. We suggest that future metabolic and liver cancer studies should screen C57BL/6 mice for TBA and exclude cholemic mice to prevent inconsistent or perplexing findings.
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Author Contributions

S.G and A.A contributed equally as first authors. S.G, A.A and M.H designed experiments and conceived the project. S.G, A.A and J.B carried out *in vivo* experiments and analyzed the data. D.H and V.K performed in-depth bile acid profiling. M.M.K performed bioinformatics analyses. D.J.W, M.V, A.T.S, T.L, J.G, E.E.I and D.M provided samples. T.L provided scientific input. S.G, A.A, J.B and M.H wrote the manuscript and all authors provided feedback.
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Main Figures

Fig. 1 | Spontaneous cholema in C57BL/6 mice upon Western diet feeding. a. Proposed protocol for screening of cholemic mice. b. Experimental scheme. c. Serum total bile acids (TBA) from 6-week-old C57BL/6J Janvier mice. Low TBA (L-TBA; <45 μM) or high TBA (H-TBA; ≥45 μM). d. Serum TBA prior to diet feeding. e. Initial body weight measurement. f–h. Serum ALT, ALP and cholesterol. i. Body weight development. j. Intraperitoneal glucose tolerance test after 30 weeks of diet. k,l. Serum ALT and ALP. m. Liver images. n. MRI scans of livers following 30 weeks of diet. o. Liver to bodyweight ratio (%). p. Liver tumor incidence. Chow L-TBA n=4; WD L-TBA n=5; WD H-TBA n=5 mice. Data are expressed as mean ± SEM. Statistical significance was calculated using either one-way analysis of variance with Tukey’s multiple comparison test (c-l,o) or Fischer’s exact test (p). (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001). N.S: non-significant.

Fig. 2 | Cholemic mice display exacerbated biliary damage, liver fibrosis and upregulation of proliferative pathways upon Western diet feeding. a. H/E and COLIV staining of livers; dashed line indicates tumor (T) border. Scale bar, 1 mm (low magnification) and 100 μm. b. CK19 and Sirius red staining. Scale bar, 100 μm. c. Quantification of CK19 and d. Sirius red positive area. e. Serum appearance. f. Serum TBIL levels. g. Serum TBA profiling. h,i. Correlation plots. j. Principal component analysis; k. gene ontology analysis and l. GSEA of H-TBA mice compared to L-TBA mice fed a WD. a-l Chow L-TBA n=4; WD L-TBA n=5; WD H-TBA n=5 mice. j-l. WD L-TBA n=3; WD H-TBA n=4 mice. Data are expressed as mean ± SEM. Statistical significance calculated by one-way analysis of variance with Tukey’s multiple comparison test (c-d,f,g) or linear regression (h,i). (* P < 0.05, *** P < 0.001, **** P < 0.0001). N.S: non-significant.
Figure 1: Comparison of serum TBA levels, ALT, ALP, and TCHOL in 6-week-old C57BL/6J males on Chow, L-TBA, and H-TBA diets. Serum TBA levels were significantly increased in the H-TBA group compared to the L-TBA group. ALT and ALP levels were also increased in the H-TBA group, while TCHOL levels were decreased. Body weight and glucose tolerance test results showed no significant differences between groups. Macroscopic MRI images showed no clear differences in liver and spleen weights between groups. Tumour incidence data indicated a higher incidence in the H-TBA group compared to the L-TBA group.
Figure 2

**WD L-TBA vs WD H-TBA**

- **a** Chow L-TBA, WD L-TBA, WD H-TBA
- **b** Chow L-TBA, WD L-TBA, WD H-TBA - Non-Tumour

**TBIL - 32 weeks of WD**

- **c** CK19
- **d** Sirius Red

**Serum bile acids - 32 weeks of WD**

- **g** Total bile acids, Unconjugated-BA, Taurin-BA, Glycine-BA

**Serum TBA vs Fibrosis**

- **h** Serum TBA vs Fibrosis

**Serum ALP vs Fibrosis**

- **i** Serum ALP vs Fibrosis

**Principal component analysis**

- **j** Principal component analysis

**GO Upregulated**

- extracellular matrix: 0.0031012
- mesenchymal cell differentiation: 0.0048762
- stem cell development: 0.0048762
- cell growth: 0.0016049
- Wnt signaling pathway: 0.0016055
- cell proliferation: 0.0028628
- MAPK cascade: 0.0021606
- Notch signaling pathway: 0.0021606

**GO Downregulated**

- carboxylic acid metabolic process: 0.0039752
- mitochondrion: 0.0058777
- lipid metabolic process: 0.0065877
- fatty acid metabolic process: 0.0065877
- fatty acid oxidation: 0.0019395
- monocarboxylic acid catabolic process: 0.0072329
- mitochondrial respiratory chain: 0.0057777
- peroxisome: 0.0072329

**GO Up in Liver Development**

- **k** Enrichment score (ES)

**Genes UP in Extracellular Matrix**

- **l** Enrichment score (ES)

**Genes UP in Liver Development**

- **m** Enrichment score (ES)

**Genes UP in Stem Cell**

- **n** Enrichment score (ES)

**Genes UP in YAP Signature**

- **o** Enrichment score (ES)

- **p** FDR q: 0.000
- **q** NES: 2.20

- **r** FDR q: 0.000
- **s** NES: 2.14

- **t** FDR q: 0.000
- **u** NES: 2.12

- **v** FDR q: 0.000
- **w** NES: 2.18

**Figure 2**