TGR5 controls bile acid composition and gallbladder function to protect the liver from bile acid overload

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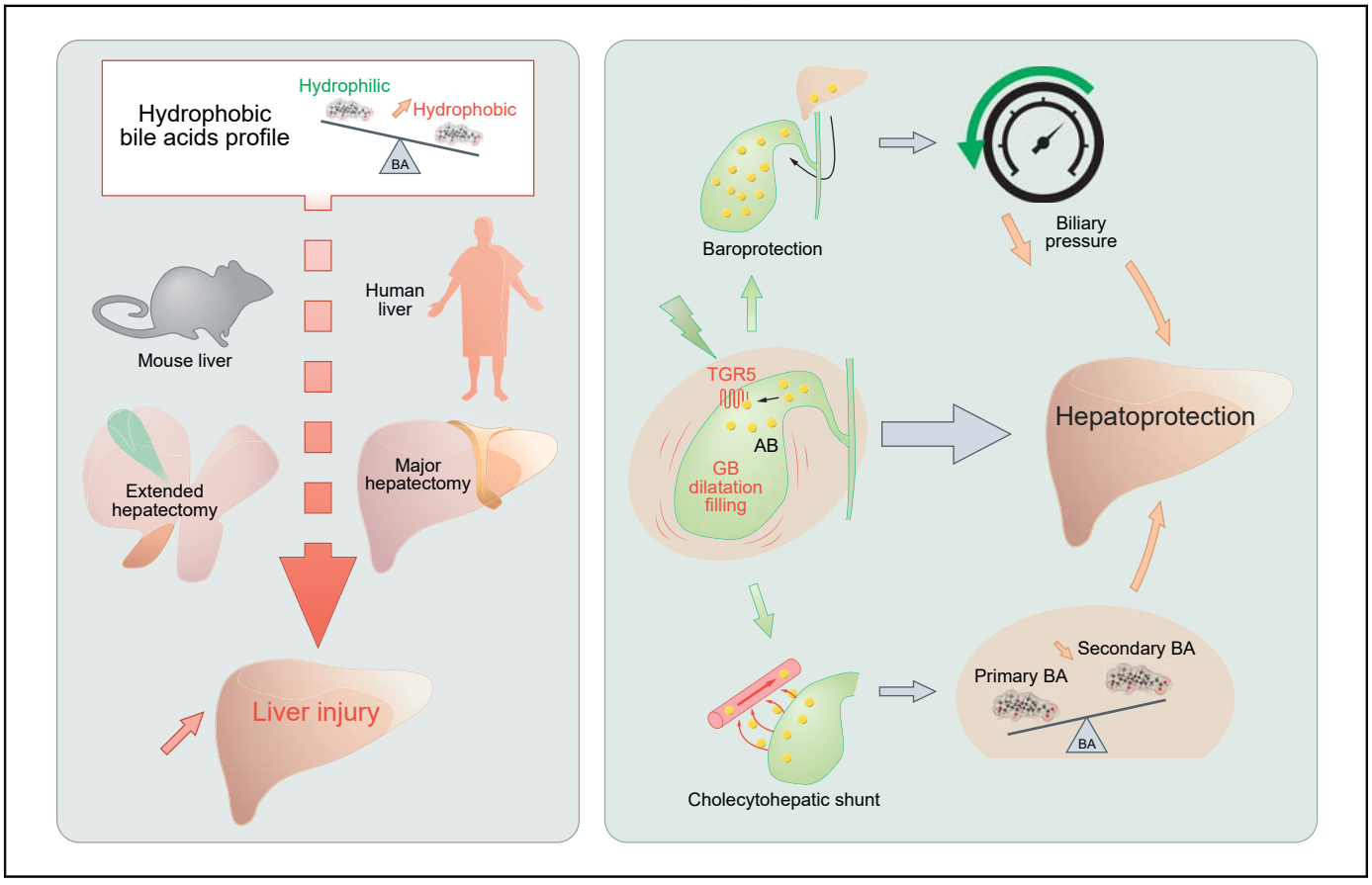
Graphical abstract



Highlights

- Reducing BA hydrophobicity improves outcomes after major hepatectomy in mice.
- The BA receptor TGR5 controls BA pool composition, which is crucial for liver repair.
- TGR5 targets the gallbladder to induce a hepatoprotective effect.
- In patients, a more hydrophobic BA pool is associated with liver injury after hepatectomy.

Lay summary

Through multiple *in vivo* experimental approaches in mice, together with a patient study, this work brings some new light on the relationships between biliary homeostasis, gallbladder function, and liver protection. We showed that hepatic bile acid composition is crucial for optimal liver repair, not only in mice, but also in human patients undergoing major hepatectomy.

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Research article

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TGR5 controls bile acid composition and gallbladder function to protect the liver from bile acid overload

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Background & Aims: As the composition of the bile acid (BA) pool has a major impact on liver pathophysiology, we studied its regulation by the BA receptor Takeda G protein coupled receptor (TGR5), which promotes hepatoprotection against BA overload.

Methods: Wild-type, total and hepatocyte-specific TGR5-knockout, and TGR5-overexpressing mice were used in: partial (66%) and 89% extended hepatectomies (EHs) upon normal, ursodeoxycholic acid (UDCA)- or cholestyramine (CT)-enriched diet, bile duct ligation (BDL), cholic acid (CA)-enriched diet, and TGR5 agonist (RO) treatments. We thereby studied the impact of TGR5 on: BA composition, liver injury, regeneration and survival. We also performed analyses on the gut microbiota (GM) and gallbladder (GB). Liver BA composition was analysed in patients undergoing major hepatectomy.

Results: The TGR5-KO hyperhydrophobic BA composition was not directly related to altered BA synthesis, nor to TGR5-KO GM dysbiosis, as supported by hepatocyte-specific KO mice and co-housing experiments, respectively. The TGR5-dependent control of GB dilatation was crucial for BA composition, as determined by experiments including RO treatment and/or cholecystectomy. The poor TGR5-KO post-EH survival rate, related to exacerbated peribiliary necrosis and BA overload, was improved by shifting BAs toward a less toxic composition (CT treatment). After either BDL or a CA-enriched diet with or without cholecystectomy, we found that GB dilatation had strong TGR5-dependent hepatoprotective properties. In patients, a more hydrophobic liver BA composition was correlated with an unfavourable outcome after hepatectomy.

Conclusions: BA composition is crucial for hepatoprotection in mice and humans. We indicate TGR5 as a key regulator of BA profile and thereby as a potential hepatoprotective target under BA overload conditions.

Lay summary: Through multiple *in vivo* experimental approaches in mice, together with a patient study, this work brings some new light on the relationships between biliary homeostasis, gallbladder function, and liver protection. We showed that hepatic bile acid composition is crucial for optimal liver repair, not only in mice, but also in human patients undergoing major hepatectomy.

Introduction

Liver repair after injury is finely tuned by a myriad of signalling molecules involved not only in cell renewal and protection, but also in maintaining differentiated functions. Within this intricate signalling network, bile acids (BA) and their receptors are particularly involved in mounting adaptive/protective responses.[1] BA are synthesised by hepatocytes, secreted in bile, and in normal conditions mostly cycle between the liver and the intestine (entero-hepatic cycle). However, during liver injury, because BA uptake capacity is overwhelmed as a result of hepatocyte loss, BA spillover and subsequent BA overload occur in the liver and in the whole organism. Consequences of this BA overload on processes of inflammation, regeneration, and biliary homeostasis[1–5] remain poorly explored. Meanwhile, biliary homeostasis has to be precisely tuned to preserve regenerating liver parenchyma from BA-induced damage. BA signal through both nuclear (mainly farnesoid X receptor, FXR) and membrane (mainly sphingosine 1-phosphate receptor 2, S1PR2, and the G protein-coupled BA receptor 1 [GPBAR-1] or TGR5) receptors, the distributions of which are large in the organism, and their activation elicits a wide array of biological responses. Although FXR is well reported to orchestrate adaptive responses protecting the

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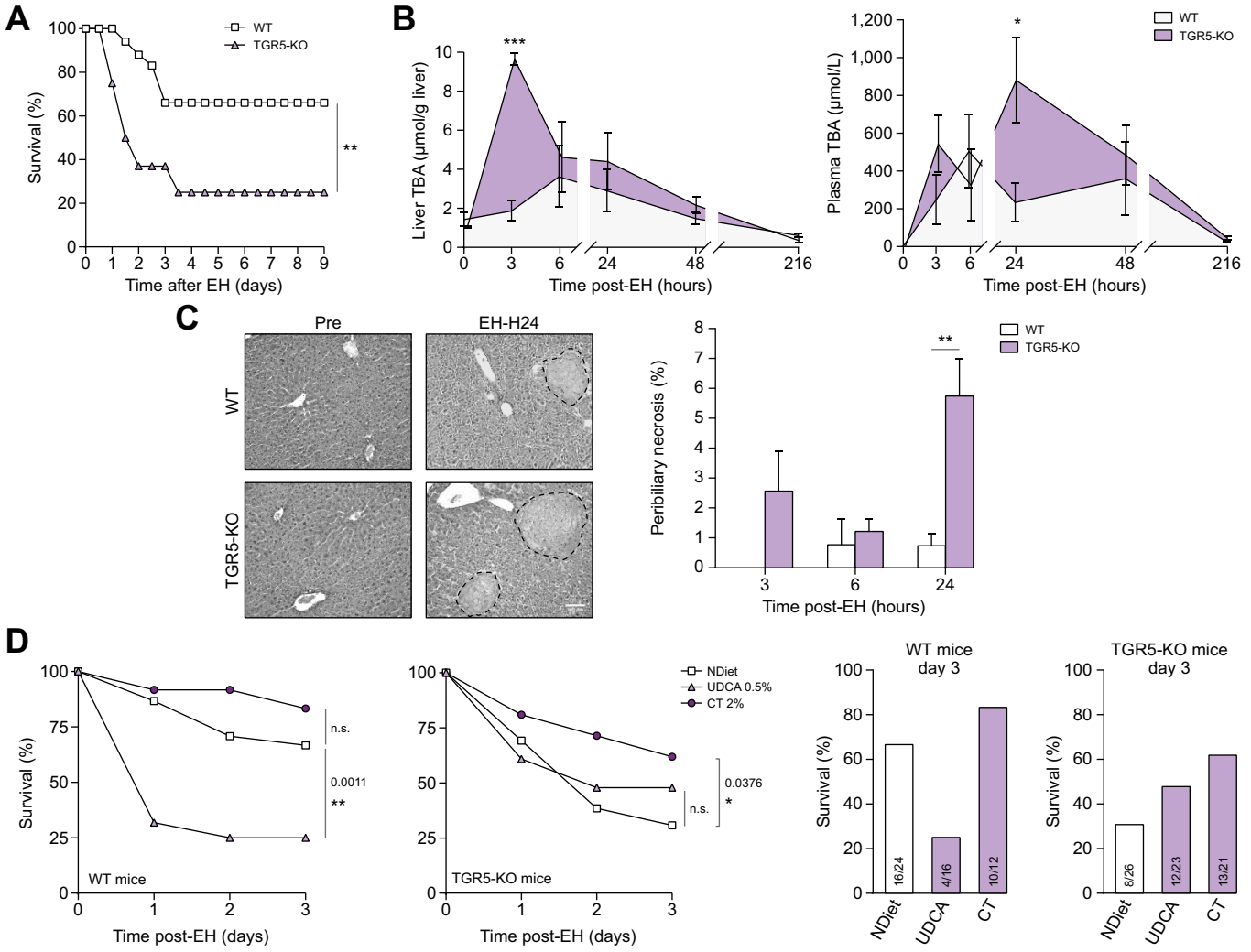


Fig. 1. BA overload as a crucial parameter for post-hepatectomy outcome. (A) Survival rates after EH were dramatically reduced in TGR5-KO (n = 10) as compared with WT (n = 13) mice. (B) Total bile acids (TBA) concentration in the liver and plasma after EH in WT and TGR5-KO mice (n = 10–13 mice/group). (C) *Left:* H&E-stained liver sections from WT and TGR5-KO mice before and after EH. Bile infarcts are delineated. Representative images, objective ×10, scale bar: 100 µm. *Right:* Semi-quantitative analysis of bile infarcts (n = 10–13 mice/group). (D) *Left graphs*: Cholestyramine (CT, 2%) treatment significantly improved survival rates after EH in TGR5-KO; non-significant improvement in WT mice. Upon 0.5% UDCA pretreatment: significantly reduced post-EH survival in WT; non-significant improvement in TGR5-KO mice. *Right histograms*: survival rates at 3 days after EH, in the 3 WT or TGR5-KO mice groups (ND, CT, UDCA). Sample sizes in histogram. Panels B and C: **p* <0.05; ***p* <0.01; ****p* <0.001; Student's *t* test. Panels A and D: log-rank (Mantel-Cox) test was used to compare the survival curves. BA, bile acid; CT, cholestyramine; EH, extended hepatectomy; KO, knockout; ND, normal diet; TGR5, Takeda G protein coupled receptor; UDCA, ursodeoxycholic acid; WT, wild-type.

liver from BA overload, TGR5 has been less explored in the liver repair field.[5,6] TGR5 is poorly expressed in hepatocytes but is highly enriched in the biliary tract in which it has been proposed to control chloride (Cl^-) secretion,[7] cholangiocyte proliferation,[8] biliary epithelial paracellular permeability,[6] and gallbladder (GB) filling.[9,10] We previously demonstrated that two-thirds partial hepatectomy (PH) was followed by an immediate and massive BA overload in rats, mice, and humans and that the lack of TGR5 in mice resulted in impaired liver regeneration mainly through an alteration of BA homeostasis.[1,2,4,5] However, the precise ways through which TGR5 may operate this control still remain incompletely understood. We hypothesised that TGR5 may protect the liver parenchyma by controlling the BA pool composition, in particular by shifting its hydrophobicity. Previous studies including ours reported that the BA pool of TGR5-KO mice was excessively hydrophobic as compared with WT mice.[5,10–12] However, how TGR5 controls BA pool hydrophobicity remains unclear, although it has been reported that TGR5 may operate a slight control on BA synthesis and gut microbiota (GM).[12,13] Importantly, the BA pool composition, in particular its hydrophobicity and the balance between primary and secondary BA, is more and more recognised as crucial for liver pathophysiology.[14–16]

In this study we uncovered that TGR5 expression was crucial for survival after extended liver resections, and found that this protection was significantly related to the TGR5-dependent control of BA composition. Importantly in human patients, a more hydrophobic BA composition was correlated with a less

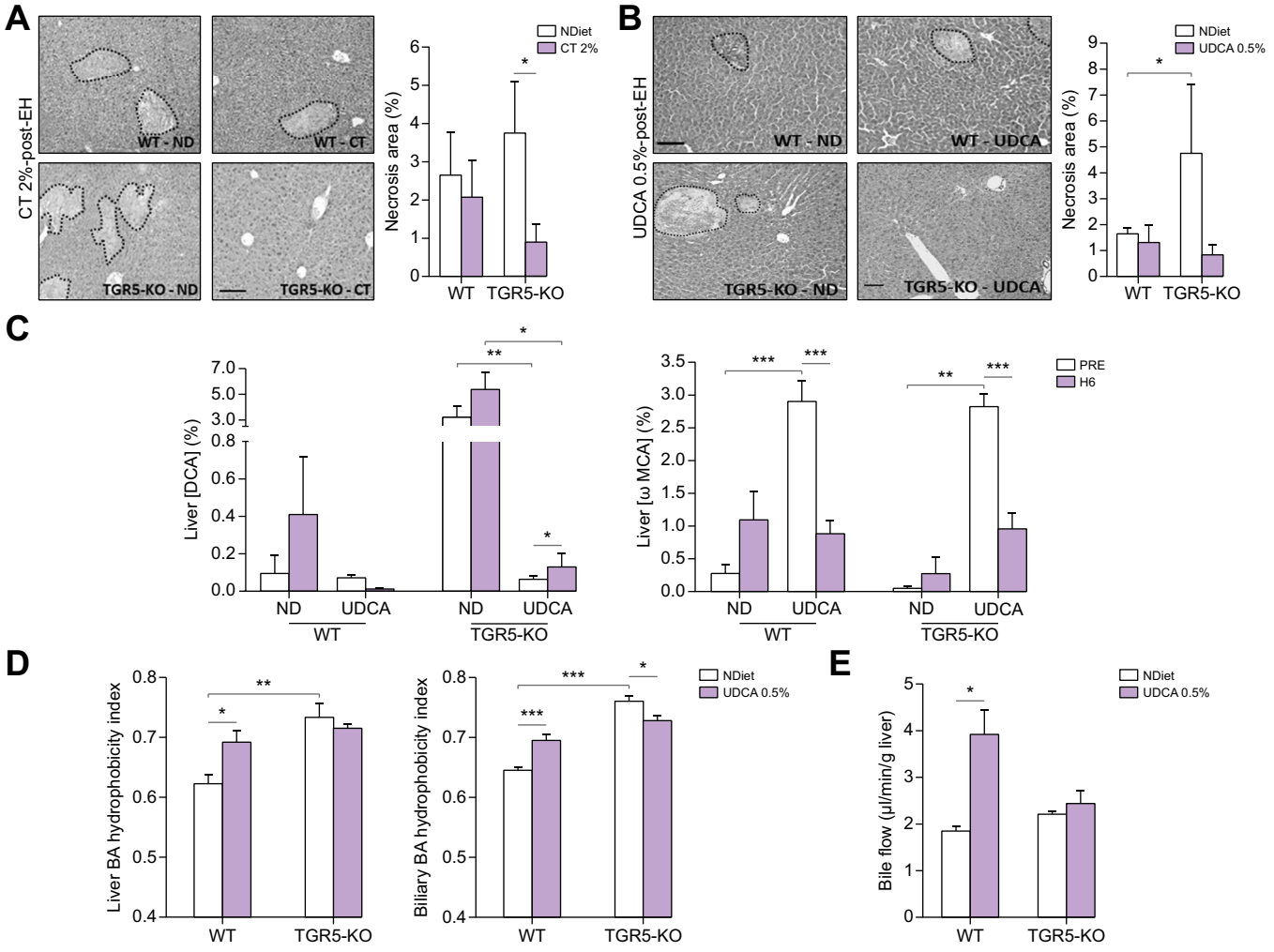


Fig. 2. CT and UDCA impact on post-EH outcome in mice. (A) CT (2%) treatment reduced post-EH liver injury (at 24 h), significantly in TGR5-KO mice, as shown on H&E-stained liver sections (left, representative images of n = 4–9 mice/group, objective ×10, scale bar: 100 µm) and semi-quantitative analysis (*right histogram*). (B) UDCA (0.5%) treatment is associated with less liver injury after EH (at 6 h) (n = 6–8 mice/group) in TGR5-KO but not WT mice; H&E-stained liver sections (*left*, representative images, objective ×10) and semi-quantitative analysis (*right histogram*). (C) Liver BA composition (DCA and ω-MCA) before and 6 h after EH in WT and TGR5-KO mice, fed with a diet enriched or not (ND) with 0.5% UDCA (n = 6–8 mice/group). (D) Hydrophobicity index in UDCA-treated WT and TGR5-KO mice. Hydrophobicity index calculated from BA analysis in liver and bile from WT and TGR5-KO mice 6 h after EH (n = 6–8 mice/group. (E) Choleretic effect of 0.5% UDCA-enriched diet (7 days), in WT but not TGR5-KO mice (n = 5 mice/group). **p* <0.05; ***p* <0.01; ****p* <0.001; Student's *t* test. BA, bile acid; CT, cholestyramine; DCA, deo xycholic acid; EH, extended hepatectomy; KO, knockout; MCA, muricholic acid; ND, normal diet; TGR5, Takeda G protein coupled receptor; UDCA, ursodeoxycholic acid; WT, wild-type.

favourable outcome after major hepatectomy. We provide further evidence in mice that TGR5 control on BA pool composition operated through an impact on GB function. TGR5 activation, by inducing GB dilatation, allows mechanical protection of the liver in obstructive conditions, and reshaping of the BA composition toward more hydrophilicity.

Materials and methods

Surgical procedures used on animals

C57Bl/6J Gpbar1$^{-/-}$ mice (referred to in this study as TGR5-KO mice) and their C57B/6J wild-type (WT) littermates, were provided by Merck Research Laboratories (Kenilworth, NJ, USA),[11] and used to found our colonies of TGR5-KO and control animals. TGR5-overexpressing mice (Tg mice) were generated at EPFL (Lausanne, Switzerland).[17] The study was performed on 10–16-week-old male mice, as detailed in the Supplementary information, Materials and methods.

Patients

Liver biopsies came from the Biological Resource Center of Kremlin-Bicêtre Hospital (CRB Paris Sud – UG 1203, Hopital Bicetre – APHP, France), as explained in more detail in Table S1. All patients signed an informed consent form, and the study on human tissues was approved by the CRB Paris Sud. Hepatic BA composition was determined on liver samples from the non-tumour removed livers (hepatectomies for tumour) as described in the Supplementary information, Materials and methods, and were correlated with the post-surgery biological follow-up (plasma alanine aminotransferase [ALT], total bilirubin [T.Bili], and alkaline phosphatase [ALP]), before hepatectomy, as well as at 0.5, 1, and 6–9 days after surgery.

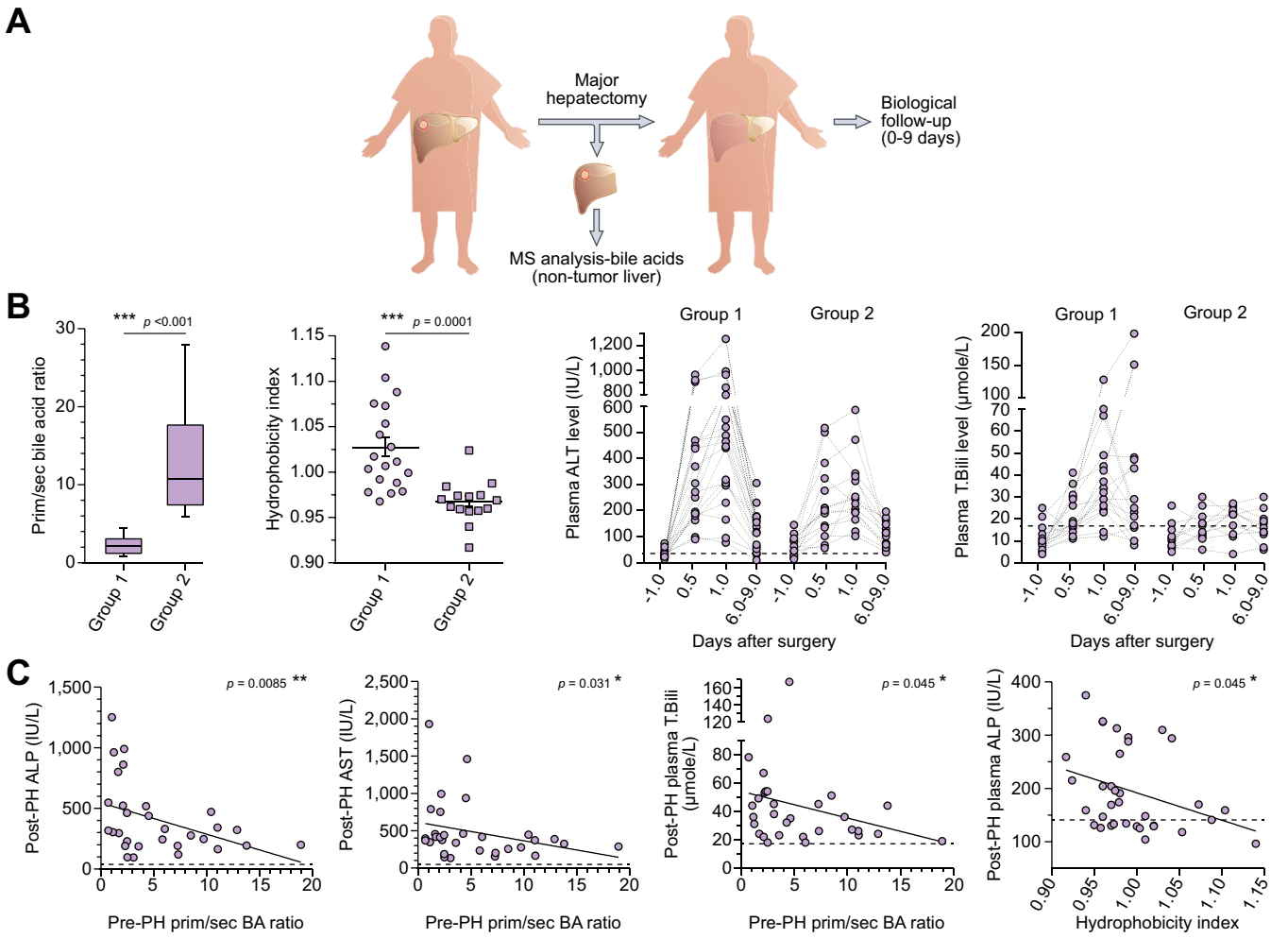


Fig. 3. BA pool composition before major hepatectomy is critical for patients after surgery. (A) Study design. In patients undergoing major hepatectomy (n = 32), non-tumoural liver samples were harvested during surgery and stored for BA mass spectrometry analysis. Peripheral blood samples for biological follow-up were taken at 12 h after hepatecomy, and every other day until discharge. (B) Patients were segregated into 2 groups (n = 18 and 14 in groups 1 and 2, respectively) in terms of pre-hepatectomy primary/secondary BA ratio and hepatic BA hydrophobicity index (2 left panels), and also for liver injury and cholestasis markers evolution (2 right panels) (see Table S1 and Fig. S7). Markers were measured the day before ('-1' time point), 12 h after ('0.5' time point), 1 day ('1' time-point), and 6–9 days ('6–9' time point) after surgery. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Student's *t* test. Dashed line in lower graphs: upper normal value. (C) Correlations between pre-hepatectomy primary/secondary BA ratio or hepatic BA hydrophobicity index, and liver injury or cholestasis markers (peak values). Spearman correlation ($p < 0.05$ considered statistically significant). ALT, alanine aminotransferase; BA, bile acid.

Immunohistochemistry

Antibodies against phospho-histone H3 (PH3), Ki67, Gr1, cytokeratin 19 (CK19), E-cadherin, as well as phalloidin, were used on ethanol/acetone-fixed 10-µm liver cryosections, and images were acquired in epifluorescence (Axiovert, Zeiss) and confocal (EZ-C2, Nikon) microscopy, and analysed with the Image J software. H&E- and Oil Red O-staining on liver sections were performed as described.

Biochemical assays and quantitative RT-PCR experiments are further described in the Supplementary information, Materials and methods.

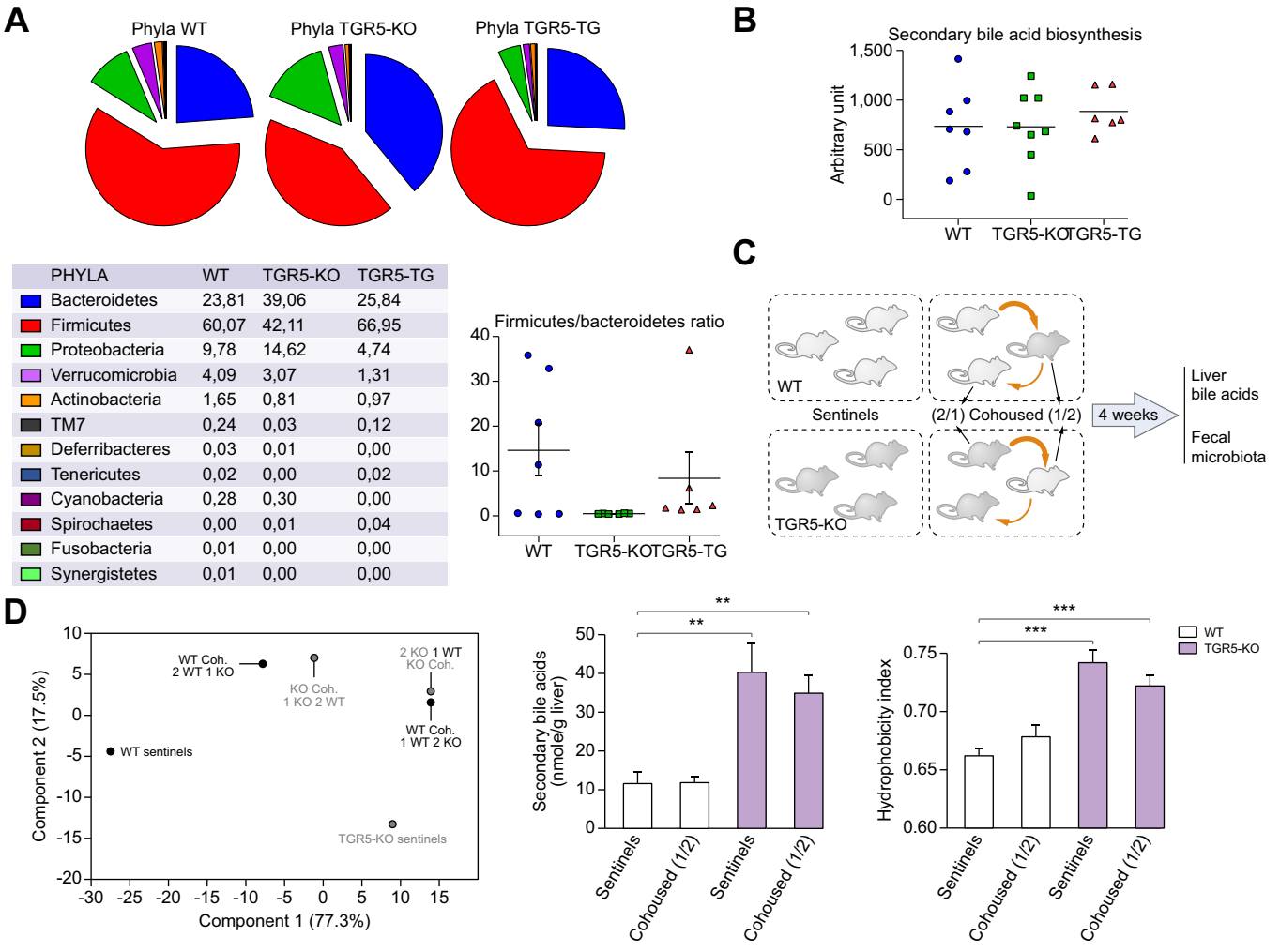
Statistical analysis

The Student's *t* test was used to compare sample means with controls. Results are expressed as means ± SEM. Spearman correlation was used to measure the degree of association between 2 variables in human studies. The log-rank (Mantel-Cox) test was used to compare survival distributions of 2 samples (survival curves). All statistical analyses were performed in GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). The *p* values ≤0.05 (*), ≤0.01 (**), and ≤0.001 (***) were considered statistically significant.

Results

TGR5-dependent control of BA overload and BA composition is critical for post-hepatectomy outcome

In TGR5-KO mice, as compared with WT mice, survival was drastically reduced in the days following EH (25% *vs.* 64% at Day 9, $p < 0.05$) (Fig. 1A). To understand the underlying TGR5-dependent processes favouring post-EH outcome, we studied a series of regeneration parameters in WT and TGR5-KO mice after EH. As shown in Fig. S1, early metabolic events (hypoglycaemia, steatosis) as well as hepatocyte hypertrophy, cell proliferation, and liver mass restoration in surviving mice, were similar in both



PHYLA	WT	TGR5-KO	TGR5-TG
Bacteroidetes	23,81	39,06	25,84
Firmicutes	60,07	42,11	66,95
Proteobacteria	9,78	14,62	4,74
Verrucomicrobia	4,09	3,07	1,31
Actinobacteria	1,65	0,81	0,97
TM7	0,24	0,03	0,12
Deferribacteres	0,03	0,01	0,00
Tenericutes	0,02	0,00	0,02
Cyanobacteria	0,28	0,30	0,00
Spirochaetes	0,00	0,01	0,04
Fusobacteria	0,01	0,00	0,00
Synergistetes	0,01	0,00	0,00

Fig. 4. The lack of TGR5 is associated with a gut microbiota dysbiosis – impact on BA pool hydrophobicity. (A) Phyla analysis on faecal microbiota from WT, TGR5-KO, and TGR5-Tg mice (n = 6–8 mice/group). (B) Microbiome analysis (PICRUST) of faecal microbiota (n = 6–8 mice/group). Predictive functional analysis on mice stools, referring to the capacity of faecal bacteria to transform primary BA into secondary BA (no significant impact of microbiota on secondary BA biosynthesis). (C) Cartoon depicting co-housing experiments between WT and TGR5-KO mice. Mice were co-housed with the aim of favouring microbiota transfer through coprophagy (Caruso *et al.*[21]). Faeces from sentinel and co-housed mice were processed for bacterial DNA and BA extractions and analysis. (D) Efficient faecal microbiota transfer through coprophagy. Based on a principal component analysis, data indicate a shift in faecal microbial community structure upon co-housing. Black and grey circles represent, respectively, WT and TGR5-KO faecal microbiota composition in either sentinel or co-housed mice. The different co-housing conditions (WT/KO mice ratios) are stated.(E) No significant impact of co-housing on hepatic composition in BA. Similar liver secondary BA and BA hydrophobicity (n = 5–6 mice/group) between in and co-housed mice (either WT or TGR5-KO). $^{**}p$ <0.01; $^{***}p$ <0.001; Student's *t* test. BA, bile acid; KO, knockout; TGR5, Takeda G protein coupled receptor; WT, wild-type.

genotypes after EH. The only strikingly different features observed after EH were peribiliary necrosis (bile infarcts on H&E-stained liver sections) and BA overload (in plasma and liver), which exhibited significantly exacerbated peak values in TGR5-KO as compared with WT mice (Fig. 1B,C). This so-called 'pseudo-obstructive phenotype' was also found after 2/3 PH.[5] To gain more insight in the potential impact of this exacerbated BA overload on post-EH survival, we treated mice before performing EH with either the BA sequestering resin cholestyramine (CT) or the BA ursodeoxycholic acid (UDCA). Both of these treatments impacted post-EH survival (Fig. 1D). CT strongly improved post-EH survival in TGR5-KO mice, whereas an improvement trend (not statistically significant) was observed in WT mice. In line with survival data, CT treatment significantly reduced the occurrence of post-EH (24 h) liver necrosis observed in TGR5-KO mice (Fig. 2A). MS analysis of liver BA revealed that CT treatment mainly shifted BA composition resulting in less secondary BA in the remnant regenerating liver in both genotypes (Fig. S2B). CT treatment also restrained post-EH total BA (TBA) overload, mainly in TGR5-KO mice (Fig. S2B) (in which post-EH TBA overload was exacerbated in the ND-fed state, see Fig. 1B). This observation partially fitted with what we observed after two-thirds PH, where TBA overload was drastically reduced upon CT in TGR5-KO mice, whereas post-PH secondary BA were neither significantly elevated nor changed by CT (Fig. S2C). These observations suggest that the beneficial effect of CT documented after two-thirds PH in TGR5-KO mice[5] would mainly be a result of a striking reduction of post-PH TBA overload, whereas after EH

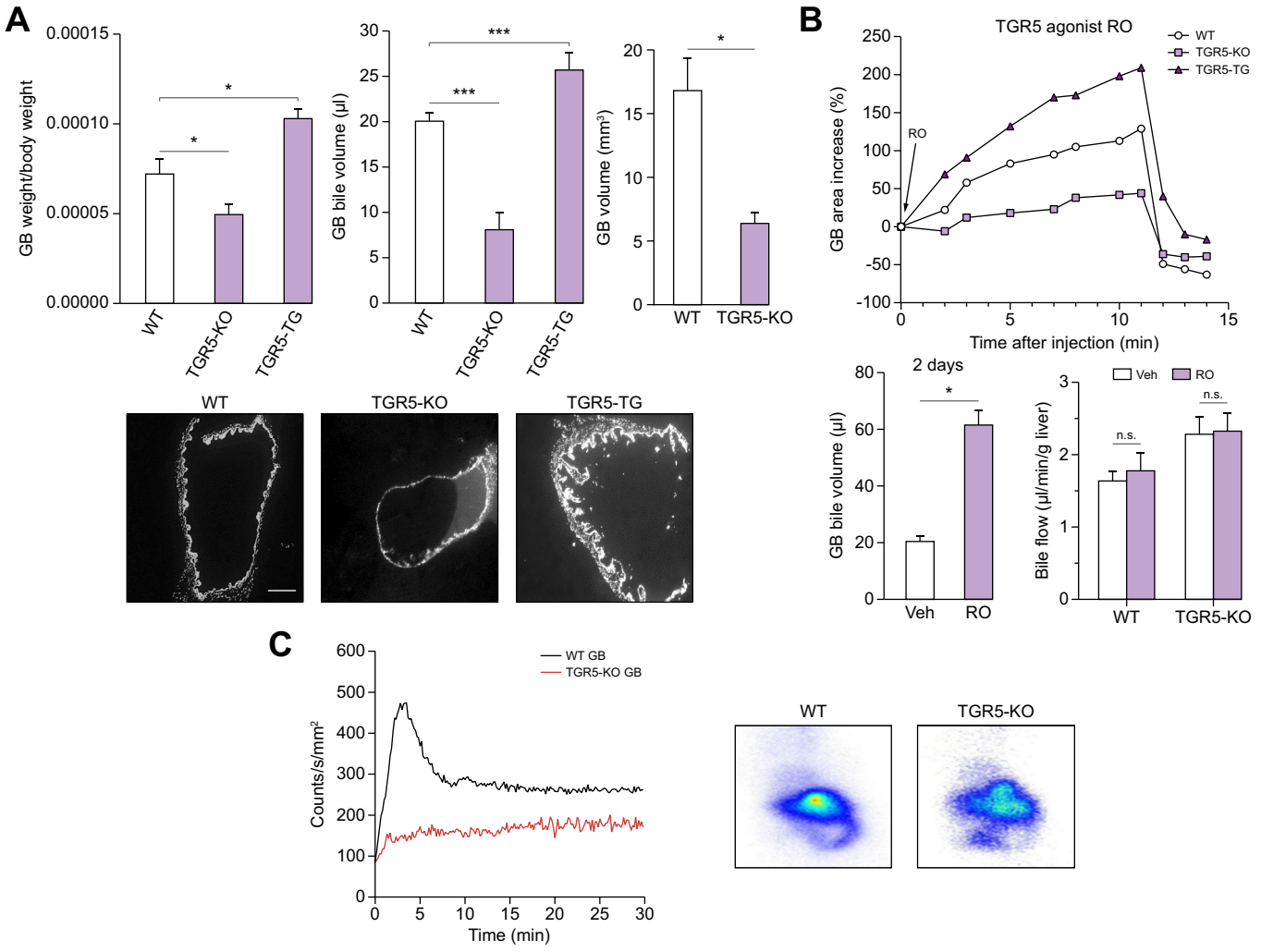


Fig. 5. TGR5 expression has major impact on gallbladder (GB) homeostasis and motor function. (A) GB weight and volume (GB bile in µl; measured GB volume in mm3) were decreased in TGR5-KO and increased in TGR5 overexpressing transgenic (Tg), as compared with WT mice (n = 6–16 mice/group). (B) TGR5 stimulation (RO5527239) increased GB filling, after i.v. injection (top) or oral gavage (10 mg/kg per day, 2 days) (bottom left), but did not impact bile flow (bottom right) (n = 4–6 mice/group). (C) Mean curves of ^{99m}Tc Mebrofenin (Choledium®) single-photon emission tomography (SPECT) performed in WT and TGR5-KO mice, as described.[22] Mice were imaged with a Beta imager and the GB area was quantified using Gammavision software (Biospace Lab, Nesles-la-Vallée, France) (n = 3–4 mice/group). *p <0.05; ***p <0.001; Student's t test. GB, gallbladder; KO, knockout; TGR5, Takeda G protein coupled receptor; WT, wild-type.

the positive impact of CT resulted from a drop in toxic secondary BA reaching the regenerating liver.

UDCA, although considered as a hydrophilic BA making a less toxic BA pool in human patients,[18] is in fact less hydrophilic than the major BA in mice.[19] Strikingly, in WT mice, post-EH survival upon UDCA treatment was strongly reduced, whereas it was slightly improved (not reaching statistical significance) in TGR5-KO mice (Fig. 1D). Associated with these phenotypes, hepatic BA analysis revealed complex modifications upon UDCA-treatment, with UDCA representing 80–90% of the TBA (Fig. 2C,D and S3A,B). Importantly, the resulting effect of UDCA treatment was a relative increase in the BA hydrophobicity index (HI), significantly detected in WT compartments (liver and bile) (Fig. 2D) but not in TGR5-KO mice which harbour already an elevated HI (higher than WT) upon normal diet[5] (Fig. 2D). This increased BA hydrophobicity, in addition with the choleretic effect upon UDCA,[18] observed in WT but lacking in TGR5-KO mice (Fig. 2E and S3C), likely contribute to the deleterious effect of UDCA on post-EH survival found only in WT mice (Fig. 1C). Of note, less post-EH hepatic injury was observed in UDCA-treated, as compared with normal diet-fed TGR5-KO mice, whereas the more severe WT phenotype was not associated with any increase in post-EH hepatocyte injury (Fig. 2B and S4). Interestingly, UDCA enhanced ion (Na^+, Cl^-, HCO_3^-) biliary output in WT but not TGR5-KO mice, whereas TBA, cholesterol, and phosphatidyl-choline secretion were similarly impacted by UDCA in the 2 genotypes (Fig. S3C,D), suggesting that TGR5-dependent UDCA effects target more likely cholangiocytes than hepatocytes, as expected.

Taken together, these data point to BA overload, BA composition, and choleresis as crucial parameters for post-hepatectomy outcome, and reveal that TGR5 operates important control on these parameters.

A recent study found significant differences in BA synthesis enzyme expression between WT and TGR5-KO mice,[12] in

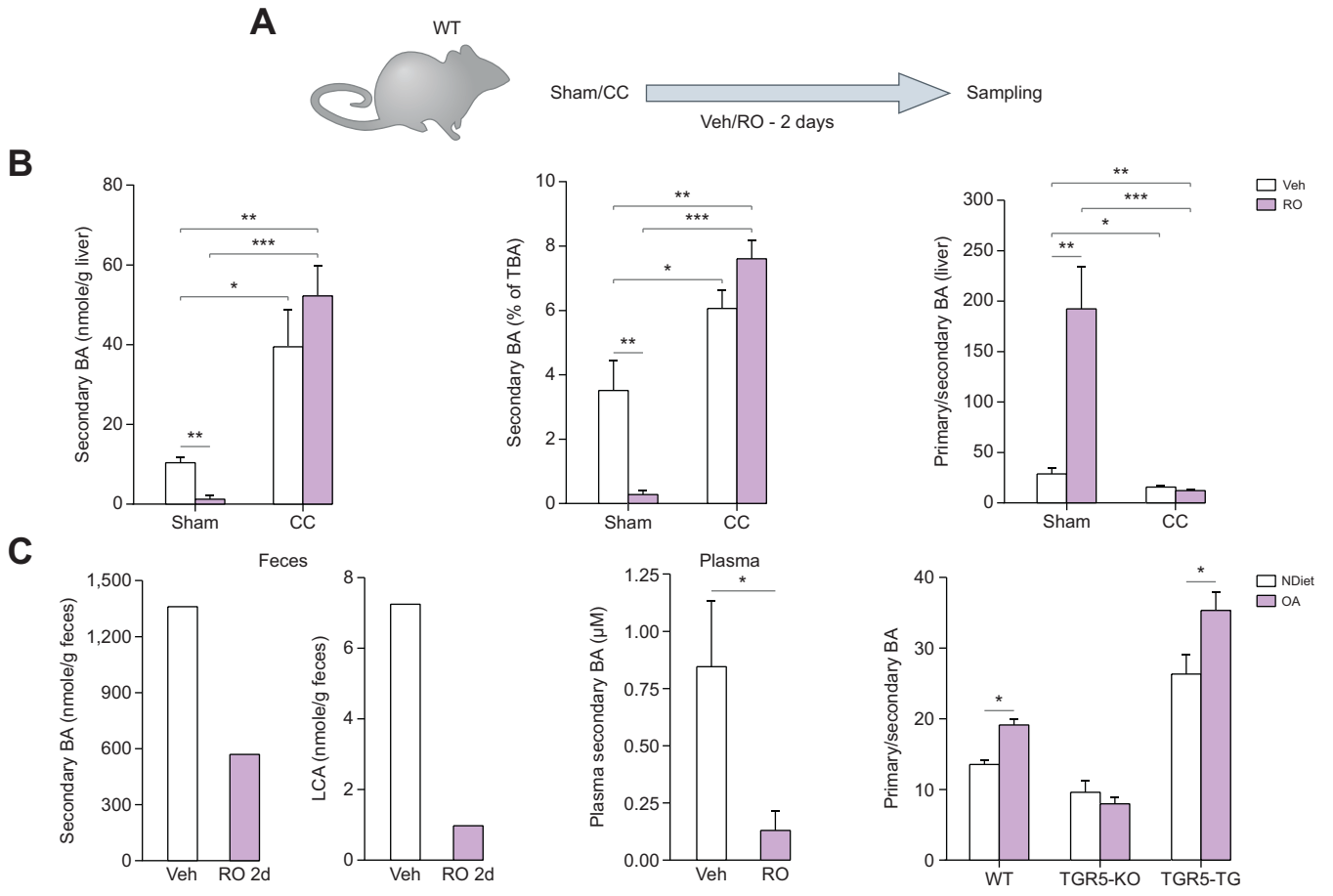


Fig. 6. TGR5 stimulation contributes to shift BA composition through an impact on GB. (A) Experimental design. After sham operation or cholecystectomy (CC), WT mice were treated (oral gavage, 10 mg/kg per day) with RO5527239) or vehicle (Veh), then sampled (plasma, liver, faeces) after 2 days. (B,C) RO5527239 treatment reduced secondary BA concentrations in liver, faeces, and plasma, and increased the primary/secondary BA ratio in the liver. Faeces from mice in the same experimental conditions were pooled. These effects were not observed after CC (n = 6–8 mice/group). $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001; Student's *t* test. BA, bile acid; GB, gallbladder; OA, oleanolic acid; LCA: lithocholic acid; TGR5, Takeda G protein coupled receptor; WT, wild-type.

discrepancy with previous reports.[5,10,11] Interestingly, we found that biliary BA composition in hepatocyte specific Alb-Cre-TGR5-KO (TGR5$^{\Delta hep}$) mice was not different from that in WT mice, suggesting that hepatocyte TGR5 expression may not be of crucial and direct impact for BA pool hydrophobicity. In line, BA synthesis enzymes expression in the different genotypes did not fit with differences in BA composition (Fig. S5A,B). Moreover, in contrast with total TGR5-KO mice, TGR5$^{\Delta hep}$ mice had similar post-PH outcome, without liver necrosis (Fig. S5C).[5] These data are also consistent with the known very low or lacking TGR5 expression in hepatocytes.[1]

BA pool composition before major hepatectomy is critical for patients after surgery

Based on mice data, we analysed the hepatic BA composition in patients undergoing major hepatectomy for tumour. A detailed description of those patients is provided in Table S1. BA were analysed, as described in the Supplementary information, Materials and methods, on the non-tumour liver removed during hepatectomy, and correlations were made with biological outcome during the follow-up in the first 9 days after surgery (Fig. 3A). Patients were segregated in 2 strikingly different groups in terms of primary/secondary BA ratio, BA hydrophobicity index, and markers of liver injury and cholestasis, without any correlation with the underlying liver condition (Table S1 and Fig. 3B). Remarkably, a pre-hepatectomy low primary/secondary BA ratio, and globally a more hydrophobic BA composition, was significantly correlated with liver injury and/or cholestasis markers after surgery (Fig. 3C). Of note, hepatic expressions of TGR5 and BA synthesis enzyme mRNAs (CYP7A1, 8B1, 27A1, 7B1, and 3A4) were similar in the 2 groups of patients, and no correlations were found with markers of liver injury (Fig. S6) or with eventual previous chemotherapy treatment (data not shown). Together these data suggest that, as observed in mice, the pre-hepatectomy BA composition would have strong impact on post-hepatectomy outcome in humans.

TGR5-related gut microbiota dysbiosis does not impact liver BA hydrophobicity

TGR5-gut microbiota interactions have only been scarcely and indirectly reported,[13,20] and until now the link between these potential interactions and the BA pool composition has not been established. Our data demonstrate that the lack of TGR5 was associated with higher abundance of Bacteroidetes and lower Firmicutes than in WT mice faecal microbiota. Interestingly, a number of bacteria classes including Clostridia and

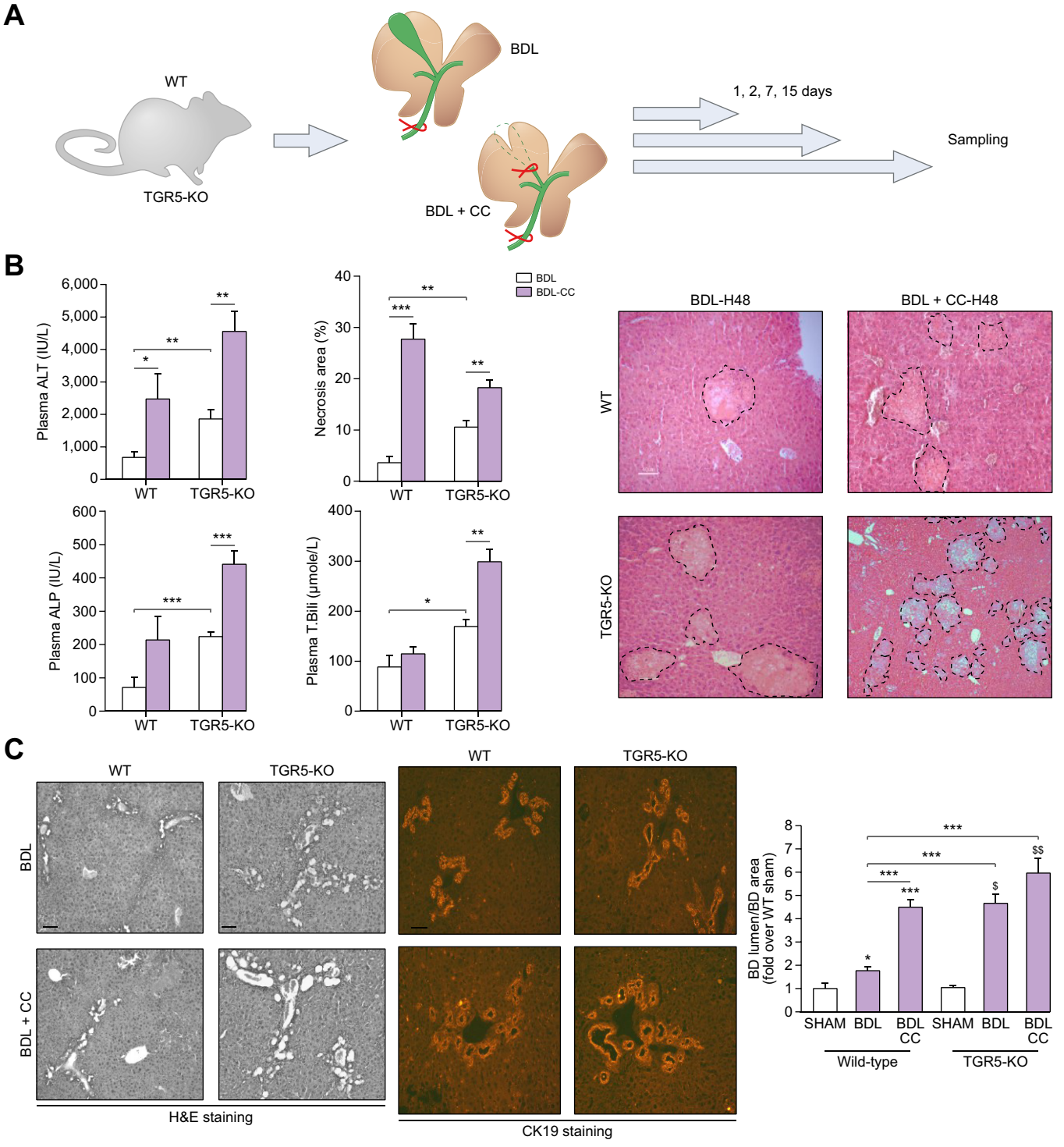


Fig. 7. GB is hepatoprotective after BDL in mice. (A) Experimental design. WT and TGR5-KO mice were submitted to either BDL alone or to BDL + CC, then sampled at 1, 2, 7, and 15 days. (B) Increased liver injury in BDL + CC as compared with BDL mice, in WT and TGR5-KO mice at H48. Plasma biochemical markers (*left*), and H&E-stained liver sections (*right*, representative images) (n = 5–7 mice/group). Bile infarcts are delineated. Obj. ×10, scale bar: 100 μm. (C) BDL-induced bile duct dilatation is increased in CC mice as compared with BDL alone. Representative H&E-stained (*left*) and CK19-immunostained (*middle*) liver sections. Semi-quantitative analysis of bile duct lumen/area on CK19-immunostained liver sections (*right*) (n = 5–7 mice/group). **Vs.* Sham WT; $: *vs.* Sham TGR5-KO. **p* <0.05; ***p* <0.01; ****p* <0.001; Student's *t* test. ALP, alkaline phosphatase; ALT, alanine aminotransferase; BDL, bile duct ligation; CC, cholecystectomy; GB, gallbladder; KO, knockout; TGR5, Takeda G protein coupled receptor; WT, wild-type.

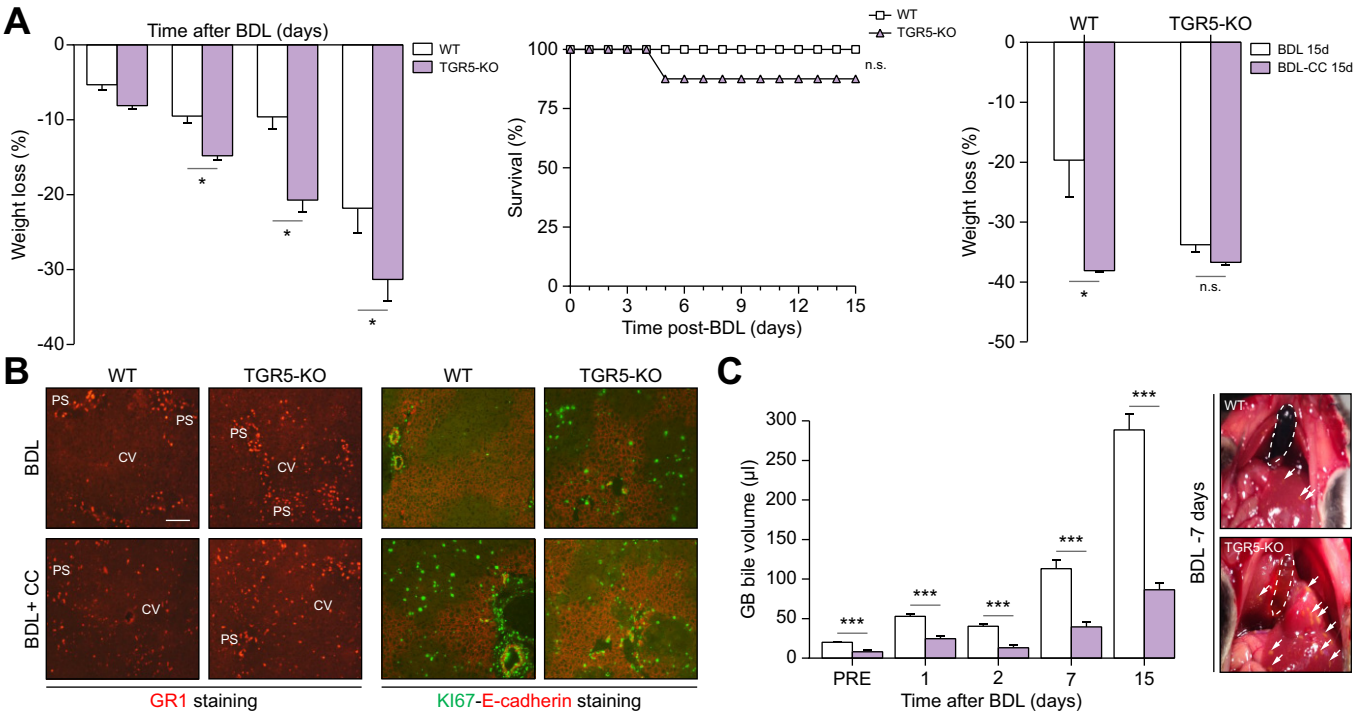


Fig. 8. Long-term protective impact of GB after BDL. (A) TGR5-KO mice are more sensitive to chronic BDL. Body weight (bw) loss was greater, and survival was only slightly impaired (not statistically different) in TGR5-KO *vs.* WT mice (*left graphs*) (n = 9–11 mice/ group). *Right histogram*: greater bw loss at 15 days after CC in BDL WT but not TGR5-KO mice (n = 4–5 mice/group). (B) CC exacerbates inflammatory infiltration and proliferative response 15 days after BDL. *Left*: Gr1 (macrophage and neutrophil polymorphonuclear cells) immunostaining on liver sections from WT and TGR5-KO mice after BDL or BDL + CC. *Right*: Ki67 and E-cadherin co-immunostaining on liver sections from WT and TGR5-KO mice after BDL or BDL + CC. Gr1+ cells and Ki67+ hepatocytes: restricted to periportal regions in WT BDL mice; more mediolobular distribution in TGR5-KO as well as in WT and TGR5-KO mice with BDL + CC. Representative images, n = 5–7 mice. Obj. ×10, scale bar: 100 µm. (C) GB bile volume in WT and TGR5-KO mice after BDL. *Left histogram*: GB bile volume analysis (n = 4–25 mice/group); representative photographs at 7 days after BDL. Dotted lines: GB area; white arrows: macroscopic bile infarcts observed on the liver surface. *p <0.05; ***p<0.001; Student's t test. Panel A middle graph: log-rank (Mantel-Cox) test was used to compare the survival curves. BDL, bile duct ligation; CC, cholecystectomy; GB, gallbladder; KO, knockout; TGR5, Takeda G protein coupled receptor; WT, wild-type.

Erysipelotrichia were inversely abundant in TGR5 overexpressing (TGR5-Tg) and TGR5-KO mice (Fig. 4A and S7). Importantly, the gut microbiome profile we found in TGR5-KO mice did not appear to be associated with any significant impact on primary to secondary BA transformation, based on predictive functional PICRUST analysis (Fig. 4B). To further investigate this question, we co-housed WT and TGR5-KO mice during 28 days to favour faecal microbiota transfer through coprophagy, as reported.[21] Mice were co-caged at WT:KO or KO:WT ratios of 2:1, and liver BA were analysed (Fig. 4C). As a control of faecal microbiota transfer, we analysed faecal bacterial DNA from co-housed mice in the different experimental groups, confirming that WT mice did acquire TGR5-KO microbiota, and that KO mice did partially acquire WT microbiota (Fig. 4D and S7C). Importantly, despite effective microbiota transfer, liver BA data did not show significant co-housing-induced change in either liver secondary BA concentration or in BA hydrophobicity (Fig. 4E). We thus suggest that the TGR5-KO gut microbiota, although different from WT, would not significantly contribute to build a more hydrophobic BA pool.

Staying focused on the link between TGR5 and BA pool composition, we studied TGR5 impact on GB function and BA pool composition. Indeed, GB is both an organ with high TGR5 expression, and in which BA pool composition can be modulated through a cholecysto-hepatic shunt.

TGR5-dependent control of GB dilatation is crucial for BA composition

In TGR5-KO as compared with WT mice, GB volume and weight were smaller, and GB filling deeply impaired. Interestingly, in TGR5-Tg mice, GB was heavier and larger than in WT mice (Fig. 5A). Treatment with the TGR5-specific agonist RO5527239 (RO, see Fig. S8) induced rapid GB dilatation in WT but not in TGR5-KO mice, whilst this effect was even stronger in Tg mice (Fig. 5B, left). This effect was also observed in more prolonged RO treatment, and did not result from any bile flow stimulation (Fig. 5B). We further built a volume–pressure curve after sequential GB lumen injections while a continuous monitoring of intraluminal pressure was performed as previously described.[6] We found that TGR5-KO had strikingly lower capacitance than WT GB, as reflected by the slope values extracted from these curves (Fig. S9). Based on these data we explored GB motor function *in vivo* by performing ^{99m}Tc mebrofenin SPECT in WT and TGR5-KO mice,[22] and consistently confirmed that GB filling capacity was deficient in the lack of TGR5 (Fig. 5C). Taken together, these data confirm and extend previous reports,[10] providing new evidence that TGR5 controls GB motor function and that in the lack of TGR5 GB may be considered as hypo- or non-functional.

Given the above data, we explored the GB involvement in TGR5-dependent impact on BA pool composition. As shown in

Fig. 6, a 2-day treatment with the TGR5-specific agonist RO resulted in a significant reduction in hepatic secondary BA concentration, as well as in an increased primary/secondary BA ratio (Fig. 6A,B). This effect was also observed, although at a lesser extent, in faeces and plasma (Fig. 6C). Accordingly, the lithocholic acid concentration in faeces was reduced upon RO treatment. Treatment with oleanolic acid, another well-reported potent TGR5 agonist,[23] similarly shifted the hepatic primary/secondary BA ratio in WT and TGR5-Tg but not TGR5-KO mice (Fig. 6C). Interestingly, cholecystectomy (CC) by itself increased secondary BA concentration in the liver after 2 days, suggesting that a physiological shunt would operate at the basal state between GB bile and the liver in mice (Fig. 6B).[22] Most importantly, the TGR5 agonist treatment impact on BA pool composition was lacking in cholecystectomised mice (Fig. 6B).

Taken together, these data suggest that TGR5 impacts on BA pool hydrophobicity at least in part through a modulation of GB function. By dilating the GB, TGR5 stimulation may favour a cholecysto-hepatic shunt[22] and thereby increase the primary/secondary BA ratio.

TGR5-mediated hepatoprotection through GB

Based on the above data, we anticipated that TGR5-mediated impact on the GB may provide protection in the setting of cholestasis. Indeed, we and others previously reported that TGR5-KO mice were more sensitive to BDL- or CA-enriched diet-induced liver injury.[5,6,8] We recently reported that TGR5-mediated signalling operated a control on the biliary epithelial barrier function, explaining at least in part why TGR5-KO mice were more prone to BA-induced parenchymal injury.[6] However, the impact of TGR5 on GB function and/or BA pool composition might also contribute to this phenotype. In the present study, we first observed that CC was associated with more severe liver injury at 48 h after BDL in WT and although at a lesser extent, also in TGR5-KO mice (Fig. 7A,B). In a more chronic setting, at 7 and 15 days after BDL, enhanced body weight loss and non significantly reduced survival were observed in TGR5-KO as compared with WT mice (Fig. 8A). Importantly, in WT but not TGR5-KO mice, body weight loss, hepatic inflammatory infiltration, medio- and centro-lobular hepatocyte proliferation, as well as systemic TBA concentration and bile duct dilatation were increased in BDL + CC as compared with BDL mice (Figs. 7C, 8A,B, and S10A), indicating a more severe liver disease when GB was removed in the WT context. However, post-BDL survival was not impaired by CC (Fig. S10B). As expected, GB volume (dilatation) rose dramatically after BDL in WT but only faintly in TGR5-KO mice (Fig. 8C), suggesting that GB operates protective impact during obstructive cholestasis at least in part through TGR5-dependent mechanisms related to GB dilatation. Importantly, as BDL completely blocked BA travel to the intestine, the liver BA in those mice were composed of more than 99% of primary BA, reflecting the lack of BA transformation in the gut (Fig. S10C). Therefore, in the BDL model, TGR5- and GB-mediated hepato-protection would not be related to an enhanced secondary BA cholecysto-hepatic shunt. It is thus likely that the TGR5- and GB-related hepato-protection would occur through GB dilatation and its linked baroprotection in completely obstructed bile ducts. In line with this view, post-BDL intrahepatic bile duct dilatation was strikingly observed in WT BDL + CC (as compared with BDL) mice, as shown on H&E and Sirius Red, and as quantified on CK19-immunostained liver sections (Fig. 7C). In TGR5-KO mice, as GB was less prone to dilatation, BDL by itself induced severe intrahepatic bile duct dilatation, and CC was not significantly associated with further post-BDL bile duct ectasia (Fig. 7C). Importantly, post-BDL differential bile duct diameter increase in WT and TGR5-KO mice could not be explained by differences in cholangiocyte proliferation (Fig. S10D). Together these data further support the hypothesis that GB alleviates post-BDL hyperpressure and dilatation in the biliary tree, and thereby protects the liver parenchyma in a TGR5-dependent manner.

Importantly, in the non-obstructive CA-enriched diet model, we also observed that removing the GB was associated with more inflammatory and necrotic liver injury. CC was associated with more liver injury, more inflammatory infiltrate, as well as with a reduced primary on secondary BA ratio (Fig. S11). Interestingly, in CA 1% fed TGR5-KO mice, CC was not associated with any hepatic BA composition shift (Fig. S11D). These data suggest that the increase in liver secondary BA (and the related liver injury) observed upon CA-enriched diet can be significantly countered by a TGR5-dependent cholecysto-hepatic shunt.

Discussion

BA signalling and BA pool composition are increasingly considered as crucial for intestine and liver pathophysiology,[14–16,24,25] and therapeutic strategies targeting BA and their receptors begin to emerge.[26] This is particularly true in the field of metabolic diseases such as obesity and diabetes,[17] as well as for cholestatic liver diseases.[27] However, in the liver repair field, BA-centred therapeutic strategies are still underexplored, awaiting for experimental substratum. In this study, we explored how the BA receptor TGR5 may regulate the BA composition, and thereby may have an impact on liver repair after different types of injury. We found that the lack of TGR5 in mice was associated with a particularly poor outcome after EH, with BA-induced parenchymal necrosis and high mortality. Importantly, shifting the BA pool composition toward less toxic BA was correlated with increased survival rates, whereas more hydrophobic BA composition was associated with more severe post-hepatectomy outcome. We further provided human data showing that, as found in mice, a more hydrophobic hepatic BA composition was associated with elevated liver injury and cholestasis markers after major hepatectomy. Finally, we uncovered in mice that TGR5, through GB dilatation, controls BA composition, reduces intrahepatic biliary pressure, and thereby protects the liver against BA-induced injury via different mechanisms depending on the type of experimental BA overload.

Although it has been recently reported that TGR5 may control the expression of BA synthesis enzymes,[12] this had not been found by previous studies.[5,10,11] Importantly, our experiments with hepatocyte specific Alb-Cre-TGR5-KO ($TGR5^{\Delta hep}$) indicate that a TGR5-dependent direct regulation of BA synthesis would be unlikely in our experimental setting, which is in line with the lack, or very low expression, of TGR5 in hepatocytes.

Interestingly, the GB is both the murine tissue most enriched in TGR5,[11,28] and a site for BA pool modification.[22] Although it is currently considered that GB can be removed without any significant consequences, we provide here some provocative data showing that GB is hepatoprotective, at least in mice, and both in obstructive and non-obstructive cholestasis experimental settings. Two main processes may be involved in this protection. Direct mechanical protection may occur, GB dilatation providing what we could call a 'baro-protective buffer' in obstructive contexts (when the obstruction is located downstream the cystic

duct); as explained above, GB dilatation may also indirectly favour the cholecysto-hepatic shunt, thereby increasing the primary/secondary BA ratio. After BDL, BA composition was not significantly changed when GB was conserved as compared with cholecystectomised mice, suggesting that in this extreme variant of obstructive cholestasis, GB-dependent baroprotection prevails. It is tempting to speculate that TGR5 activation in the GB, and possibly in the biliary tree, may elicit similar processes in humans than in mice. This would be in line with previous data suggesting TGR5-induced apical sodium-dependent bile acid transporter translocation to the apical membrane in human cholangiocytes.[7] Precise signalling and molecular mechanisms operating in this shunt remain to be explored. Whatever the mechanisms involved in the control of BA pool hydrophobicity in humans (whether dependent or not on TGR5), our data on hepatectomised patients suggest that BA composition is critical for liver repair in both mice and humans, this concept being still debated.[29,30]

Our data showed that even though the lack of TGR5 was associated with a gut microbiota dysbiosis, we did not identify any significant impact of it on hepatic BA composition. However, further studies will be necessary to explore mechanisms underlying the TGR5-dependent dysbiosis, as well as its fine consequences on biliary homeostasis. Of course, the TGR5-KO hydrophobic BA pool may, in return, shape a modified gut microbiota, a hypothesis needing further specific investigations.

It emerges from our data that stimulating choleresis in a small remnant liver after EH should be deleterious, in keeping with data reporting that UDCA may aggravate obstructive cholestasis in several experimental and clinical settings.[18] The underlying reasons why UDCA-induced choleresis appeared at least in part dependent on TGR5 remain unclear. However, the fact that UDCA-induced chloride and bicarbonate biliary secretion is impaired in TGR5-KO as compared with WT mice is reminiscent of previously reported data suggesting that TGR5 regulates this secretory activity.[5,7,31] We also showed that UDCA treatment in mice, in complete opposition to humans, increases BA pool hydrophobicity, pointing out species differences in BA metabolism.[19]

We recently reported that TGR5-mediated BA signalling in the biliary epithelium strengthened paracellular barrier function, protecting the liver parenchyma against BA-induced injury during cholestasis.[6] The present study provides evidence that TGR5 contributes to a more hydrophilic BA pool by targeting the GB compartment in the enterohepatic cycle and modulating GB function (dilatation) (Fig. S12). Based on our mice and human data, future clinical studies will determine if a pre-surgery BA pool hydrophobicity index might prove to be an accurate predictive marker for post-hepatectomy outcome. As a whole, TGR5 exerts hepatoprotective effects through biliary epithelial barrier function and BA pool composition; this may open new avenues for treatment in the fields of cholestasis and liver repair.

Abbreviations

ALP, alkaline phosphatase; ALT, alanine aminotransferase; BA, bile acid; BDL, bile duct ligation; CA, cholic acid; CC, cholecystectomy; CT, cholestyramine; CYP, cytochrome P450; EH, extended hepatectomy; GB, gallbladder; GM, gut microbiota; GPBAR1, G protein-coupled bile acid receptor 1; HI, hydrophobicity index; KO, knockout; ND, normal diet; OA, oleanolic acid; PH, partial hepatectomy; TBA, total BA; TGR5, Takeda G protein coupled receptor; UDCA, ursodeoxycholic acid; WT, wild-type.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Authors' contributions

Conceptualisation: TT, GM, VBJ
Formal analysis: TT, GM, VBJ
Writing – original draft: TT
Writing – review and editing: TT, GM
Funding acquisition: TT
Investigation and methodology: VBJ, IG, ID, GM, MG, LH, NP, DR, JUB, RB, MS, AA, TT
Project administration: TT
Resources: CU, EV, NG, JCDV

Data availability

When possible, experimental data could be shared via contact with the corresponding author.

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Supplementary data

Supplementary data to this article can be found at https://doi.org/10.1016/j.jhepr.2020.100222.

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