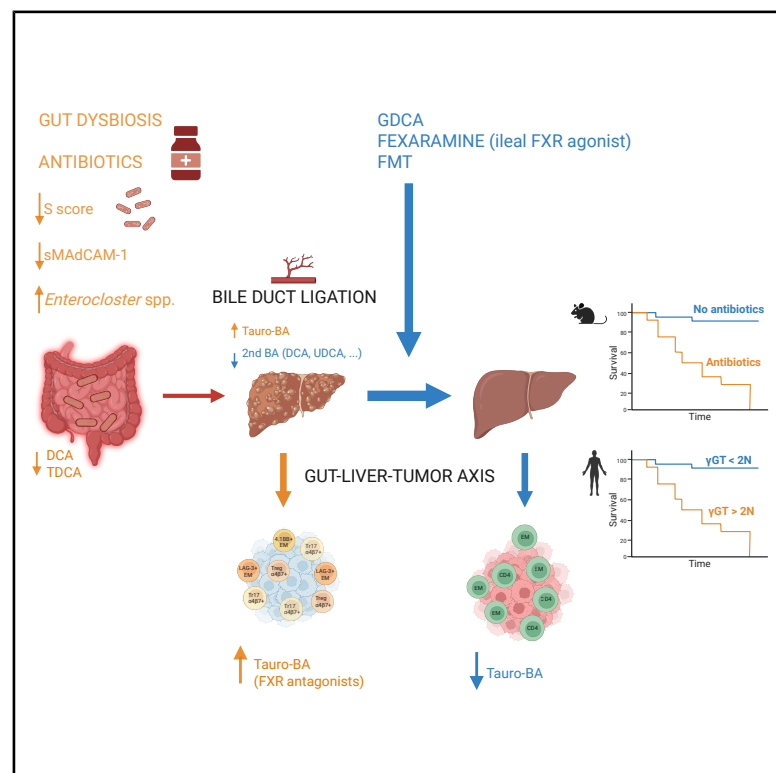


Subclinical cholestasis is a hallmark of gut dysbiosis causing resistance to cancer immunotherapy

Graphical abstract



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In brief

Mallard de La Varende et al. demonstrate that gut dysbiosis impairs tumor immunosurveillance through the gut-liver axis, causing subclinical cholestasis and immunotherapy resistance in tumors distant from gastrointestinal and hepatic tissues. Gut dysbiosis triggers plasma elevation of γ -glutamyl transferase, increase in TCDCA in humans (TMCA in mice), and drop in sMAdCAM-1 levels.

Highlights

- Gut dysbiosis reshapes bile acids and weakens cancer immunotherapy
- Metabolic or microbiota interventions compensate dysbiosis-related bile acid shifts
- Glycodeoxycholic acid compensates ileal MAdCAM-1 loss and TIL exhaustion
- Subclinical cholestasis predicted primary resistance to cancer immunotherapy

Article

Subclinical cholestasis is a hallmark of gut dysbiosis causing resistance to cancer immunotherapy

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SUMMARY

Gut dysbiosis compromises cancer immunosurveillance by downregulating ileal mucosal addressin cell adhesion molecule 1 (MAdCAM-1), but the metabolic landscape associated with gut dysbiosis remains elusive. Here, we show that antibiotics (ABX) or ABX-associated *Enterocloster* species lead to the loss of secondary bile acids (BAs) including deoxycholic acid (DCA) and the accumulation of tauro-conjugated primary BAs (tauro-chenodeoxycholic acid [TCDCA] and tauro- β -muricholic acid [T- β MCA]) from the alternative pathway in the plasma of patients and mice. Fecal microbial transplantation (FMT), the ileum-specific farnesoid X receptor (FXR) agonist fexaramine, or glycodeoxycholic acid (GDCA) compensated dysbiosis-associated BA abnormalities and circumvent primary resistance to PD-1 blockade. GDCA curtailed ABX-induced MAdCAM-1 downregulation and T cell exhaustion in tumors. Subclinical cholestasis defined by elevation of γ -glutamyl transferase (γ GT) correlated with increased TCDCA and decreased soluble MAdCAM-1 in plasma and predicted poor survival in multivariate analyses in six cohorts of patients who received immunotherapy. Hence, subclinical cholestasis accompanies gut dysbiosis, paving the way to immunoresistance.

INTRODUCTION

Among metabolic pathways, the lipid metabolism has emerged as a key determinant of T cell responses, with extracellular lipid

uptake contributing to their activation status, differentiation, and adaptation to environmental stress.^{1–3} Bile acids (BAs) are abundant cholesterol-derived metabolites essential for lipid absorption. Primary BAs such as cholic acid (CA) and chenodeoxycholic



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acid (CDCA) are synthesized in the liver from cholesterol via the classical pathway under the action of hepatic enzymes (CYP7A1 and CYP8B1) and conjugated with taurine, glycine or other amino acids and then excreted into the small intestine.¹ Under physiological conditions, the liver-specific classical pathway is dominant, while the alternative (or acidic) pathway dependent on the enzymes CYP27A1 and CYP7B1 is marginal and not liver specific.¹ BA composition is determined by not only the activity of hepatic microsomal enzymes but also that of microbial enzymes in the small and large intestines.² Bacterial bile salt hydrolases (BSHs) mediate the deconjugation of BAs, whereas bacterial hydroxysteroid dehydrogenases (HSDHs) mediate oxidation and epimerization of the 3-, 7-, and 12-hydroxy groups of BA. Liver-derived BAs are further modified by the gut microbiota to generate intestinal secondary BAs, such as CA-derived deoxycholic acid (DCA), CDCA-derived lithocholic acid (LCA), and ursodeoxycholic acid (UDCA).¹ In mice, but not humans, an additional C6 hydroxylation of CDCA (by murine Cyp2c70) gives rise to muricholic acids (MCAs) and gut microbiota-modified derivatives.¹ In germ-free mice, the absence of microbiota leads to an increase in the BA pool and farnesoid X receptor (FXR) antagonists, including tauro- β MCA (T- β MCA).¹

These hepatic and intestinal BAs along with their conjugated or hydroxylated derivatives profoundly influence T cell functions. Intestinal BAs regulate Th17 and peripheral Foxp3⁺ regulatory T cell differentiation directly or indirectly through dendritic cells.^{3–7} Liver cancers exhibit elevated levels of BAs in their parenchyma, presumably as a result of deregulated BA synthesis or defects in BA export.⁸ A recent report unveiled the immunosuppressive effect of primary conjugated and secondary BAs accumulating in hepatocellular carcinomas on T cell responses and cancer immunosurveillance during PD-1 blockade,⁹ echoing a pioneering study showing the inhibition of hepatic natural killer T (NKT) cell chemotaxis by secondary BAs during liver carcinogenesis.¹⁰ Beyond their local effects on the gut-liver axis, BAs may exert systemic immunomodulation.^{11–13} Given the critical role of intestinal commensalism in the metabolic transformation of hepatic BAs, dysbiosis, which is a prolonged deviation of the taxonomic composition of the gut microbiota,¹⁴ may affect both local and systemic adaptive T cell functions.^{15–17} Carcinogenesis¹⁸ and cancer treatments, co-morbidities, and comedications may lead to dysbiosis.^{19,20} Typically, cessation of antibiotics (ABX) induces the relative dominance of the genus *Enterocloster*, associated with immunosuppression and

resistance to PD-1 blockade.²¹ This immunosuppressive effect of *Enterocloster* species involves the downregulation of mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), expressed on high endothelial venules of the ileal lamina propria and gut lymphoid organs. Specifically, *Enterocloster clostridioformis* reshuffles the composition of BAs.²¹ However, deviations in BA metabolism and their links with gut dysbiosis and immune dysfunction in cancer patients during immunotherapy remain poorly characterized and were the focus of the present study.

RESULTS

Gut dysbiosis-associated shifts in BA metabolism

ABX alter the BA composition, but their effects in mice bearing extraintestinal tumors have not been comprehensively studied.²² Mass spectrometry-based targeted metabolomics was performed on ileal and colonic contents, liver tissue, and plasma from C57BL/6 mice inoculated with subcutaneous MCA205 fibrosarcoma. Mice received a broad-spectrum ABX cocktail (ampicillin, colistin, and streptomycin) orally for two weeks prior to analysis (Figures 1A and S1A–S1C) in two geographically distinct animal facilities (France and Canada) but harbored similar plasma BA profiles at baseline (Figures S1A and S1B). ABX caused significant perturbations in BA metabolism (Figures 1B and S1D; Table S1). At both sites, ABX increased the plasma BA pool (Figures S1E and S1F) and drastically decreased secondary BA plasma concentrations, including DCA (Figure 1C, upper; Figure S1G, left), UDCA, ω -MCA, hyo-DCA (HDCA), as well as their conjugated forms (tauro-DCA [TDCA]) (Figure 1C, lower; Figures S1D and S1G, right), 7-ketoDCA, and nor-UDCA (Figures 1B and S1D). In plasma, the levels of primary unconjugated BAs (CA and CDCA) dropped (Figures 1B and S1D), while the concentrations of conjugated primary BAs (tauro- α - β - ω -muricholic acid [T- α - β - ω -MCA], tauro-cholic acid [TCA], and tauro-chenodeoxycholic acid [TCDCA]) rose with ABX, together with the tauro-conjugated UDCA (TUDCA) for about 2 weeks (Figures 1D, S1D, and S1H). In the liver, ABX caused a significant reduction in α -MCA, ω -MCA, TDCA, and tauro-lithocholic acid (TLCA), as well as a rise in β -MCA, its tauro-conjugated derivative (T- α - β - ω -MCA), and TUDCA (Figure 1B). Similarly, in the colon and to a lesser extent in the ileum, ABX depleted most BAs, such as DCA, glyco-DCA (GDCA), UDCA, and primary BA, but increased tauro-conjugated primary BAs, T- α - β - ω -MCA, TCA, TCDCA, and TUDCA (Figure 1B). To establish a causal link between gut dysbiosis and BA perturbations, we subjected ABX-pretreated mice to oral gavage with fecal microbial transplantation (FMT) from four cancer patients responding to immunotherapy in the ONCOBIOTICS cohort to revert dysbiosis (Figures S1I–S1K). Each of the four FMTs prevented the loss of DCA, HDCA, 7-ketoDCA, nor-UDCA (Figures S1I and S1J) and unconjugated primary BAs (CDCA, CA) after ABX treatment, while blunting the recirculation of tauro-conjugated primary BAs, T- α - β - ω -MCA, TCDCA, and TCA (Figures S1I and S1K).

Next, we replaced ABX by iterative gavages with the ABX-associated *Enterocloster clostridioformis* strain 141.²¹ In such conditions, we generated a “gut dysbiosis” as revealed by 16S rRNA targeted sequencing, unveiling a profound shift in the stool taxonomic composition, characterized by the enrichment

of strains from the *Enterocloster* genus (Figures S2A and S2B), increased alpha diversity (Figure S2C), and significant alterations in the dynamics of beta diversity (Figure S2D), along with a relative overrepresentation of Eggerthellaceae and Clostridiaceae family members, coinciding with the depletion of immunogenic *Alistipes* spp. (Figure S2E) over 4 days after gavaging with *E. clostridioformis*. In these conditions, *E. clostridioformis* tended to mimic some of the effects of broad-spectrum ABX on BA composition, inducing the accumulation of β -MCA and its derivatives, such as HDCA (Figures S3A and S3B), as well as T- α - β - ω -MCA and TUDCA in the plasma of both naive (Figure 1E) and tumor-bearing mice treated with anti-PD1 antibodies (Abs) (Figures S3A, S3C, and S3D). Importantly, such tauro-conjugated primary BAs reached the tumor microenvironment (TME), as indicated by their increased relative abundance among all BAs (Figures 1F and 1G).

We next analyzed the transcriptional profile of BA rate-limiting enzymes in the liver by quantitative reverse-transcription PCR (RT-qPCR) in animals receiving ABX, followed by 4 days of recolonization with or without two gavages of *E. clostridioformis* in naive mice. The expression of the enzymes of the classical pathway, *Cyp7a1* and *Cyp8b1*, was drastically decreased by ABX and did not recover after ABX cessation (Figures S3E and S3F). However, ABX +/- *E. clostridioformis* tended to increase the expression of enzymes of the alternative pathway, *Cyp27a1* and *Cyp7b1* (Figure S3G), in line with the increase in CDCA derivatives, such as TCDCA, β -MCA, and T- α - β - ω -MCA after oral gavage with *E. clostridioformis* or ABX. Of note, the mouse-specific enzyme converting CDCA into α -MCA (*Cyp2c70*) was upregulated in the liver post oral gavage with ABX and *E. clostridioformis* (Figure S3H). The activity of bile acid-CoA:amino acid *N*-acyltransferase (*Baat*) enzyme involved in the glyco- or tauro-conjugation tended to increase following ABX and *E. clostridioformis* in the liver and the ileum, albeit at low levels (Figure S3I). We confirmed the downregulation of the enzymes of the BA classical pathway and the upregulation of *Cyp2c70* using bulk RNA sequencing of cell-sorted hepatocytes as well as the upregulation of *Baat* gene product in ileal enterocytes (albeit at very low expression levels) (Figures S3J–S3M). Gut dysbiosis increased *Vdr* transcription in ileal enterocytes but not that of other BA receptors in hepatocytes, cholangiocytes, or enterocytes (Figures S3N–S3P). Of note, the sodium/BA co-transporter family member *Slc10a1*, which is involved in the uptake of BA for the enterohepatic cycle, was not significantly affected by ABX (Figures S3Q–S3S).

Altogether, ABX caused a metabolic shift in the BA composition, typically featured by an abnormal accumulation of primary BAs derived from CDCA, such as the tauro-conjugated primary BAs (TCDCA), and β -MCA and its tauro-conjugated derivative (T- α - β - ω -MCA), as well as the loss of intestinal and plasma secondary BAs derived from CA, i.e., DCA and G/TDCA. These ABX-induced metabolic shifts in the BA composition were prevented by FMT and partially mimicked by intestinal colonization with *E. clostridioformis*.

GDCA circumvents resistance to PD-1 blockade during dysbiosis

To draw causal links between gut dysbiosis-associated BA abnormalities and resistance to immunotherapy, we attempted

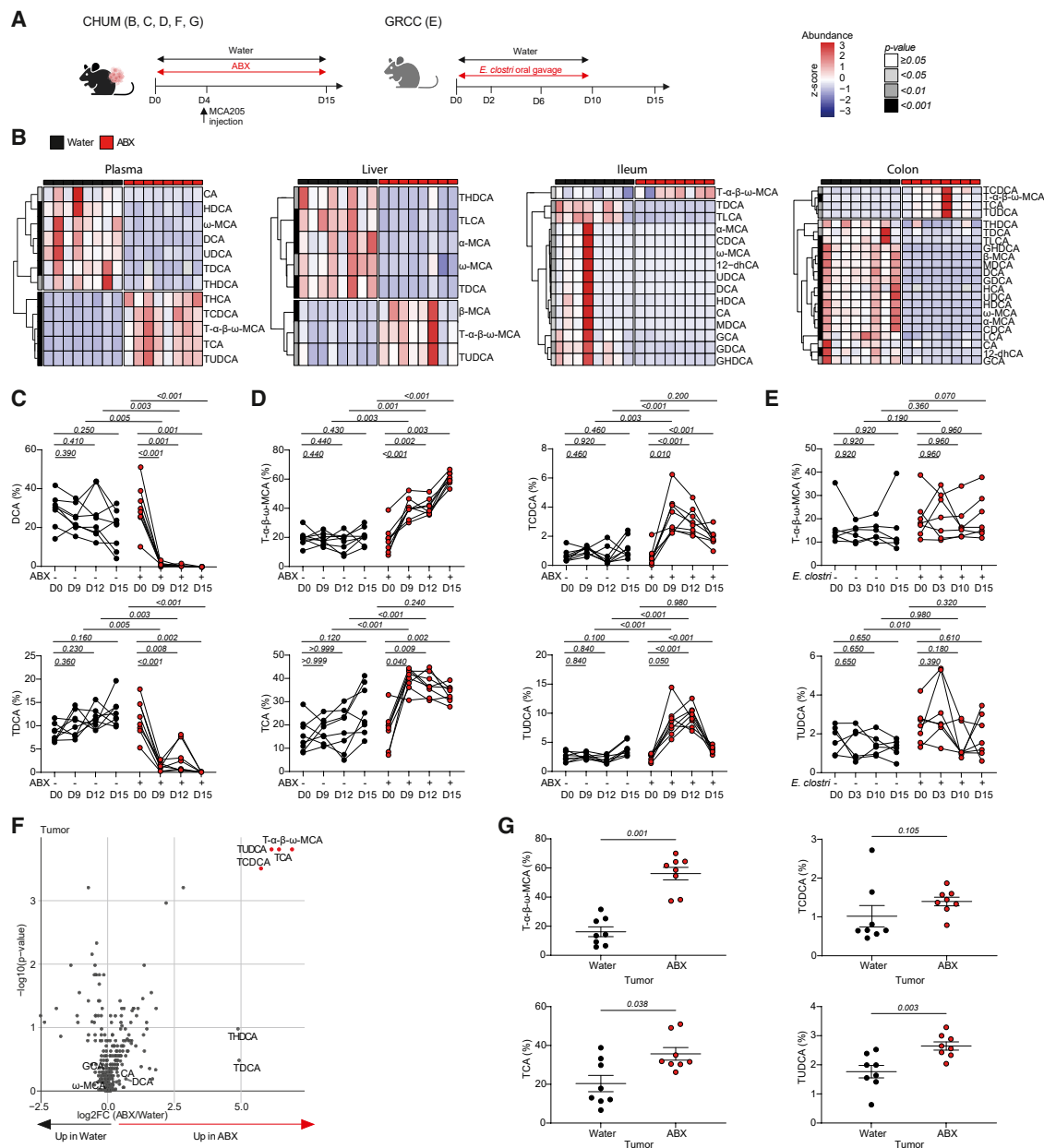


Figure 1. Gut dysbiosis perturbs BA composition

(A) Experimental settings at two different centers; D, day.

(B) Supervised hierarchical clustering of BAs significantly different between ABX and water-fed tumor bearers assessed by metabolomics.

(C and D) Kinetics of the percentages of distinct circulating BAs among the total BA.

(E) Id. in the plasma of naive mice at GRCC and orally gavaged with *E. clostridioformis* or water controls.

(F and G) Metabolite levels in tumor beds after 15 days of ABX. Volcano plot comparing ABX vs. drinking water (F), red dots indicate metabolites significantly increased after ABX. Dot plots depicting the percentages of distinct BA (G). Error bars indicate mean $\pm$ SEM (G).

One representative experiment containing 8 mice/group (B, C, D, F, and G) and 6 and 7 mice/group (E) is depicted. Comparisons between groups were performed using non-parametric Mann-Whitney U test (2 groups), followed by multiple-comparisons test controlling the false discovery rate (FDR) (B, F, and G), or non-parametric Friedman test (>2 paired groups for longitudinal metabolomics analysis), followed by two stage set-up method of Benjamini, Krieger, and Yekutieli (C, D, and E).

See also [Figures S1–S3](#) and [Table S1](#).

to compensate dysbiotic tumor-bearing mice with oral delivery of secondary BAs during PD-1 blockade. C57BL/6 mice were orally gavaged with *E. clostridioformis*, then inoculated with MCA205 sarcoma cells or RET melanoma cells, and treated five days later intraperitoneally (i.p.) with α -PD-1 monoclonal antibody (mAb) alone or together with anti-CTLA4 Abs. At immunotherapy start, mice were fed with selected secondary BAs (GDCA, LCA, and UDCA) at a physiological concentration four times every other day (Figure 2A). Tumor growth retardation induced by immunotherapy was markedly inhibited by *E. clostridioformis* (Figure 2B left, $p = 0.023$) and restored by GDCA (Figure 2B right, $p = 0.021$) but neither by UDCA nor by LCA (Figure 2C). Similarly, the *Ret* transgenic melanoma model responded to the combination of α -CTLA-4 + α -PD-1 mAbs, but the gut colonization with *E. clostridioformis* tended to compromise the efficacy of immunotherapy (Figure 2D, $p = 0.06$), unless GDCA or UDCA (but not LCA) was co-administered (Figure 2D, $p = 0.002$; Figure 2E left, $p = 0.009$; right, $p = 0.044$). Even in the context of full-fledged gut dysbiosis provoked by ABX followed by two *E. clostridioformis* oral gavages, other secondary BAs from the classical pathway, TDCA and DCA, could control the progression of MCA205 sarcomas to some extent (Figure 2F, $p = 0.035$ with TDCA; $p = 0.065$ with DCA on day 13).

To investigate the pharmacokinetics of these exogenously delivered BA, we performed longitudinal mass spectrometry-based metabolomics of plasma and liver on day 7, 24 h after the 3rd administration, or on day 12, 4 days after the 4th oral administration of BA. Oral gavage with GDCA and TDCA (but not with LCA or UDCA) increased or tended to increase the recirculation of GDCA and/or DCA but decreased that of TCDCA (Figure 2G; Figures S4A and S4B; Table S1). Oral LCA and/or UDCA (but not GDCA) increased the plasma concentrations of LCA and UDCA as well as those of MDCA and microbial-CA or TLCA, TUDCA, and tauro-HDCA (THDCA) (Figure 2G; Figures S4A and S4B; Table S1). In the liver, GDCA decreased α -MCA, while UDCA increased T/G-UDCA, T/G-HDCA, and TLCA (Figure S4C). The direct comparison of the BA metabolic profile of animals gavaged with GDCA versus LCA confirmed the dominance of GDCA in the plasma at day 7 in the former group, while the LCA-fed group exhibited not only a dominance of LCA and TLCA but also an ABX-like profile (TCDCA, THDCA, and TUDCA) in the liver and plasma (Figures 2G and 2H; Figure S4C). Tauro-conjugated primary BAs, TCDCA and TCA, were dampened by GDCA (Figure 2G, left) and UDCA, respectively (Figure 2G, right) in the plasma.

To better understand the kinetics of orally fed BA and their downstream derivatives, we analyzed the bile salt hydrolase/ aminoacyltransferase (*bsh*) sequences and activity of our *E. clostridioformis* strain 141 that are required to deconjugate acyl-bound amines from the sterol core of the primary BAs.²³ *Bsh* sequencing of our strain revealed that its enzyme is more closely related to Penicillin V acylases, a group of N-terminal nucleophile hydrolases that are similar to classical cholyglycine hydrolases. This is somewhat in contrast to many other *Enterocloster* species, which contain classical enzymes (Figure S4D). Activity analysis of the *E. clostridioformis* strain 141 showed that it has a limited capacity to hydrolyze tauro-conjugated BAs, such as TCDCA or T- β -MCA, compared with

other *Enterocloster* species but drastically hydrolyzes GDCA (Figures S4E and S4F), in accordance with the dysbiosis-associated BA abnormalities. Moreover, using 16S rRNA targeted sequencing, we did not observe major differences in the fecal taxonomic composition across BA recipient groups (Figures S5A–S5C). GDCA and TDCA triggered similar microbial shifts (Figures S5A and S5B), and GDCA significantly induced a mild relative overrepresentation of immunogenic *Bifidobacterium pseudolongum*^{24,25} and Erysipelatotrichaceae family members²⁶ (Figures S5C and S5D; Table S2).

We conclude that oral GDCA supplementation of dysbiotic mice causes a rise in GDCA and DCA levels and a drop in TCDCA level in the plasma while decreasing α -MCA level in the liver, culminating in the restoration of sensitivity to PD-1 blockade, though without correcting dysbiosis.

Inhibitory effects of experimental cholestasis on PD-1 blockade

Attempting to draw causal links between the accumulation of tauro-conjugated primary BAs and resistance to immunotherapy, we used two procedures. First, we conducted a surgical bile duct ligation (BDL) (versus sham surgery) in mice transplanted with MCA205 fibrosarcoma three days before (Figure 3A), which resulted in cholestatic liver inflammation and injury (Figure 3B) associated with a brisk rise in plasma T α - β - ω MCA and TCA (Figure 3C; Table S1), paralleled by a drastic drop in CA and DCA (Figure 3D; Table S1). Consequently, BDL culminated in complete cancer resistance to PD-1 blockade (Figure 3E). Secondly, using fexaramine, an ileum-selective FXR agonist (Figure 3F), we markedly rescued the efficacy of α -PD-1 Ab in ABX-treated tumor bearers (Figure 3G), restoring T cell functions in tumor-resident lymphocytes (Figures 3H and S6A) and decreasing plasma T α - β - ω -MCA compared with ABX alone treatment ($p = 0.015$ without vs. $p = 0.422$ with fexaramine on day 7) and TCA ($p = 0.001$ without vs. $p = 0.422$ with fexaramine on day 7; Figure 3I; Table S1), albeit with inter-individual variability (Figure S6B). Indeed, fexaramine induced the ileal and colonic rise in the FXR antagonist T α - β - ω -MCA,²⁷ potentially for a subsequent elimination (Figure S6C) but did not act on ABX-induced liver expression of *Baat*, the enzyme inducing tauro-conjugation of primary BAs (Figure S6D). In parallel, fexaramine tended to increase UDCA ($p = 0.052$) and DCA ($p = 0.054$) (Figures 3J and S6B; Table S1) as well as other secondary BAs (Figure S6E). Driven by a recent report showing that TCDCA can promote exhaustion of T lymphocytes infiltrating liver cancers¹² and by the capacity of fexaramine to restrain LAG3 expression on CD8⁺ tumor-infiltrating lymphocytes (TILs) (Figure 3H, right), we assessed whether cholestasis and dysbiosis-associated BAs could directly affect T cell functions *in vitro*. Indeed, we confirmed that TCDCA and T- β -MCA (but not all the other BAs) markedly reduced the viability of T cell receptor (TCR)-engaged splenic CD3⁺ T cells, while GDCA maintained the proliferation of CD8⁺ T cells (Figures 3K, S6F, and S6G). Hence, mechanical cholestasis or gut dysbiosis could lead to plasma accumulation of tauro-conjugated primary BAs, including the strong T- β -MCA FXR antagonist, culminating in T cell dysfunction and resistance to cancer immunotherapy that the ileal FXR agonist fexaramine could antagonize.

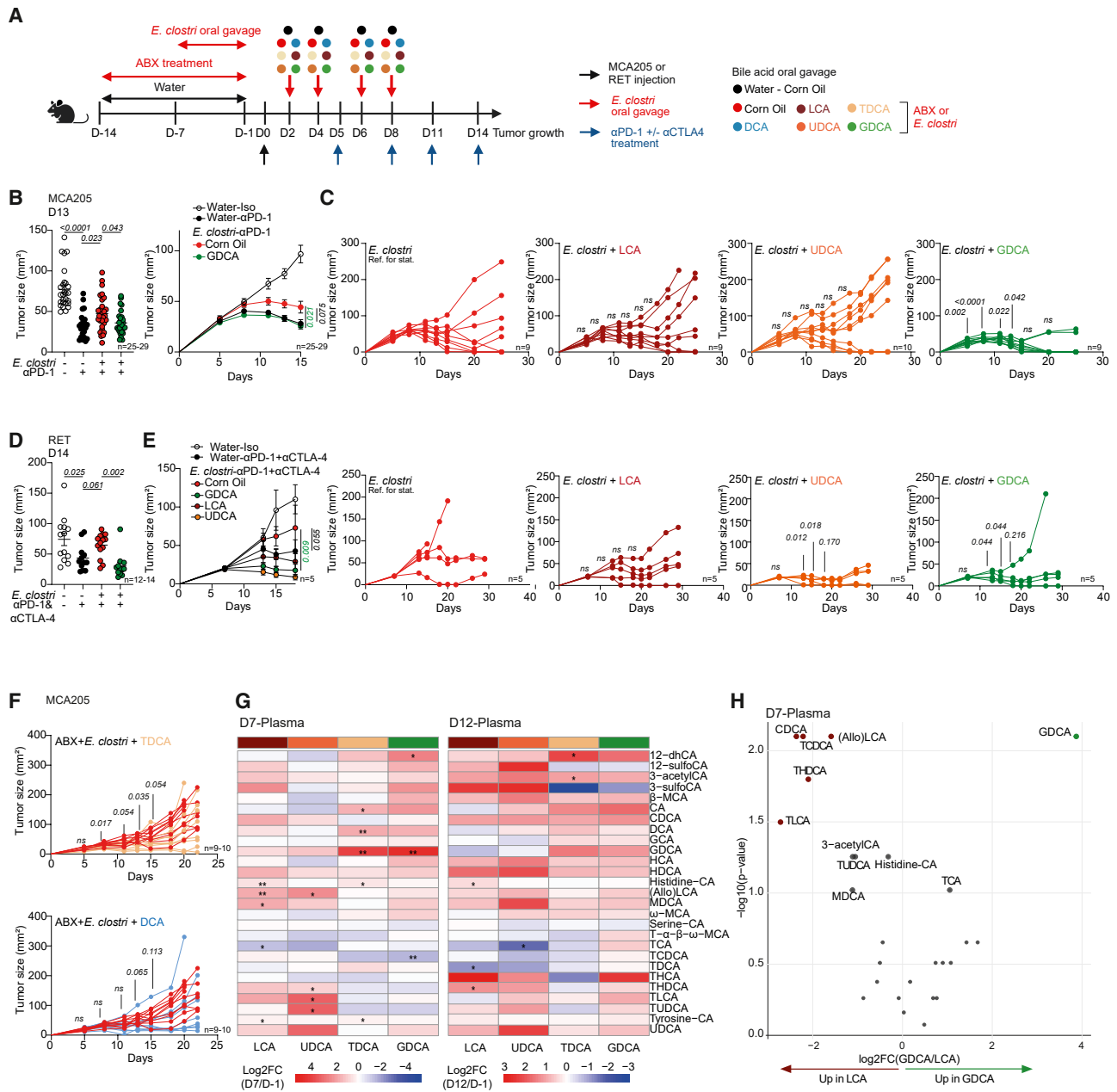


Figure 2. GDCA rescues gut dysbiosis-associated resistance to immunotherapy

(A) Experimental setting at GRCC.

(B–F) Effect of *E. clostridioformis* with or without GDCA on the response to PD-1 blockade (B and C) or PD-1+CTLA-4 blockade (D and E) against MCA205 fibrosarcomas (B and C) or RET (D and E). Tumor size at day 13–14 (B and D, left) or tumor growth kinetics (B right and E left) in three (C) or two (D) concatenated experiments comprising 25–29 (B), 9–10 (C), and 12–14 or 5 mice/group (E) and one representative experiment (E). (F) MCA205 tumor growth kinetics in mice treated with ABX (2 weeks) and orally gavaged twice with *E. clostridioformis* and BA (TDCA and DCA) treated with α -PD-1. Two pooled independent experiments of 4–5 animals/group. Comparisons between groups were performed using non-parametric Kruskal-Wallis H test (>2 groups), followed by multiple-comparisons test controlling the false discovery rate (FDR). *p* values were calculated at each time point comparing each group with *E. clostridioformis* + corn oil (red curves) with the Mann-Whitney U test (2 groups) (C, E right, and F). The tumor growth kinetics were analyzed using the online tool “TumGrowth” (see STAR Methods) (B right and E left). Mean $\pm$ SEM (B, D, and E left) or individual tumor growth kinetics (C, E four right panels, and F) are depicted.

(G) Heatmaps representing the log₂FC of BA levels between before (at D-1) and after oral BA at day 7 (left) and day 12 (right) in 5 mice/group. *p* value (*, *p* < 0.05; **, *p* < 0.01) on the log₂FC between oral BA versus corn oil (Mann-Whitney U test, 2 groups).

(H) Volcano plot contrasting plasma metabolites after GDCA versus LCA in dysbiotic mice at day 7 (*n* = 5 mice/group). Green and red dots are significant metabolites (Mann-Whitney U test, 2 groups).

See also Figures S4 and S5 and Tables S1 and S2.

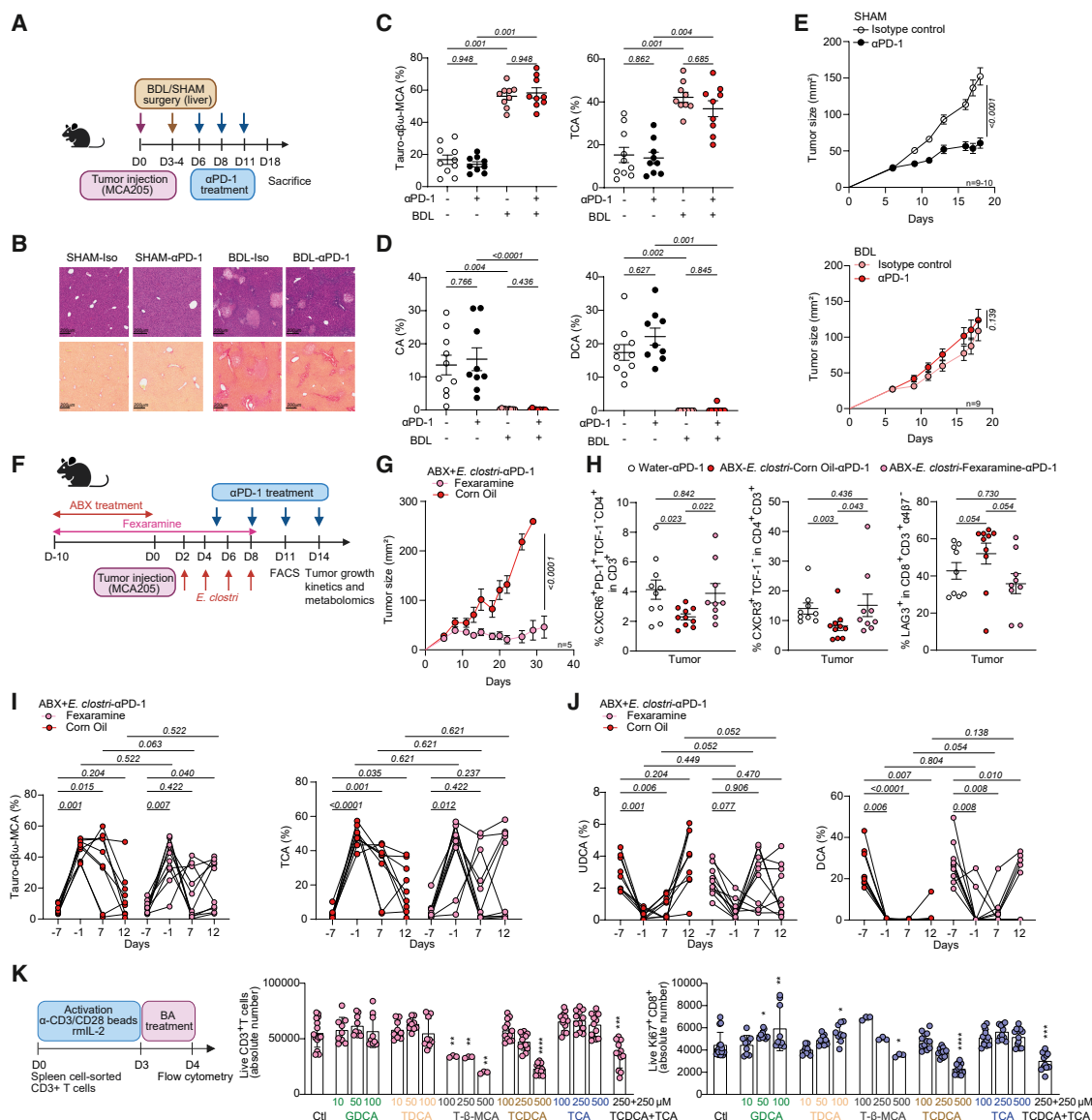


Figure 3. Modulations of tauro-conjugated primary BAs and efficacy of PD-1 blockade

(A) Surgical bile duct ligation (BDL) in MCA205 tumor-bearing mice treated with anti-PD-1 Abs at Centre de Recherche des Cordeliers.

(B) Livers were stained with hematoxylin and eosin (top) as well as Sirius Red (bottom). One representative photograph of each liver in each group of 8–10 mice is shown.

(C and D) Percentages of distinct BAs influenced by BDL.

(E) Mean tumor growth curves in both groups. The tumor growth kinetics were analyzed using the online tool “TumGrowth” (see STAR Methods).

(F) Experimental setting of ABX + *E. clostridioformis*-induced gut dysbiosis compensated by fexaramine in MCA205 tumor bearers at GRCC.

(G) Tumor growth kinetics were analyzed as in (E). A representative experiment comprising 5 mice/group is shown, out of two yielding similar results.

(H) Flow cytometric analysis of TILs in mice receiving ABX and/or fexaramine and treated with αPD-1 mAb. One representative experiment comprising 10 mice/group is depicted. One dot represents one tumor.

(I and J) Longitudinal plasma metabolomics analyzing various BAs (in percentages of the total BA) over time. Each dot represents one mouse housed at GRCC animal facility.

(K) Ex vivo stimulation of CD3⁺ T splenocytes exposed to various concentrations of BAs (left), followed by flow cytometry analysis of the co-culture for cell viability and proliferation. The absolute number of live CD3⁺ T cells or percentage of Ki67⁺ in CD8⁺ T cells recovered at 24 h of BA exposure is shown (middle and right, respectively). Also refer to Figures S6F and S6G for CD4⁺ T cells and fold change calculations. Each dot represents one well, and each experiment was performed in triplicate wells and performed between three to five times ($n = 9–15$ wells), except for T-β-MCA condition that was performed once ($n = 3$ wells). $n = 9–10$ mice/group (B–E and H–J). Error bars indicate mean ± SEM (C, D, E, G, H, and K). Comparisons between groups were analyzed using non-parametric Mann-Whitney U test (2 groups) or Kruskal-Wallis H test (>2 groups), followed by multiple-comparisons test controlling the false discovery rate (FDR) (C, D, H, and K), or non-parametric Friedman test (>2 paired groups for longitudinal metabolomics analysis), followed by two stage set-up method of Benjamini, Krieger, and Yekutieli (I and J). The tumor growth kinetics (E and G) were analyzed using the online tool “TumGrowth” (see STAR Methods).

See also Figure S6 and Table S1.

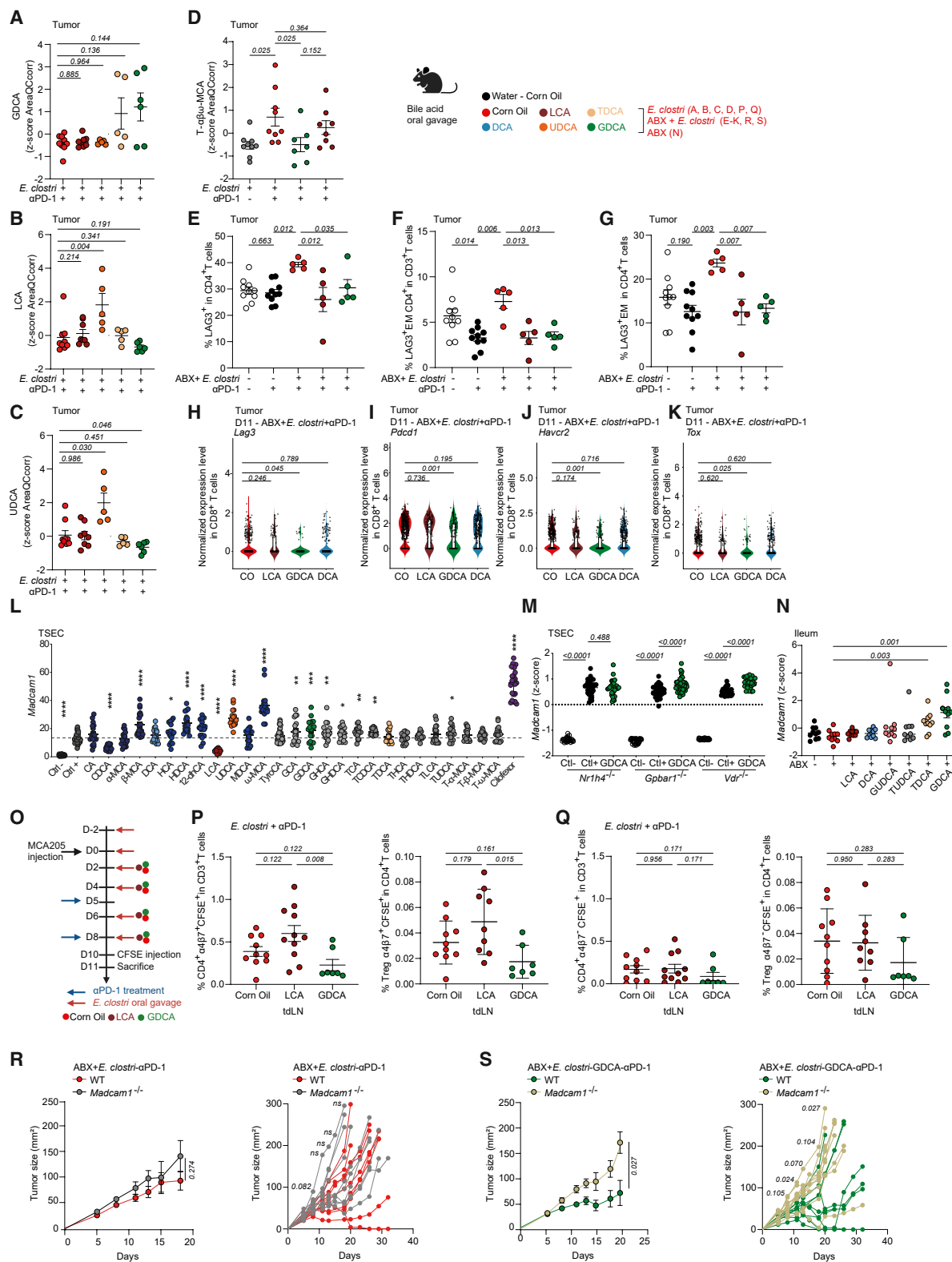


Figure 4. GDCA acts at sites distal from the liver to reprogram the TME

(A–D) Intratumoral abundance of orally gavaged BAs determined by mass-spectrometry-based metabolomics in *E. clostridioformis* dysbiotic mice treated with αPD-1 on day 12 post MCA205 injection, comparing BA vs. water + corn oil controls using Z scores of AreaQCorr. Shown is the concatenation of 2 experiments including 3–5 mice/group.

(legend continued on next page)

Gut BAs reach the subcutaneous TME to reprogram local immunity

Natural and orally fed BAs reached distal tissues by day 12, as revealed by tumor metabolomics (Figures 1F and 1G and 4A–4D; Table S1). Indeed, oral gavage with GDCA increased GDCA tumoral levels only in one-third of the animals (Figure 4A, $p = 0.144$), while that of UDCA induced a significant rise in LCA and UDCA (Figures 4B and 4C, $p = 0.004$ and 0.03 , respectively). This might be explained by the capacity of *E. clostridioformis* strain 141 to metabolize GDCA into DCA in the intestines (Figures S4E and S4F). Of note, oral GDCA could prevent the tumoral accumulation of T α - β - ω -MCA observed with *E. clostridioformis* (Figure 4D, $p = 0.025$).

Flow cytometric analyses of TILs and myeloid cells unveiled an *E. clostridioformis*-induced rise in the LAG3 exhaustion marker in CD4⁺ T cells (Figure 4E, $p = 0.01$; Figure S6A), most specifically in effector memory (TEM) CD4⁺ T cells (Figures 4F and 4G, $p = 0.006$). Conversely, GDCA significantly prevented this dysbiosis-associated T cell exhaustion, dampening LAG3 in CD4⁺ (Figure 4E, $p = 0.035$) and in TEM CD4⁺ T cells (Figures 4F and 4G, $p = 0.013$). We confirmed these findings using single-cell RNA sequencing of tumor-infiltrating CD45⁺ cells on day 11 post oral gavage with GDCA, DCA, or LCA (Figures 4H–4K; Figure S7). Single-cell-resolved levels of gene expression of the TME were determined after isolating 18,669 and 14,842 cells from the tumor beds and tumor-draining lymph nodes (tdLNs), respectively, across all experimental conditions. We identified 20 clusters in tumors (including 6 T cell clusters) and 19 clusters in tdLNs (including 12 T cell clusters) (Figures S7A–S7C). Focusing on GDCA versus corn oil (CO) control-imprinted TME, we did not find a quantitative impact of GDCA on the distribution of T cells within clusters (Figures S7B and S7C), but rather observed significant variations in gene expression levels in the proliferating CD4⁺ and/or CD8⁺ T cell subset in the tumor beds and in the naive/memory CD8⁺ T cell cluster in the tdLNs (Figure S7D, lower and upper panels). This naive/memory CD8⁺ T cell subset from GDCA-imprinted tdLNs was characterized by a type 1 IFN signature (*Stat1*, *Ifit3*, *Ifi2712a*, *Gimap7*, *Gbp2*, and *B2m*) associated with the upregulation of the prototypic translation machinery (including many *Rps* and

Rpl ribosomal proteins) and the cytoskeleton remodeling (*Actb*), with a downregulation of the mitochondrial oxidative metabolism (*Lgals1*, *mt-Cytb*, *mt-Co-1/2/3*, and *mt-Atp6*) (Figure S7D, upper panel). This signature has been described during the T cell clonal expansion phase, notably followed by inhibition of the translation of those transcripts in CD8⁺ T effectors,²³ what we indeed observed in the TME (Figure S7D, lower panel). The cluster of proliferating CD4⁺ and/or CD8⁺ T cells in the GDCA-imprinted TME (defined by markers featuring in Table S3, including *Nkg7*, *Cxcr6*, *Skap1*, *Ablim1*, *Sh2d2a*, *Mki67*, *Cdk1*, and *Top2a*) that may contain progenitor exhausted T cells (Tpex), downregulated *Ptpn22*,²⁸ *Gzmb*²⁹ (Figure S7D, lower panel), *Tox*, *Havcr2*, *Pdcd1*, and *Lag3* (Figures 4H–4K) gene transcripts, while upregulating the AP1 family member *Fos*, *Il21r*, *Il1r2*, *Cd40*, and *Cd70* gene products (Figure S7D, lower panel), suggesting retardation in the T cell terminally differentiation status. Interestingly, one of the top gene transcripts upregulated by GDCA on this proliferating CD4⁺ and/or CD8⁺ T cell subset is the creatine transporter *Slc6a8*, critical for TCR-mediated activation of the mTORC1 signaling relevant in cancer immunosurveillance (Figure S7D, lower panel).^{30,31} The proliferating CD4⁺ and/or CD8⁺ T cells of the “GDCA tumors” also had a myeloid chemoattracting secretory profile (Figure S7D, lower panel). Intriguingly, the dominant CX3CR1 monocytic/macrophage cluster within the myeloid compartment of “GDCA tumors” was characterized by the expression of the nonsense mediated mRNA decay (NMD), a cellular quality control pathway degrading mutant transcripts, potentially involved in tumor antigenicity and immunoediting³² (Figure S7E). In the “DCA-imprinted TME”, the CD8⁺ cytotoxic T cell cluster was characterized by the functions associated with proficient antitumor responses, including the upregulation of *Bhlhe40*, *Trbv2*, *Ccr7*, *Irf8*, *Il21r*, and *Sema4c*³³ (Table S3). The “GDCA imprinted” gene profiles contrasted with that observed with LCA-gavaged mice where NOS2⁺ and/or CX3CR1⁺ macrophages lost transcription of their antigen-processing machinery (*H2-K1*, *H2-D1*, *H2-Ab1*, *Ctss*, *Ccl5*, and *Ifitm3*) (Table S3). Of note, “LCA imprinted” tdLN-derived naive/memory CD8⁺ T cells upregulated *Adamts17*, a sheddase blunting the CD122 signaling in CD8⁺ T cells³⁴ (Table S3). Moreover, the gene

(E–G) Flow cytometric analysis of LAG-3 expression on various CD4⁺ T subsets infiltrating the tumor of dysbiotic mice receiving BA and α -PD-1 mAb. One representative experiment comprising 5–10 mice/group is depicted.

(H–K) Violin plots showing the expression levels of selected genes encoding the exhaustion signature in all CD8⁺ TILs in each treatment group on day 11. See also Figure S7.

(L and M) *In vitro* screening of several BAs at 100 μ M or cilofexor at 50 μ M on *Madcam1* relative expression by RT-qPCR in TSECs exposed to TNF- α (vs. negative control [Ctrl-]) (L) or TSECs genetically modified by means of CRISPR-Cas9 gene editing (M). Results from 3–4 independent experiments were pooled, and each dot represents one well; $n = 18$ –42 (L) and 24 (M).

(N) *In vivo* effects of orally gavaged BA on *Madcam1* expression in the ileum of mice treated with ABX. Results from 2 independent experiments are pooled using Z score, and each dot represents one mouse; $n = 10$.

(O–Q) Experimental setting (O) and flow cytometric determination of the percentage of CFSE⁺ T cell subsets reaching the tdLN in day 12 tumor-bearing mice at 24 h after CFSE injection into mLN (P and Q). Results from 2 independent experiments are pooled, and each dot represents one mouse; $n = 7$ –11.

(R and S) Differential effects of corn oil (R) versus GDCA (S) in WT vs. *Madcam1*^{−/−} MCA205-bearing mice treated with PD-1 blockade. Two pooled experiments comprising $n = 9$ –10 mice/group, showing individual kinetics and mean $\pm$ SEM tumor growths. p values were calculated at each time point, comparing the two groups.

Error bars indicate mean $\pm$ SEM (A–N and P–S). Comparisons between groups were performed using non-parametric Kruskal-Wallis H test (>2 groups), followed by multiple-comparisons test controlling the false discovery rate (FDR), except written otherwise (A–G, L–N, P, and Q), or significance was calculated with Wilcoxon rank-sum test and adjusted for multiple testing using Benjamini-Hochberg correction (H–K). The tumor growth kinetics (R left and S left) were analyzed using the online tool “TumGrowth” (see STAR Methods). For R (right) and S (right), p values were calculated at each time point comparing the two groups together using the Mann-Whitney U test.

See also Figures S4–S8 and Tables S1 and S3.

expression fingerprint of LCA TIL revealed a profound down-regulation of the type 1 and 2 IFN responses compared with the negative control-imprinted TME (Figure S7F; Table S3). Altogether, these results showed that BAs can act at distal sites to reprogram the TME and tdLNs, influencing the outcome of tumor-specific immune responses.

Because dysbiosis facilitates the exodus of terminally differentiated and exhausted $\alpha 4\beta 7$ -expressing CD4⁺ Tregs and their emigration to the tdLNs by downregulating ileal MAdCAM-1 in high endothelial venules,²¹ we wondered whether BAs might downregulate MAdCAM-1 expression, thereby favoring the emigration of enterotropic Tregs to tdLNs. For this, transformed endothelial sinusoidal cells (TSECs) were cultured in the presence of physiological concentrations of an array of commercially available primary and secondary, tauro- and glyco-conjugated BAs, followed by the quantification of *Madcam1* mRNA by RT-qPCR.²¹ As reported, LCA downregulated *Madcam1* expression.²¹ In sharp contrast, DCA and its derivatives (GDCA, HDCA, and UDCA) upregulated *Madcam1*. Conversely, most tauro-conjugated BAs failed to affect *Madcam1* expression (Figure 4L). CRISPR-Cas9-based knockout of 3 potential BA receptors (Fxr, Vdr, and Tgr5) in TSECs led to the conclusion that *Fxr/Nr1h4* is essential for the GDCA-induced upregulation of *Madcam1* (Figure 4M). *In vivo*, GDCA efficiently increased *Madcam1* mRNA in the ileum from ABX-treated mice, validating the *in vitro* results (Figure 4N). Next, we injected carboxyfluorescein succinimidyl ester (CFSE) into the mesenteric lymph nodes (mLNs) and then monitored the emigration of CFSE-labeled cells to distant sites at 24 h in tumor-bearing mice fed with *E. clostridioformis* under PD-1 blockade (Figure 4O). Oral administration of GDCA decreased the migration of CFSE⁺ $\alpha 4\beta 7$ ⁺ CD4⁺ T cells (and more specifically $\alpha 4\beta 7$ ⁺ CD4⁺ Foxp3⁺ CD25⁺ Treg cells) (Figure 4P) but not that of $\alpha 4\beta 7$ ⁺ CD4⁺ Treg to tdLNs (Figure 4Q), compared with *E. clostridioformis* + LCA, albeit non-significantly compared with the *E. clostridioformis* group without BA gavage.

Finally, we compared the capacity of GDCA to restore sensitivity to PD-1 blockade in MCA205 tumor-bearing *Madcam1*-proficient mice versus deficient mice in settings of gut dysbiosis (as detailed above, Figure 2F). GDCA lost its adjuvant properties in the *Madcam1*^{-/-} mice (Figures 4R and 4S) but not in *Tgr5/Gpbar*^{-/-} animals (Figure S8A).

Incidentally, to exclude possible direct effects of BAs on cancer cells, we analyzed the expression patterns of the five BA receptors in MCA205 fibrosarcoma by RT-qPCR. In comparison with ileal mucosa, MCA205 sarcoma tissues expressed low levels of *Fxr/Nr1h4*, *Vdr*, and *Tgr5/Gpbar1* gene transcripts (Figure S8B). At physiological concentrations, BAs failed to kill HT29 cells, which readily succumbed to oxaliplatin or mitoxanthrone (Figure S8C). CRISPR-Cas9-mediated knockout of *Fxr/Nr1h4* or *Tgr5/Gpbar1* failed to affect tumor outgrowth, but the *Fxr/Nr1h4* knockout sensitized MCA205 fibrosarcomas to α -PD-1 blockade (Figures S8D and S8E, $p = 0.001$).

In summary, oral GDCA reached tumor bed and prevented the exhaustion of TIL effector memory lymphocytes. It is, at least in part, a consequence of the MAdCAM-1-dependent blockade of tumor homing of immunosuppressive enterotropic Tregs.

Subclinical cholestasis in cancer patients treated with immune checkpoint blockade

To investigate the clinical significance of these findings, we analyzed the effects of ABX on mass spectrometry-detectable circulating BAs in a prospective cohort (ONCOBIOTICS cohort [NCT04567446]), which included 133 patients with lung, kidney, or bladder cancer treated with immune checkpoint blockade (ICB)-based first-line regimens.³⁵ ABX exposure prior to plasma sampling significantly decreased the plasma concentration of UDCA (Figure 5A, $p = 0.021$ compared with no ABX exposure), while increasing TCDCA (Figure 5A, $p = 0.038$ compared with no ABX exposure). These ABX effects were significant within a 30-day time window (Figures 5B–5D) and led to a disruption of the normal pattern of correlations between unconjugated primary and secondary BAs (Figure 5E). In the 61 patients with paired plasma metabolomics and stool metagenomics data, S-score (reflecting gut dysbiosis when closest to 0)³⁶ showed significant, albeit weak, positive correlations with DCA (Figure 5F, left) and negative correlations with total tauro-conjugated BAs (Figure 5F, right).

To dissect the patterns of circulating BAs in the ONCOBIOTICS cohort of cancer patients, we performed an unsupervised hierarchical clustering of the plasma concentrations of BAs, monitored before or within the first 42 days of PD-1/PD-L1 blockade in 111 patients. Three clusters of patients could be clearly identified (Figure 6A; Table S4). Cluster 1 was characterized by the relative dominance of unconjugated primary and secondary BAs (Figure 6B). In cluster 2, patients harbored higher levels of glyco-conjugated primary BAs than those of the others (Figure 6C), while cluster 3 was typified by the accumulation of tauro-conjugated primary and secondary BAs, leading to a ratio between glyco-/tauro-conjugated BAs of <3, as well as by elevated plasma levels of the inflammatory marker calprotectin (S100A9) (Figures 6D and 6E). A similar clustering pattern was found in metastatic melanoma (Figures S9A–S9C) and NSCLC patients (Figures S9D–S9F) in Canada (as described by Routy et al.³⁷ and Duttagupta et al.³⁸; Table S4). Univariate Cox regression analysis of the overall survival (OS) of ONCOBIOTICS patients revealed that cluster 3 tends to be associated with dismal outcome (Figure 6F; Table S4). Notably, these 3 clusters exhibited different immune profiles. Cluster 2 was enriched in naive CD8⁺ T cells, while cluster 3 showed a relative scarcity of total and memory CD27⁺ and CD127⁺ CD4⁺ T cells (Figures S10A and S10B).

Given the limited effect size of ONCOBIOTICS and the demonstration of a potential clinical relevance of subclinical cholestasis¹ brought up in rare retrospective studies in renal cell carcinoma patients treated with nivolumab,^{39,40} we determined whether the routine biomarkers of cholestasis, which are gamma-glutamyl transferase (γ GT) and alkaline phosphatases (ALP), might correlate with immunotherapy resistance across six independent and pan-cancer cohorts gathering 599 patients: ONCOBIOTICS ($N = 111$),³⁵ PREMIS ($N = 185$),⁴¹ G2R ($N = 100$), MEDITREME ($N = 54$),⁴² METIMMOX ($N = 38$),⁴³ and AtezoTRIBE ($N = 111$) amenable to PD-1/PD-L1 and/or CTLA-4 blockade, alone or combined with other therapeutic modalities (Figure 7A; Table S5). An elevation of γ GT (defined as twice the upper limit of normal [ULN]: ≥ 45 U/L for

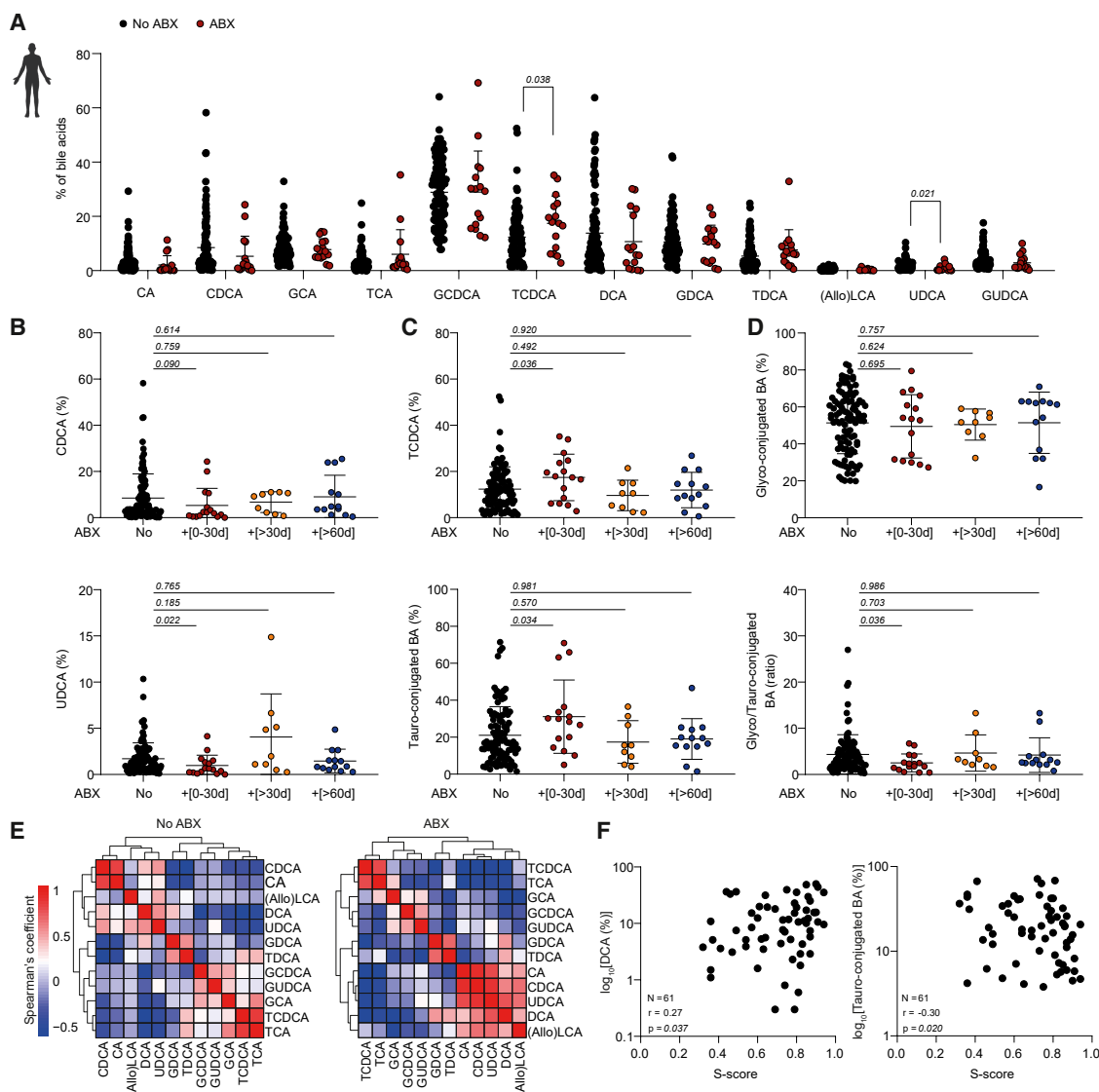


Figure 5. Gut dysbiosis alters BA composition in cancer patients

(A–D) Percentages of each BA among the total BAs in the plasma, assessed using metabolomics in 133 cancer patients ($n = 138$ samples) from the ONCOBIOTICS cohort who took ABX ($n = 16 + [0-30]$ days) (A–D); $n = 9 + [30-60]$ days) (B–D) or did not take ABX ($n = 100$) (A–D) within different time windows post-ABX cessation as well as for plasma ratio of glyco- over tauro-conjugated BAs (D, bottom). Error bars indicate mean $\pm$ SD. Comparisons between groups were performed using non-parametric Mann-Whitney U test (2 groups) in (A) and non-parametric Kruskal-Wallis H test (>2 groups) in (B)–(D).

(E) Clustering of Spearman's correlation coefficients of plasma BAs in patients who took ABX ($n = 13 + [0-30]$ days, left) or did not take ABX ($n = 85$, right).

(F) Spearman's correlations between distinct plasma BAs (in percentages of total BA) and the paired stool S-score, calculated in 61 ONCOBIOTICS patients before and until 15 days after immunotherapy initiation.

See also Figures S9–S11 and Tables S4, S5, and S6.

males and ≥ 35 U/L for females) was observed at baseline in $25.7\% \pm 3.9\%$ (mean $\pm$ SEM) of patients across these cohorts (Figure 7A; Tables S5). Among the 561 patients across the five cohorts, those above this threshold at immunotherapy initiation exhibited reduced progression-free survival (PFS) and/or OS in univariate analyses (Table S5; Figures 7B–7G; Figure S11A), except the AtezoTRIBE arm “no ICB” (Figure 7F). The Cox regression multivariate analyses adjusting for liver metastases and other prognostic factors (but not for co-medications or auto-

immune hepatitis) confirmed that γ GT was an independent negative prognostic parameter for PFS in ONCOBIOTICS ($p = 0.024$, Table S6), G2R ($p = 0.015$, Table S6), and MEDITREME ($p = 0.013$, Table S6), as well as for OS in PREMIS ($p = 0.014$, Table S6), MEDITREME ($p = 0.048$, Table S6), and AtezoTRIBE ($p = 0.039$, Table S6).

Similar results were obtained for elevated ALPs (ALP ≥ 1 ULN) that were associated with a dismal prognosis in univariate analyses (Figures S11B–S11D for ONCOBIOTICS,

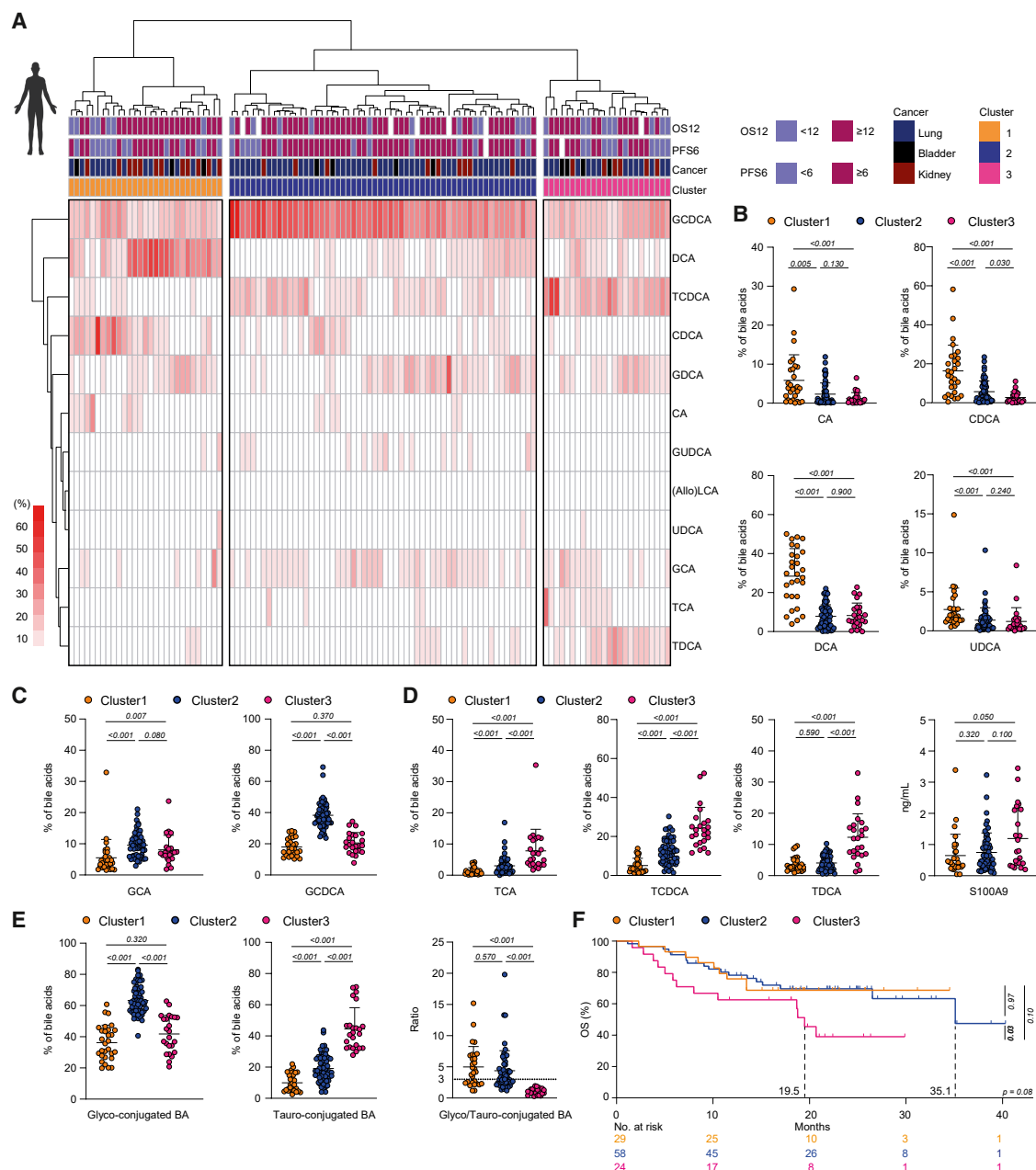


Figure 6. Cancer patients can be stratified based upon plasma BA composition

(A) Non-supervised hierarchical clustering of the percentages of plasma BAs in 111 ONCOBIOTICS patients before and until 42 days post ICB initiation. (B–E) Dot plots of the percentages of BAs or conjugated BAs and calprotectin (S100A9) measured in the plasma according to the three clusters illustrated in (A). Also refer to Table S4 and Figures S9 and S10. Each dot represents plasma levels in one patient ($n = 111$). Error bars indicate mean $\pm$ SD. Comparisons between groups were performed using non-parametric Kruskal-Wallis H test (>2 groups), followed by multiple-comparisons test controlling the false discovery rate (FDR). (F) Kaplan-Meier survival curves and univariate Cox regression to assess the prognostic value of each BA cluster (Cox-Mantel test). See also Figures S9–S11 and Tables S4, S5, and S6

METIMMOX, and MEDITREME) and in multivariate analyses, for PFS and OS in ONCOBIOTICS ($p = 0.002$ and 0.042 , respectively; Table S6) and for OS in MEDITREME (Table S6, $p = 0.017$). Immunotherapy tended to decrease γ GT concentrations in patients with PFS of ≥ 6 months, while the levels remained ≥ 2 ULN in patients who progressed within 6 months after ICB initiation (Figures 7H and S11E). This trend was not

observed for ALP levels (Figures S11F and S11G), although patients with elevated ALP at the start of immunotherapy had significantly reduced PFS in both ONCOBIOTICS and METIMMOX cohorts (Figures S11B and S11C). Moreover, circulating liver enzymes (γ GT, ALP, and transaminases) and soluble MAdCAM-1 (sMAdCAM-1) at baseline were positively and negatively correlated with tauro-conjugated BAs,

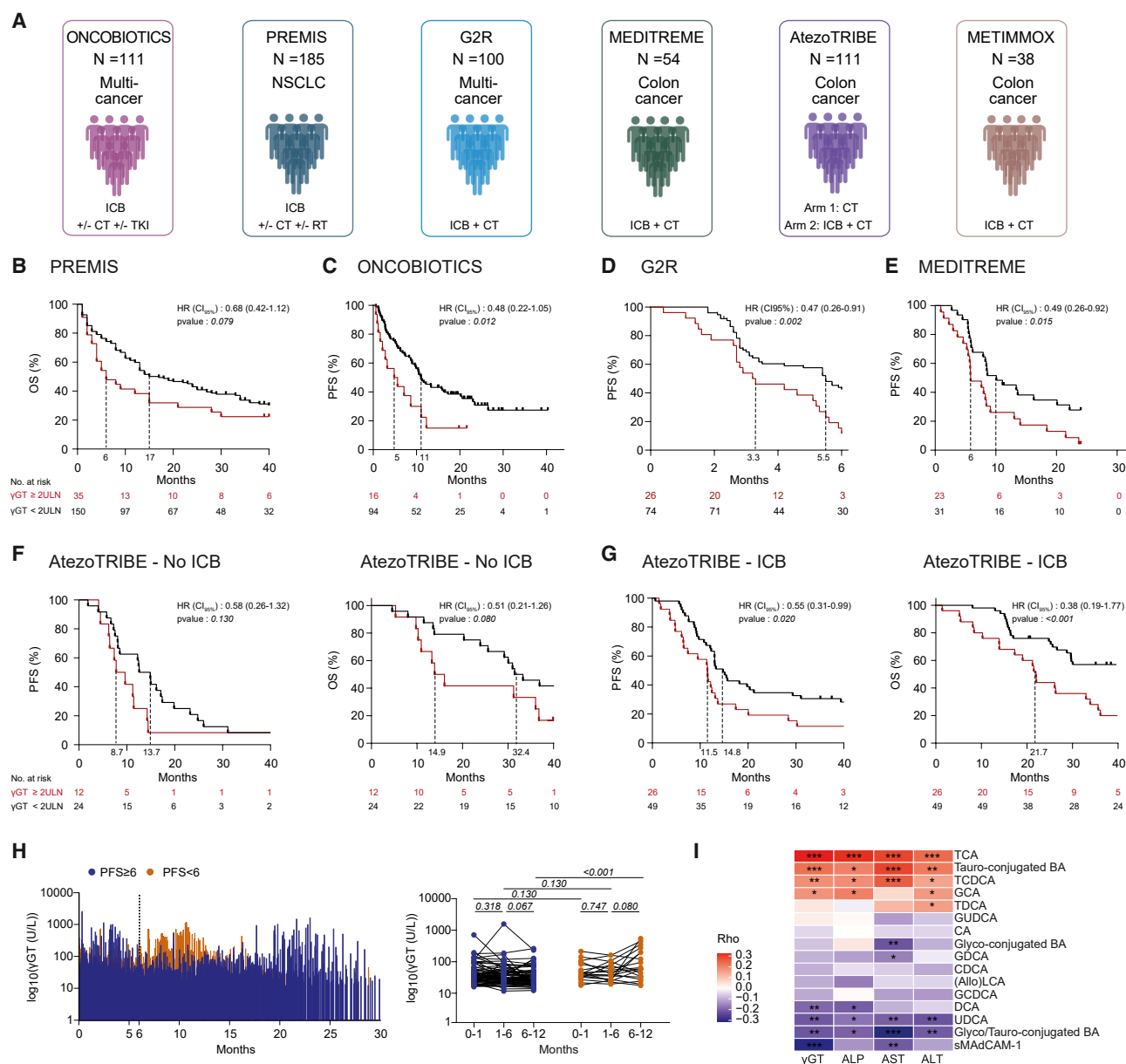


Figure 7. Subclinical cholestasis reduces cancer patient survival

(A) Patient cohorts amenable to immunotherapy (ICB), +/- chemotherapy (CT), radiotherapy (RT), or tyrosine kinase inhibitors (TKI) and randomized in 2 arms (AtezoTRIBE) for whom γ GT and alkaline phosphatase (ALP) levels were assessed in the plasma.

(B–G) Univariate analyses for overall survival (OS) (B, F, and G) and progression-free survival (PFS) (C–G), using the Kaplan-Meier estimator and Cox regression with indicated hazard ratios (HRs) and log rank (Mantel Cox) test p values to assess the prognostic value of γ GT in each cohort aligned in (A). Also refer to [Tables S5](#) and [S6](#) for univariate and multivariate analyses, respectively.

(H) Longitudinal evolution of the mean γ GT levels in U/L (scale in $\log_{10}$) for 111 patients who progressed (PFS < 6, orange) or not (PFS $\geq$ 6, blue) within 6 months of ICB initiation in the ONCOBIOTICS cohort, as well as for different periods of time. Comparisons between time points (0–1, 1–6, and 6–12 months) were performed using non-parametric Wilcoxon matched-paired test for each PFS group. Comparisons between groups (PFS < 6 or $\geq$ 6) were performed using non-parametric Kruskal-Wallis H test (>2 groups), followed by multiple-comparisons test controlling the false discovery rate (FDR).

(I) Clustering of Spearman's correlation coefficients between BAs (%), liver parameters, and sMAdCAM-1 (in ng/mL) in the plasma of patients from ONCOBIOTICS, FMT-LUMINATE, MIMIC, and AtezoTRIBE cohorts (p values: *, <0.05; **, <0.01; ***, <0.001). High γ GT levels were defined as $\geq$ 2ULN (ULN: male patients $\geq$ 45U/L; female patients $\geq$ 35U/L).

See also [Figures S9–S11](#) and [Tables S4, S5, and S6](#).

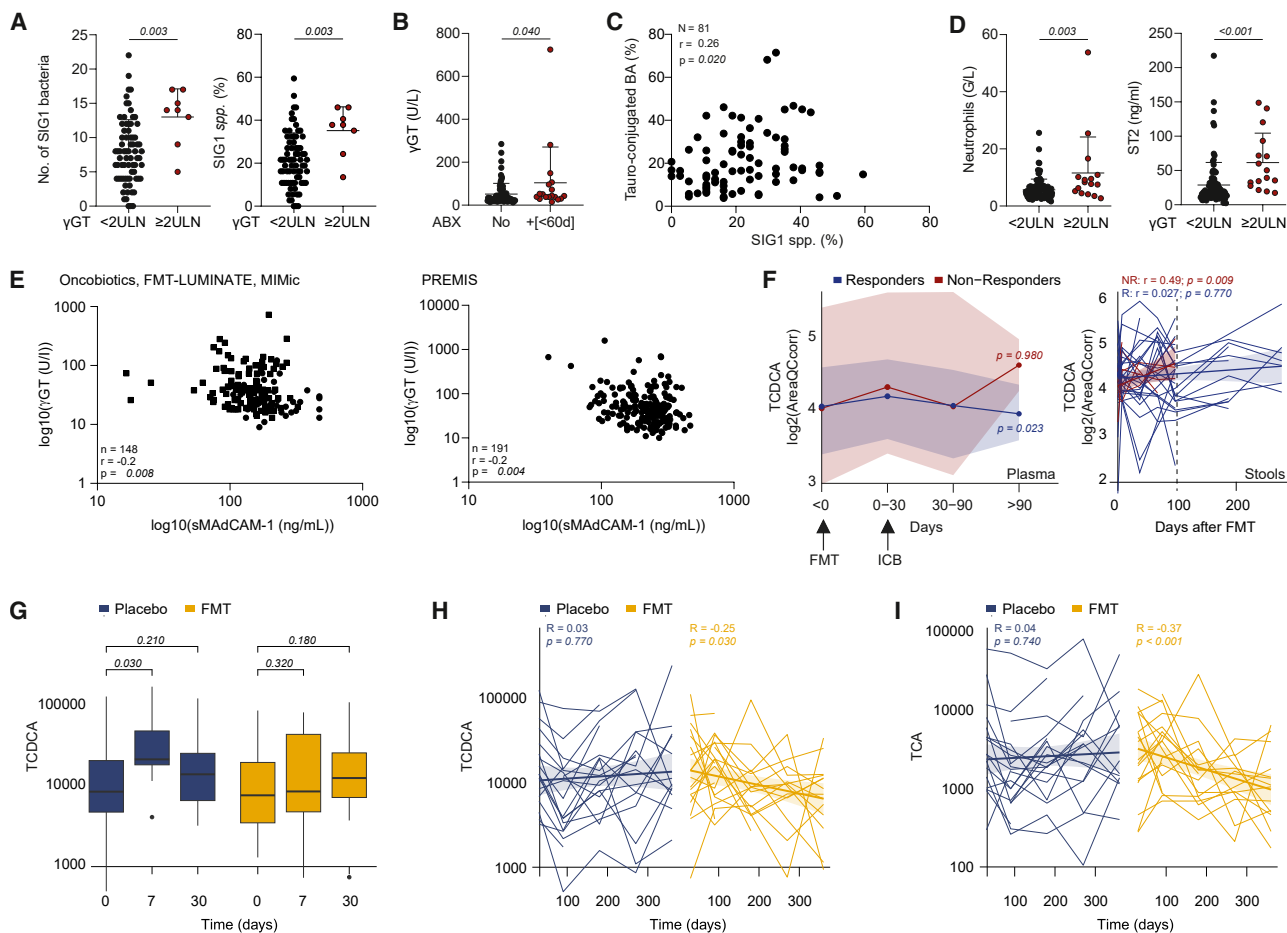


Figure 8. Subclinical cholestasis is a surrogate marker of gut dysbiosis

(A) Absolute number and percentages of SIG1 bacteria among the 37 SIG1³⁶ in patient feces according to plasma γ GT $<$ or $\geq$ 2 ULN ($n = 73$ and 8 samples, respectively).

(B) Plasma γ GT levels (in U/L) in patients treated with ABX according to the window of blood sampling before ABX ($n = 85$ in no ABX and 18 samples in ABX cessation less than 60 days, respectively).

(C) Spearman's correlation between percentages of tauro-conjugated BAs and SIG1 bacteria species among 37 SIG1 at baseline.³⁶

(D) Increase in neutrophil blood counts and plasma soluble ST2 levels in patients with γ GT $\geq$ 2 ULN versus those with γ GT $<$ 2 ULN ($n = 16$ and 95, respectively).

(E) Spearman's correlation between γ GT (U/L) level and sMadCAM-1 (ng/mL) in ONCOBIOTICS, FMT-LUMINATE, and MIMIC pooled cohorts and PREMIS.

(F) Trajectory of TCDCA (in mean and $CI_{95\%}$ Log2AreaQCCorr) in the plasma ($n = 32$ patients) or in the stools ($n = 34$ patients) of pooled cohorts and PREMIS. Comparison between responders and non-responders were performed using a longitudinal slope estimation approach. Left: the significance of temporal changes of plasma TCDCA levels within responders and non-responders was assessed from the estimated time effect in each group, and p values are annotated (related to STAR Methods). Right: Spearman's correlations between fecal TCDCA levels and days after FMT.

(G) Boxplots of raw values of TCDCA (AreaQCCorr) at three time points in TACITO ($n = 45$). The box shows the interquartile range (Q1–Q3), with the median marked inside. Whiskers extend up to $1.5 \times$ IQR beyond Q1 and Q3. Values outside the whiskers are plotted separately as outliers.

(H and I) Idem as in (F) right in the 2 arms of TACITO for TCDCA (H) and TCA (I), depicted in trajectories. Also refer to Figure S11I to visualize the differential evolution of all BAs in the two treatment arms. The significance of temporal changes of plasma TCDCA (H) and TCA (I) levels within placebo and FMT arms after the treatment start was assessed using Spearman's correlations, and p values and coefficients correlation (Rho) are annotated.

Comparisons between groups were performed using non-parametric Mann-Whitney U test (2 groups) (A, B, and D) or Kruskal-Wallis H test (>2 groups) (G), followed by multiple-comparisons test, controlling the false discovery rate (FDR). Error bars represent mean $\pm$ SD in (A)–(D).

See also Figures S9–S11 and Tables S4, S5, and S6.

respectively, in all patients from the pooled ONCOBIOTICS, as well as in the individual FMT-LUMINATE, MIMIC, and AtezoTRIBE cohorts (Figure 7I). The increased prevalence of immunosuppressive SIG1 bacteria (as defined by the S-score and Toposcore³⁶) in patients presenting elevated γ GT or tauro-conjugated BAs at baseline is in line with the observation

that γ GT levels are higher in patients after ABX uptake (Figures 8A–8C; Figure S11H). Moreover, γ GT levels correlated with blood markers of inflammation (neutrophils) and gut permeability (soluble ST2) (Figure 8D). In accordance with our previous report²¹ and Figure 7I, sMadCAM-1 and γ GT baseline plasma levels exhibited a negative correlation in all three

cohorts pooled together and PREMIS (Figure 8E, $p = 0.008$ and 0.004 , respectively). Altogether, subclinical cholestasis defined by γ GT can be considered as a proxy of gut dysbiosis.

Finally, we addressed whether FMT might avoid the accumulation of tauro-conjugated BAs associated with gut dysbiosis and resistance to immunotherapy in cancer patients. Using a linear mixed-effect model approach to predict trajectory slope, we analyzed the evolution of each plasma BA pre- and post-FMT in responders versus non-responders from the MIMIC and FMT-LUMINATE trials, using three time points of plasma metabolomics.^{37,38,44} Notably, tauro-CDCA (TCDCA) followed an ascending slope in non-responders but a stable or descending slope in responders (Figure 8F, left). Concomitantly, the levels of TCDCA were increased in the stool of the non-responder patients (Figure 8F, right; $p = 0.009$). We validated this observation in the TACITO trial randomizing metastatic renal cell carcinoma (mRCC) patients into FMT versus placebo, in addition to the combination of tyrosine kinase inhibitor and ICB, showing a 30% increase in patients without progression at 12 months in the FMT arm.⁴⁵ Plasma levels of TCDCA increased in the first week of therapy in the placebo but not in the FMT arm (Figure 8G, $p = 0.03$), and a descending slope of TCDCA and TCA was observed in the FMT group over the first year of follow-up, but not in placebo (Figures 8H and 8I; Figure S11).

Altogether, FMT can be considered as a therapy preventing dysbiosis-associated accumulation of tauro-conjugated BAs over the course of the disease and/or treatment.

DISCUSSION

This study links gut dysbiosis with abnormalities in BA recirculation to the tumor, compromising tumor immunosurveillance and hence causing resistance to ICB in murine malignancies distant from the enterohepatic cycle. In particular, the abnormal synthesis or recirculation of TCDCA or α - β - ω -MCA (FXR antagonists) and the loss of secondary BAs of the classic pathway (DCA, an FXR agonist, and GDCA/TDCA derivatives) act in concert to compromise cancer immunosurveillance during ABX uptake or *E. clostridioformis*-enforced gut dysbiosis.

Our data extend previous findings using BA oral gavage in mice^{19,21} and others demonstrating the aberrant activity of the BAAT enzyme in liver cancers culminating in TCDCA synthesis and exhaustion of TILs.⁹ In mice, GDCA (and its derivative DCA, an FXR agonist), FMT, or the ileal FXR agonist fexaramine could dampen plasma levels of TCDCA and/or α - β - ω -MCA, notoriously FXR antagonists, while surgical BDL did the contrary, phenocopying the effects of ABX. Accordingly, fexaramine and GDCA supplementation of dysbiotic mice prevented CD4⁺ effector T cell and T_{pex} exhaustion (characterized by Lag-3, Tox, and Tim-3 expression) in TILs. Hence, the balance between FXR agonists and antagonists in the ileum might regulate immunosurveillance of tumors located at distal sites from the intestines.

The prognostic value of baseline γ GT levels had long been underestimated until Winter et al. reported their independent negative impact on OS.⁴⁶ Our results across six independent cohorts of cancer patients corroborated this original study, suggesting that γ GT elevations may be added to the routine panels that identify patients unlikely to respond to immunotherapy, presumably as a consequence of a disabled gut-liver

axis. Therapeutic strategies (such as UDCA⁴⁷ or obeticholic acid¹) to curtail liver cholestasis aim at increasing BA flow, reducing BA reabsorption from the intestine, shutting down BA synthesis in the liver, and/or triggering intestinal FGF15/FGFR- β Klotho signaling. However, in our hands, UDCA inconsistently circumvented primary resistance to α -PD-1 mAb induced by ABX/*E. clostridioformis*. In contrast, oral GDCA supplementation restored immunotherapy responses in dysbiotic mice. A proof-of-concept phase 1 trial showed that GDCA-containing capsules repressed primary BA synthesis without impacting glucose and energy metabolism.⁴⁸ FMT represents another option for dysbiotic cancer patients. Indeed, subclinical cholestasis correlated with low Toposcore or ABX uptake, downregulation of sMAdCAM-1, and systemic inflammation in our study, in accordance with previous reports suggesting that intestinal microbiota plays an important role in the pathophysiology of cholestatic liver disease.^{49,50} Pioneering studies have evaluated the interceptive function of probiotics, such as *Lactobacillus rhamnosus* GG, in cholestatic liver diseases.^{51,52} Probiotics could decrease the T- β -MCA FXR antagonist, unleashing liver FXR activity. Similarly, gut microbiota not only regulates secondary BA metabolism but also inhibits BA synthesis in the liver by alleviating FXR inhibition in the ileum.⁵³ Therefore, restoring a healthy intestinal commensalism may avoid cancer-associated subclinical cholestasis to facilitate adaptive anticancer immune responses. Prospective clinical trials may stratify cancer patients based on various proxies of gut dysbiosis including subclinical cholestasis to predict primary resistance to cancer immunotherapy.

Limitations of the study

In murine models, *Enterocloster* spp.-induced gut dysbiosis was not *stricto sensu* mimicking that mediated by ABX. The use of stools from ABX-treated patients for a transfer into avatar mouse models would have been an additional line of evidence that gut dysbiosis is clinically significant to dampen ICB efficacy. Demonstration of a causal link between GDCA compensatory effects on dysbiosis and endothelial MAdCAM-1 function would have required endothelium-restricted *Nr1h4/Fxr* gene deletion. Therefore, this study cannot exclude alternative BA sensing routes. Multivariable analyses testing the prognostic value of γ GT in cancer patients took into account only ABX and liver metastases but failed to consider other confounding factors such as hepatic autoimmune reactions,⁴⁶ alcohol consumption, several co-medications, or the socioeconomic status. Prospective studies including these extrinsic factors should confirm these findings.

RESOURCE AVAILABILITY

Lead contact

Request for further information and resources should be directed to and will be fulfilled by the lead contact, Laurence Zitvogel (laurence.zitvogel@gustaveroussy.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The bulk RNA sequencing of murine hepatocytes, cholangiocytes, and enterocytes (refer to Figure S3) and the 16S sequencing data of murine fecal samples (refer to Figures S2 and S5) have been deposited in the ENA (European

Nucleotide Archive) database under submission number [ERA35991104](#), with project number ENA: [PRJEB109926](#). Raw and processed data of the single-cell RNA sequencing of murine immune cells from tumors and tDLNs (refer to [Figures 4](#) and [S7](#)) have been deposited in the Gene Expression Omnibus (GEO) under accession number GEO: [GSE326943](#). Human and mouse metabolomic data supporting the claims of the manuscript are available in Mendeley Data: <https://doi.org/10.17632/wsydn677rw.1>. Human metagenomic data for S-score and Toposcore calculations for [Figures 5F](#), [8A](#), and [8C](#) are available in Mendeley Data: <https://doi.org/10.17632/wsydn677rw.1>. No new code was generated in this study. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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Conceptualization, L.Z.; data curation, A.L.M.d.L.V., M.F., M.A.V., C.d.I.C.-F., F.-X.D., Y.H., and M.D.; formal analysis, A.L.M.d.L.V., M.F., C.T., I.L., A.-L.T., S.T., M.A.V., Y.H., T.-H.P., D.S., V.I., S.J., G.V., D.K., Y.F., and R.A.Q.; investigation, A.L.M.d.L.V., M.F., C.T., I.L., M.M., A.-L.T., S.T., S.L., O.M., H.G.d.T., M.A.V., P.L., E.R., B.T.A., S.D., G.V. F.A., M.L., S.F., O.K., C.d.I.C.-F., P.-E.F., F.G., D.K., Y.F., R.A.Q., C.A.C.S., L.D., G.K., L.Z., F.-X.D., C. Ciccarese, and R.I.; methodology, L.Z., G.K., A.L.M.d.L.V., M.F., C.T., I.L., M.M., A.-L.T., S.T., S.L. S.D., F.A., M.A.V., M.L., S.F., O.K., C.d.I.C.-F., A.S., N.W., D.K., Y.F., and R.A.Q.; resources, F.M., C. Cremolini, S.M.V., J.G.L., A.M., F.-X.D., M.D., C.T., F.G., A.H.R., P.A.B., S.M., S.P., A.E., B.R., C. Ciccarese, R.I., G.I., A.S., and N.W.; software, M.F., M.A.V., C.d.I.C.-F., Y.H., T.-H.P., D.S., and V.I.; supervision, L.Z., G.K., M.F., and L.D.; validation, all authors; visualization, A.L.M.d.L.V., M.F., A.-L.T., S.T., M.A.V., S.L., S.F., Y.H., M.L., C.d.I.C.-F., D.K., Y.F., R.A.Q., and V.I.; writing – review & editing, L.Z., G.K., M.F.; A.L.M.d.L.V., A.-L.T., S.T., L.D. T.-H.P., and Y.H.; project administration, L.Z., G.K., A.L.M.d.L.V., M.F., A.-L.T., and S.T.; funding acquisition, L.Z. and G.K.

DECLARATION OF INTERESTS

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STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- [EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS](#)
 - Human
 - Experimental model

METHOD DETAILS

- Blood sample collection for ONCOBIOTICS, MIMic and FMT-LU-MINATE
- Stool samples collection for ONCOBIOTICS
- Human and mouse plasma sample preparation and bile acid detection by UHPLC-MS
- sST2, sMAdcAM-1 and S100A9 quantification in patients
- Spectral flow cytometry on PBMC
- Slope-prediction method
- Clinical cox models
- Patients clustering based on circulating bile acids composition
- Oral bacterial gavage with commensal species or BAs
- FMT experiments
- Subcutaneous cancer mouse models
- Statistical analysis of tumor growth
- Surgical bile duct ligation
- MCA205 fibrosarcoma knockout mouse model
- Flow cytometry
- Tracing migration of leukocytes from the gut to the TME: CFSE staining of mLN cells up to TME or tDLNs
- RNA extraction and RT-qPCR lysis and extraction protocols for mouse samples
- Quantitative gene expression assay
- Bulk RNA-sequencing for hepatocytes, cholangiocytes and enterocytes
- Single cell RNA-sequencing for tDLN and tumors
- Ileal, colonic, liver, tumor tissues preparation and bile acid detection by UHPLC-MS
- Mouse clustering based on bile acids composition
- Cell lines and treatment
- PCR amplification and TIDE analysis
- *In vitro* T cell culture and bile acid exposure
- *Enterocloster* spp. treatment with BA
- Genome assembly and BSH identification of strain *Enterocloster clostridioformis* 141
- Distribution and diversity analysis of BSH in *Enterocloster* genomes
- Cancer cell viability after BA *in vitro* treatment
- DNA extraction and 16S rRNA targeted sequencing of mouse stools
- Bioinformatics, statistics and reproducibility of 16S rRNA targeted sequencing data

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor(R) 700 anti-mouse CD45	Biolegend	Cat# 103128; RRID:AB_493715
PerCP anti-mouse CD45	Biolegend	Cat# 103130; RRID:AB_893339
CD3 ϵ Antibody, anti-mouse, APC-Vio® 770, REAfinity™	Miltenyi Biotec	Cat# 130-117-676; RRID:AB_2733115
CD4 Antibody, anti-mouse, VioBlue®, REAfinity™	Miltenyi Biotec	Cat# 130-118-568; RRID:AB_2733943
PE/Dazzle(TM) 594 anti-mouse CD8a	BioLegend	Cat# 100762; RRID:AB_2564027
Brilliant Violet 650(TM) anti-mouse CD25	BioLegend	Cat# 102038; RRID:AB_2563060
ROR gamma (t) Monoclonal Antibody (B2D), PE-Cyanine7, eBioscience	Thermo Fisher Scientific	Cat# 25-6981-82; RRID:AB_2784671
FOXP3 Monoclonal Antibody (FJK-16s), APC, eBioscience	Thermo Fisher Scientific	Cat# 17-5773-82; RRID:AB_469457
APC/Cyanine7 anti-mouse CD3	Biolegend	Cat# 100222; RRID:AB_2242784
Brilliant Violet 605(TM) anti-mouse CD4	BioLegend	Cat# 116027; RRID:AB_2800581
FITC anti-mouse CD279 (PD-1)	BioLegend	Cat# 135214; RRID:AB_10680238
Brilliant Violet 421(TM) anti-mouse CD186 (CXCR6)	BioLegend	Cat# 151109; RRID:AB_2616760
Brilliant Violet 650(TM) anti-mouse CD183 (CXCR3)	BioLegend	Cat# 126531; RRID:AB_2563160
TIGIT Monoclonal Antibody (GIGD7), PerCP-eFluor™ 710, eBioscience	Thermo Fisher Scientific	Cat# 46-9501-82; RRID:AB_11150967
TOX Antibody, anti-human/mouse, APC, REAfinity™	Miltenyi Biotec	Cat# 130-118-335; RRID:AB_2751485
PE anti-mouse TCF-1 (C63D9)	Cell signaling Technology	Cat# 14456; RRID:AB_2798483
PE anti-TCF1 (TCF7)	BioLegend	Cat# 655208; RRID:AB_2728492
Brilliant Violet 421(TM) anti-mouse CD223 (LAG-3)	BioLegend	Cat# 125221; RRID:AB_2572080
Brilliant Violet 650(TM) anti-mouse/human CD44	BioLegend	Cat# 103049; RRID:AB_2562600
CD137 (4-1BB) Monoclonal Antibody (17B5), APC, eBioscience	Thermo Fisher Scientific	Cat# 17-1371-82; RRID:AB_2573162
Alexa Fluor(R) 700 anti-mouse CD62L	BioLegend	Cat# 104426; RRID:AB_493719
CD278 (ICOS) Monoclonal Antibody (C398.4A), PE-Cyanine7, eBioscience	Thermo Fisher Scientific	Cat# 25-9949-82; RRID:AB_2573566
FITC anti-mouse Ki-67	BioLegend	Cat# 652410; RRID:AB_2562141
Brilliant Violet 421(TM) anti-human CD197 (CCR7)	BioLegend	Cat# 353208; RRID:AB_11203894
Human Integrin alpha 4 beta 7/LPAM-1 (Research Grade Vedolizumab Biosimilar) Alexa Fluor® 647-conjugated Antibody	R and D Systems	Cat# FAB10078R; RRID:AB_3644725
PE/Dazzle(TM) 594 anti-human CD274 (B7-H1, PD-L1)	BioLegend	Cat# 329732; RRID:AB_2616889
Hu CD273 RB780 MIH18 50 μ g	BD Biosciences	Cat# 755831; RRID:AB_3688138
CD194 (CCR4)	BD Biosciences	Cat# 562906; RRID:AB_2737882
Hu CD196 (CCR6) BUV661 11A9 50 μ g	BD Biosciences	Cat# 750696; RRID:AB_2874817
PE/Fire™ 640 anti-human CD103 (Integrin α E)	BioLegend	Cat# 350243; RRID:AB_2924550
Hu CD1c BUV737 F10/21A3 50 μ g	BD Biosciences	Cat# 748723; RRID:AB_2873127

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
PE/Fire(TM) 810 anti-human CD183 (CXCR3)	BioLegend	Cat# 353759; RRID:AB_2894484
Hu CXCR5 BUV615 RF8B2 50 µg	BD Biosciences	Cat# 751293; RRID:AB_2875306
Hu Fc Epsl R1 Alp BV480 AER-37 50 µg	BD Biosciences	Cat# 747779; RRID:AB_2872243
Spark Red(TM) 718 anti-human/mouse/rat CD278 (ICOS)	BioLegend	Cat# 313563; RRID:AB_2936693
IgA Antibody, anti-human, APC-Vio® 770, REAfinity™	Miltenyi Biotec	Cat# 130-116-884; RRID:AB_2727742
IgG BV480	BD Biosciences	Cat# 746341; RRID:AB_2743662
Brilliant Violet 510(TM) anti-human IgM	BioLegend	Cat# 314522; RRID:AB_2562916
Brilliant Violet 750(TM) anti-human CD279 (PD-1)	BioLegend	Cat# 329965; RRID:AB_2810504
Spark Violet(TM) 423 anti-human CD123	BioLegend	Cat# 396719; RRID:AB_2910457
CD148 BUV395	BD Biosciences	Cat# 745674; RRID:AB_2743163
Brilliant Violet 570(TM) anti-human CD16	BioLegend	Cat# 302035; RRID:AB_10915988
Hu CD21 BUV805 B-ly4 50 µg	BD Biosciences	Cat# 742008; RRID:AB_2871306
CD24 (Heat Stable Antigen)	BD Biosciences	Cat# 564521; RRID:AB_2738834
PE/Fire(TM) 700 anti-human CD25	BioLegend	Cat# 356145; RRID:AB_2876678
Brilliant Violet 650(TM) anti-human CD3	BioLegend	Cat# 300468; RRID:AB_2629574
Brilliant Violet 711(TM) anti-human CD38	BioLegend	Cat# 303528; RRID:AB_2563811
Brilliant Violet 650(TM) anti-human CD40	BioLegend	Cat# 334338; RRID:AB_2566209
Spark Blue(TM) 574 anti-human CD45	BioLegend	Cat# 368557; RRID:AB_2904402
Hu CD5 BUV563 UCHT2 50 µg	BD Biosciences	Cat# 741354; RRID:AB_2870855
Hu CD71 BUV496 M-A712 50 µg	BD Biosciences	Cat# 750652; RRID:AB_2874780
CD88 (C5a Receptor)	BD Biosciences	Cat# 742320; RRID:AB_2740700
Cytek® 25-Color Immunoprofiling Assay, cFluor® Reagent Kit (18C)		Cat# R7-40002
IgD anti-human cFluor® BYG667	Cytek Bioscience	Cat# R7-40002
CD11c anti-human cFluor® BYG781	Cytek Bioscience	Cat# R7-40002
CD14 anti-human cFluor® B548	Cytek Bioscience	Cat# R7-40002
CD19 anti-human cFluor® R685	Cytek Bioscience	Cat# R7-40002
CD4 anti-human cFluor® YG584	Cytek Bioscience	Cat# R7-40002
CD45RA anti-human cFluor® V450	Cytek Bioscience	Cat# R7-40002
CD8 anti-human cFluor® B532	Cytek Bioscience	Cat# R7-40002
HLA-DR anti-human cFluor® B690	Cytek Bioscience	Cat# R7-40002
CD127 anti-human cFluor® R659	Cytek Bioscience	Cat# R7-20078
CD27 anti-human cFluor® R840	Cytek Bioscience	Cat# R7-20082
TruStain FcX(TM) PLUS (anti-mouse CD16/32)	Biolegend	Cat# 156604; RRID:AB_2783138
Pacific Blue(TM) anti-mouse CD4	BioLegend	Cat# 100531; RRID:AB_493374
Brilliant Violet 650(TM) anti-mouse CD8a	BioLegend	Cat# 100742; RRID:AB_2563056
FITC anti-human/mouse Granzyme B	BioLegend	Cat# 515403; RRID:AB_2114575
PE/Cyanine7 anti-mouse IFN-γ	BioLegend	Cat# 505826; RRID:AB_2295770
Ki-67 Monoclonal Antibody (SolA15), APC, eBioscience	Thermo Fisher Scientific	Cat# 17-5698-82; RRID:AB_2688057
InVivoMAb anti-mouse PD-1 (CD279)	BioXCell	Cat# BE0146; RRID:AB_10949053
InVivoMAb rat IgG2a isotype control, anti-trinitrophenol	BioXCell	Cat# BE0089; RRID:AB_1107769
InVivoMAb anti-mouse CTLA-4 (CD152)	BioXCell	Catalog #BE0164; RRID:AB_10949609

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
InVivoMAb mouse IgG2b isotype control, unknown specificity	BioXCell	Cat# BE0086; RRID:AB_1107791
Anti-mouse CD16/CD32	eBioscience	Cat# 14-0161-85; RRID:AB_467134
TruStain FcX™ PLUS (anti-mouse CD16/32) antibody	Biolegend	Cat# 156604; RRID:AB_2783137
Bacterial and virus strains		
<i>Enterocloster clostridioformis</i>	This lab	Fidelle, 2023
EB BAA613 - <i>Enterocloster bolteae</i>	ATCC	BAA-613
EC 25537 - <i>Enterocloster clostridioformis</i>	ATCC	25537
Chemicals, peptides, and recombinant proteins		
Zombie Aqua™ Fixable Viability Kit	Biolegend	Cat# 423102
LIVE/DEAD™ Fixable Aqua dye	Thermo Fisher Scientific	Cat# L34966
CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry	Thermo Fisher Scientific	Cat# C34554
LIVE/DEAD™ Fixable Blue Dead Cell Stain	Invitrogen™	Cat# L23105
BD Horizon™ Brilliant Stain Buffer Plus	BD Biosciences	Cat# 566385
PE Streptavidin	Biolegend	Cat# 405203
Anti-SynCAM (TSLC1/CADM1) mAb-Biotin	MBL	Cat# CM004-6
Cholic acid, CA	Avanti	Cat# 700212P
Chenodeoxycholic acid, CDCA	Sigma-Aldrich	Cat# C8261
α-muricholic acid, α-MCA	Avanti	Cat# 700232P
β-muricholic acid, β-MCA	Avanti	Cat# 700233P
Deoxycholic acid, DCA	Sigma-Aldrich/Thermo Fisher Scientific	Cat# 30960/148140250
Hyochoic acid, HCA	Avanti	Cat# 700159P
Hyodeoxycholic acid, HDCA	Avanti	Cat# 700214P
12-dehydrocholic acid, 12-dDCA	Avanti	Cat# 700215P
Lithocholic acid, LCA	Thermo Scientific Chemicals	Cat# 229090050
Ursodeoxycholic acid, UDCA	Thermo Scientific Chemicals	Cat# 208590250
Tyrosine-conjugated cholic acid, Tyro-CA	Generously provided by Kenya Honda, Keio University School of Medicine, Tokyo, Japan	NA
Glycocholic acid, GCA	Avanti	Cat# 700265P
Glycodeoxycholic acid, GDCA	Sigma-Aldrich	Cat# G9910
Glycohyocholic acid, GHCA	MedChemExpress	Cat# HY-122141
Glycohyodeoxycholic acid, GHDC	Avanti	Cat# 700264P
Taurocholic acid sodium salt hydrate, TCA	Sigma-Aldrich	Cat# T4009
Sodium taurochenodeoxycholate, TCDCA	Sigma-Aldrich	Cat# T6260
Taurodeoxycholic acid sodium salt, TDCA	Millipore	Cat# 580221
Taurohyodeoxycholic acid sodium salt, THDCA	Avanti	Cat# 700248P
Tauroolithocholic acid sodium salt, TLCA	Avanti	Cat# 700252P
Tauroursodeoxycholic acid sodium salt, TUDCA	Millipore	Cat# 580549
Cilofexor	MedChemExpress	Cat# HY-109083
Murideoxycholic acid, MDCA	Avanti	Cat# 700270P
ω-muricholic acid, ω-MCA	Avanti	Cat# 700231P
Tauro-α-muricholic acid sodium salt, T-α-MCA	Avanti	Cat# 700243P
Tauro-β-muricholic acid sodium salt, T-β-MCA	Avanti	Cat# 700244P

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tauro- ω -muricholic acid sodium salt, T- ω -MCA	Avanti	Cat# 700245
Tauro- γ -muricholic acid sodium salt, THCA	Avanti	Cat# 700246P
Recombinant mouse IL-2	BioLegend	Cat# 575406
Ampicillin	Sigma-Aldrich	Cat# A9518
Streptomycin	Sigma-Aldrich	Cat# A9137
Colistin	Sigma-Aldrich	Cat# C4461
Fexaramine	MedChemExpress	Cat# 574013-66-4
Liberase	Roche	Cat#05 401 119 001
DNase1	Roche	Cat# 04 716 728 001
Recombinant murine TNF- α	PeptoTech	Cat# 315-01A
Mitoxantrone	Sigma-Aldrich	Cat# M6545
Oxaliplatin	Accord	NA
Hoechst 33342	Thermo Scientific	Cat# R37165
Propidium iodide	Sigma-Aldrich	Cat# P4864

Critical commercial assays

eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat# 00-5523-00
MojoSort™ Mouse CD3 T cell Isolation Kit	BioLegend	Cat# 480031
PureLink™ Genomic DNA Mini Kit	Thermo Fisher Scientific	Cat# K182002
Platinum™ SuperFi II PCR Master Mix	Thermo Fisher Scientific	Cat# 12368010
PureLink™ PCR Micro Kit	Thermo Fisher Scientific	Cat# K310250
CD45 microbeads	Miltenyi	Cat# 130-052-30
BD™ Mouse Single Cell Sample Multiplexing Kit	BD	Cat# 633793
BD Rhapsody™ Whole Transcriptome Analysis (WTA) Amplification Kit	BD	Cat# 633801
BD Rhapsody™ Whole Transcriptome Analysis (WTA) Reagent Kit	BD	Cat# 665915
Lamina Propria Dissociation kit	Miltenyi	Cat# 130-097-410
CD326 (EpCAM) MicroBeads	Miltenyi	Cat# 130-105-958
Liver Perfusion kit	Miltenyi	Cat# 130-128-030
RNAeasy Mini kit	Qiagen	Cat# 74106
Dynabeads® Magnetic Beads	Gibco	Cat# 11452D
eBioscience™ Cell Stimulation Cocktail	Thermo Fisher Scientific	Cat# 00-4975-93

Deposited data

The bulk RNA sequencing of murine hepatocytes, cholangiocytes, and enterocytes from the murine experiment	This paper	ENA (European Nucleotide Archive): PRJEB109926
16S rRNA sequencing data of murine fecal samples from the murine experiment	This paper	ENA (European Nucleotide Archive) : PRJEB109926
Single cell RNA sequencing of immune cells from tumor and tumor draining lymph nodes from the murine experiment	This paper	GEO: GSE326943
Metabolomic data in mouse from the murine experiments	This paper	Mendeley Data: https://doi.org/10.17632/wsydn677rw.1
Metabolomic data in human from the ONCOBIOTICS, MIMIC and FMT-LUMINATE trials	This paper	Mendeley Data: https://doi.org/10.17632/wsydn677rw.1

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
MCA205 fibrosarcoma	American Type Culture Collection	N/A
RET melanoma	Provided by Viktor Umansky (DKFZ—German Cancer Research Center)	N/A
Transformed sinusoidal endothelial cells (TSECs)	Provided by B.P. Fennimore (University of Colorado Anschutz Medical Campus, Aurora, CO, USA)	N/A
TSECs with knockout of three bile acid receptors-TGR5 (gene: Gbpar1), FXR (gene: Nr1h4), and VDR (gene: Vdr)	This paper	N/A
HT29	ATCC	HTB-38
Oligonucleotides		
<i>Nr1h4</i> ^{-/-} detection primer A	Eurofins	ATGGGCCTTTTCAGAATGCCG
<i>Nr1h4</i> ^{-/-} detection primer B	Eurofins	TCAAGGCTCATCCGGGTGAT
<i>Gpbar1</i> ^{-/-} detection primer A	Eurofins	ACCATCAGGGCTACTGGTCC
<i>Gpbar1</i> ^{-/-} detection primer B	Eurofins	TGCTGCATTGGCTACTGGTG
<i>Vdr</i> ^{-/-} detection primer A	Eurofins	GCCAGAACCTCAGCTTGGTC
<i>Vdr</i> ^{-/-} detection primer B	Eurofins	CATGATAAGGCATCGGCGCT
Software and algorithms		
Statistical environment R	http://www.R-project.org/	N/A
Prism 10	GraphPad, San Diego, CA, USA	N/A
Other		
Edit-R All-in-one Lentiviral (knockout) Mouse <i>Nr1h4</i> sgRNA	Horizon Discovery	Cat# catalog no. VSGM11942-247929487 Target sequence: AGTGCCAGGAGTGCCGGCTA
Edit-R All-in-one Lentiviral (knockout) Mouse <i>Gpbar1</i> sgRNA	N/A	Cat# VSGM11942-247972009 Target sequence: ATGAGCGTCGCCCACCACTA
Edit-R All-in-one Lentiviral (knockout) Mouse <i>Vdr</i> sgRNA	N/A	Cat# VSGM11942-248142193 Target sequence: TTCGTGCAGACGTAAGTACA

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human

Study participant details

Data were derived from eight prospective clinical trials, comprising both observational and interventional design.

1. ONCOBIOTICS

- **ClinicalTrials.gov ID:** [NCT04567446](https://clinicaltrials.gov/ct2/show/study/NCT04567446)
- **Type:** Observational design
- **Description:** ONCOBIOTICS is a multicenter observational study conducted across 14 sites in France and Canada. It investigates the impact of the microbiome on clinical outcomes in patients with advanced non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), and bladder cancer (BC) treated with anti-PD-(L)1 therapies, either alone or combined with chemotherapy or tyrosine kinase inhibitors. Patients were followed according to standard care until disease progression, intolerable toxicity, or up to two years of immune checkpoint inhibitor (ICI) treatment. Detailed eligibility criteria, baseline data, and laboratory tests including baseline gamma-glutamyl transferase (GGT), are provided in the trial protocol and recorded via electronic case report forms. All participants provided written informed consent. Blood and stool samples were collected at baseline. Available samples were used in this analysis, with availability varying based on patient consent, sample pairing, volume constraints, and site-level data reporting. Blood samples were used for metabolomics analysis, specifically for TDCA quantification in the MVA (multivariate analysis), following Alves Costa Silva et al., 2024.⁵⁴ Stool samples were used to calculate the Toposcore via metagenomic analysis, following the methodology described in Derosa et al., *Cell*, 2024.³⁶

2. MEDITREME

- ClinicalTrials.gov ID: NCT03202758
 - Type: Interventional design
 - Description: Clinical data were obtained from the phase II clinical trial NCT03202758, which enrolled 54 patients with histologically confirmed metastatic colorectal cancer harboring a **KRAS mutation**. All patients received a combination treatment consisting of Durvalumab (750 mg every 2 weeks), **Tremelimumab (75 mg every 4 weeks)**, and **FOLFOX chemotherapy**. Eligible patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. All participants provided written informed consent and any locally required authorizations prior to undergoing protocol-related procedures, including screening assessments. Eligibility criteria, baseline characteristics, and concomitant medications were recorded in **eCRFs** and are detailed in the trial protocol available on [ClinicalTrials.gov](https://clinicaltrials.gov). For this study, we used de-identified clinical data collected from the trial, focusing in particular on **GGT** levels. These data were used for Kaplan–Meier survival analyses and multivariable analyses (MVA) to explore associations between GGT levels and clinical outcomes.
3. METIMMOX
- ClinicalTrials.gov ID: NCT03388190
 - Type: Interventional design
 - Description: Clinical data were obtained from the NCT03388190 study, a multicenter, open-label, randomized phase II trial evaluating first-line treatment in patients with microsatellite stable (MSS)/mismatch repair-proficient (pMMR) metastatic colorectal cancer. Patients were randomized to receive either the oxaliplatin-based standard-of-care Nordic FLOX regimen (control arm) or a sequential treatment with Nordic FLOX followed by nivolumab (experimental arm). The primary objective was to determine whether the experimental arm improved progression-free survival (PFS), with secondary endpoints including safety, tolerability, and quality of life. The trial was approved and conducted as per Norwegian legislation, and all participants provided written informed consent. Eligibility criteria, baseline characteristics, and concomitant medications were documented in eCRFs, with full criteria available in the trial protocol on [ClinicalTrials.gov](https://clinicaltrials.gov). For the purpose of this study, we used de-identified clinical data from this trial, focusing particularly on the ALP variable for Kaplan–Meier survival analyses.
4. G2R
- ClinicalTrials.gov ID: NCT04169841
 - Type: Interventional design
 - Description: The NCT04169841 phase II Study Evaluate the Efficacy of a Double Immunotherapy by Durvalumab and Tremelimumab Combined With Olaparib in Patients With Solid Cancers and Carriers of Homologous Recombination Repair Genes Mutation in Response or Stable After Olaparib Treatment. Eligibility criteria and baseline data, including recent medications, are in the trial protocol and recorded in electronic case reports. Full eligibility criteria are listed in the trial protocol (available at www.clinicaltrials.gov). All patients must give signed informed consent. For this study, we used de-identified clinical data collected from the trial, focusing in particular on **GGT** levels. These data were used for Kaplan–Meier survival analyses and multivariable analyses (MVA) to explore associations between GGT levels and clinical outcomes.
5. ATEZOTRIBE
- **ClinicalTrials.gov:** [NCT03721653](https://clinicaltrials.gov/ct2/show/study?term=NCT03721653)
 - Type: Interventional design
 - Description: AtezoTRIBE is a phase II trial enrolling 218 patients with unresectable stage IV colorectal cancer. Participants were randomized 2:1 to receive FOLFOXIRI plus bevacizumab with or without atezolizumab (anti-PD-L1). Eligibility criteria and baseline data, including recent medications, are in the trial protocol and recorded in electronic case reports. Full eligibility criteria are listed in the trial protocol (available at www.clinicaltrials.gov). All patients signed a written informed consent to study procedures. For this study, we used de-identified clinical data collected from the trial, focusing in particular on **GGT** levels. These data were used for Kaplan–Meier survival analyses and **MVA** to explore associations between GGT levels and clinical outcomes.
6. PREMIS
- **ClinicalTrials.gov:** [NCT03984318](https://clinicaltrials.gov/ct2/show/study?term=NCT03984318)
 - Type: Observational design
 - Description: Patients enrolled in PREMIS were ≥ 18 years of age, with histologically proven solid malignancy and at least one tumor evaluation by imaging after immunotherapy onset. Eligibility criteria and baseline data, including recent medications, are in the trial protocol and recorded in electronic case reports. Full eligibility criteria are listed in the trial protocol (available at www.clinicaltrials.gov). Patient should understand, sign, and date the written informed consent form prior to any protocol-specific procedures performed. For this study, we used de-identified clinical data collected from the trial, focusing in particular on **GGT** levels. These data were used for Kaplan–Meier survival analyses and MVA to explore associations between GGT levels and clinical outcomes.
7. MIMic
- ClinicalTrials.gov ID: NCT03772899
 - Type: Interventional design
 - Description: The NCT03772899 study is a phase I open-label single-arm trial for 20 patients with a confirmed diagnosis of unresectable or metastatic cutaneous melanoma (BRAF wild type or mutant) treated with an association of ICB (pembrolizumab or nivolumab) and FMT from healthy donors. The purpose of this study is to examine the safety of combining these

two therapies in melanoma patients. The investigator will use fecal material from a healthy donor selected via our stringent protocol that is Health Canada approved. In addition to assessing the safety of the combination, the investigator will also study the effect of FMT on the immune system and microbial ecosystem of the gut. Full eligibility criteria are listed in the trial protocol (available at www.clinicaltrials.gov). Patients must be able to provide written informed consent prior to enrollment in the study. Blood samples were collected at baseline and used for metabolomics analysis.

8. FMT-LUMINATE

- ClinicalTrials.gov ID: NCT04951583
- Type: Interventional design
- Description: The NCT04951583 study is a multi-center single-arm, open-label Simon two-stage, phase II trial in 45 patients with metastatic or unresectable non-small cell lung cancer (NSCLC) and melanoma treated with ICB. Patients will receive standard-of-care ICB therapy in combination with fecal microbial transplantation (FMT). Eligibility criteria, baseline characteristics, and concomitant medications were documented in **eCRFs**, with full criteria available in the trial protocol on ClinicalTrials.gov. All patients signed an informed consent prior to enrollment in the study. Blood samples were collected at baseline and used for metabolomics analysis.

9. TACITO

- ClinicalTrials.gov ID: NCT04758507
- Type: Interventional design
- Description: The NCT04758507 trial is an investigator-initiated, randomized, double-blind, placebo-controlled phase 2a study. It aims to evaluate whether fecal microbiota transplantation (FMT) from donors who achieved a complete response to immune checkpoint inhibitors (ICB) can improve the efficacy of first-line therapy with pembrolizumab combined with axitinib in patients with metastatic renal cell carcinoma (mRCC). The trial was carried out in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonisation Good Clinical Practice (ICH-GCP) guidelines, while also complying with applicable local and institutional regulations. The study protocol received approval from the institutional review board/local ethics committee (ID: 2664). **Written informed consent was obtained from all participants prior to enrollment in the study.** Full eligibility criteria are listed in the trial protocol (available at www.clinicaltrials.gov).

Ethics approval and consent to participate. The study design and conduct complied with all relevant regulations regarding the use of human participants and were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP) guidelines. All participants provided written informed consent. Consent to publish clinical information potentially identifying individuals (e.g., age and sex) was also obtained. Data and sample collection adhered to applicable regulatory and ethical requirements, as well as the ICH E6(R2) GCP guidelines.

Ethical committees.

- ONCOBIOTICS “IML1” (NCT04567446): Translational analyses were performed under the study ONCOBIOTICS (sponsor protocol N: CSET 2017/2619, ID800 RCB N: 2017-A02010-53) according to the ethical guidelines and approval of the local ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB) of the Kremlin Bicêtre Hospital).
- MIMIC (NCT03772899): Patients were enrolled and treated at three academic centers in Canada to include the London Regional Cancer Program (LRCP, Ontario), the Center hospitalier de l'Université de Montréal (CHUM, Québec), and the Jewish General Hospital (JGH, Québec). The research ethics board (REB) at each institution approved the conduct of the trial in accordance with the current federal regulations to include the Canadian Food and Drug Regulations (C.05.001); the US Code of Federal Regulations (21CFR part 56); International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Good Clinical Practice Guidelines (ICH GCP); and the Declaration of Helsinki. Refer to <https://doi.org/10.1038/s41591-023-02453-x> for this information.
- FMT-LUMINATE (NCT04951583): The clinical trial and correlative analyses were approved by the CHUM Ethics Review Board and each participating center, ethics number: MP-02-2022-10121/21.173. Refer to <https://doi.org/10.1038/s41591-025-04186-5> for this information.
- ATEZOTRIBE (NCT03721653): The study has been approved by 25 ethics committees and is currently ongoing at 25 Italian oncology units. The protocol is conducted in accordance to the standards of Good Clinical Practice, in agreement with the principles laid down by the 18th World Medical Assembly (Helsinki 1964) and subsequent amendment (Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996, Edinburgh 2000). The protocol version 1.2 (6th October 2017) was approved on 20th September 2018 by the Ethics Committee of Coordinating Center (Comitato Etico Area Vasta Nord Ovest, CEAVNO) and by the local Ethics Committees of participating centers. The list is provided as Additional file 1. Then, the study was amended in order to regulate additional tissue, blood and fecal samples collection and the protocol version 2.1 (19th February 2019) was approved on 13th June 2019 by the Ethics Committee of Coordinating Center (Comitato Etico Area Vasta Nord Ovest, CEAVNO) and by the local Ethics Committees of participating centers. Refer to <https://doi.org/10.1186/s12885-020-07169-6> for this information (Supplementary information).
- TACITO (NCT04758507): The study was performed in compliance with the Declaration of Helsinki. Ethical approval was granted by Ethics Committee of the Fondazione Policlinico Gemelli IRCCS (ID #2664). Written informed consent was obtained from all

participants. Refer to <https://doi.org/10.1038/s41591-025-04189-2> for this information.

- The PREMIS study was approved by an ethical committee (Comité de Protection des Personnes Sud-Ouest et Outre Mer I; ID-RCB: 2018-A01257-48) and the institutional review board (CSET no. 2018/2728). All enrolled patients provided a signed informed consent.
- The MEDITREME study was approved by the Ethics Committee CPP TOURS Région Center – Ouest 1 on 27 March 2017 under the number 2017T1-03 and was registered with the French national health products agency (ANSM). The French ethical authorities asked us to include only RAS-mutated metastatic CRC because RAS WT patients must receive an anti-EGFR in first-line of treatment, and it would be unethical to give a protocol without anti-EGFR for these patients. The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization for Good Clinical Practice guidelines and the CONSORT 2010 guidelines. All patients provided written informed consent to study procedures before enrollment. The last version of the protocol is available as supplementary information.
- Protocol approval for the METIMMOX trial was obtained from the Regional Committee for Medical and Health Research Ethics of South-East Norway (2017/1850), the Norwegian Medical Agency (17/12752) and the institutional review boards. The trial was conducted in accordance with the guidelines of Good Clinical Practice and the Declaration of Helsinki. All patients provided written informed consent.
- The G2R (GUIDE2REPAIR) study was approved by the Ethics Committee CPP île de France 8 on 18 July 2019 under the EudraCT number 2018-002971-17 and was registered with the French national health products agency (ANSM).

Human participant details.

- ONCOBIOTICS “IML1” (NCT04567446): Biological sex and gender were considered in the study, and we had 69% male and 31% female participants as referred in [Table S5](#).
- MIMIC (NCT03772899) and FMT-LUMINATE (NCT04951583): Biological sex was considered in the study and we had 62% male and 38% female participants, and refer to [Table S4](#).
- ATEZOTRIBE (NCT03721653): Biological sex and gender were considered in the study, and refer to [Table S5](#).
- The PREMIS : Biological sex and gender were considered in the study, and refer to [Table S5](#).
- METIMMOX : Biological sex and gender were considered in the study, and refer to [Table S5](#).
- MEDITREM : Biological sex and gender were considered in the study, and refer to [Table S5](#).
- TACITO (NCT04758507): Biological sex and gender were considered in the study.
- G2R (GUIDE2REPAIR): Biological sex and gender were considered in the study, and refer to [Table S5](#).

Race, ethnicity, or other socially relevant groupings were not included in this study. OS and PFS were assessed from the date of treatment start to the date of death or disease progression/death, respectively.

Experimental model

Mice

All animal experiments were performed in compliance with French and European laws and regulations or the Canadian Council on Animal Care guidelines. The institutional animal ethics committee (Direction départementale de la protection des populations (DDPP) du Val-de-Marne) and the French Ministère de la Recherche approved all procedures in this study (permission number: 2023-024-42156 (all mice experiments except written otherwise) and 2024_010_48794 (FMT experiments) or #46833–20240111607908 v6 (BDL experiment)). Experiments were performed in accordance with Government and institutional guidelines and regulations. Seven-weeks old female C57BL/6 mice were purchased from Harlan or Envigo (France). Canadian animal studies were approved by the Institutional Animal Care Committee (CIPA) (Ethics numbers: C18025BRs). Murine experiments were conducted using seven-week-old female C57BL/6 mice, obtained from Charles River. All mouse experiments were performed at the animal facility at the GRCC or at CHUM in Montreal ([Figure 1](#) except D; [Figures S1A](#) and [S1C](#)) except for the bile duct ligation performed at the Center de Recherche des Cordeliers. The animals were housed in SPF conditions with a 12 hour-day/night rhythm and naive at baseline. Before every experiment, we randomized the mice to avoid bias. Each experiment was at least duplicated. No samples were excluded from the experiments. The plasma and the stool were drawn or harvested in the afternoon and morning respectively. If not otherwise indicated, mice were treated with a broad-spectrum ABX solution containing ampicillin (1mg/mL), streptomycin (5 mg/mL), and colistin (1 mg/mL) (Sigma-Aldrich) (ABX) added to sterile drinking water. Solutions and bottles were changed three times weekly. ABX were administered during 7–14 days in the drinking water of naive or MCA205 tumor-bearing mice. In accordance with our institutional ethics committee guidelines, the maximal permitted tumor burden was 1500 mm³ corresponding to 300 mm² (calculation was L x l). Tumor sizes were measured and controlled three times a week and when the tumor size exceeded 250 mm², mice were euthanized immediately to avoid reaching the permitted size of 300 mm². Mice were also controlled by technicians every day. Predefined humane endpoints included tumor ulceration or body weight loss greater than 20% relative to baseline. If either endpoint was reached, mice were euthanized within 24 h.

Madcam1^{−/−} (B6.129-Madcam1tm1.2Nwag/J) mice were a kind gift from A. Schippers (University Hospital Aachen, Aachen, Germany).

Tgr5/Gpbar1^{−/−} mice were a kind gift from Kristina Schoonjans, PhD, Laboratory of Metabolic Signaling (LMS), EPFL, Switzerland. The mice were bred at Gustave Roussy in SPF conditions with a 12 hour-day/night rhythm and naive at baseline. The genotyping was performed by western-blot.

METHOD DETAILS

Blood sample collection for ONCOBIOTICS, MIMic and FMT-LUMINATE

Blood samples were drawn from patients. Plasma was collected after centrifugation of lithium heparin tubes without EDTA and peripheral blood mononuclear cells (PBMC) were separated using a density gradient medium. Blood samples were used for the detection of bile acids by UHPLC-MS.

Stool samples collection for ONCOBIOTICS

Stool samples were self-collected by the participants at home according to the IHMS SOP 004 (<https://human-microbiome.org/index.php?id=Sop&num=004>). Immediately after the collection, they were stored at −20°C and transported on dry ice. Upon arriving at the biobank of the recruitment center (between 4 and 24 h later), they were stored at −80°C in plastic tubes. Two aliquots of the same sample were used for metaproteomic and metagenomic analyses. All the samples were processed according to International Human Microbiome Standards (IHMS) guidelines (SOP 03 V1).

DNA was extracted from an aliquot of the same stool samples used for metaproteomic analysis using the IHMS SOP 07 V2 H and sequenced with an Ion proton sequencer (Thermo Fisher Scientific). The sequencing was performed at a single site (MetaGenoPolis). Reads were cleaned using Alien Trimmer (version 0.4.0) to (1) remove resilient sequencing adapters and (2) trim low-quality nucleotides at the 3' side using a quality cutoff of 20 and a length cutoff of 45 bp. Cleaned reads were subsequently filtered from human and other possible food contaminant DNA using human genome (GRCh38-p13), with an identity score threshold of 95%. For each metagenome we profiled the taxonomic composition with MetaPhlAn-4.⁵⁵ For alpha diversity, we computed the per-sample Richness and Shannon indexes⁵⁶ using the vegan R package.⁵⁷ Differences between groups were assessed using the Wilcoxon-Mann-Whitney test. For beta-diversity, we computed the between-samples Bray-Curtis dissimilarities using the implementation available in the Vegan R package 51. Differential abundance analysis on taxonomic profiles between groups were performed using the LefSE software.

Human and mouse plasma sample preparation and bile acid detection by UHPLC-MS

For each patient, 50 µL of human plasma was mixed with 500 µL of ice-cold extraction mixture (methanol/water, 9/1 spiked with a cocktail of internal standards) and centrifuged (15 min at 10,000g, 4°C). Regarding murine plasma, 25 µL of biological sample was mixed with 250 µL of the same ice-cold extraction mixture and treated as described above. Mice plasma was harvested at the same time for each time point. Supernatants were collected and several fractions were split to be analyzed by different liquid and gas chromatographies coupled with mass spectrometers (LC-MS and GC-MS).⁵⁸ For human samples, 150 µL of supernatant (50 µL for murine) were transferred to glass vials and evaporated to be used for GC-MS. Then, 50 µL of methoxyamine (20 mg/mL in pyridine) was added on dried extracts, which were stored at room temperature in the dark, overnight. The day after, 80 µL of N-Methyl-N-trimethylsilyl-trifluoroacetamide were added, and the final derivatization was made at 40°C for 30 min. Samples were then transferred into vials and directly injected into the GC-MS system – 7890A gas chromatograph coupled with a triple quadrupole 7000C (Agilent Technologies) – equipped with a high sensitivity electronic impact source (EI) operating in positive mode. Others aliquots (polyamines, bile acids and short chain fatty acids) were analyzed by LC-MS 1290 UHPLC (Ultra-High Performance Liquid Chromatography) coupled to a QQQ 6470 (Agilent Technologies). Biliary acids and polyamines were analyzed by MRM analysis.⁵⁴ Small organic acids and ketones were analyzed by MRM analysis in both positive and negative polarity, alternatively, gas temperature was set to 300°C with a gas flow of 12 L/min. Capillary voltage was set to 4 kV in positive and 5 kV in negative polarity mode. Ten (10) µL of sample were injected on a Zorbax Eclipse XDB- column (P/N 981758-902, 100 mm × 2.1 mm particle size 1.8 µm 1200 bars) from Agilent technologies, protected by a guard column (XDB-C18; 5 mm × 2.1 mm particle size 1.8 µm) and heated at 50°C by a Pelletier oven. Gradient mobile phase consisted of water with 0.1% of formic acid (A) and 0.1% of formic acid in acetonitrile (B) freshly made. Flow rate was set to 0.7 mL/min, and gradient as follow: initial condition was 80% phase A and 20% phase B for 3 min, changing to 45% phase B over 4 min before rinsing phase and equilibrium before next injection. Peak detection and integration of the analytes were performed using the Agilent Mass Hunter quantitative software (B.07.01).

Pseudo-targeted analysis by UHPLC-HRAM (Ultra-High Performance Liquid Chromatography – High Resolution and Accurate Mass) was performed on a U3000 (Dionex)/Orbitrap q-Exactive (Thermo) coupling, previously described in Durand et al. 2021⁵⁹ and Abdelatif et al.⁶⁰ All targeted treated data were merged and cleaned with a dedicated R package (version 4.0) (<https://github.com/kroemerlab/GRMeta>).

sST2, sMAdCAM-1 and S100A9 quantification in patients

sST2, sMAdCAM-1 and S100A9 were quantified in patient's plasma samples with the Bio-Plex 200 systems (Bio-Rad) and sMAdCAM-1/ST2/S100A9 kit from R&D Systems (Human Luminex Discovery Assay LXSAM).

Spectral flow cytometry on PBMC

Aliquots of $5\text{--}10 \times 10^6$ PBMC cryopreserved in fetal calf serum (FCS)/DMSO solution were defrost, washed and resuspended in FACS Buffer (Brilliant Stain Buffer Plus BD + 5% FCS + 2mM EDTA). Dead cells were excluded using LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit from Invitrogen. CCR7 (BV421), LPAM1 (Alexa 647), PDL1 (PE-Dazzle594), PDL2 (RB780), CADM1 (PE), CCR4 (BV605), CCR6 (BUV661), CD103 (PE-Fire640), CD1c (BUV737), CXCR3 (PE-Fire810), CXCR5 (BUV615), FcεRIa (BV480), ICOS (Spark R718), IgA (APC-Vio700), IgD (cF BYG667), IgG (BV480), IgM (BV510), PD1 (BV750), CD11c (cF BYG781), CD123 (Spark V432), CD127 (cF R659), CD14 (cF B548), CD148 (BUV395), CD16 (BV570), CD19 (cF R685), CD21 (BUV805), CD24 (BB515), CD25 (PE-Fire700), CD27 (cF R840), CD3 (BV650), CD38 (BV711), CD4 (cF YG584), CD40 (BV650), CD45 (Spark B574), CD45RA (cF V450), CD5 (BUV563), CD71 (BUV496), CD8 (cF B532), CD88 (BV786), HLA-DR (cF B690), Streptavidin (PE) were purchased from BD Biosciences, R&D systems, BioLegend, Miltenyi Biotec and Cytex and used to stain cells. (see [STAR Methods](#)) Stained samples were acquired on Cytex Aurora 5-laser spectral analyzer and analyses were performed on FlowJo v10.8.1.

Slope-prediction method

Responders and non-responders were compared using a longitudinal slope estimation approach. For each metabolite, individual longitudinal slopes were estimated using Best Linear Unbiased Predictors (BLUP) derived from linear mixed-effects models fitted to repeated measurements over time. Estimated slopes were subsequently compared between responders and non-responders to evaluate differences in metabolite trajectories. The significance of temporal changes within responders and non-responders was assessed from the estimated time effect in each group. (Related to [Figure 8F](#), left).

Clinical cox models

Kaplan-Meier survival curves were generated using Prism 10 (GraphPad, San Diego, CA, USA) and the statistical difference between groups was compared using the Log rank test. Multivariate analysis and hazard ratio evaluation were performed using the Cox proportional hazard regression model.

Patients clustering based on circulating bile acids composition

Unsupervised hierarchical clustering was performed to identify groups of patients with similar circulating bile acid profiles. A matrix containing bile acid percentages or log2foldchange values for each patient was used as input. Clustering analysis was conducted in R using the “pheatmap” package (version 1.0.13). Hierarchical clustering was applied to both patients and bile acid variables, or only for bile acid variables. Pairwise distances between patients were calculated using the Euclidean distance metric. Clusters were generated using Ward’s minimum variance method (Ward.D2 linkage), which minimizes the total within-cluster variance at each step of the agglomerative clustering process. No data scaling or normalization was applied prior to clustering. The resulting dendrograms and heatmaps were generated using the “pheatmap” function, with patient annotations displayed according to the available clinical metadata. The clustering output was used to define patient subgroups exhibiting distinct circulating bile acid signatures. (related to [Figure 6A](#); [Figures S9A](#), [S9D](#), and [S11I](#)).

Oral bacterial gavage with commensal species or BAs

E. clostridioformis was isolated from the ileum of mice that received ABX in drinking water for 7 days followed by 4 days ABX-free at GRCC.²¹ *E. clostridioformis*, were grown on Columbia agar with 5% sheep blood (COS) plates in an anaerobic atmosphere created using three anaerobic generators (Biomérieux) at 37°C for at least 48 h. Colonization of SPF C57BL/6 mice was performed by oral gavage with 100 μL of suspension containing 1×10^8 to 1×10^9 bacteria. The optical density of bacterial solution in 0.9% NaCl was measured using a fluorescence spectrophotometer (Eppendorf) at wave lengths of 600 nm in 0.9% NaCl. Mice were gavaged one to eight times. The identification of specific bacteria was accomplished using a matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometer (Bruker, France). Mice were gavaged four times every 2 days with 120 mg/kg body weight of LCA (Thermo-Fisher Scientific, 229090050), UDCA (ThermoFisher, 208590250), GDCA (Sigma Aldrich, G9910-5G), TDCA (EMD Millipore Corp, 580221-5GM), DCA (ThermoFisher, 148140250) diluted in corn oil. For Fexaramine treatment (MCE, 574013-66-4), mice were gavaged every day during 10 days (starting at day 0 of ABX treatment) and then every 2 days for one more week (after ABX cessation) at the dose of 120mg/kg of mice.

FMT experiments

In FMT experiments, mice received 3 days of ABX before undergoing FMT (from 4 different cancer patients) the next day by oral gavage. Fecal samples collected from patients in the ONCOBIOTICS study were used for FMT. FMT was performed by thawing fecal material. As described before, frozen fecal samples were brought back to room temperature and resuspended in NaCl at a density of 0.1g/mL. Mice were placed in a new cage after the procedure. Two hundred microliters of the suspension were then transferred by oral gavage into each ABX-pretreated recipient using animal-feeding needles. In addition, another 100 μL was applied to the fur of each animal.

Subcutaneous cancer mouse models

MCA205 fibrosarcoma was purchased from the American Type Culture Collection and. The RET melanoma cell line, generated by transgenic expression of the *RET* proto-oncogene under the control of the metallothionein-1 promoter driving spontaneous

melanogenesis, was provided by Viktor Umansky (DKFZ—German Cancer Research Center; syngeneic from C57BL/6J mice). Both cell lines (syngeneic to C57BL/6J mice) were cultured at 37°C in the presence of 5% CO₂ in RPMI 1640 containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/mL penicillin–streptomycin, 1 mM sodium pyruvate, and minimum essential medium nonessential amino acids (henceforth referred to as complete RPMI 1640). Cell lines were regularly tested for *Mycoplasma* contamination and were not used after more than 10 passages. MCA205 sarcoma and RET syngeneic to C57BL/6J mice were implanted subcutaneously with 0.8×10^6 and 0.5×10^6 cells/mouse for MCA205 and RET respectively and treated intraperitoneally when tumors reached 25 to 35 mm² in size with α PD-1 mAb (250 μ g/mouse, clone RMP1-14) or isotype control (250 μ g/mouse, clone 2A3) for MCA205 and α PD-1 + α CTLA-4 mAb (250 μ g/mouse, clone RMP1-14 and 100 μ g/mouse, clone 9D9 respectively) or isotype control (250 μ g/mouse, clone 2A3 and 100 μ g/mouse, clone MPC-11 respectively) for RET. Mice were injected four times at a 3-day interval with α PD-1/ α CTLA-4 (Bio X Cell, clone RPMI-14, 250 μ g per mouse; clone 9D9, 100 μ g/mouse respectively) or isotype control (Bio X Cell, clone 2A3, 250 μ g per mouse; clone MPC-11, 100 μ g/mouse respectively) mAbs. Tumor length and width were routinely monitored three times a week by means of a caliper. All antibodies were purchased from Bio X Cell.

Statistical analysis of tumor growth

Longitudinal tumor growth data were analyzed using the online tool “TumGrowth” available at <https://kroemerlab.shinyapps.io/TumGrowth/>, which is an open-access platform specifically developed for the statistical analysis of experimental tumor growth curves. Tumor volume measurements collected over time were modeled using linear mixed-effects models, allowing incorporation of repeated measurements within individual animals and inter-animal variability. Tumor growth kinetics were fitted using a two-phase piecewise linear regression model (linear-linear model). When appropriate, the breakpoint between growth phases was estimated automatically by the software. Treatment group and time were included as fixed effects, while individual animals were modeled as random effects. Model adequacy was assessed using the diagnostic plots provided by the application. Differences in tumor growth between treatment groups were assessed by pairwise comparisons of the estimated growth slopes derived from the mixed-effects model. Statistical significance was evaluated using Wald tests, and *p*-values were adjusted for multiple comparisons when appropriate. A two-sided *p*-value <0.05 was considered statistically significant.

Surgical bile duct ligation

Cell culture. MCA205 murine fibrosarcoma cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PS). Animal experiments. Nine-week-old female C57BL/6J mice were housed under specific pathogen-free conditions with *ad libitum* access to food and water on a 12 h light/dark cycle. All animal experiments were approved by the institutional animal care and use committee (ethical protocol #46833) and conducted in accordance with applicable guidelines. On day 0 (D0), mice were subcutaneously injected in the right posterior flank with 0.8×10^6 MCA205 cells suspended in 50 μ L 1 $\times$ PBS under 2% isoflurane anesthesia. Tumor dimensions were measured with calipers at the time points indicated in the scheme, and tumor area (mm²) was calculated as length $\times$ width. On D3–D4 after tumor inoculation, mice underwent bile duct ligation (BDL) or sham surgery under 2% isoflurane anesthesia. For BDL, the common bile duct was exposed and doubly ligated. For sham surgery, the bile duct was exposed and manipulated without ligation. Perioperative care and postoperative monitoring were performed in accordance with institutional guidelines. Anti-PD-1 treatment was initiated when tumors reached approximately 25 mm². Mice then received intraperitoneal injections of anti-PD-1 or isotype control (Iso) antibody under 2% isoflurane anesthesia on D6, D8, and D11, at 250 μ g per mouse in 100 μ L PBS (1 $\times$) per injection. Entire tumor growth curves were analyzed using TumGrowth (<https://kroemerlab.shinyapps.io/TumGrowth/>) with independent sample *t* test. A *p* value <0.05 was considered statistically significant. Data are presented as mean $\pm$ SEM unless otherwise stated.

MCA205 fibrosarcoma knockout mouse model

Establishment of BA receptors knockout cell lines

Edit-R All-in-one Lentiviral (knockout) Mouse *Nr1h4* sgRNA (target sequence: AGTGCCAGGAGTGCCGGCTA; catalog no. VSGM11942-247929487), *Gpbar1* sgRNA (target sequence: ATGAGCGTCGCCCCACCACTA; catalog no. VSGM11942-247972009), and *Vdr* sgRNA (target sequence: TTCGTGCAGACGTAAGTACA; catalog no. VSGM11942-248142193) were purchased from Horizon Discovery.

For transduction, MCA205 fibrosarcoma cells were expanded and seeded at 4×10^5 cells per well in tissue culture-treated 6-well plate with complete RPMI-1640 medium. TSECs were expanded and seeded at 1.4×10^5 cells per well in 6-well plate with complete DMEM/F-12 medium supplemented with ECGS, and cultured overnight.

The following day, the culture supernatant was removed, and the transduction mixture was prepared by combining 10 μ g/mL polybrene (catalog no. TR-1003-G, Sigma-Aldrich) with 200 μ L of lentiviral particles and 800 μ L of complete medium (without serum and antibiotics) per well, corresponding to a multiplicity of infection (MOI) of 0.3. The mixture was added to the cells and incubated for 6 h at 37°C in a 5% CO₂ incubator. Subsequently, 1 mL of complete medium (containing 2 $\times$ serum and 2 $\times$ antibiotics) was added, and cells were cultured for an additional two days. An additional control well, treated identically but without lentiviral particles, was included. Forty-eight hours post-transduction, cells were detached with trypsin and transferred to T75 tissue culture flasks. Selection was performed using puromycin: 2 μ g/mL for TSEC knockouts and 6 μ g/mL for MCA205 knockouts, maintained for 7 days until all cells in the control flask were eliminated. Surviving cells were collected and subjected to single-cell sorting using a BD FACSAria™ Fusion flow cytometer (BD, Franklin Lakes, NJ, USA). Immediately prior to sorting, 1 μ g/mL DAPI was added to the cells to assess

viability. Viable (DAPI-negative), puromycin-resistant single clones were isolated and cryopreserved at -80°C . These KO tumor cells were inoculated into mice, which were subsequently treated with either $\alpha\text{PD-1}$ or an isotype control antibody as previously described. Mice received four intraperitoneal injections at a 3-day interval with $\alpha\text{PD-1}$ (Bio X Cell, clone RPMI-14, 200 μg per mouse) or isotype control (Bio X Cell, clone 2A3, 200 μg per mouse) monoclonal antibodies. Tumor length and width were measured three times per week using a caliper.

Flow cytometry

Tumors and tdLNs, were harvested at different time points as indicated in the individual experiments. Excised tumors were cut into small pieces and digested in RPMI medium containing Liberase at 25 mg/mL (Roche) and DNase1 at 150 IU/mL (Roche) for 30 min at 37°C , and then crushed and filtered twice using 70 μm and 30 μm cell strainers (Miltenyi Biotec). Lymph nodes and spleens were crushed in RPMI medium and subsequently filtered twice through a 70- μm cell strainer. Four million cells from each sample were pre-incubated with purified anti-mouse CD16/CD32 (clone 93; eBioscience) for 10 min at 4°C before membrane staining (30 min at 4°C). For intracellular staining, the FoxP3 staining kit (eBioscience) was used. Dead cells were excluded using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Biolegend). Information on the antibodies used to stain murine cells is found in [STAR Methods](#). Stained samples were acquired on CytoFLEX S 13 colors (Beckman Coulter), and analyses were performed with Kaluza software 2.1 (Beckman Coulter). Treg cells were defined after gating on FoxP3 $^{+}$ CD25 $^{+}$ CD4 $^{+}$ cells.

Tracing migration of leukocytes from the gut to the TME: CFSE staining of mLN cells up to TME or tdLNs

C57BL/6J tumor-bearing mice were anesthetized with 2–2.5% isoflurane and administered meloxicam (1 mg/kg) intraperitoneally for analgesia. Abdominal skin and peritoneum were cut at the midline to access the mLNs. The mLNs were gently mobilized through the midabdominal incision onto a sterile plastic-coated surgical drape. Ileum draining mLNs were visually identified according to their vasculature. The two most prominent mLNs were injected with 100 μM CFSE diluted in 5 μL of PBS using a 30-Ga insulin syringe. After repositioning the mLNs, the peritoneal membrane was closed by continuous clips.

RNA extraction and RT-qPCR lysis and extraction protocols for mouse samples

Tumor or intestinal samples were snap-frozen in liquid nitrogen. On the day of extraction, samples were thawed at 4°C and put in RLT Plus buffer containing 0.1% beta mercaptoethanol then homogenized in a microtube homogenizer (Benchmark Scientific) in RNA-free glass bead tubes (Dutscher). Total RNA extraction and genomic DNA removal were performed with the RNeasy Mini kit (Qiagen) following the manufacturer's recommendations. A maximum of 1 μg of RNA, measured using a NanoDrop Spectrophotometer (ThermoFisher Scientific), was reverse transcribed into cDNA with a mixture composed of SuperScript III Reverse Transcriptase (Life Technologies), RNaseOUT Recombinant Ribonuclease Inhibitor (Life Technologies), random primers (Promega), and Deoxynucleoside Triphosphate Set, PCR grade (Roche Diagnostics).

Quantitative gene expression assay

The expression of mouse *Ppia*, *Madcam1*, *Nr1h4*, *Gpbar1*, *Vdr*, *Nr1i2*, *Nr1i3* genes (all from Life Technologies) was analyzed with the TaqMan Gene Expression Assay using the Universal Master Mix II on a QuantStudioTM 3 Real-Time PCR System (Life Technologies). Amplifications were performed using the following ramping profile: one cycle at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, and 60°C for 1 min. RT-qPCR data were normalized to the expression levels of the housekeeping genes *Ppia*, as indicated in each figure, by means of the $2^{-\text{DCt}}$ method multiplied by 10^6 .

Bulk RNA-sequencing for hepatocytes, cholangiocytes and enterocytes

For enterocytes isolation, we used the Lamina Propria Dissociation kit from Miltenyi followed by magnetic separation using the CD326 (EpCAM) MicroBeads mouse from Miltenyi. For hepatocytes and cholangiocytes, we used the Liver Perfusion kit from Miltenyi. The hepatocytes were obtained from the pellet at the end of the protocol. We used the supernatant and performed a magnetic separation using the CD326 (EpCAM) MicroBeads mouse from Miltenyi to obtain the cholangiocytes. We extracted the RNA from each population with the RNeasy Mini kit from Qiagen. The Bulk RNA-sequencing was performed at the Genomic Platform at Gustave Roussy, France.

RNA-seq data generation and analysis

RNA-seq libraries were generated in five replicates for each condition and sequenced in paired-end mode with a read length of 100 bp. Raw sequencing reads were processed with fastp (v0.23.2)⁶¹ to trim adapters and remove low-quality bases (Phred score <30), and, when necessary, downsampled using seqkit (v2.5.1)⁶² to obtain comparable read depths across samples (20–60 million high-quality reads per sample). Fastq files were subsequently assessed with FastQC (v0.12.1)⁶³ and MultiQC (v1.16),⁶⁴ and pseudoaligned to the GRCh38 transcriptome reference with kallisto (v0.50.1)⁶⁵ using default parameters. Genes with an absolute expression of less than 20 reads across all samples were filtered out.

Differential expression analysis was performed with DESeq2⁶⁶ (v1.40.2). Logarithm fold changes were modified by the lfcShrink algorithm with the “Ashr” option. Differentially expressed gene (DEG) cutoff was defined at an FDR less than 0.05 and an absolute log2 fold change (log2FC) value greater than 0.5 in pairwise comparisons between experimental conditions. For visualization purposes, variance stabilizing transformation (VST) values and transcripts per million (TPM) were obtained with DESeq2 functions and used where indicated in the figures.

Single cell RNA-sequencing for tdLN and tumors *scRNA-sequencing whole transcriptome using BD rhapsody*

BD Rhapsody experiments were performed following manufacturer's (BD Biosciences) protocol. Prior to experiment, tumor cells were enriched using mouse CD45 microbeads from Miltenyi (130-052-30) following the Miltenyi's protocol. Two independent cartridges were used to capture 77,821 cells (tumor) and 76,529 cells (lymph nodes). Experimental conditions were pooled prior to analysis using the BD™ Mouse Single Cell Sample Multiplexing Kit (Cat No: 633793), with 9 and 11 sample barcodes per cartridge, respectively. Libraries were processed according to the BD Rhapsody™ Whole Transcriptome Analysis (WTA) Amplification Kit (Cat No: 633801) and the BD Rhapsody™ Whole Transcriptome Analysis (WTA) Reagent Kit (Cat No: 665915). Libraries were then subjected to indexed paired-end sequencing, 2×100 bp setting, on a DNBSEQ-T7 (BGI Genomics, Shenzhen, China) with 30% PhiX spike-in control. A total of 1,415 million reads and 2,068 million reads were sequenced for the tumor and lymph node libraries, respectively.

Alignment and pre-processing of single-cell RNA sequencing data

Transcriptomic FASTQ files were processed using the standard Rhapsody analysis pipeline (BD Biosciences), following the manufacturer's recommendations. For read quality control, recursive substitution error correction (RSEC) and distribution-based error correction (DBEC) were applied; these manufacturer-developed algorithms correct for PCR and sequencing errors. A cell was defined as a singlet if the minimum read count associated with a single sample tag exceeded a threshold of 75%. A cell was classified as a multiplet if it exceeded this threshold for more than one sample tag. Cells that did not meet the threshold were labeled as undetermined. Both multiplets and undetermined cells were excluded from downstream analysis. Samples were demultiplexed using the Sample Tag Call matrix generated by the Seven Bridges BD Rhapsody alignment pipeline, and metadata were manually assigned to each tag.

Analysis of single cell RNA-sequencing

Filtered gene-expression matrices were subjected to processing using Seurat R Package (v 5.4.0). Quality control measures were implemented to remove debris, damaged cells and doublets (min_genes_per_cell = 600; max_genes_per_cell = 5000; max_%mitochondrial_reads = 20; max_%ribosomal_reads = 30). After filtering, each dataset was normalized using Seurat's log-normalization method, and the top 3,000 highly variable genes were identified. The data were then scaled while regressing out technical sources of variation, including mitochondrial and ribosomal content and total UMI counts. Principal component analysis (PCA) was subsequently performed using the selected variable genes, and the first 30 principal components were retained for downstream analyses. UMAP dimensionality reduction was generated based on these 30 components, followed by neighbor identification and clustering using the Louvain algorithm (resolution = 0.5). Differential expression analysis was performed using FindAllMarkers to identify cluster-specific upregulated genes, which were used for manual annotation of each cell population. For T cells, FindSubCluster (resolution = 0.1, Louvain algorithm) was applied to distinguish CD4 and CD8 subsets. Statistical comparisons were performed using Wilcoxon tests with FDR correction.

For the differential expression analysis of total CD8 lymphocytes in tumor single-cell data, all clusters containing CD8 lymphocytes were merged into a single cluster and filtered to remove cells containing Cd4 reads ($Cd3e > 0$ & $Cd8a > 0$ & $Cd4 = 0$). Gene expression was extracted using the FetchData function from Seurat. Pairwise statistical comparisons were performed between experimental conditions using Wilcoxon tests with FDR correction, comparing each bile acid-treated group against the EC control. Results were visualized as violin plots overlaid with boxplots and individual data points using ggplot2.

GSEA was performed using the fgsea R package (v1.32.4) on ranked gene lists derived from differential expression results. Genes were ranked by average $\log_2$ fold change, with a small random noise term (mean = 0, $sd = 1 \times 10^{-6}$) added to break ties. Genes with non-finite $\log_2$ fold change values were excluded prior to ranking. Pathways from the msigdb_v2024.1.Mm_GMTs collection were used as the reference gene sets, with a minimum gene set size of 15 and a maximum of 500. GSEA was run with $\epsilon = 0.0$ and 100,000 permutations. Pathways with an adjusted p -value < 0.05 were considered significant, and results were visualized as dot plots displaying the top 50 pathways ranked by absolute normalized enrichment score (|NES|).

Ileal, colonic, liver, tumor tissues preparation and bile acid detection by UHPLC-MS

Ileal, colonic, liver, tumor mouse tissues. Approximately 30 mg of collected tissue was weighed and placed in a 2-mL homogenizer tube with ceramic beads (Precellys, Bertin Technologies, France) with 1 mL of ice-cold extraction mixture (9:1 methanol-water, -20°C , with labeled ^{13}C -glycocholic acid as internal standard). To facilitate the extraction of endogenous metabolites, samples were then completely homogenized (3 cycles of 20 s/2380g; Precellys 24, Bertin Technologies, Montigny-le Bretonneux, France) and centrifuged (10 min at 15,000g, 4°C). To detect bile acids, 300 μL of supernatant was collected and treated following a previously described protocol.⁵⁸ Bile acids were analyzed by LC-MRM (Multiple Reaction Monitoring) with a 1290 UHPLC (Ultra-High Performance Liquid Chromatography, Agilent Technologies) coupled to a mass spectrometer 6470 TQ (Triple Quadrupole, Agilent Technologies), as previously described.⁵⁹ Targeted data were cleaned with a R (version 4.0) dedicated package (<https://github.com/kroemerlab/GRMeta>).

Mouse clustering based on bile acids composition

Metabolomic profile analysis and visualization were performed in R (version ≥ 4.4), with data normalization and heatmap organization adapted to the biological objective of each figure using the "pheatmap" package (version 1.0.13). For multi-compartment analyses (plasma, liver, ileum, and colon; related to Figures 1B and S3A), raw metabolite intensities were normalized using row-wise Z score

scaling and hierarchically clustered were done across rows using Ward's minimum variance method (ward.D2). Pairwise group comparisons were performed using two-sided non-parametric Wilcoxon rank-sum tests. Statistical significance was represented as lateral row annotations using discretized *p*-value categories (≥ 0.05 , <0.05 , <0.01 , and <0.001). Assessment of the impact of antibiotic treatment in Figure S1D was based on an initial selection of metabolites significantly altered relative to the Water group (Wilcoxon test, $p \leq 0.05$). Selected metabolites were subsequently normalized using row-wise Z score scaling and hierarchically clustered across rows using Ward's minimum variance method (ward.D2). Bile acids were highlighted in red, and *p*-value categories from the Water versus ABX comparison were displayed as lateral annotations. Unsupervised exploration of the bile acid composition across the Water, ABX, and FMT groups (related to Figure S1I) or with Fexaramine treatment (related to Figure S6B) was performed after inverse hyperbolic sine (asinh) transformation of metabolite intensities and removal of zero-variance variables. Hierarchical clustering was applied to both metabolites and samples using Euclidean distance and complete linkage (ward.D2). In Figure S6B, lateral annotation of *p*-value classes derived from a two-sided Wilcoxon test between the two groups. (related to Figures 1B; S1D and S1I, and S3A, S6B).

Cell lines and treatment

Culture media and supplements were obtained from Life Technologies (Carlsbad, CA, USA), and plasticware was sourced from Corning (Corning, NY, USA). Transformed sinusoidal endothelial cells (TSECs) were generously provided by B.P. Fennimore (University of Colorado Anschutz Medical Campus, Aurora, CO, USA). TSECs and TSECs with knockout of three bile acid receptors-TGR5 (gene: *Gbpar1*), FXR (gene: *Nr1h4*), and VDR (gene: *Vdr*)-were cultured in DMEM/F-12 medium supplemented with GlutaMAX™, 10% fetal bovine serum (FBS), 10 U/mL penicillin sodium, 10 mg/mL streptomycin sulfate, and Endothelial Cell Growth Supplement (ECGS; catalog no. 1052, ScienCell). Cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Recombinant murine TNF- α (catalog no. 315-01A, PeproTech) was diluted into culture medium from a 40 μ g/mL stock solution immediately prior to treatment. Various bile acids and related compounds were prepared as follows: cholic acid (CA; catalog no. 700212P, Avanti), chenodeoxycholic acid (CDCA; catalog no. C8261, Sigma-Aldrich), α -muricholic acid (α -MCA; catalog no. 700232P, Avanti), β -muricholic acid (β -MCA; catalog no. 700233P, Avanti), deoxycholic acid (DCA; catalog no. 30960, Sigma-Aldrich), hyocholic acid (HCA; catalog no. 700159P, Avanti), hyodeoxycholic acid (HDCA; catalog no. 700214P, Avanti), 12-dehydrocholic acid (12-dDCA; catalog no. 700215P, Avanti), lithocholic acid (LCA; catalog no. 229090050, Thermo Scientific Chemicals), ursodeoxycholic acid (UDCA; catalog no. 208590250, Thermo Scientific Chemicals), and tyrosine-conjugated cholic acid (Tyro-CA, generously provided by Kenya Honda, Keio University School of Medicine, Tokyo, Japan), as well as glycocholic acid (GCA; catalog no. 700265P, Avanti), glycodeoxycholic acid (GDCA; catalog no. G9910, Sigma-Aldrich), glycohyocholic acid (GHCA; catalog no. HY-122141, MedChemExpress), glycohyodeoxycholic acid (GHDCA; catalog no. 700264P, Avanti), taurocholic acid sodium salt hydrate (TCA; catalog no. T4009, Sigma-Aldrich), sodium taurochenodeoxycholate (TCDCA; catalog no. T6260, Sigma-Aldrich), taurodeoxycholic acid sodium salt (TDCA; catalog no. 580221, Millipore), taurohyodeoxycholic acid sodium salt (THDCA; catalog no. 700248P, Avanti), tauroolithocholic acid sodium salt (TLCA; catalog no. 700252P, Avanti), and taurourso-deoxycholic acid sodium salt (TUDCA; catalog no. 580549, Millipore). The FXR agonist Cilofexor (catalog no. HY-109083, MedChemExpress) was also included.

These compounds, dissolved in DMSO, were diluted 1:1000 into the culture medium to a final concentration of 100 μ M (or 50 μ M for Cilofexor). Additionally, murideoxycholic acid (MDCA; catalog no. 700270P, Avanti), ω -muricholic acid (ω -MCA; catalog no. 700231P, Avanti), tauro- α -muricholic acid sodium salt (T- α -MCA; catalog no. 700243P, Avanti), tauro- β -muricholic acid sodium salt (T- β -MCA; catalog no. 700244P, Avanti), tauro- ω -muricholic acid sodium salt (T- ω -MCA; catalog no. 700245P, Avanti), and tauro- γ -muricholic acid sodium salt (THCA; catalog no. 700246P, Avanti) were similarly prepared and diluted to a final concentration of 10 μ M.

Cells were treated with the bile acids and/or FXR agonists, together with TNF- α (final concentration of 10 ng/mL), for 48 h. Following treatment, cell pellets were harvested for RNA extraction and subsequent RT-qPCR analysis to assess Madcam1 expression as previously described.

PCR amplification and TIDE analysis

Genomic DNA was isolated from transduced TSECs or MCA205 fibrosarcoma cell clones using the PureLink™ Genomic DNA Mini Kit (catalog no. K182002, Invitrogen). Polymerase chain reaction (PCR) was conducted to amplify the relevant regions using the Platinum™ SuperFi II PCR Master Mix (catalog no. 12368010, Invitrogen) with primers designed to generate approximately 500–800 bp fragments encompassing the target SNP (see STAR Methods). PCR products were purified with the PureLink™ PCR Micro Kit (catalog no. K310250, Invitrogen) and assessed by agarose gel electrophoresis to confirm clean, single-band amplification prior to Sanger sequencing (Eurofins Genomics). DNA editing efficiency was evaluated by uploading sequencing chromatograms to the Tracking of Indels by DEcomposition (TIDE) online platform (<https://tide.nki.nl/>)⁶⁷ to calculate indel percentages. Decomposition windows and left boundaries were optimized according to the distance from the cut sites to maximize alignment quality for each target. Indel analysis was limited to a maximum of 10 indel sizes, with a significance threshold set at $p < 0.001$ for all analyses.

In vitro T cell culture and bile acid exposure

Mouse T cells were isolated from the spleens of C57BL/6J mice using the MojoSort™ Mouse CD3 T cell Isolation Kit (catalog no. 480031, BioLegend) and activated with Dynabeads Magnetic Beads (catalog no. 11452D, Gibco) according to the manufacturer's instructions. Cells were cultured in complete RPMI 1640 medium supplemented with 50 μ M 2-mercaptoethanol (catalog no. ES-

007-E, MERCK) and 30 U/mL recombinant mouse IL-2 (carrier-free; catalog no. 575406, BioLegend). On day 3, activated T cells were resuspended and cultured for an additional 2 days in fresh medium containing 30 U/mL IL-2. Subsequently, CD3⁺ T cells were plated at 3×10^5 cells per well in 48-well plates and exposed overnight to bile acids dissolved in sterile H₂O, including glycodeoxycholic acid (GDCA; catalog no. G9910, Sigma-Aldrich), taurodeoxycholic acid sodium salt (TDCA; catalog no. 580221, Millipore), sodium taurochenodeoxycholate (TCDCA; catalog no. T6260, Sigma-Aldrich), taurocholic acid sodium salt hydrate (TCA; catalog no. T4009, Sigma-Aldrich), or tauro- β -muricholic acid sodium (T- β -MCA; catalog no. HY-135103, MedChemExpress). Cells were then restimulated with eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors; catalog no. 00-4975-93, Invitrogen) for 5 h, followed by Fc receptor blocking using TruStain FcX™ PLUS (anti-mouse CD16/32) antibody (catalog no. 156604, BioLegend) and viability staining with LIVE/DEAD™ Fixable Aqua dye (catalog no. L34966, Invitrogen). Surface and intracellular staining were performed using the following antibodies: Pacific Blue™ anti-mouse CD4 (catalog no. 100531, BioLegend), Brilliant Violet 650™ anti-mouse CD8a (catalog no. 100742, BioLegend), FITC anti-human/mouse Granzyme B (catalog no. 515403, BioLegend), PE/Cyanine7 anti-mouse IFN- γ (catalog no. 505826, BioLegend), and APC-conjugated Ki-67 (clone SolA15; catalog no. 17-5698-82, Invitrogen). Intracellular cytokine staining was carried out using the eBioscience™ Foxp3 Transcription Factor Staining Buffer Set (catalog no. 00-5523-00, Invitrogen) according to the manufacturer's protocol. Cells were resuspended and analyzed on a CytoFLEX S flow cytometer (Beckman Coulter).

Enterocloster spp. treatment with BA

All three bacteria were incubated with 500 μ M of either TCDCA, TBMCA, or GDCA. Each bacteria was grown in 2 mL of RCM medium overnight at 37 C. Each overnight culture's OD600 was measured and for each strain, the OD was standardized to 0.05 in 1 mL of RCM. Samples were prepared in a 24 well plate containing 1 mL of RCM and 500 μ M of one of the bile acids. The 24 well plates were incubated at 37 C for 24 h. After 24 h, each sample's endpoint OD600 was measured and 300 μ L of each culture was incubated in ice-cold methanol, vortexed briefly, and then sat on the benchtop overnight. The methanol extracts were then centrifuged and the supernatant was frozen at -80 C until it was run on the LC-MS/MS. Sterile RCM was used as a control and also received the bile acid treatment(s). Samples used for LC-MS/MS were prepared in a 96 well plate. Each well contained a 50 μ L aliquot of each extracted culture and 50 μ L of 50% methanol/phenol red (2.5 μ g/mL). Injection blanks were prepared as 100% methanol and were the first well in each row. Quality control wells were located in column 12 of each row and contained 10 μ L from each non-injection blank well in each row.

Genome assembly and BSH identification of strain *Enterocloster clostridioformis* 141

For the *Enterocloster clostridioformis* strain 141, genomic sequences underwent rigorous quality control by Trimmomatic (version 0.27) before being assembled using MEGAHIT⁶⁸ (version 1.2.9). Protein-coding sequences were predicted by Prodigal (version 2.6.1) from the resulting contigs. To identify bile salt hydrolase (bsh) genes, a BLASTp search was performed against the predicted protein sequences using known BSH sequences as queries.

Distribution and diversity analysis of BSH in *Enterocloster* genomes

To investigate the distribution of BSH isoforms across the *Enterocloster* genus, a total of 151 *E. clostridioformis* and 158 *E. bolteae* genomes were retrieved from the NCBI database (2026.03.01). Potential bsh gene sequences were initially identified using Bakta⁶⁹ (version 1.6.1) genome annotation and subsequently filtered based on protein length, retaining sequences between 300 and 400 amino acids to ensure functional integrity. Representative BSH sequences were then clustered using MMseqs2 with a 95% identity threshold to analyze the diversity and prevalence of specific isoforms within each species. For phylogenetic reconstruction, multiple sequence alignment of the identified BSH sequences was performed using MUSCLE within the MEGA (version 11), followed by the construction of a Maximum Likelihood (ML) tree to delineate the evolutionary relationships between these BSH isoforms.

Cancer cell viability after BA *in vitro* treatment

HT29 cells were purchased and grown as per ATCC. HT29 cells were left untreated or were exposed to BA (LCA, CDCA, TDCA, GDCA, UDCA, DCA) at different dosages as indicated for 48 h. Mitoxantrone (1 and 3 μ M; Sigma-Aldrich; catalog No. M6545) and oxaliplatin (250 and 500 μ M; Accord), known ICD inducers, were the positive control.⁷⁰ Cells were treated with the bile acids for 48 h, then stained with Hoechst (33342; H3570, Thermofisher; 1/5000) + propidium iodide (PI; P4864-10mL; Sigma; 1/1000) for 30 min at 37°C before acquisition with an ImageXpress XL automated microscope (Molecular Devices, Sunnyvale, CA, US), equipped with a 20 $\times$ PlanApo objective (Nikon, Tokyo, Japan) with a minimum of 6 images per well. Nuclei were segmented using Hoechst fluorescence, and cell parameters (nuclear area, Hoechst and PI fluorescence intensities) were measured. Cell data were cleaned using a gating based on nuclear area and Hoechst intensity to separate "cells", "healthy cells" and "debris", and a threshold was determined on PI intensity to identify PI⁺ cells. Data were then summarized to a cell count and a percentage of PI cells by image. A one way unpaired Mann-Whitney U-test was performed.

DNA extraction and 16S rRNA targeted sequencing of mouse stools

Preparation and sequencing of mouse fecal samples were performed at IHU Méditerranée Infection, Marseille, France. Briefly, DNA was extracted using two protocols. The first protocol consisted of physical and chemical lysis, using glass powder and proteinase K, respectively, then processing using the Macherey-Nagel DNA Tissue Extraction Kit.⁷¹ The second protocol was identical to the first

protocol, with the addition of glycoprotein lysis and deglycosylation steps.⁷² The resulting DNA was sequenced,^{73,74} targeting the V3–V4 regions of the 16S rRNA gene.

Bioinformatics, statistics and reproducibility of 16S rRNA targeted sequencing data

Microbial species which did not have a biological meaning (environmental, rumen, extra-mammals, food, etc.) were excluded from raw data. In addition, a strengthened prevalence cutoff of 5% was employed in order to enrobust the differential analysis (DA) thus resulting in 172 species that were considered for subsequent statistical analyses. Measurements of α diversity (within sample diversity) such as Richness, Shannon and Inverse Simpson indexes, were calculated at species level using the SciKit-learn package v1.0.1, starting from raw reads counts. For beta-diversity, data matrices were first transformed with pseudocount and centered-log-ratio (CLR), then normalized and standardized using QuantileTransformer and StandardScaler methods from Sci-Kit learn package v1.0.1. Normalization using the output_distribution = 'normal' option transforms each variable to a Gaussian-like shaped distribution, whilst the standardization results in each normalized variable having a mean of zero and variance of one ($10.1080/2162402 \times .2025.2484880$). Exploratory analysis of β -diversity (between sample diversity) was calculated using the Bray-Curtis measure of dissimilarity and represented in principle Coordinate Analyses (PCoA), along with methods to compare groups of multivariate sample units (analysis of similarities - ANOSIM, permutational multivariate analysis of variance - PERMANOVA) to assess significance in data points clustering. ANOSIM and PERMANOVA were automatically calculated after 999 permutations, implemented with custom scripts (Python v3.8.2, Seaborn v0.11.2, SciKit-learn v1.0.1) ($10.1080/2162402 \times .2023.2237354$). We implemented Partial Least Square Discriminant Analysis (PLS-DA) and the subsequent Variable Importance Plot (VIP) as a supervised analysis, wherein the VIP values (equal or higher than 1, default settings) are used to identify the most discriminant bacterial species among the cohorts. Mann-Whitney U test and Kruskal-Wallis test were employed to assess significance for pairwise or multiple comparisons, respectively, considering a p value ≤ 0.05 as significant. Clustermaps of microbial abundances were generated with Euclidean distance and Ward linkage algorithms by means of custom scripts (Python v3.8.2, Seaborn v0.11.2, SciKit-learn v1.0.1). Where clearly stated, all p values were corrected for multiple hypothesis testing using a two-stage Benjamini-Hochberg FDR at 10% (<https://doi.org/10.1016/j.ejca.2023.112948>).

QUANTIFICATION AND STATISTICAL ANALYSIS

Overall survival and progression-free survival analyses of patients were performed using the Kaplan-Meier estimator, and Cox regression in uni- or multivariate analyses (Table S4) and the patient groups were compared using the log rank (Mantel-Cox) test. In *in vitro* and *in vivo* experiments, unless otherwise stated, statistical significance was determined by non-parametric Mann-Whitney U test (two unpaired groups), Wilcoxon matched-pairs signed-rank test (two groups of paired samples), Kruskal-Wallis H test (more than two groups), correcting the statistical significance for multiple comparisons and FDR using the two-stage step-up method of Benjamini and Hochberg using GraphPad Prism 10 (GraphPad, San Diego, CA, USA) and the statistical environment R (<http://www.R-project.org/>). For tumor growth analysis, we used Tumor Growth available at <https://kroemerlab.shinyapps.io/TumGrowth/>. p -value and FDR p -value adjusted <0.05 was considered statistically significant. All values are mean $\pm$ (SD) or (SEM) as stated in the legends. The number of independent biological replicates for each experiment and the sample size of each experimental group or condition are provided in the figure legends.