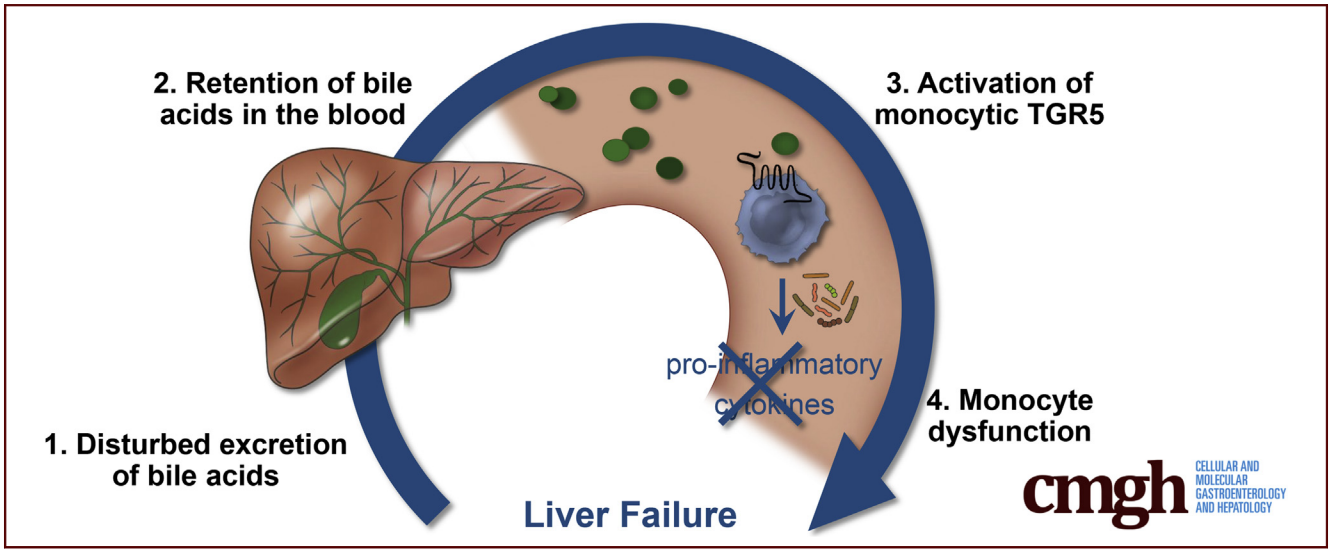
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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Circulating Bile Acids in Liver Failure Activate TGR5 and Induce Monocyte Dysfunction

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SUMMARY

Serum bile acids accumulate in liver failure. We show that, depending on their composition and quantity, these bile acids activate Takeda G-protein–coupled receptor 5, an immunomodulatory receptor that is highly expressed in monocytes. Takeda G-protein–coupled receptor 5 activation leads to monocyte dysfunction and correlates with mortality.

BACKGROUND & AIMS: Retention of bile acids in the blood is a hallmark of liver failure. Recent studies have shown that increased serum bile acid levels correlate with bacterial infection and increased mortality. However, the mechanisms by which circulating bile acids influence patient outcomes still are elusive.

METHODS: Serum bile acid profiles in 33 critically ill patients with liver failure and their effects on Takeda G-protein–coupled receptor 5 (TGR5), an immunomodulatory receptor that is highly expressed in monocytes, were analyzed using tandem mass spectrometry, novel highly sensitive TGR5 bioluminescence resonance energy transfer using nanoluciferase (NanoBRET, Promega Corp, Madison, WI) technology, and in vitro assays with human monocytes.

RESULTS: Twenty-two patients (67%) had serum bile acids that led to distinct TGR5 activation. These TGR5-activating serum bile acids severely compromised monocyte function. The release of proinflammatory cytokines (eg, tumor necrosis factor α or interleukin 6) in response to bacterial challenge was reduced significantly if monocytes were incubated with TGR5-activating serum bile acids from patients with liver failure. By contrast, serum bile acids from healthy volunteers did not influence cytokine release. Monocytes that did not express TGR5 were protected from the bile acid effects. TGR5-activating serum bile acids were a risk factor for a fatal outcome in patients with liver failure, independent of disease severity.

CONCLUSIONS: Depending on their composition and quantity, serum bile acids in liver failure activate TGR5. TGR5 activation leads to monocyte dysfunction and correlates with mortality, independent of disease activity. This indicates an active role of TGR5 in liver failure. Therefore, TGR5 and bile acid metabolism might be promising targets for the treatment of immune dysfunction in liver failure. *(Cell Mol Gastroenterol Hepatol 2021;12:25–40; https://doi.org/10.1016/j.jcmgh.2021.01.011)*

Keywords: Liver Failure; Bile Acids; TGR5; GPBAR1.

Cholestasis is a condition that complicates not only liver but also nonhepatic diseases such as critical illness, and is related to increased mortality.[1–3] Recent studies have shown that the disturbed excretion of bile acids and their accumulation in the blood are associated especially with infections and fatal outcomes in these diseases.[4–7] The mechanisms by which circulating bile acids influence patient outcomes still are elusive.

Bile acids, secreted by the liver into the bile, have long been believed to be simple emulsifiers of dietary fats.[8] However, increasing evidence has indicated that bile acids are complex signaling molecules that activate various nuclear and membrane-bound receptors.[9,10] One of these receptors, the Takeda G-protein–coupled receptor 5 (TGR5, also known as G-protein–coupled bile acid receptor 1), is an immunomodulatory receptor that fine-tunes innate immune function and has been suggested to mediate immune tolerance in the gut.[11,12] Monocytes and macrophages express TGR5.[9,13,14] In general, these myeloid cells produce high amounts of proinflammatory cytokines in response to bacterial pathogens. The same cells also may be involved in tolerance toward the plethora of bacteria colonizing the human gut. This tolerance is supposed to be mediated, at least in part, by intestinal bile acids, that activate TGR5 on myeloid cells, thereby preventing the production of proinflammatory cytokines and subsequent inflammation.[11,12]

In various liver diseases, the bile acid flow from the liver into the bile is disturbed, leading to an accumulation of bile acids in the blood.[15,16] To date, massively increased serum bile acid levels in liver diseases were considered mainly as a surrogate parameter for excretory liver failure. We hypothesized that the quantity and composition of circulating bile acids may impact myeloid cell functionality via TGR5. Therefore, we investigated the bile acid profiles of patients with liver failure requiring admission to the intensive care unit and analyzed the effect of the complex bile acid profiles on monocyte function. Furthermore, our study determined the relative potency of several bile acids to activate TGR5 and analyzed how complex bile acid mixtures affect TGR5 activity.

Results

Patients With Liver Failure Show Serum Bile Acids Activating TGR5

To investigate TGR5 activity in response to circulating bile acids, we established a TGR5 reporter assay using a recently described highly selective and specific nanoluciferase bioluminescence resonance energy transfer assay (NanoBRET, Promega Corp, Madison, WI) in combination with a mini-G_s-protein.[17] Mini-G_s-protein recruitment by the G-protein–coupled receptor TGR5 was measured via NanoBRET (Figure 1*A*). TGR5, C-terminally tagged with a nanoluciferase (Nluc), served as the donor, and the mini-G_s-Venus fusion protein was the acceptor. Human embryonic kidney (HEK) 293T cells were transiently transfected with both vectors and NanoBRET change was monitored before and after ligand addition. As shown in Figure 1*B*, stimulation with taurolithocholic acid (TLCA), the most potent naturally occurring TGR5 agonist,[9] showed a dose-dependent recruitment of the mini-G_s-protein. The NanoBRET change induced by 100 μmol/L TLCA was set to 100%, reflecting maximal TGR5 activity. To control the specificity of our assay, the β_2 adrenergic receptor (ADRB2), another G-protein–coupled receptor recruiting G_s proteins, also was fused genetically to a nanoluciferase. The TGR5 agonist did not induce an ADRB2 NanoBRET change, and the ADRB2 agonist isoprenaline did not activate TGR5 NanoBRET (Figure 1*C*), indicating specificity of the NanoBRET assay. Using this method, we evaluated whether serum bile acids would activate TGR5. We assessed the individual serum bile acid profiles of 33 critically ill patients with liver failure and 33 healthy controls by tandem mass spectrometry. To exclude unspecific serum effects, the pure serum bile acid profile from each subject was remixed in buffer and measured for their potential to activate TGR5. Serum bile acids from healthy human volunteers hardly induced any TGR5 activation (means ± SD, 1.3% of maximal activation ± 9.0%). By contrast, serum bile acids from patients with liver failure activated TGR5 significantly (means ± SD, 37.4% ± 19.1%) (Figure 1*D*).

In Acute-on-Chronic Liver Failure Patients, 95% Show Serum Bile Acids Activating TGR5

Serum bile acids from our patients induced TGR5 activity ranging from 0.8% to 76.1% (Table 1). Some patients showed levels that were comparable with healthy controls, while others had a serum bile acid composition potently activating TGR5. To identify patients with serum bile acids that activate TGR5 relevantly, we defined the mean value of healthy individuals ± 3× SD as the reference range. Consequently, serum bile acid profiles inducing a TGR5 activity of ≥28.3% were defined as pathologic and were classified as TGR5-activating serum bile acids. By using this cut-off value, none of the healthy volunteers had TGR5-

Abbreviations used in this paper: **ACLF, acute-on-chronic liver failure; ADRB2, β_2 adrenergic receptor; DMSO, dimethyl sulfoxide; EV, empty vector; IFN, interferon; LPS, lipopolysaccharide; NanoBRET, bioluminescence resonance energy transfer using nanoluciferase; Nluc, nanoluciferase; TGR5, Takeda G-protein–coupled receptor 5; TLCA, taurolithocholic acid; UDCA, ursodeoxycholic acid.**

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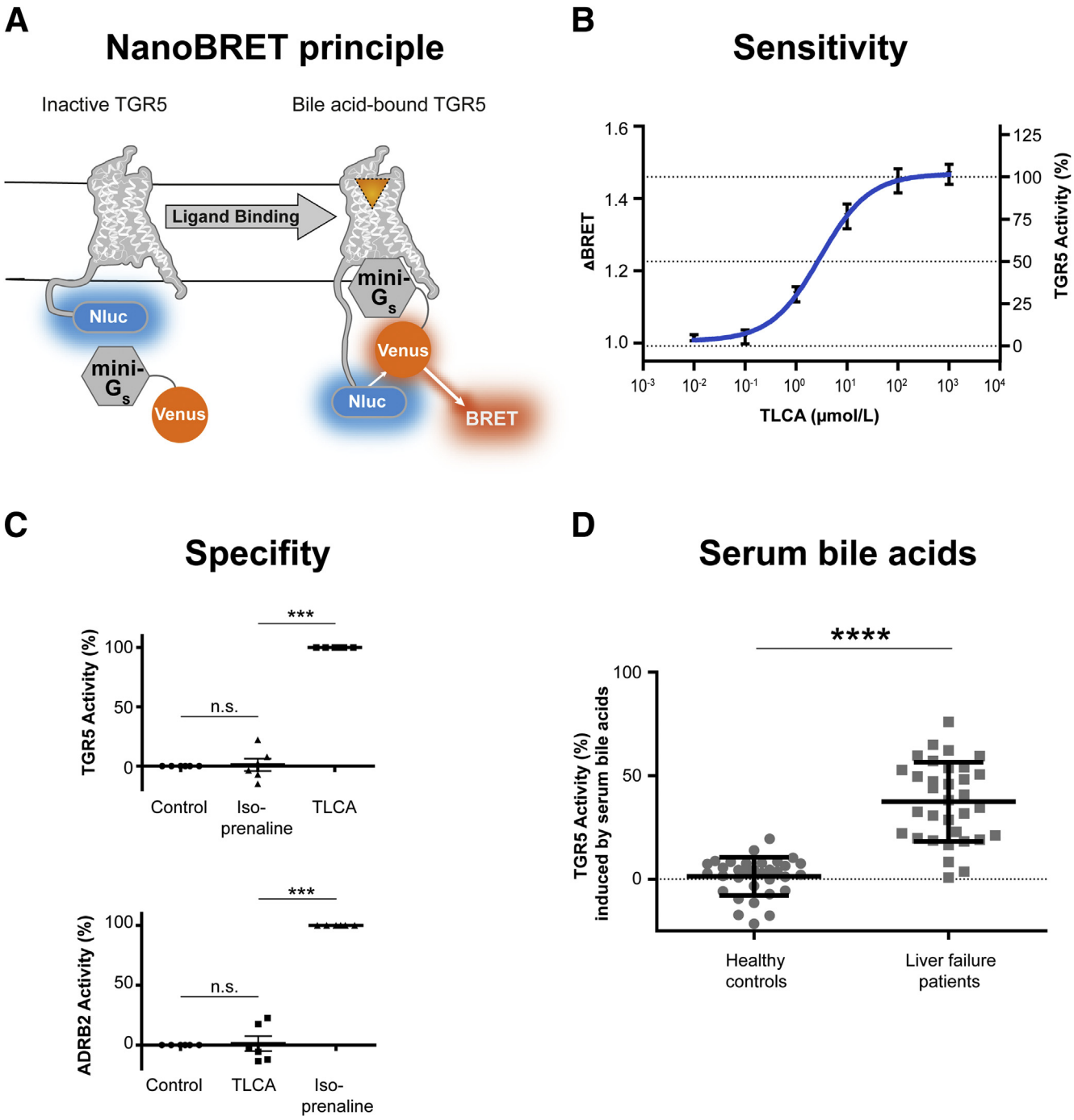


Figure 1. Patients with liver failure show serum bile acids activating TGR5. (*A* and *B*) The TGR5 NanoBRET assay measured dose-dependent G_s recruitment by bile acids. N = 10. Data are means ± SEM. (*C*) TLCA selectively activated TGR5 NanoBRET. The ADRB2 agonist isoprenaline selectively activated ADRB2 NanoBRET. N = 6. Data are means ± SEM, 1-way repeated-measures analysis of variance with Bonferroni correction. (*D*) Patients with liver failure show serum bile acids significantly activating TGR5. n = 33/group. Data are means ± SD, Student *t* test. ***$P < .001$, ****$P < .0001$.

activating serum bile acids. By contrast, 67% of our critically ill patients with liver failure had serum bile acids that activated TGR5 relevantly (22 of 33 patients) (Table 1).

Liver failure in critically ill patients is a heterogeneous condition with different etiologies and characteristics. Interestingly, almost all patients with acute-on-chronic liver failure (ACLF) showed bile acid profiles activating TGR5 (18 of 19 patients; 95%) (Figure 2*A* and Table 2). Although pathologically increased TGR5 activity also was induced by bile acid profiles of patients with acute liver failure (33% with TGR5-activating serum bile acids) or liver graft failure (20% with TGR5-activating serum bile acids), the

Table 1. TGR5 Activity Induced by Serum Bile Acids From Patients With Liver Failure

Patient ID	TGR5 activity,[a] %	Patient ID	TGR5 activity,[a] %	Patient ID	TGR5 activity,[a] %
01	3.7	12	**76.1**	23	**54.2**
02	**30.7**	13	16.5	24	**45.9**
03	**57.2**	14	8.3	25	**38.2**
04	**48.3**	15	19.9	26	**28.7**
05	**65.0**	16	23.0	27	**53.7**
06	**34.6**	17	19.1	28	**18.3**
07	**59.5**	18	**59.7**	29	**40.9**
08	**44.1**	19	18.7	30	0.8
09	**31.8**	20	**52.9**	31	**49.6**
10	**50.7**	21	**32.7**	32	22.2
11	**62.3**	22	21.2	33	**47.2**

[a]Pathologically increased values are shown in bold (ie, bile acids that mediate TGR5 activity ≥28.3%).

proportion was significantly lower. Accordingly, the mean TGR5 activity induced by serum bile acids was highest in patients with ACLF (Figure 2*B*).

Increased serum bile acids are commonly understood as an indicator for excretory liver failure.[15,16] Consequently, TGR5 activation by serum bile acids might correlate with bilirubin, alkaline phosphatase, or other liver function tests indicative of cholestasis. We therefore compared blood tests of patients with and without TGR5-activating serum bile acids (Table 2). Blood tests indicated severe liver failure, but there was no difference between both groups. Consequently, none of the routine blood tests we used could predict TGR5 activation induced by serum bile acids. This suggests that the TGR5 activation by complex serum bile acid profiles is an independent factor in the pathogenesis of liver failure.

Only the total serum bile acid concentration assessed by a colorimetric assay was correlated to TGR5 activity (Figure 2*C* and Table 2).

TGR5-Activating Serum Bile Acids Induce Monocyte Dysfunction

TGR5 activation has been linked closely to immune suppression in monocytes.[9,18] TGR5-activating serum bile acids therefore might modulate monocyte function in patients. To investigate this hypothesis, we treated monocytes from healthy human volunteers producing proinflammatory cytokines in response to bacterial lipopolysaccharide (LPS) with bile acids. We remixed the mean bile acid profile from the patients with TGR5-activating serum bile acids. We compared its effect with the mean bile acid profile of matched healthy controls

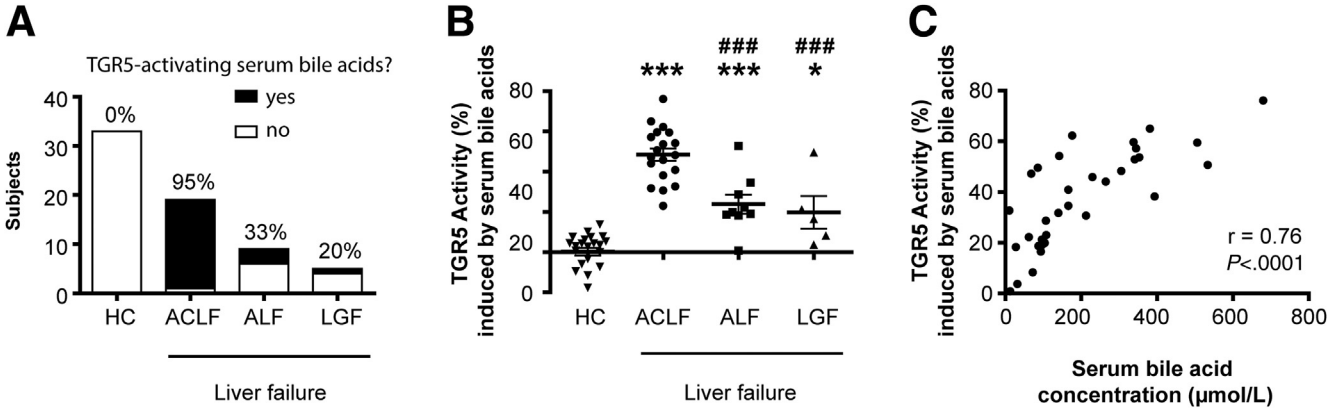


Figure 2. In acute-on-chronic liver failure patients, 95% show serum bile acids activating TGR5. (*A*) Proportion of subjects with serum bile acids activating TGR5. (*B*) TGR5 activity induced by serum bile acid profiles. Data are means ± SD. One-way repeated-measures analysis of variance with Bonferroni correction. $^{*}P < .05$ compared with HC, $^{***}P < .001$ compared with HC, and $^{\#\#\#}P < .001$ compared with ACLF. (*C*) Correlation between serum bile acid concentration and TGR5 activity induced by serum bile acids, Spearman correlation rank test. ACLF, acute-on-chronic liver failure; ALF, acute liver failure; HC, healthy control; LGF, liver graft failure.

Table 2. Characteristics of Liver Failure Patients With and Without TGR5-Activating Serum Bile Acids

Characteristics	TGR5-activating serum bile acids? Yes n = 22	No n = 11	*P*
Age, *y*	55 ± 12	56 ± 13	NS
Male sex, % (n)	50 (11)	64 (7)	NS
Etiology of liver failure, % (n)			
Acute-on-chronic	81.8 (18)	9.1 (1)	
Alcoholic cirrhosis	50.0 (11)		
Viral cirrhosis	4.5 (1)		
Viral and alcoholic cirrhosis	4.5 (1)		
Cryptogenic cirrhosis	9.0 (2)	4.5 (1)	
Other cirrhosis[a]	13.6 (3)		
Acute	13.6 (3)	54.5 (6)	
Liver graft failure	4.5 (1)	36.4 (4)	
Hemodialysis, % (n)	55 (12)	45 (5)	NS
Serum levels			
ALT, *U/L*	42 (27–99)	54 (40–88)	NS
AST, *U/L*	86 (64–353)	78 (57–126)	NS
AST/ALT	2.2 (1.4–3.2)	1.6 (1.3–2.1)	NS
ALP, *U/L*	118 (79–161)	147 (70–444)	NS
Total bilirubin, *μmol/L*	357 ± 120	350 ± 81	NS
Albumin, *g/L*	18 (17–25)	19 (16–23)	NS
Bile acid serum parameter			
Total serum bile acids, *μmol/L*	285 (166–476)	72 (46–96)	<.001
TGR5 activity by bile acids, %	48.4 ± 12.4	15.6 ± 7.7	<.001

NOTE. Values are means ± SD or median with 95% CI.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a]Other causes of cirrhosis included the following: 1 patient with primary sclerosing cholangitis, 1 patient with primary biliary cholangitis, and 1 patient with Alagille–Watson syndrome.

Table 3. Serum Bile Acid Profile of Healthy Controls vs Liver Failure Patients With TGR5-Activating Serum Bile Acids

Bile acid profile, *μmol/L*	Healthy controls (n = 22)	Patients (n = 22)	*P*	TGR5 activity,[a] %
Primary bile acids				
Cholic acid	0.3 ± 0.5	0.1 ± 0.1	NS	0.0
Taurocholic acid	0.0 ± 0.1	7.8 ± 9.9	NS	0.9
Glycocholic acid	0.2 ± 0.2	15.4 ± 15.8	< .01	1.6
Chenodeoxycholic acid	0.4 ± 0.7	0.2 ± 0.2	NS	0.1
Taurochenodeoxycholic acid	0.1 ± 0.1	23.7 ± 12.3	< .001	16.5
Glycochenodeoxycholic acid	0.9 ± 0.6	29.2 ± 21.1	< .001	17.4
Secondary bile acids				
Lithocholic acid	0.0 ± 0.0	0.0 ± 0.0	NS	0
Taurolithocholic acid	0.0 ± 0.0	0.0 ± 0.1	NS	0
Deoxycholic acid	0.5 ± 0.8	0.0 ± 0.0	NS	0
Taurodeoxycholic acid	0.1 ± 0.1	0.2 ± 0.8	NS	0.4
Glycodeoxycholic acid	0.3 ± 0.3	0.4 ± 1.3	NS	0.6
Tertiary bile acids				
Ursodeoxycholic acid	0.1 ± 0.3	6.0 ± 6.4	NS	1.0
Tauroursodeoxycholic acid	0.0 ± 0.0	21.0 ± 14.3	< .001	3.2
Glycoursodeoxycholic acid	0.1 ± 0.2	81.2 ± 56.9	< .001	8.3
Total				50.0

NOTE. Values are means ± SD, 1-way analysis of variance with Bonferroni correction.
[a]TGR5 activity caused by single serum bile acids from patients; calculated according to the formula shown in Figure 6*C* (eg, glycocholic acid in serum from patients was increased significantly and caused a TGR5 activity of 1.6%).

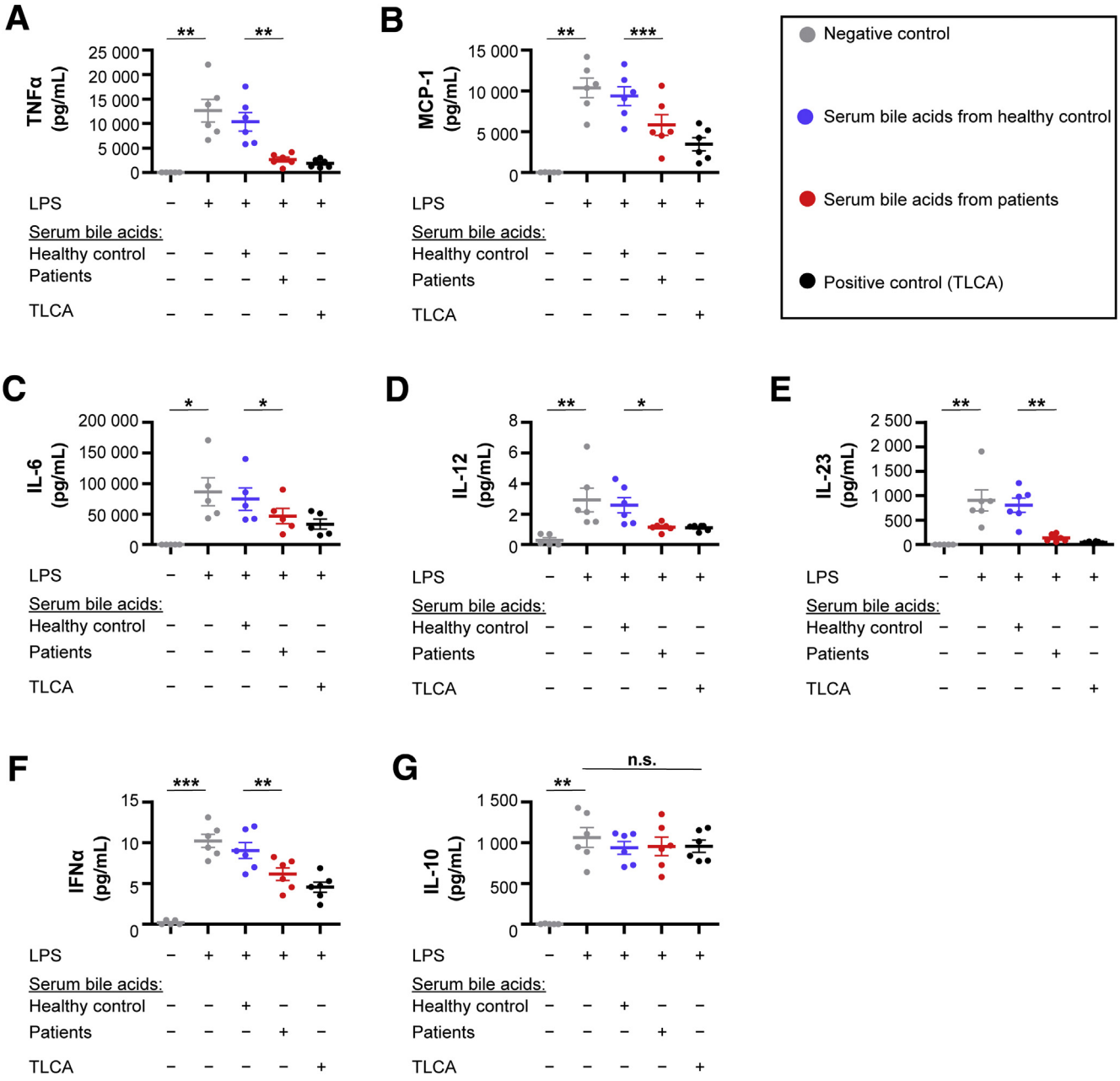


Figure 3. TGR5-activating serum bile acids induce monocyte dysfunction. (*A–G*) Monocytes were incubated with the mean bile acid profile from either healthy controls or liver failure patients with TGR5-activating serum bile acids. TGR5-activating serum bile acids significantly inhibited the release of proinflammatory cytokines induced by LPS. Anti-inflammatory interleukin (IL)10 was not altered. Data are means ± SEM, N = 5–6. Two-way repeated-measures analysis of variance with post hoc paired *t* test. **P* < .05, ***P* < .01, and ****P* < .001. IFN, interferon; MCP-1, monocyte chemoattractant protein-1; TLCA, taurolithocholic acid; TNFα, tumor necrosis factor α.

(both mean bile acid profiles are shown in Table 3). Monocytes were preincubated with bile acid mixtures and subsequently stimulated with LPS. Cells left untreated served as a negative control and the TGR5 agonist TLCA as a positive control.

Monocytes stimulated with TGR5-activating serum bile acids from patients showed a significant immune dysfunction (Figure 3*A–F*). The release of proinflammatory cytokines and chemokines, such as tumor necrosis factor α, interleukin 6, and monocyte chemoattractant protein-1, was reduced significantly. Interestingly, the anti-inflammatory cytokine interleukin 10 was not affected (Figure 3*G*).

To assure that the effects of the serum bile acids on monocytes were mediated by TGR5, we repeated our experiments using the human monocytic cell line THP-1, which does not express TGR5 endogenously.[9,19] THP-1 monocytes were transduced with TGR5 or empty vector

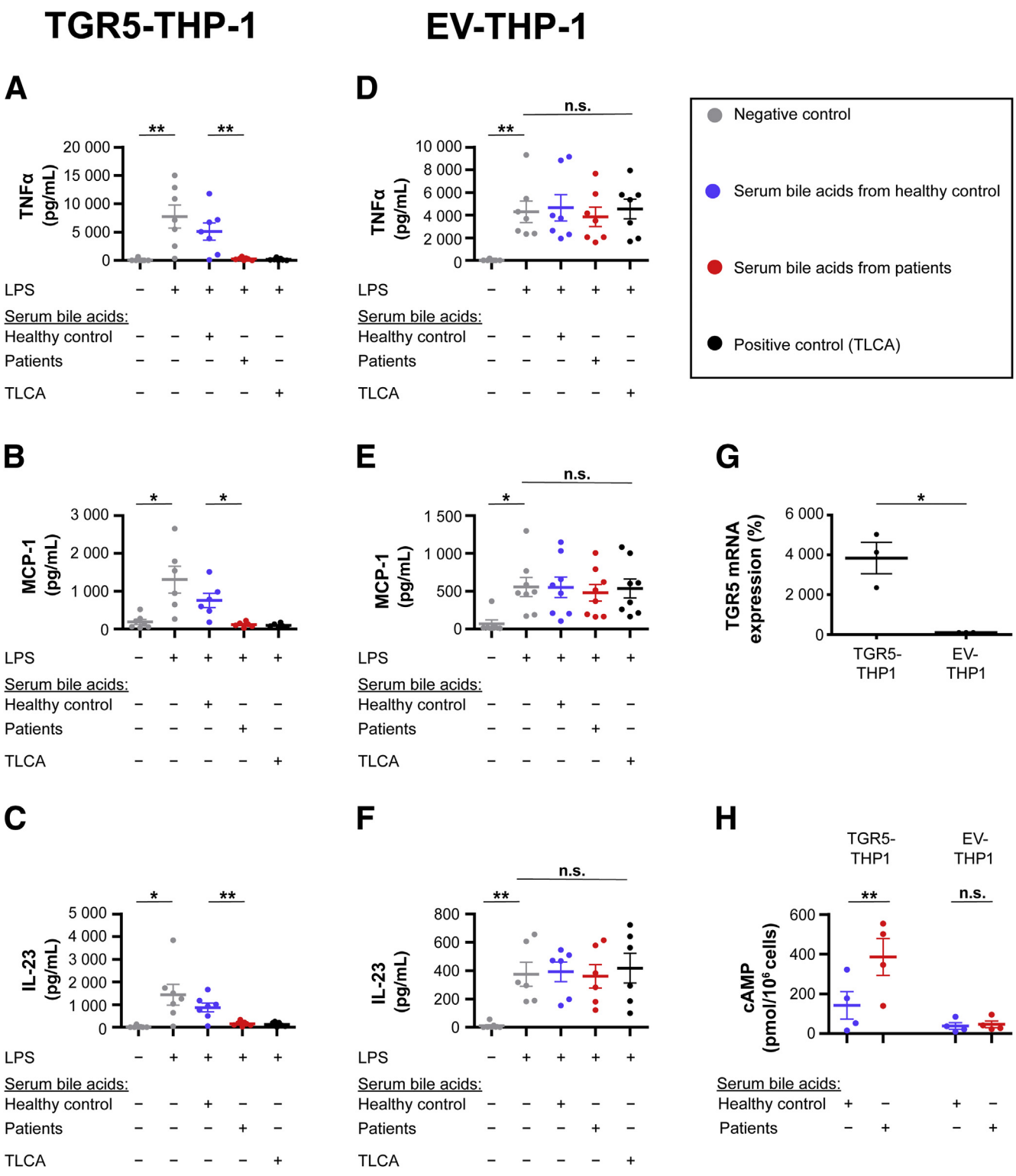


Figure 4. Effects of serum bile acids are mediated by TGR5. THP-1 monocytes were transduced with TGR5 or EV and incubated with the mean bile acid profile from either healthy controls or liver failure patients with TGR5-activating serum bile acids. Serum bile acids from patients significantly inhibited the secretion of proinflammatory cytokines in (*A–C*) TGR5–THP-1, but not in (*D–F*) EV–THP-1. Two-way repeated-measures analysis of variance with post hoc paired *t* test, N = 6–8. (*G*) TGR5–THP-1 showed a significantly higher TGR5 expression compared with EV–THP-1, paired *t* test, N = 3. (*H*) Serum bile acids from patients induced significant adenosine 3′,5′-cyclic monophosphate (cAMP) production in TGR5-THP-1, but not in EV-THP-1. Two-way repeated-measures analysis of variance with the post hoc Holm–Sidák test, N = 4. All data are means ± SEM. **P* < .05, ***P* < .01. MCP-1, monocyte chemoattractant protein-1; mRNA, messenger RNA; TNFα, tumor necrosis factor α.

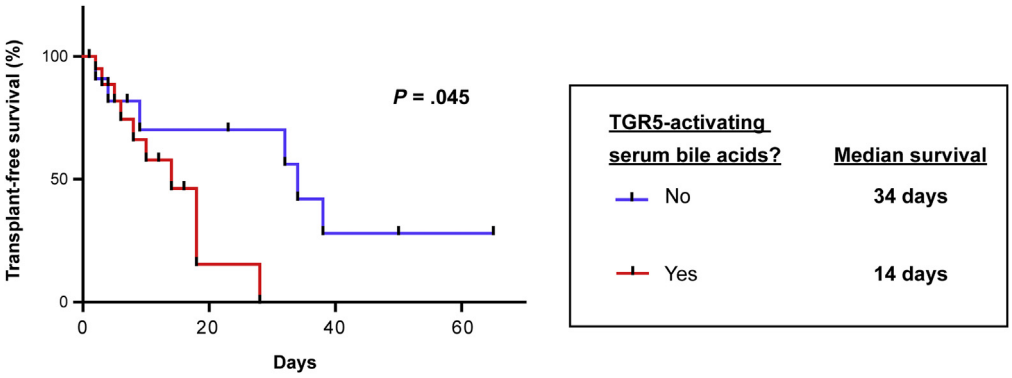


Figure 5. TGR5-activating serum bile acids are associated with increased mortality. Critically ill patients with liver failure were grouped according to their serum bile acid profiles. Patients with TGR5-activating serum bile acids had a significantly shorter survival (n = 22, *red line*) as compared with patients without (n = 11, *blue line*).

(EV). TGR5-activating serum bile acids from liver failure patients triggered adenosine 3′,5′-cyclic monophosphate production (Figure 4*H*) and immune suppression (Figure 4*A–C*) in TGR5-expressing THP-1. By contrast, EV–THP-1 did not show any bile acid effects (Figure 4*D–F* and *H*), indicating that the immunosuppressive effects of serum bile acids were mediated by TGR5.

TGR5-Activating Serum Bile Acids Are Associated With Increased Mortality

Monocyte dysfunction in liver failure is associated with a poor outcome. In particular, an inhibited ex vivo tumor necrosis factor α secretion after LPS challenge has been linked to high mortality.[20] From our 33 liver failure patients investigated, 22 showed serum bile acids activating TGR5. We therefore compared the outcome of these 22 patients with the other 11 patients. Indeed, TGR5-activating serum bile acids were associated with significantly increased mortality (Figure 5). Serum bile acids already have been shown to be a risk factor for a fatal outcome in other studies, but were considered mainly as a surrogate parameter for liver failure.[4–6] To exclude that the increased mortality of the 22 patients with TGR5-activating serum bile acids was the result of confounding factors, we assessed severity scores that have been described to predict mortality in liver failure and intensive care patients. As indicated by multivariable Cox proportional hazards regression, severity scores and TGR5 activation by serum bile acids predicted mortality independently (Table 4). Furthermore, TGR5-activating serum bile acids were a risk factor for fatal outcome independent of the patients' total serum bile acid concentration (Table 5), suggesting an active role of TGR5 in the pathogenesis of liver failure.

TGR5 Activation Is Caused by the Additive Effect of Conjugated Primary and Tertiary Serum Bile Acids

The human serum bile acid profile consists of circulating primary bile acids (bile acids, which are produced in the liver), secondary bile acids (primary bile acids, which have been modified by gut microbiota), and tertiary bile acids (ursodeoxycholic acid derivatives, ie, secondary bile acids that have been metabolized further by the liver or were administered directly as a drug). To assess which serum bile acids contribute to TGR5 activation, we used the TGR5 NanoBRET assay to determine the TGR5 potency of the 15 most abundant human serum bile acids. Therefore, we quantified the concentration of each bile acid that was required to achieve the half-maximal effect of TLCA (the most potent naturally occurring bile acid).[9] One exemplary calculation is shown in Figure 6*A*. The curves for all bile acids are shown in Figure 7 and Figure 8. The potency of each bile acid to activate TGR5 was expressed as equivalent of cholic acid (Figure 6*B*).

Secondary bile acids were the most potent activators of TGR5 (up to 243.5 times as potent as cholic acid). Primary

Table 4. Mortality Hazard Ratios for TGR5-Activating Serum Bile Acids and Severity Scores

Predictors	HR	95% CI	*P* value
TGR5-activating serum bile acids	7.785	1.294–46.853	.025
MELD-Na	1.300	1.075–1.570	.007
SAPS II	0.887	0.808–0.974	.012
APACHE II	1.279	1.054–1.552	.013
SOFA	1.386	1.052–1.827	.020

NOTE. Cox proportional hazard regression.
APACHE II, Acute Physiology And Chronic Health Evaluation II; HR, hazard ratio; MELD-Na, Model for End-Stage Liver Disease-Sodium; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment Score.

Table 5. Mortality Hazard Ratios for TGR5-Activating Serum Bile Acids and Total Serum Bile Acid Concentration

Predictors	HR	95% CI	*P* value
TGR5-activating serum bile acids	10.600	1.801–62.392	.009
Total serum bile acid concentration	0.995	0.990–1.001	.078

NOTE. Cox proportional hazard regression.

and tertiary bile acids only showed mild to moderate TGR5 potency (1.0–8.4 times as potent as cholic acid). This is in line with previous studies comparing the effects of primary, secondary, and tertiary bile acids.[9,21,22]

We hypothesized that the effect of different bile acids might be additive in mixtures. Consequently, the total TGR5 activity induced by bile acid mixtures might be calculated by the sum of effects of the single bile acids. To address this question, we calculated the TGR5 activity induced by each single serum bile acid using the product of (1) its bile acid concentration, (2) its respective TGR5 potency, and (3) k, a constant determined by previous measurements. The products of all serum bile acids were added for the total TGR5 activity (Figure 6*C*).

By using this formula, we calculated the TGR5 activity induced by the serum bile acids for each patient and healthy volunteer. Each calculated TGR5 activity was compared with the TGR5 activity measured by TGR5 NanoBRET. Interestingly, there was a significant and close correlation of calculated and measured TGR5 activity (Figure 6*D*). This indicates that bile acids found in serum indeed had an additive effect.

We now compared the serum bile acid profiles of the 22 patients with TGR5-activating serum bile acids with their age- and sex-matched controls (Table 3). The bile acid profiles showed significantly enhanced levels of conjugated cholic acid, chenodeoxycholic acid, and ursodeoxycholic acid. Using our formula (Figure 6*C*), we calculated the TGR5 activity induced by each single bile acid (Table 3, last column). Chenodeoxycholic acid conjugates, although only moderate activators of TGR5, caused most of the serum TGR5 activation in our patients (16.5% and 17.4% of a total of 50% serum TGR5 activity). Furthermore, the weak TGR5 activators glycocholic acid, glycoursodeoxycholic acid, and tauroursodeoxycholic acid contributed to TGR5 activation.

Discussion

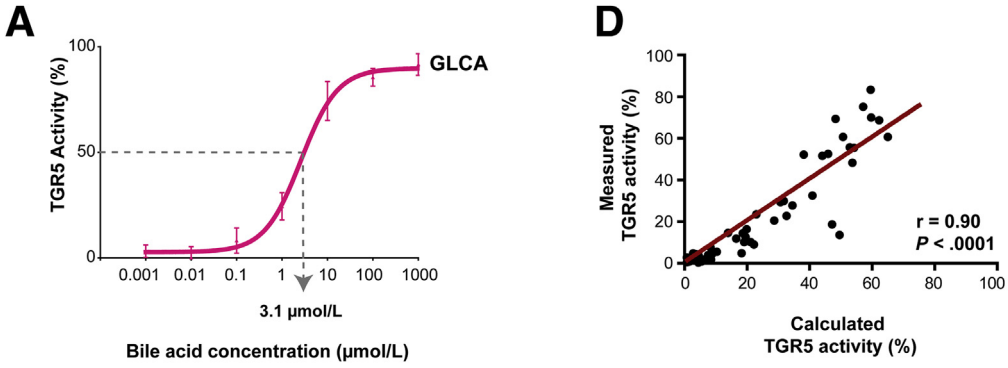
The immunomodulatory effects of the bile acid receptor TGR5 were first described in 2003.[9] Since then, many studies have confirmed immunosuppressive actions of single bile acids on monocytes and macrophages mediated by TGR5.[9,14,23] However, studies investigating the effects of complex bile acid profiles are lacking. To overcome the challenge of multiple TGR5-activating bile acids in human serum, we established a highly specific and sensitive TGR5 NanoBRET assay. This assay showed that none of the tested healthy individuals, but 67% of our patients, had serum bile acid profiles activating TGR5. These bile acid profiles inhibited monocyte function significantly (Figure 3). In line with previous studies investigating single bile acids in macrophages, we showed that the proinflammatory cytokine secretion upon LPS stimulation was reduced significantly, while the anti-inflammatory functions were preserved.[14,23]

Functionally impaired monocytes are an abundant hallmark of liver failure, which makes patients highly susceptible to infections and fatal outcome.[24] Impaired monocytes in liver failure are characterized by their inability to produce proinflammatory cytokines upon LPS challenge,[25,26] a feature that can be induced in healthy monocytes by incubating them with plasma from liver failure patients.[27,28] Our study showed a link between increased serum bile acids, which frequently are seen in liver failure, and immune dysfunction. Bile acids that accumulate in the blood of patients with liver failure activate TGR5 in monocytes and thus impair monocyte function. Because monocyte dysfunction leading to infection is a major complication of liver dysfunction and an important driver of mortality,[24] TGR5 and bile acid metabolism might be interesting targets for pharmacologic approaches. Diets, changes of the microbiome, certain medications, and treatments alter the bile acid composition in the intestine and serum.[29–32] Consequently, therapeutic bile acids (ursodeoxycholic acid, obeticholic acid), inhibitors of reabsorption, and liver dialysis might be evaluated for their potential to reduce TGR5 activity and improve immune function.

Our study showed that TGR5-activating serum bile acids were associated with increased mortality in liver failure. Strikingly, these serum bile acids were a risk factor for a fatal outcome independent of disease severity and commonly used prognostic scores (Table 4). Predicting the mortality in liver failure is of utmost importance because it is the base for allocation of donor livers worldwide.[33] A detailed knowledge of individual risk factors is pivotal for an equitable allocation policy. In most countries, deceased donor livers are allocated to the patients with the Model for End-Stage Liver Disease score.[33] TGR5 activation by serum bile acids might reflect the increased waitlist mortality seen in some cholestatic liver diseases (eg, primary biliary cirrhosis).[34,35] Nevertheless, our observation on mortality was conducted in only 33 patients. Whether studies with larger numbers of subjects will confirm this correlation remains to be investigated.

In our patients, TGR5 activation was not owing to the occurrence of a single bile acid, but was the sum of effects of

several bile acids (Table 3). (Patho-)Physiological conditions, in which mixtures of ligands bind to the same receptor with different affinities, frequently are observed in clinical practice. One strategy to handle the pharmacologic complexity is to define the potencies of the ligands. Prominent examples are glucocorticoid potencies and the relative strength of opioids. These potencies allow an estimate of the effect of drug combinations. We assessed the potency of each bile acid relative to cholic acid. In line with the literature, secondary bile acids, that is, lithocholic acid, deoxycholic acid, and their conjugates, had the highest potency to activate TGR5.[9,22] Using these potencies, we calculated the TGR5 activity induced by bile acid mixtures as the sum of TGR5 activity of single bile acids (Figure 6*C*). Calculated TGR5 activity in 66 individuals correlated closely with the measured TGR5 activity (Figure 6*D*). The formula therefore



B

	Bile acid concentration to achieve 50% of max. TGR5 activity (µmol/L)	TGR5 potency P_b
Primary bile acids		
1. Cholic acid (CA)	560.0	1.0
2. Taurocholic acid (TCA)	390.0	1.4
3. Glycocholic acid (GCA)	485.0	1.2
4. Chenodeoxycholic acid (CDCA)	67.0	8.4
5. Taurochenodeoxycholic acid (TCDCA)	69.0	8.1
6. Glycochenodeoxycholic acid (GCDCA)	80.0	7.0
Secondary bile acids		
7. Lithocholic acid (LCA)	20.0	28.0
8. Taurolithocholic acid (TLCA)	2.3	243.5
9. Glycolithocholic acid (GLCA)	3.1	180.6
10. Deoxycholic acid (DCA)	20.0	28.0
11. Taurodeoxycholic acid (TDCA)	24.0	23.3
12. Glycodeoxycholic acid (GDCA)	31.0	18.1
Tertiary bile acids		
13. Ursodeoxycholic acid (UDCA)	290.0	1.9
14. Tauroursodeoxycholic acid (TUDCA)	320.0	1.8
15. Glycoursodeoxycholic acid (GUDCA)	470.0	1.2

C

$$T = \sum_{b=1}^{15} (c_b \times P_b \times k)$$

T	calculated TGR5 activity (%)
c_b	concentration of respective bile acid (µmol/L)
P_b	TGR5 potency of respective bile acid
k	0.085 (% x L/µmol)
b	respective bile acid in mixture

Figure 6. TGR5 activation is caused by the additive effect of conjugated primary and tertiary bile acids. (*A*) TGR5 potency was measured for each bile acid (eg, glycolithocholic acid [GLCA]) at increasing concentrations. The concentration that was required to achieve the half-maximal effect of TLCA was calculated. (*B*) TGR5 potencies of bile acids were expressed as a multiple of cholic acid potency. (*C*) Formula to calculate the TGR5 activity induced by serum bile acids using the bile acid concentrations and TGR5 potencies (potencies of bile acids). (*D*) Calculated and measured TGR5 activity correlated closely, N = 66, Spearman correlation rank test. max., maximum.

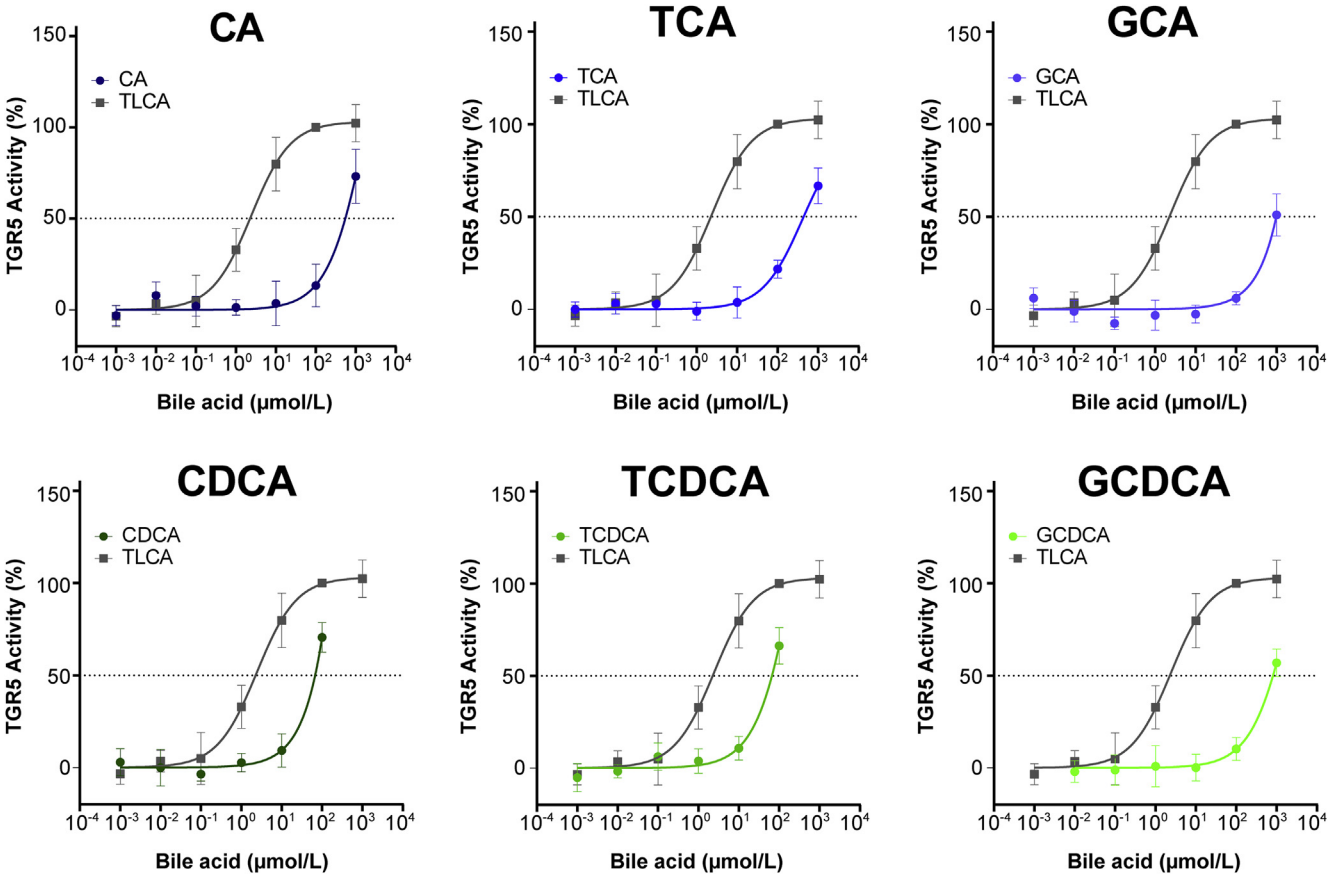


Figure 7. TGR5 activation by primary bile acids. TGR5 activity was measured for each bile acid at increasing concentrations. The concentration that was required to achieve the half-maximal effect of TLCA was calculated. CA, cholic acid; CDCA, chenodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; TCA, taurocholic acid; TCDCA, taurocholic acid.

might be a useful tool to understand the complex bile acid profiles seen in patients. It allows calculation of the effect of bile acids on TGR5 directly from serum bile acid profiles and could be used to compare bile acid effects before and after distinct therapies.

This study had several limitations: first and foremost, our study was limited to 33 patients and further studies will be needed to ascertain the TGR5-mediated effects of bile acid profiles in liver failure. Second, according to departmental standard operating procedures, all patients with liver failure received UDCA therapy (3 × 250 mg/d). Although the weak TGR5 activator UDCA and its conjugates accumulate in serum, UDCA therapy lowers serum concentration of bile acids that are potent TGR5 activators.[29,36] The effect of bile acid profiles therefore might be underestimated in our study.

Taken together, TGR5-activating serum bile acids were abundant in critically ill patients with liver failure and associated with a fatal outcome. In addition to other recently identified mechanisms,[25,37] serum bile acids may contribute to immune dysfunction in liver diseases. Therefore, circulating bile acids and TGR5 are interesting targets for new therapeutic approaches.

Materials and Methods

Patients and Healthy Volunteers

Patients with liver failure treated in the surgical intensive care unit of Jena University Hospital were screened for study inclusion. Inclusion criteria were as follows: (1) age ≥18 years; (2) plasma disappearance rate of indocyanine green <10%/minute; (3) plasma bilirubin level >170 μmol/L; and (4) international normalized ratio >1.5 and/or overt hepatic encephalopathy (grades II–IV according to West Haven Criteria). Liver failure included ACLF, acute liver failure, and liver graft failure. According to our departmental standard operating procedures, all patients with liver failure received UDCA therapy (3 × 250 mg/d). All clinical data including scores (Acute Physiology And Chronic Health Evaluation II, Simplified Acute Physiology Score II, and Sequential Organ Failure Assessment Score) and laboratory data were obtained on the day of blood sampling. Model for End-Stage Liver Disease including serum sodium score was calculated from these data. Survival was monitored until death, discharge from the hospital, or liver transplantation. Follow-up time was censored at the date of discharge from the hospital or liver transplantation. For each patient, an age- and sex-matched

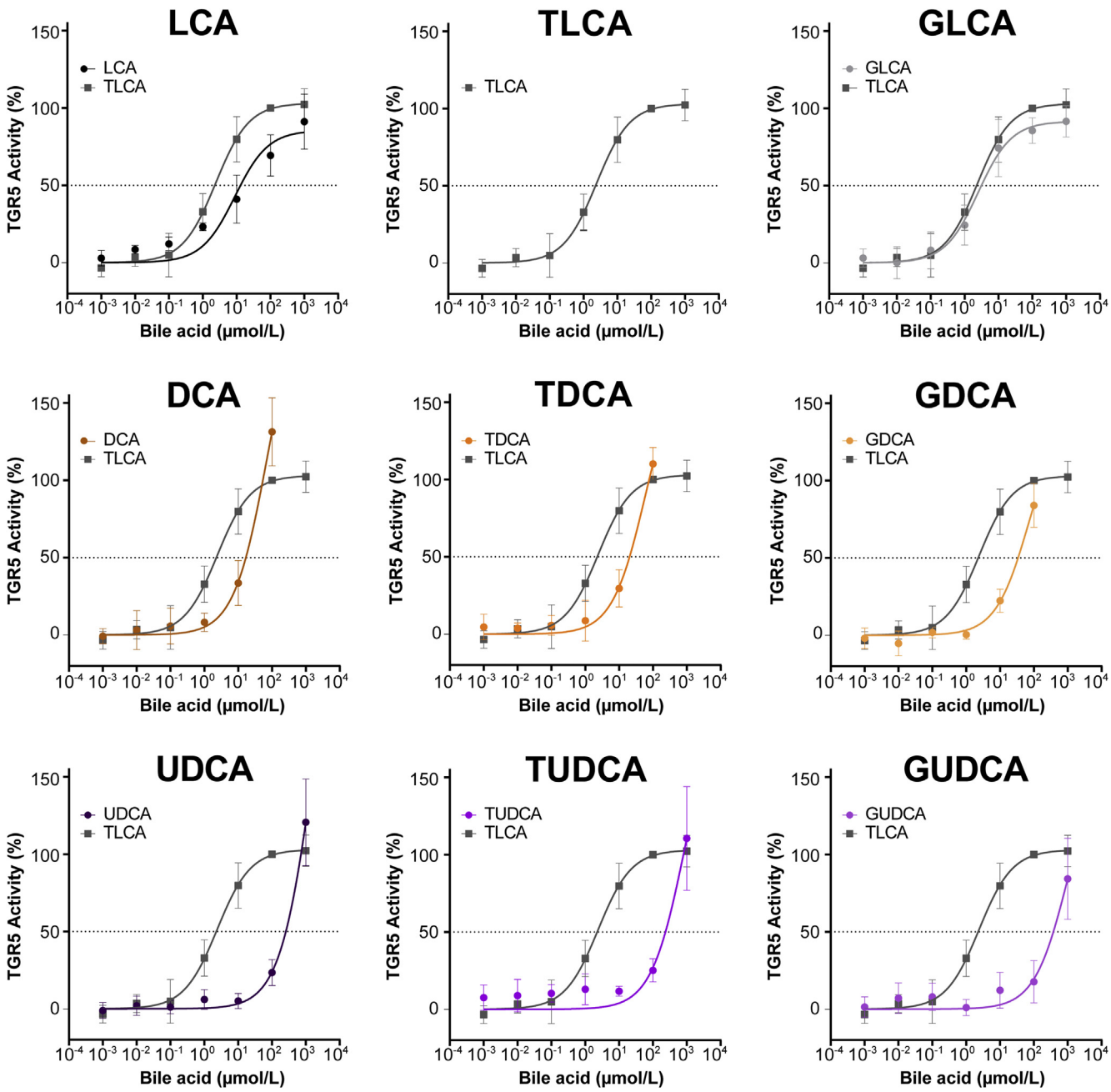


Figure 8. TGR5 activation by secondary and tertiary bile acids. TGR5 activity was measured for each bile acid at increasing concentrations. The concentration that was required to achieve the half-maximal effect of TLCA was calculated. DCA, deoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; LCA, lithocholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

healthy volunteer was selected. Healthy volunteers had no past medical history of cholestatic or liver diseases and were classified according to American Society of Anesthesiology (ASA) physical status classification system as ASA I or II.

Study Approval

The protocol was approved by the Ethical Committee of the Jena University Hospital. All patients or their legal representatives and healthy volunteers provided written informed consent before inclusion in the study.

Bile Acids

Total bile acid levels in serum were quantified by an enzymatic colorimetric kit (Abbott Laboratories, Abbott Park, IL). Bile acid profiles were measured after protein precipitation and filtration from serum samples. Samples were loaded on an Agilent 1200 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) with a CTC-PAL autosampler coupled to an API 4000 Triple Quadrupole mass spectrometer with electrospray ionization source (AB SCIEX, Framingham, MA). All

Table 6. Primers Used for Quantitative Real-Time Polymerase Chain Reaction

Gene name	Gene symbol	Gene ID	Forward primer, 5' to 3'	Reverse primer, 5' to 3'
Target gene				
TGR5	*GPBAR1*	151306	CTCCCAGGCTATCTTCCCAG	ACAGAGAGGAAGGCAGCAG
Housekeeping genes				
Calnexin	*CANX*	821	AAGGTTACTTACAAAGCTCC	ACTCCTTCATTTCCTCTACC
Ubiquitin C	*UBC*	7316	TCGCAGCCGGGATTTGG	TCACGAAGATCTGCATTGTCAAG
Proteasome 20S subunit β3	*PSMB3*	5691	GTCCACATCATCGAGAAGGACA	GGCTCTGGGAACAGGGTTAG

chromatographic separations were performed with a reverse-phase analytical column.

Pure bile acids and bile acid profiles for NanoBRET assays or cell culture experiments were remixed in the respective assay buffer or medium. Tauroursodeoxycholic acid was purchased from VWR International (Darmstadt, Germany), glycolithocholic acid was purchased from Santa Cruz Biotechnology (Dallas, TX), and all other bile acids were obtained from Sigma-Aldrich (Taufkirchen, Germany). Bile acid stock solutions were dissolved in dimethyl sulfoxide (DMSO).

NanoBRET

TGR5 plasmid was purchased from Sino Biological (Beijing, China). ADRB2 plasmid already has been used before by our group.[38] Nanoluciferase (Promega Corp, Madison, WI) was appended directly to the respective C terminus of the receptors via an isothermal assembly reaction (New England Biolabs, Ipswich, MA) using polymerase chain reaction products, which introduced 20– to 40-base pair overlap. Plasmid sequences were controlled by restriction enzyme digest and sequencing (Eurofins, Ebersberg, Germany). The plasmid encoding a fragment of $G_{s\alpha}$ subunit (mini-G_s) tagged with Venus fluorophore was generated as described previously.[17] HEK293T cells were transiently transfected with mini-G_s-Venus and either TGR5-Nluc or ADRB2-Nluc in a 7:3 ratio using Effectene Transfection Reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. After incubation for 24 hours at 37°C and 5% CO_2, transfected cells were seeded into white opaque 96-well plates. The following day, medium was replaced with BRET buffer supplemented with Nano-Glo luciferase substrate (Promega Corp). Pure bile acids or bile acid profiles were remixed in BRET buffer. NanoBRET measurements were conducted using the following: (1) 0.5% DMSO solvent control (negative control); (2) 100 μmol/L TLCA (maximal stimulation; later set to 100%); and (3) bile acid or bile acid profile. All samples contained 0.5% DMSO. The emission of the NanoBRET donor and acceptor was measured on a Synergy Neo2 plate reader (BioTek, Winooski, VT), using a custom-made filter (excitation bandwidth, 541–550 nm; emission, 560–595 nm; fluorescence filter, 620/15 nm). BRET ratios were calculated by the division of the acceptor emission by the donor emission, individually corrected for baseline measurements and buffer conditions and normalized to the maximal stimulation. Maximal stimulation induced by 100 μmol/L TLCA for TGR5 and 100 μmol/L isoprenaline for ADRB2 was set to 100%. Each measurement was performed in triplicate and repeated at least 2 times.

Cell Culture

Primary human monocytes were isolated from healthy donors by negative selection (Thermo Fisher Scientific, Waltham, MA) as described previously.[39] Monocyte purity was 92.5% ± 0.9%, and viability was 99.1% ± 0.5%. Monocytes suspended in RPMI-1640 (Dutch modification) including 10% fetal calf serum, 100 U/mL penicillin, 100 U/mL streptomycin (all Sigma-Aldrich), 1 mmol/L pyruvate and 2 mmol/L GlutaMax (both Thermo Fisher Scientific), were seeded into a 96-well plate (5×10^5 cells/well). After 60 minutes, cells were stimulated with the following: (1) 0.1% DMSO solvent control (negative control); (2) 100 μmol/L TLCA (positive control); (3) mean bile acid profile of the 22 patients with TGR5-activating serum bile acids; or (4) mean bile acid profile of 22 respective healthy controls. All samples contained 0.1% DMSO. After 60 minutes of incubation, 100 ng/mL LPS from *Escherichia coli* (Sigma-Aldrich) was added to the medium for 6 hours. Supernatants were frozen at -80°C for further analysis. Cytokine levels were assessed by a bead-based immunoassay (BioLegend, San Diego, CA). Each experiment was performed in duplicate.

pLV-C-GFPSpark (EV, LVCV-01) and pLV–G-protein–coupled bile acid receptor 1–GFPSpark (TGR5 vector, HG11472-ACGLN) were purchased from Sino Biological, Inc. THP-1 cells (TIB-202; ATCC, Manassas, VA) were stably transduced with both vectors and sorted for equal GFP expression. TGR5 expression was verified by quantitative real-time polymerase chain reaction. Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany). Primer sequences are listed in Table 6. Cytokine experiments were performed as described earlier. Adenosine 3′,5′-cyclic monophosphate was measured using an enzyme-linked immunosorbent assay kit (Cayman, Ann Arbor, MI) after incubation of cells in serum-free medium including 500 μmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich).

Statistics

Statistical analysis was conducted as indicated in the respective figure legends using GraphPad Prism (La Jolla, CA) or SPSS (IBM, Chicago, IL).

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Raphael Silvanus Haider (Formal analysis: Supporting; Investigation: Supporting; Methodology: Supporting; Visualization: Supporting; Writing – review & editing: Supporting)

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Conflicts of interest

The authors disclose no conflicts.

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