Bile Acid Metabolome after an Oral Lipid Tolerance Test by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Abstract

Context
Besides their role in intestinal resorption of lipids, bile acids are regarded as endocrine and metabolic signaling molecules. The detailed profile of bile acid species in peripheral blood after an oral lipid tolerance test (OLTT) is unknown.

Objective
We quantified the regulation of 18 bile acids after OLTT in healthy individuals.

Material and methods
100 volunteers were characterized by anthropometric and laboratory parameters and underwent OLTT. Venous blood was drawn in the fasted state (0 h) and at 2h, 4h, and 6 h after OLTT. Serum concentrations of 18 bile acids were measured by LC-MS/MS.

Results
All of the 6 taurine-conjugated bile acids (TUDCA, THDCA, TCA, TCDCA, TDCA, TLCA) and all of the 6 glycine-conjugated bile acids (GUDCA, GHDCA, GCA, GCDCA, GDCA, GLCA) rose significantly at 2h and remained elevated during OLTT. Of the primary bile acids, CA remained unchanged, whereas CDCA significantly decreased at 4h. Of the secondary bile acids, DCA, UDCA and HDCA were not altered, whereas LCA decreased. There was a significant positive correlation between the intestinal feed-back regulator of bile acid synthesis FGF-19 and bile acids. This correlation seems to depend on all of the six taurine-conjugated bile acids and on GCA, GDCA, and GCDCA. Females and users of hormonal contraception displayed higher levels of taurine-conjugated bile acids.

Conclusions
The novelty of the study is based on the identification of single bile acids during OLTT. LC-MS/MS-based quantification of bile acids in serum provides a reliable tool for future investigation of endocrine and metabolic effects of bile acids.
Introduction

Beyond their established role in facilitating the intestinal resorption of lipids and beyond the well-known mechanism of ileal reabsorption and enterohepatic recirculation to the liver, there is increasing data on pleiotropic and systemic effects of bile acids. Thus, bile acids have been shown to affect carbohydrate metabolism, lipid metabolism, immune regulation, growth and differentiation [1–3]. Based on this observation, it is intriguing to speculate that bile acids circulating in the peripheral blood might act as endocrine and metabolic signaling molecules upon food intake. Regarding metabolic function, postprandial regulation of systemic bile acids might be of importance but has been only poorly investigated.

Bile acids exert their effects via two main pathways, the small heterodimeric partner (SHP)-dependent nuclear receptor farnesoid X receptor (FXR\(\alpha\)) pathway and the SHP-independent G-protein-coupled bile acid receptor (TGR5) pathway. Thus, bile acid effects resemble those of hormones. Bile acids act as ligands to FXR\(\alpha\). In the liver, FXR\(\alpha\)-activated target gene transcription controls bile acid synthesis, insulin-sensitivity, glycolysis, gluconeogenesis, lipogenesis, carbohydrate metabolism and lipid metabolism via SHP- and sterol regulatory element binding protein-1c (SREBP-1c)-dependent mechanisms [4–7].

Fibroblast growth factors (FGFs) represent a family of 22 proteins resembling the function of classical hormones acting on FGF receptors (FGFR1-4). Whereas a dominant role of FXR\(\alpha\)-regulated FGF-19 in the control of bile acid metabolism has been widely accepted, a physiological role of peroxisome proliferator-activated receptor-\(\alpha\) (PPAR\(\alpha\))-regulated FGF-21 in metabolic homeostasis is less clear [8–10]. Tissue-specific metabolic activities of FGF-19 and FGF-21 are modulated by beta Klotho, a homologous single-pass transmembrane protein binding to FGF receptors, and by particular FGF receptor isoform expression [11].

FGF-19 has been regarded as a regulator of hepatic bile acid synthesis [12, 13]. FGF-19 gene transcription and secretion by enterocytes of the ileum is induced by FXR\(\alpha\) activation upon bile acid binding. FGF-19 then circulates to the liver via the portal vein and downregulates hepatic bile acid synthesis by a feedback inhibitory circuit [7, 14] through fibroblast growth factor receptor-4 (FGFR-4)-mediated transcriptional repression of the enzyme cholesterol 7 alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid synthesis [15]. Bile acids as FXR\(\alpha\) activators are able to induce hepatic expression and secretion of FGF-21 [16].

Key FXR pathways have been described in multiple animal models and include molecular and pathogenetic implications for hepatic fibrosis, inflammation, atherosclerosis, lipid metabolism, carbohydrate metabolism, endothelial function, thyroid function and bile acid homeostasis [17].

In a metabolic and endocrine point of view, alterations of peripheral blood bile acid concentrations can be investigated by mixed meals given to individuals. However, mixed meals containing proteins, carbohydrates and lipids in different combinations represent a disadvantage when interpreting data. Recently, our group has investigated the isolated effect of carbohydrates on bile acid concentrations by an oral glucose tolerance test [18]. However, there is no single study available investigating the isolated effect of orally ingested lipids on systemic bile acid concentrations in healthy individuals.

Therefore, it was the aim of the present study to investigate by LC-MS/MS

- whether 18 primary, secondary and tertiary conjugated and non-conjugated bile acids are altered in serum of \(n = 100\) healthy individuals during an oral lipid tolerance test (OLTT) by using a carbohydrate- and protein-free lipid solution
- whether anthropometric parameters influence the time course of bile acids after OLTT
• whether alterations of the serum bile acid metabolome during OLTT correlates to variations in serum FGF-19 levels

**Material and Methods**

**Study cohort**

The present study cohort has been characterized earlier for anthropometric and laboratory parameters as well as for the regulation of adipokines and FGF-19/FGF-21 upon OLTT [19, 20]. Briefly, participants were examined at the University Hospital of Regensburg. 100 healthy volunteers (58 females and 42 males; 66 with normal weight and 34 overweight/obese) gave their written informed consent to the study approved by the ethical committee of the Medical Faculty, University of Regensburg. Exclusion criteria were a positive history of any kind of illness, evidence of acute or chronic infection within 10 days prior to the OLTT, age < 18 years or > 55 years, and any kind of medication (except oral contraceptives). Pregnant and menstruating women were not admitted to the study. Age, BMI, hip circumference, waist circumference, waist/hip ratio, triceps skinfold thickness, and blood pressure were recorded. Patients' history regarding type 2 diabetes and cardiovascular diseases as well as smoking habits or hormonal contraception were documented. Table 1 summarizes the predominant characteristics of the study population as published earlier in detail [19].

**Oral lipid tolerance test (OLTT)**

OLTT was conducted after an overnight fast of 12 h. During OLTT, the participants had to rest and not to eat or smoke. The ingestion of 500 ml of pure water was allowed over the 6 hours. Venous blood samples were drawn at 0h (fasting), 2h, 4h, and 6 h. Serum was prepared by 10 min of centrifugation at 4000 rotations per min (rpm). As reported recently by our group in detail [19], we used a solution that was free of proteins and carbohydrates. Briefly, the OLTT solution (160 ml; 758.1 kcal; 75 g vegetable fat as pure vegetable oils) comprised the following components: **Component 1**: 150 ml water/fat solution (Calogen® NUTRICIA-Neutral, Pfrimmer Nutricia, Erlangen, Germany): 75 g vegetable fat as pure triglycerides containing 7.95 g saturated fatty acids, 45.6 g mono-unsaturated fatty acids, 21.45 g polyunsaturated fatty acids, 69 g water, 0.15 g carbohydrates (insignificant trace amount), 675 kcal. **Component 2**: 5 ml (4.6 g; 41.5 kcal) sunflower oil containing 10.2% saturated fatty acids, 26.3% mono-unsaturated fatty acids, and 63.5% poly-unsaturated fatty acids. **Component 3**: 5 ml (4.6 g; 41.6 kcal) olive oil containing 14% saturated fatty acids, 78% mono-unsaturated fatty acids, and 8% poly-unsaturated fatty acids.

**Quantification of human bile acids in serum by HPLC-MS/MS**

Bile acid species were quantified as described previously by LC-MS/MS [21]. Briefly, serum samples were spiked with a mixture of deuterated bile acids as internal standard prior to protein precipitation. Serum extracts were subjected to LC-MS/MS detection in negative ion mode after base-line separation of isobaric species. The LC-MS/MS system consisted of an API 4000 QTrap (AB Scie, Darmstadt, Germany) that was coupled with electrospray ionization. Chromatographic separation was achieved by an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany). Quantification was achieved using a matrix calibrator generated by standard addition.
Statistical analysis

For calculating mean values ± standard deviation (± SD) or ± standard error of the mean (± SEM), a statistical software package (SPSS 22.0) was used. Mean values were compared by the non-parametric Mann-Whitney U-test for 2 independent samples and the Kruskal-Wallis-H-test for k independent samples. For subgroup analysis of mean values, Bonferroni’s correction was performed. Correlation analysis was done by using the Spearman-Rho test for linear variables. A p-value below 0.05 (two tailed) was considered as statistically significant. In the figures, the circles are showing the mean values and the whiskers are giving the 95% confidence interval (CI) of the mean.

Results

Effect of OLTT on main bile acid species

Table 2 gives an overview of the main bile acid species and their systemic response in serum during OLTT. All of the main bile acid species, total/free bile acids, primary/secondary bile acids, and taurine-/glycine-conjugated bile acids show significant alterations during OLTT.

Table 1. Demographic and clinical statistics of the study population.

| Study population (n = 100)                      |
|-----------------------------------------------|
| Age (years)                                   | 28.1 ± 7.7 [18–54] |
| Males n (%)                                   | 42 (42) |
| Females n (%)                                 | 58 (58) |
| **Anthropometric parameters**                 |
| Mean BMI (kg/m²)                               | 24.2 ± 5.0 [14.8–46.1] |
| BMI < 25 kg/m² (n, %)                         | 66 (66) |
| BMI ≥ 25 kg/m² (n, %)                         | 34 (34) |
| Waist circumference (cm)                      | 83.9 ± 13.0 [63.0–122.0] |
| Hip circumference (cm)                        | 99.5 ± 10.1 [77.0–131.0] |
| Waist-Hip Ratio                               | 0.84 ± 0.09 [0.68–1.07] |
| Skin fold thickness (mm)                      | 11.3 ± 8.9 [1.0–47.0] |
| **Circulation**                               |
| Systolic blood pressure (mm Hg)               | 121 ± 15 [90–174] |
| Diastolic blood pressure (mm Hg)              | 78 ± 9 [60–105] |
| Heart rate (min⁻¹)                            | 73 ± 12 [50–105] |
| **Carbohydrate-Metabolism**                   |
| Plasma glucose 0h (mg/dl)                     | 74 ± 13 [40–110] |
| Plasma insulin 0h (mU/l)                      | 8.9 ± 5.7 [2.3–33.3] |
| Plasma C-peptide 0h (μg/l)                    | 1.3 ± 0.5 [0.5–3.8] |
| **Lipoprotein-Metabolism**                    |
| Total cholesterol 0h (mg/dl)                  | 191 ± 39 [52–297] |
| Triglycerides 0h (mg/dl)                      | 120 ± 50 [24–299] |
| LDL cholesterol 0h (mg/dl)                    | 111 ± 33 [21–196] |
| HDL cholesterol 0h (mg/dl)                    | 60 ± 17 [23–107] |
| **Inflammation**                              |
| CRP (mg/l)                                    | 3.9 ± 3.2 [2.9–27] |

Data are presented as means ± SD (standard deviation) and ranges (square brackets). Demographic data of the present study cohort were under investigation earlier and were reported elsewhere in detail [19, 20]. BMI was not correlated significantly with bile acid or FGF-19 levels. There was a marginal, physiologically irrelevant correlation between age and free bile acid concentration (p = 0.049; r = -0.200).

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Total bile acids increased (~3fold) with a maximum at 2h and remained elevated at 4h and 6h when compared to the fasted state. In contrast, free bile acids decreased significantly (~50%) at 6h after OLTT. Both, primary and secondary bile acid species significantly increased (~3-fold) with a maximum at 2h and remained at higher levels when compared to the fasted state. Similarly, the taurine- and glycine-conjugated bile acids significantly increased (~4 to 5-fold) with a maximum level at 2h and also remained at a higher plateau when compared to 0h.

Quantitative profiling of 18 human single bile acids during OLTT

Since changes in main bile acid species do not allow a deeper insight into bile acid physiology and potential endocrine effects of circulating single/specific bile acids after oral lipid ingestion, 18 bile acids were quantified at all points of time (0h, 2h, 4h, 6h) during OLTT in all participating individuals (n = 100). Table 3 summarizes the individual changes of each of the 18 bile acids during OLTT. Taurine-conjugated bile acids: All of the 6 taurine-conjugated bile acids (TUDCA, THDCA, TCA, TCDCA, TDCA, TLCA) rose significantly (~5-fold) and early (at 2h) and remained at higher levels until the end of the OLTT.

Glycine-conjugated bile acids: All of the 6 glycine-conjugated bile acids (GUDCA, GHDCA, GCA, GCDCA, GDCA, GLCA) rose significantly (~3 to 4-fold) and early (at 2h) and remained at higher levels until the end of the OLTT.

Primary bile acids: Of the two primary bile acids (CA and CDCA), CA remained unchanged during OLTT, whereas CDCA significantly decreased at 4h.

Secondary bile acids: Of the secondary bile acids, DCA and HDCA were not significantly altered during OLTT. LCA decreased during OLTT in a stepwise manner. However, significance was only reached at 2h when compared to 0h.

Tertiary bile acids: Of the tertiary bile acids, UDCA was not altered significantly.

Fig 1 exemplarily depicts the time course of total bile acids, taurine- and glycine-conjugated bile acids as well as CDCA.

Correlation analysis of postprandial FGF-19 and FGF-21 serum concentrations with bile acid species after oral lipid ingestion

Table 4 summarizes the results of the correlation analysis of postprandial FGF-19 concentrations with bile acid species. Our previous work [20] demonstrated a significant (p = 0.006)
Table 3. Profile of 18 human bile acid species in serum before and during oral lipid tolerance test (OLTT) measured by HPLC-MS/MS.

| Bile acid species | OLTT 0h (nmol/l) | OLTT 2h (nmol/l) | OLTT 4h (nmol/l) | OLTT 6h (nmol/l) | p-Value Kruskal-Wallis test |
|-------------------|------------------|------------------|------------------|------------------|---------------------------|
| **Primary bile acids** |                  |                  |                  |                  |                           |
| Non-conjugated    |                  |                  |                  |                  |                           |
| CA                | 873.479 ± 204.050| 898.947 ± 424.138| 465.679 ± 148.421| 481.794 ± 177.428| 0.2542                    |
| CDCA              | 569.232 ± 132.654| 453.927 ± 72.115 | 258.856 ± 45.992 | 261.614 ± 50.172 | 0.001                     |
| Taurine-conjugated|                  |                  |                  |                  |                           |
| TCA               | 69.491 ± 11.138  | 353.543 ± 58.511*| 291.204 ± 66.445*| 192.564 ± 41.567* | <0.001                    |
| TCDCA             | 188.461 ± 20.865 | 922.491 ± 104.140*| 787.482 ± 114.169*| 531.767 ± 74.298* | <0.001                    |
| Glycine-conjugated|                  |                  |                  |                  |                           |
| GCA               | 321.045 ± 52.853 | 1370.278 ± 140.969**| 995.840 ± 111.553**| 712.917 ± 85.597** | <0.001                    |
| GCDCA             | 741.949 ± 92.486 | 3099.537 ± 273.912**| 2474.421 ± 238.492**| 1672.319 ± 138.267**| <0.001                    |
| **Secondary bile acids** |              |                  |                  |                  |                           |
| Non-conjugated    |                  |                  |                  |                  |                           |
| DCA               | 403.422 ± 36.961 | 534.504 ± 54.941  | 400.323 ± 40.126  | 341.823 ± 30.930  | 0.065                     |
| HDCA              | 51.293 ± 11.781  | 50.838 ± 4.923    | 42.022 ± 4.159    | 36.430 ± 3.067    | 0.088                     |
| LCA               | 142.273 ± 53.182 | 128.878 ± 18.523* | 103.149 ± 17.075  | 82.148 ± 11.409  | 0.004                     |
| Taurine-conjugated|                  |                  |                  |                  |                           |
| TDCA              | 80.859 ± 11.849  | 428.328 ± 71.909**| 412.929 ± 103.339**| 241.241 ± 54.809**| <0.001                    |
| THDCA             | 5.040 ± 0.893    | 22.608 ± 4.483**  | 21.426 ± 4.720**  | 13.582 ± 2.259**  | <0.001                    |
| TLCA              | 7.595 ± 1.339    | 34.069 ± 6.728**  | 32.182 ± 7.081**  | 20.204 ± 3.360**  | <0.001                    |
| Glycine-conjugated|                  |                  |                  |                  |                           |
| GDCA              | 353.597 ± 46.483 | 1438.036 ± 145.168**| 1193.556 ± 143.518**| 787.910 ± 85.000**| <0.001                    |
| GHDCA             | 126.382 ± 17.910 | 378.963 ± 61.405**| 356.581 ± 63.921**| 268.137 ± 38.275**| <0.001                    |
| GLCA              | 162.795 ± 25.813 | 521.973 ± 87.772**| 489.985 ± 91.349**| 363.635 ± 54.707**| <0.001                    |
| **Tertiary bile acids** |              |                  |                  |                  |                           |
| Non-conjugated    |                  |                  |                  |                  |                           |
| UDCA              | 142.273 ± 53.182 | 128.878 ± 18.523* | 103.149 ± 17.075  | 82.148 ± 11.409  | 0.195                     |
| Taurine-conjugated|                  |                  |                  |                  |                           |
| TUDCA             | 7.595 ± 1.339    | 34.069 ± 6.728**  | 32.182 ± 7.081**  | 20.204 ± 3.360**  | <0.001                    |
| Glycine-conjugated|                  |                  |                  |                  |                           |
| GUDCA             | 162.795 ± 25.813 | 521.973 ± 87.772**| 489.985 ± 91.349**| 363.635 ± 54.707**| <0.001                    |

Data are given as mean concentrations ± SEM (standard error of the mean) in nmol/l. For overall correlation analysis of data, the non-parametric Kruskal-Wallis-H test was applied. For direct comparison of mean bile acid levels from 0h to 2h, 4h, and 6h respectively, the non-parametric Mann-Whitney U-Test was used (* p<0.01; ** p<0.001). Since 4 subgroups (timepoints) were compared, significance is reached at a p-value < 0.0125 due to Bonferroni’s correction. Primary bile acids: CA, cholic acid; CDCA, chenodeoxycholic acid. Secondary bile acids: DCA, deoxycholic acid, HDCA, hyodeoxycholic acid. LCA, lithocholic acid; Tertiary bile acids: UDCA, ursodeoxycholic acid; Taurine-conjugated bile acids: TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; THDCA, taurohyodeoxycholic acid. Glycine-conjugated bile acids: GCA, glycocoholic acid; GCDCA, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycursoxdeoxycholic acid; GDHC, glycohyoxydeoxycholic acid.

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increase of triglycerides between 2h and 4h (Table 1) and a significant rise of FGF-19 at 4h during OLTT in this cohort [20]. In detail, basal FGF-19 levels were 105 ± 81 pg/ml and rose up to 141 ± 102 pg/ml (p = 0.006) at 4 h [20]. In comparison, the maximum rise of bile acids occurred early at 2h during OLTT and the 1–2h delay between the postprandial rise of bile acids and FGF-19 is in accordance with data from the literature [18, 22]. However, this time
In the present study, we could detect significant positive correlations between FGF-19 levels and bile acids which were most striking at 6h after lipid ingestion. With respect to main bile acid species, there was a highly significant and positive correlation between FGF-19 levels and total bile acids, free bile acids, primary bile acids, secondary bile acids, taurine-conjugated bile acids and glycine-conjugated bile acids. Since it was our aim to identify those bile acids that are mainly responsible for these class effects, correlation analysis was performed for each of the 18 bile acids measured. No significant correlations were found for GUDCA, UDCA, GHDCA, HDCA, CA, CDCA, GLCA, and LCA. The positive correlation between FGF-19 and bile acids was strongest for the taurine-conjugated bile acids ($r = +0.8$). All of the six taurine-conjugated bile acids TUDCA, THDCA, TCA, TCDCA, TDCA, and TLCA showed this positive correlation with FGF-19. The correlation between FGF-19 and the glycine-conjugated bile acids is caused only by GCA, GDCA, and GCDCA, since GUDCA, GHDCA, and GLCA had no effect. For interested readers, the correlation analysis of FGF-19 with bile acid species is presented for gender subgroups and for contraceptive users/non-users under Online Supporting Information.

**Fig 1.** Time-dependent variations of bile acids during an oral lipid tolerance test (OLTT) are shown in healthy individuals ($n = 100$) for total bile acids (A), taurine-conjugated bile acids (B), glycine-conjugated bile acids (C), and chenodeoxycholic acid (D). The bars are showing the median values and the whiskers are giving the 95% confidence interval (CI) of the mean. CDCA, chenodeoxycholic acid.

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However, subgroups might be too small for profound or extensive interpretation. In our previous study [20], FGF-21 levels were shown to decrease significantly and transiently during OLTT. In detail [20], basal FGF-21 concentrations decreased from 158.8 ± 203.6 pg/ml at 0h down to 127.6 ± 184.4 pg/ml at 2h (p = 0.03). We now retrieved these data and calculated a correlation analysis for FGF-21 and bile acids. In contrast to FGF-19 mentioned above, FGF-21 was negatively correlated to some of the bile acids. In detail, FGF-21 concentrations at 6h were significantly and negatively correlated with taurine-conjugated bile acids in total (p = 0.017; r = -0.24) and with TCCA (p = 0.001; r = -0.7), TDCCA (p = 0.002; r = -0.25), and TLCA (p = 0.004; r = -0.21).

Effects of gender and BMI on postprandial bile acid concentration

Subgroup analysis displayed differential effects of sex on postprandial bile acid levels at 6h after OLTT. Table 5 summarizes the effects of gender on postprandial bile acids. Taurine-conjugated bile acids in general were significantly higher in females when compared to males (~2.5-fold). In detail, all of the taurine-conjugated bile acid species were significantly higher (~1.5 to 3-fold) in females when compared to males. Since bile acids were not significantly different between males and females at baseline (data not shown), these sex differences are not due to differences in overall levels but possibly to differences in sensitivity to lipid challenge. None of the glycine-conjugated bile acids was gender-dependent. UDCA was the only bile acid identified with significantly lower levels in females when compared to males. The remaining bile acids were not significantly different between females and males. When individuals were subdivided into lean subjects (BMI < 25 kg/m²) and overweight/obese subjects (BMI ≥ 25 kg/m²),

| Bile acid species                  | p-value | correlation coefficient r |
|------------------------------------|---------|--------------------------|
| FGF-19 total bile acids            | <0.001  | + 0.41                   |
| FGF-19 free bile acids             | 0.012   | + 0.25                   |
| FGF-19 primary bile acids          | 0.001   | + 0.34                   |
| FGF-19 secondary bile acids        | <0.001  | + 0.7                    |
| FGF-19 taurine-conjugated bile acids | <0.001  | + 0.8               |
| FGF-19 glycine-conjugated bile acids | 0.001   | + 0.34                |

Table 5. Correlation analysis of postprandial FGF-19 serum concentrations with bile acid species.
mean values of all bile acids measured were not significantly different (data not shown). Importantly, when taurine-conjugated bile acid levels of males were compared to those of women not using contraceptives, we could not detect significant changes between genders. Thus, the effect of hormonal contraception was further investigated.

### Effects of hormonal contraception on postprandial bile acid concentration

Users of hormonal contraception displayed differential effects regarding their postprandial bile acid levels at 6h after OLTT. Table 5 summarizes the effects of hormonal contraception on postprandial bile acids. Taurine-conjugated bile acids were higher in users when compared to non-users (~3-fold). In detail, all of the taurine-conjugated bile acid species were affected by contraceptives and showed significantly higher concentrations. Since taurine-conjugated bile acids were also slightly higher (p = 0.026; data not shown) at baseline in users vs. non-users, the marked differences at 6h after lipid ingestion might be due to differences in overall levels as well as to differences in sensitivity to lipid challenge. CDCA was the only bile acid identified whose concentrations were significantly lower under hormonal contraception. The remaining bile acids were not significantly different between users and non-users of hormonal contraception (data not shown).

### Discussion

The present study was conducted in order to gain systematic insight into changes of serum bile acids and their regulator proteins FGF-19 and FGF-21 during OLTT in healthy individuals. Since the uptake of bile acids by the liver via the enterohepatic circulation is highly efficient, nano/micro-molar fractions enter the systemic circulation and might exert yet unknown endocrine and metabolic effects on organs other than the liver. Systemic bile acid concentrations...
might result from a passive spillover by the enterohepatic circulation (portal vein) or from direct hepatic release via the hepatic veins. The latter is a matter of speculation but would offer the fascinating hypothesis of bile acids as hepatokines exerting multi-organ systemic effects. For example, molecular studies have shown that some bile acid species are able to interact with lipid bilayers at sub-micellar concentrations [23]. Thus, disturbance of liposomal membranes by bile acids might affect a plethora of ligand-receptor interactions and subsequent signal transduction as well as intracellular protein trafficking.

The novelty of the present study is based on the identification of single bile acids that are responsible for the systemic alterations of main bile acid species such as total/free bile acids, primary/secondary bile acids, and taurine-/glycine-conjugated bile acids during OLTT. This was only possible by applying a sophisticated technical approach for the measurement of 18 different human bile acids. HPLC-MS/MS is suitable for rapid and precise quantification of human bile acids, as shown earlier by our group [18]. Whereas all of the main bile acid species showed alterations during OLTT, it is now possible to detect single bile acids that cause these species effects and to exclude single bile acids not contributing to the postprandial systemic alterations. Moreover, the present study including n = 100 participants is large enough to be statistically robust against outliers when compared to similar studies with less participants [24–26].

SHP-independent bile acid signaling can be investigated by targeting FGF-19. Our group and others have shown that serum levels of FGF-19 rise significantly 4h after oral lipid ingestion [20] and this response is blunted in patients with non-alcoholic fatty liver disease and insulin resistance [24]. We observed a rapid onset increase of bile acids after 2h during OLTT, similar to a bile acid increase at 1h after a glucose challenge [18]. After this increase, bile acids remained significantly elevated up to 6h when compared to the fasted state. The postprandial increase of bile acids is followed by a rise of FGF-19 after 90–120 min [22] in normal subjects. Since we could recently describe a significant rise of FGF-19 at 4h after OLTT in the presented cohort of healthy volunteers [20], we retrieved these data and tested for a possible correlation of basal and postprandial FGF-19 levels with each of the bile acids measured. Whereas basal FGF-19 and bile acid concentrations showed no correlation in the fasted state, we were able to describe significant positive correlations of postprandial (6h) FGF-19 levels with a distinct pattern of bile acids. A widely accepted mechanism for the postprandial FGF-19 rise in plasma is based on the release of FGF-19 from enterocytes following bile acid binding to FXRα and its activation. Since FGF-19 plasma levels rise significantly later (after 4h upon OLTT) when compared to plasma bile acids (after 2h) [20, 22], the positive correlation between FGF-19 and postprandial bile acids might indicate the existence of an alternative route of FGF-19 secretion upon oral lipid uptake. It currently remains unclear why only a distinct type of bile acids is correlated postprandially with FGF-19 whereas others are not. Future studies have to address this interesting question. Since fatty liver disease and insulin resistance are known factors [24] that are able to blunt FGF-19 secretion, an individual metabolic constellation might affect the correlation between bile acids and FGF-19 release.

In contrast, FGF-21 concentrations significantly declined during OLTT and there was a negative correlation between FGF-21 and taurine-conjugated bile acids during OLTT. These results obtained in vivo challenge the results obtained in primary hepatocytes in vitro [16], where a bile acid-induced and FXRα-dependent secretion of FGF-21 has been reported. The exact mechanism and the physiological background how and why bile acids change after OLTT remain unclear. There are two principle mechanisms for a postprandial increase of systemic bile acids, a spillover phenomenon from the intestinal circulation to the systemic circulation or a direct release by the liver. Since FGF-19 is upregulated typically late after 4h during OLTT, bile acids are upregulated relatively fast after 2h. This might argue against a spillover
phenomenon from the intestinal circulation and favors a direct release by the liver upon oral lipid uptake. The latter potential mechanism might provide the potential basis for a role of bile acids as “hepatokines” that has to be investigated by further mechanistic studies. The observed decrease of free bile acid concentrations contrary to conjugated bile acid species suggests an elevated turnover rate of free bile acid conjugation with glycine or taurine, respectively, as a consequence of oral lipid ingestion. As discussed earlier by Matysik et al. investigating bile acid profiles during an oral glucose tolerance test [18], postprandial FXRα activation by bile acids might induce a rise in bile acid amidation rate. However, since neither the rate of bile acid resorption from the small intestine nor of hepatic bile acid synthesis was quantified in the presented study, no proof of principle can be given for this speculation.

Not all the bile acid species measured activate the receptors FXR and TGR5 with the same potency. This fact complicates the interpretation of data implying that different bile acids patterns might have different biological impact according to receptor activation and signaling.

Taken together, after a pure lipid ingestion the time kinetics of bile acids, triglycerides and FGF-19 in healthy individuals could be clarified by the present study. Bile acids are upregulated very fast after 2h of ingestion. This rise in bile acids precedes the increase of triglycerides (Table 1) which typically occurs between 2h and 4h. After 4h, FGF-19 becomes upregulated. Whereas there is an established and unquestioned role of the enterohepatic circulation as a route for bile acids and FGF-19, future studies have to investigate the exact routes of bile acids, FGF-19, and FGF-21 from the intestine and/or form the liver into the systemic circulation. It would be of interest to conduct studies on a cellular level investigating whether postprandially upregulated bile acids and FGF-19 differentially regulate the activation of beta Klotho in a given metabolic context such as hyperlipidemia or hyperglycemia.

Summary

- Total bile acids as well as taurine- and glycine-conjugated bile acids show an early (2h) rise after OLTT and remain elevated up to 6h. This bile acid rise in peripheral blood precedes the postprandial rise of FGF-19 seen at 4h and the postprandial rise of triglycerides seen between 2h to 4h.
- Of the primary bile acids, CA remained unchanged after OLTT, whereas CDCA decreased at 4h.
- Of the secondary bile acids, LCA decreased and the others remained unchanged.
- There is a significant and positive correlation between FGF-19 and several bile acids. The strongest correlation was documented for taurine-conjugated bile acids.
- There is a significant and negative correlation between FGF-21 and taurine-conjugated bile acids.
- Higher levels of taurine-conjugated bile acids in females are caused by hormonal contraception whereas glycine-conjugated bile acids were not affected by gender or contraception.

Conclusions

To our knowledge, this is the first study quantifying 18 different bile acid species in peripheral blood of healthy individuals by LC-MS/MS after oral lipid ingestion by using a solution free of carbohydrates and proteins. Moreover, the novelty of the present study lies on the identification of single bile acids that are responsible for the systemic alterations of main bile acid.
species. LC-MS/MS-based quantification of bile acids in peripheral blood serum provides a reliable tool for future investigation of endocrine and metabolic effects of circulating bile acids beyond their function in enterohepatic circulation and liver physiology.

Supporting Information

S1 Table. Correlation analysis of postprandial FGF-19 serum concentrations with bile acid species in gender subgroups.

(S1 Table)

S2 Table. Correlation analysis of postprandial FGF-19 serum concentrations with bile acid species in subgroups of hormonal contraception.

(S2 Table)

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Author Contributions

Conceived and designed the experiments: ASchmid TK ASchäffler. Performed the experiments: HN GL. Analyzed the data: ASchmid ASchäffler. Contributed reagents/materials/analysis tools: GL ASchäffler. Wrote the paper: ASchmid ASchäffler.

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