Asynchronous rhythms of circulating conjugated and unconjugated bile acids in the modulation of human metabolism

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Abstract. Al-Khaifi A, Straniero S, Voronova V, Chernikova D, Sokolov V, Kumar C, Angelin B, Rudling M (Karolinska Institutet at Karolinska University Hospital Huddinge; Karolinska Institutet/AstraZeneca Integrated CardioMetabolic Center (KI/AZ ICMC), Novum, Stockholm, Sweden; Sultan Qaboos University, Muscat, Oman; M&S Decisions, Moscow, Russia; AstraZeneca, Gothenburg, Sweden). Asynchronous rhythms of circulating conjugated and unconjugated bile acids in the modulation of human metabolism. J Intern Med 2018; 284: 546–559.

Background and Objectives. Bile acids (BAs) traversing the enterohepatic circulation (EHC) influence important metabolic pathways. By determining individual serum BAs in relation to markers of metabolic activity, we explored how diurnal variations in their EHC relate to hepatic metabolism in normal humans.

Methods. Serum BAs, fibroblast growth factor 19 (FGF19), lipoproteins, glucose/insulin and markers of cholesterol and BA syntheses were monitored for 32 h in 8 healthy males. Studies were conducted at basal state and during initiation of cholestyramine treatment, with and without atorvastatin pretreatment. Time series cross-correlation analysis, Bayesian structural model and Granger causality test were applied.

Results. Bile acids synthesis dominated daytime, and cholesterol production at night. Conjugated BAs peaked after food intake, with subsequent FGF19 elevations. BA synthesis was reduced following conjugated BA and FGF19 peaks. Cholestyramine reduced conjugated BAs and FGF19, and increased BA and cholesterol production; the latter effects attenuated by atorvastatin. The relative importance of FGF19 vs. conjugated BAs in this feedback inhibition could not be discriminated. Unconjugated BAs displayed one major peak late at night/early morning that was unrelated to FGF19 and BA synthesis, and abolished by cholestyramine. The normal suppression of serum triglycerides, glucose and insulin observed at night was attenuated by cholestyramine.

Conclusions. Conjugated and unconjugated BAs have asynchronous rhythms of EHC in humans. Post-prandial transintestinal flux of conjugated BAs increases circulating FGF19 levels and suppresses BA synthesis. Unconjugated BAs peak late at night, indicating a non-postprandial diurnal change in human gut microflora, the physiological implications of which warrants further study.

Keywords: cholesterol, cholestyramine, diurnal variation, fibroblast growth factor 19, lipoproteins, triglyceride.

Introduction

Bile acids (BAs) are amphiphilic molecules made from cholesterol in the liver. They solubilize lipophilic molecules, promoting both their...
secretion into bile and absorption from intestine, as well as being the major driver of bile flow [1, 2]. Due to an efficient uptake mainly in the lower small intestine, >95% of BAs are reabsorbed and returned to the liver through the portal vein. Their efficient clearance and resecretion into bile conserves the BA pool within the enterohepatic circulation (EHC), so that even if circulating 5–15 times/day, the daily loss that is compensated by de novo synthesis is only about 15–45% of the pool size [1–6]. The EHC of BAs can be regarded as a semi-closed dynamic system where their flux from liver to gallbladder, intestines and back to the liver via the portal vein is propagated by mechanical pumps in the form of gallbladder contraction and intestinal propulsion, working together with transporters for BA membrane transfer and enzymes involved in BA synthesis and conjugation. All these steps are subject to genetic, dietary and microbial diversity, contributing to a wide interindividual variation [1]. Furthermore, since the EHC includes the digestive system, it is evident that there is a marked influence of diurnal rhythms, both related to fasting/feeding and to neuroendocrine regulation. Some of the critical points of regulation are notoriously difficult to access in healthy humans, and a large part of our knowledge of basal physiology is still inferred from animal models.

The BA pool is composed of many different BA species, all with particular properties. In humans, cholic acid (CA) and chenodeoxycholic acid (CDCA) are produced by the liver as primary BAs, which can be converted by bacteria to the secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA), primarily in the colon [7]. Ursodeoxycholic acid (UDCA) is formed by subsequent epimerization. Essentially all BAs in human bile are secreted conjugated with either glycine or taurine [1, 2, 8], but may be deconjugated by intestinal bacterial bile salt hydrolases [7]. Conjugated BAs are mainly taken up by active transport in the distal ileum, although some – particularly glycine-conjugated CDCA and DCA – can also be taken up by passive diffusion, which accounts for a major part of intestinal uptake of unconjugated BAs [1, 9, 10]. These are less efficiently cleared by the liver [5, 11], and thus relatively enriched in the peripheral circulation.

BAs regulate their own synthesis [1, 2], but the exact mechanisms are still incompletely known. Interruption of the EHC by biliary diversion or ileal resection, or by treatment with BA-binding resins, markedly stimulates BA production. Conversely, oral administration of most human BAs suppresses BA synthesis. Many enzymes contribute to the production of BAs, with cholesterol 7α-hydroxylase (CYP7A1) being rate-limiting in humans [1, 2]. Changes in the rate of BA synthesis also induce compensatory responses in hepatic cholesterol metabolism via modulation of the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase, and of LDL receptors [12]. By monitoring the serum marker of CYP7A1 activity, 7α-hydroxy-4-cholesten-3-one (C4), we [13] and others [14, 15] have described that BA synthesis has a diurnal rhythm in humans, with higher production during the feeding period than at night. Cholesterol synthesis – reflected by circulating lathosterol – has an asynchronous pattern, peaking after midnight [13]. In animals, feedback inhibition by BAs is dependent on their interaction with nuclear receptors in liver and intestine, predominantly farnesoid X receptors (FXRs) [16, 17], although there may also be other mechanisms involved. Analysis of serum fibroblast growth factor 19 (FGF19) levels indicate that this FXR-controlled protein is influenced by the transintestinal flux of BAs [18], but its role in the physiological regulation of BA synthesis in humans is still incompletely understood [19].

The physicochemical properties of the individual BAs seem to be important for their interaction with FXR. Thus, in vitro studies indicate that hydrophobic BAs, such as CDCA and DCA, are stronger FXR-activators than CA, as are conjugated vs. unconjugated BAs [16, 17]. Some BAs such as UDCA, and particularly the muricholic acids (MCAs) which are abundant in mice, may even act as antagonists [20]. Changes of the BA pool composition may thus alter BA and cholesterol metabolism, but information on this is still sparse [8]. In addition to regulating overall body cholesterol balance and lipid absorption, BAs may act as potent signalling molecules in other aspects of metabolic regulation [8, 21]. Thus, interruption of the EHC of BAs stimulates the production of VLDL triglycerides, whereas feeding CDCA suppresses hepatic triglyceride production [22]. Such modulations of EHC can also influence the metabolism of lipoproteins, insulin and glucose [21]. In a recent human study [23], we characterized the dynamic metabolic responses in relation to serum BAs and FGF19 levels following interruption of BA EHC by cholestyramine treatment, and unexpectedly found
increased serum triglycerides and glucose late at night. This focused our interest on exploring possible unknown patterns of metabolic regulation during this less well-studied period of the diurnal cycle in normal humans.

Here, we present data from a series of clinical experiments on healthy males during a 32-h period of normal dietary intake in the basal state and during the initiation of interruption of BA EHC by cholestyramine treatment, both under basal conditions and during inhibition of cholesterol synthesis by atorvastatin pretreatment. Our aim was to explore the relationships between individual BA species and circulating FGF19, BA and cholesterol synthesis and serum lipoprotein and glucose/insulin levels. Specifically, we asked: (i) Is there a difference between the pattern of EHC of conjugated and unconjugated BAs? (ii) Does the transintestinal flux of different BAs relate to circulating FGF19 levels? (iii) How do changes in BA synthesis relate to the serum levels of different BAs and FGF19? (iv) How do these patterns change when the intestinal absorption of BAs is blocked by cholestyramine treatment (CME)? and (vi) Are the responses to CME treatment altered when hepatic cholesterol levels are suppressed by concomitant treatment with atorvastatin (CME+STAT)?

Materials and methods

Subjects

The study included 8 healthy males (age 20–45 year, BMI 22–28) recruited by local advertisement. They had normal working hours and no signs of cardiovascular, liver, metabolic, gastrointestinal or kidney disease upon physical examination and basal laboratory screening; no one was on medication. Basal data on the individual subjects are presented in Table S1. All participants gave written informed consent to participation in the study, which had been approved by the Regional Ethics Committee, Karolinska Institutet, Stockholm.

Study design

Participants were scheduled for 3 sessions: no treatment (basal), cholestyramine treatment only (CME) and combined with atorvastatin (CME+STAT) (Fig. 1). The sessions were separated by a washout period of at least 1 month.

For each session, the subjects stayed at the metabolic ward from 08:00 day 1 until 16:00 day 2, and blood samples were drawn every 90 min from an indwelling forearm catheter. Standardised meals (breakfast, lunch, dinner, supper; Table S2) were provided at the indicated times, and they slept in darkness from 22:00 to 07:30 (Fig. 1). CME (4 g; Questran, Bristol-Myers Squibb, Princeton, NJ) was given as an oral drink before each meal day 1 (Fig. 1b). In the CME+STAT experiment, they were also given four daily doses of 40 mg atorvastatin (Lipitor, Pfizer). The first and second doses were taken in the morning 2 days before CME administration, and the third and fourth in the morning days 1 and 2 (Fig. 1c).

Serum analyses

Individual BAs and markers of BA and cholesterol synthesis

Combined quantitation of C4 and the 15 major human BAs (free, glycine- and taurine-conjugated CA, CDCA, DCA, LCA, UDCA) was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). All chemicals and solvents were of the highest purity available. Methanol, acetonitrile and formic acid were obtained from VWR (Radnor, Pennsylvania). Deuterium-labelled standards of C4 and unconjugated, glycine- and taurine-conjugated BAs were from Steraloids, Inc. (Newport, RI, USA).

Serum (100 µL) was mixed with 400 µL of acetonitrile and 10 µL of each of the deuterium-labelled C4 and BA internal standards (10 ng d7-C4 and 25 ng d4-BA) and vortexed for 5 s. After 15 min centrifugation at 14 000 g, supernatants were transferred to glass tubes and dried under a stream of nitrogen at 40 °C. The residue was dissolved in 200 µL of MeOH-H2O (1 : 1, v/v), and 5 µL of this solution was injected.

C4 and individual BAs were analysed by LC-MS/MS using a Xevo TQ-XS Triple Quadrupole Mass Spectrometer (Waters Corp., Mass., USA) operated in the ion evaporation mode with an ion spray ionization probe. Data were acquired and processed using MassLynx MS Software. Chromatography was achieved using a reverse-phase C18 column, 1.7 µm, 2.1 x 50 mm internal diameter (Waters Corporation, Mass, USA). The column flow was 0.5 mL min⁻¹. C4 levels were normalised for total cholesterol [13]. Free lathosterol in serum was
determined by GC-MS, and expressed as mg mol\(^{-1}\) cholesterol [24].

**Lipids and lipoproteins**

Total cholesterol and triglycerides (TGs) were determined using reagents from Roche/Hitachi (Mannheim, Germany; TG 12016648, Chol 12016630). Lipoprotein profiles were analysed by fast performance liquid chromatography [25].

**Serum FGF19, insulin and glucose**

A sandwich enzyme-linked immune sorbent assay was used for serum FGF19 and insulin following the manufacturer’s instructions (FGF19 Quantikine ELISA kit, Cat. No. DF1900; R&D Systems, Minneapolis, MN), (Mercodia Insulin ELISA, 10–1113–01, Uppsala, Sweden). Serum glucose was determined with reagents from Roche/Hitachi (GLU 1.1448668). All samples were assayed in duplicate; the intra- and inter-assay coefficients of variation were <10%.

**Statistical analyses**

Data are presented as mean ± SEM. Wilcoxon signed rank test was used for pairwise comparisons using 08 : 30 h’ data of day 2 as comparison point. An estimate of cross-correlation function \([r_{p12}(\text{lag})]\) between two time series was used to investigate time correlations of FGF19/C4, FGF19/BA and BA/C4 at the different time lags (0 h, ±2 h, ±3.5 h, ±4.5 h, etc.), as described in legend to Figure S3. A Granger causality test [26] and a Bayesian structural time-series model [27] were used to determine if one of the time series provides statistically significant information about future values of other.

**Results**

**Diurnal rhythms of BA and cholesterol synthesis in relation to individual BAs, FGF19 and lipoproteins at the basal state**

Data obtained during the basal state (Fig. 1a) confirmed that the diurnal rhythms of both serum BAs and FGF19 are linked to food intake, and that the postprandial increases in serum BAs appear about 2 h prior to serum FGF19 increases (Fig. 2a, b). During day time, serum C4 generally peaked at ~13 : 00 and ~22 : 00 (Fig. 2c). Also, a third C4 peak was seen between 5 : 30 and 7 : 00 at the end of the sleeping phase. Although the diurnal changes in C4 were more discrete on day 1 of the present study, they are comparable to those previously reported [13]. As expected [13, 15, 28], levels of BAs, C4 and FGF19 showed considerable interindividual variation, but the patterns observed were consistent (Figure S1A-C).

Total serum BAs increased after each meal, while the relative BA composition remained essentially unchanged (Fig. 2d). At night, serum total BAs were reduced, but a peak presented at 5 : 30. At least 50% of serum total BAs in untreated subjects were CDCA, 40% CA + DCA, whereas UDCA and
Fig. 2 Diurnal variations at basal state. Diurnal variations of serum (a) Total BAs, (b) FGF19, (c) C4, (d) Individual BAs, (e) Conjugated BAs and (f) Unconjugated BAs in 8 healthy volunteers at basal condition. Data presented as mean ± SEM. Arrows indicate the time of meal intake.
LCA constituted <5% (Fig. 2d). When data on conjugated and unconjugated BAs were analysed, markedly different profiles were observed (Figs 2e-f, S2A-B). At least 70% of total serum BAs at daytime were glycine-conjugates, while taurine-conjugates were <1% of total BAs. This may relate to the inclusion of only males who have a lower fraction of taurine-conjugates [28]; these BA values were not subject to further analysis. Whereas the conjugated BAs showed typical postprandial increases (Figs 2e, S2A), the levels of unconjugated BAs were marginally influenced by food intake (Figs 2f, S2B). Instead they displayed a robust increase at 5:30 fully accounting for the peak of total BAs observed at this time point. In this peak, unconjugated BAs constituted 55% of total BAs, mostly CDCA and CA (Figs 2f, S2C-D).

Measurements of serum lathosterol showed the lowest levels during the day and highest during the night phase, gradually returning to initial levels the next morning (Fig. 3a). This is in line with previous findings on the circadian rhythm of cholesterol production in humans [13, 29].

Serum total, LDL, HDL and VLDL cholesterol levels remained constant at daytime and declined by ~5% during the sleeping period (Fig. 3b), presumably due to hemodilution caused by the supine position [30]. Serum total and VLDL TG levels showed anticipated postprandial peaks; from 5:30 onwards they had returned to values similar to those in the overnight fasting state (Fig. 3c). Similar to serum TGs, insulin and glucose levels peaked postprandially and levelled off overnight from 2:30 onwards (Fig. 3d, e). The interindividual variations in the levels of lathosterol, total cholesterol and TGs at basal state are shown in Figure S1D-F.

To examine the relationships between individual BAs and FGF19 and C4 levels in the basal state, we performed cross-correlation analysis which is an established method for measurement of similarities between time-dependent signals that have identical sampling rates. As noted (Fig. 2a, b), the postprandial increases in total serum BAs precede the increases in serum levels of FGF19. Further analysis of the cross-correlation function between time-dependent conjugated/unconjugated individual BAs and FGF19 revealed the presence of positive correlations with a time lag of about 2 h (0–3.5 h) between FGF19 and conjugated BAs (Figure S3). No correlation within biologically relevant time interval (shaded area in Figure S3) was detected between FGF19 and any of the unconjugated individual BAs. These findings were confirmed by a Bayesian structural time-series analysis where the combination of GDCA, GCDCA and GUDCA was used as predictor of the time evolution of circulating FGF19 levels (Legend to Figure S3).

**Effects of interruption of EHC on BA and cholesterol metabolism and the influence of inhibition of cholesterol synthesis**

To explore the influence of disrupting transintestinal and transhepatic fluxes of BAs, we repeated the experiment in the same individuals while initiating CME treatment (Fig. 1b). With the aim to create an even stronger deficiency of BAs in the EHC, an additional third experiment was performed which included 4 days of treatment with the HMG-CoA reductase inhibitor, atorvastatin (CME+STAT) (Fig. 1c).

As expected, CME intake reduced serum total BAs already after the first dose with markedly attenuated postprandial serum BA peaks (Fig. 4a). Further, the composition of serum BAs was significantly altered during both CME and CME+STAT treatments (Figure S2, Table 1). While CA levels were comparable to those seen at the untreated state (Figure S2C), the postprandial peaks of CDCA, DCA and UDCA were abolished by both treatment regimens (Figure S2D-F).

Serum BA profiles during CME treatment showed that the levels of unconjugated BAs were reduced by at least 60% compared to the basal state at 8:30 day 1, and they were somewhat further reduced in the CME+STAT experiment (Figure S2, Table 1). Of particular interest was that the unconjugated BA peak at night vanished during CME administration (Figure S2B). Likewise, the levels of conjugated BAs were markedly attenuated in the CME and in the CME+STAT experiments (Figure S2A).

In accord with the BA responses, FGF19 serum levels started to decline from day 1 of CME intake, with diminished postprandial peaks (Fig. 4b). By the end of day 1, at 17:30, the level of FGF19 was reduced by >90%, and did not show any trend to normalize at the end of the experiment. The changes in FGF19 serum levels in the CME+STAT experiment were similar to those seen following CME only (Fig. 4b, Table 1).
Fig. 3 Diurnal variations at basal state. Diurnal variation of serum (a) Lathosterol, (b) Cholesterol, (c) Triglycerides, (d) Insulin and (e) Glucose at basal state of 8 healthy volunteers. Data presented as mean ± SEM. Arrows indicate the time of meal intake.
Fig. 4 Effects of treatments with cholestyramine only (CME) and combined with statin (CME+STAT) on the diurnal variations. (a) Total BAs, (b) FGF19, (c) C4, (d) Lathosterol, (e) Total cholesterol, (f) Total triglycerides, (g) Insulin and (h) Glucose in 8 healthy volunteers. Data presented as mean ± SEM. Arrows indicate the time of meal intake.
After the first dose of CME, C4 increased gradually and on day 2 the increase was ~4-fold compared to baseline ($P = 0.001$; Table 1; Fig. 4c). However, atorvastatin pretreatment reduced this stimulation, resulting in a 2-fold increase in serum C4, presumably due to a more severe deficiency in hepatic cholesterol. Nevertheless, C4 remained higher compared to the basal state during CME+STAT treatment throughout the study (Fig. 4c).

Consistent with increased serum C4 following CME, serum lathosterol was doubled when compared to the basal situation (8 : 30) (Table 1). This increase was observed from late afternoon day 1, and remained elevated throughout the study (Fig. 4d). The effects of atorvastatin were noticeable from the baseline levels of both lathosterol and total cholesterol (Fig. 4d and e). As expected, atorvastatin treatment reduced lathosterol levels by 66%, as compared to treatment with CME only (Fig. 4d; Table 1).

Previously, CME-treated subjects showed increased serum TG, glucose and insulin levels mainly at late night after commencing treatment [23]. In the present study, CME treatment did not induce such increases. However, it was evident that the reduction in TG, glucose and insulin levels observed overnight in the basal state was abolished during treatment (Fig. 4f-h). Our current data indicate that during CME treatment the mean TG level was 23% higher, and slightly higher also during CME+STAT. Similarly, both insulin and glucose levels tended to be higher during the same period with CME or CME+STAT treatment. During day time, total TGs, glucose and insulin instead tended to be lower in response to both CME and CME+STAT (Fig. 4f-h).

**Discussion**

In this work, we aimed to characterize the integrated regulation of some metabolic pathways related to BA EHC in normal humans, both during the diurnal phases and following controlled pharmacologic perturbations. It was obvious that even when performing repeated studies in the same well-characterized individuals, there are large interindividual variations in several aspects, making it difficult to quantify changes of smaller magnitude that may still be of physiological importance. However, a number of robust patterns could be observed, giving a basis for conclusions as well as discussion of future human studies.

The present study confirms and expands previous work characterizing the diurnal variations of BA and cholesterol synthesis in humans [13]. Thus, serum C4 levels peaked at ~13 : 00 and ~22 : 00. Compared to previous data, we now also observed a third C4 peak at 5 : 30, which is in agreement with data from a previous report using another

**Table 1** Measurements of serum samples collected at 8 : 30 day 2 of the experiment under basal and treatment conditions

| Measurement                  | Basal       | CME         | CME+Statin  |
|------------------------------|-------------|-------------|-------------|
| Total BAs (μmol L⁻¹)         | 2.1 (±1.3)  | 1.5 (±0.7)  | 1.1 (±0.4)* |
| CA (μmol L⁻¹)                | 0.4 (±0.3)  | 0.6 (±0.3)  | 0.4 (±0.2)  |
| CDCA (μmol L⁻¹)              | 1.0 (±1.0)  | 0.6 (±0.6)* | 0.4 (±0.3)* |
| DCA (μmol L⁻¹)               | 0.5 (±0.5)  | 0.2 (±0.1)  | 0.1 (±0.1)**|
| Total conjugated BAs (μmol L⁻¹) | 1.1 (±0.8)  | 1.0 (±0.5)  | 0.7 (±0.4)  |
| Total unconjugated BAs (μmol L⁻¹) | 0.9 (±0.6)  | 0.5 (±0.4)* | 0.3 (±0.3)* |
| FGF19 (pg mL⁻¹)              | 148.2 (±42) | 16.9 (±4.3)**| 14.8 (±4.0)**|
| C4/c (mg mol⁻¹)              | 2.0 (±1.6)  | 14.1 (±7.6)**| 8.9 (±3.9)**|
| Lathosterol/c (mg mol⁻¹)     | 254.8 (±120) | 486.7 (±206)**| 169.1 (±71)**|
| Total Cholesterol (mmol L⁻¹) | 4.5 (±0.9)  | 4.2 (±0.7)  | 3.8 (±0.9)**|
| Total TGs (mmol L⁻¹)         | 1.5 (±0.5)  | 1.5 (±0.8)  | 1.5 (±0.4)  |
| Glucose (mmol L⁻¹)           | 5.0 (±0.5)  | 5.2 (±0.4)  | 5.2 (±0.4)  |
| Insulin (mU L⁻¹)             | 7.6 (±5.6)  | 4.9 (±2.0)  | 6.5 (±2.7)  |

Wilcoxon matched-pairs test was used to evaluate the difference in the measurements between 8 matched pairs before and after treatments. Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$. 
approach to study BA synthesis in humans [31]. In contrast to the pattern observed in rats and mice, where the diurnal changes in cholesterol synthesis are synchronous with those of BAs, we could demonstrate a clear disassociation between BA and cholesterol synthesis in humans. It is tempting to speculate that this marked species difference may be relevant for the known susceptibility to dietary cholesterol and conditions such as atherosclerosis and gallstones in humans [1]. The diurnal variations of both BA and cholesterol syntheses are abolished following CME (Fig. 4c, d).

Serum conjugated BAs increase postprandially, followed by peaks in circulating FGF19 levels. Fractional uptakes of conjugated and unconjugated individual BAs by human liver have been calculated from parallel measurements of individual BAs in portal and peripheral venous blood [4, 5, 11], which makes it possible to roughly estimate the presumed portal venous fluxes of BAs in the present work [32] (Fig. 5a). As shown in Fig. 5, ~300 μmol h⁻¹ of conjugated BAs are extracted (and resecreted) by the liver during fasting, and up to ~900 μmol h⁻¹ postprandially. These numbers are in reasonable agreement with previous measurements of fasting and stimulated hourly biliary total BA secretion in humans, 750 and 1400 μmol h⁻¹, respectively [33]. The corresponding uptake of unconjugated BAs is limited in both situations, ~10 μmol h⁻¹.

A major and unexpected finding in this study was a distinct peak of serum unconjugated BAs late at night, without any concomitant changes in conjugated BAs. This was evident in 6 of the 8 subjects studied (Figure S4A) and abolished by CME or CME+STAT treatment (Figures S2, S4). Information on circulating BA levels during the night in humans is limited, but similar observations have been reported in 3 healthy controls [15]. Based on calculations as above, the amount of unconjugated BAs entering the portal vein at this time can be estimated to ~100 μmol h⁻¹, corresponding to an hepatic uptake (and need for reconjugation) of ~50 μmol h⁻¹ (Fig. 5b). Although we cannot fully explain this interesting finding, a likely mechanism is an increased input of bacterially deconjugated BAs from the small intestine. This is supported by the small contribution of DCA (mostly formed in the large intestine), by the rapid return of this peak to normal, and by its elimination by CME. Based on studies in mice, the presence of a diurnal variation in the activity of the intestinal microbiota has recently been proposed [34, 35].

While there was no indication that these changes in unconjugated BAs had any influence on circulating FGF19 levels or BA synthesis, consistent with their lesser activation of FXR [17, 36], it is tempting to propose that the high venous levels reached may influence other BA-responsive structures. One such candidate is the ubiquitously expressed transmembrane G protein-coupled receptor TGR5 [37, 38] which is activated by unconjugated BAs [39]. Its role in human physiology is still unclear, however [37]. Another interesting speculation relates to that unconjugated BAs seem to cross the blood–brain barrier, and have been detected in the central nervous system of animals [40]. In fact, in vitro studies indicate that BAs may influence the hypothalamic-pituitary-adrenal axis influencing diurnal rhythms [41, 42], and BA-mediated effects have also been proposed in the pathogenesis of hepatic encephalopathy [43, 44]. Further exploration of the possible physiological importance of the nightly increase in unconjugated BAs will thus be of great interest.

In the basal situation, the temporal relationships between individual BAs and FGF19 indicate that the circulating levels of FGF19 vary in response to the transintestinal flux of conjugated BAs, particularly CDCA and DCA. In contrast, this was not seen for unconjugated BAs. These findings were confirmed by cross-correlation analysis and Bayesian statistics, and are compatible with the concept that synthesis and secretion of FGF19 occurs in the distal small intestine under regulation of FXR. In contrast to BAs, the difference between portal and peripheral concentrations of FGF19 is very low or absent [45, 46]. This indicates that the FGF19 levels reaching the liver under physiological conditions are far below those needed to demonstrate suppression of BA synthesis in cell culture and animal models [19], or in humans infused with an FGF19 analogue [47]. An attempt of applying cross-correlation analysis and non-linear mixed effects modelling to predict whether the conjugated BA or FGF19 level is the major driver for suppressing BA synthesis (C4 levels) partially failed, however. There are at least two reasons for this: the strong dependence of circulating FGF19 on individual BA levels, and the presence of a diurnal variation in BA synthesis independent of food intake, compatible with a basal (‘clock-related’) regulation of CYP7A1
Fig. 5 Simulation of dynamics of unconjugated and conjugated BAs based on data presented in this study. (a) Systemic and portal venous serum levels, (b) Estimated hepatic uptake. Coloured areas represent individual BA levels in the compartments, food intake is marked by arrows. Modelling described in Supplementary discussion [Ref. Figure S3] (c) Regulation of BA synthesis under normal and interrupted enterohepatic circulation. Postprandial absorption of conjugated BAs in the distal ileum induces FGF19 expression, both conjugated BAs and FGF19 signal to liver to suppress BA synthesis from cholesterol. Unconjugated BAs mainly peak at late night and may signal to the brain to contribute to regulation of clock genes. Treatment with cholestyramine eliminates the diurnal variations of conjugated/unconjugated BAs and FGF19.
activity also in humans [13, 14]. After cessation of CME, BA synthesis starts to return towards normal before the levels of FGF19 increase (Fig. 4c). Thus, the role of circulating FGF19 in the physiological regulation of BA synthesis in humans is still uncertain [19, 48]. However, considering the marked reduction of FGF19 during CME treatment, together with the strong evidence from studies of the FGF15-FGFR4-βKlotho axis in genetically modified mice [49–51], the presence of a critical level of circulating FGF19 may well be essential for maintaining normal mechanisms of feedback inhibition [19].

As for the regulation of TG synthesis by BAs, the present work does not support the concept that this is mediated by FGF19 [19]. In contrast to our previous results [23], serum TGs or glucose/insulin did not markedly increase during the late night phase following CME treatment. However, the lowering of TGs and glucose/insulin occurring during late night in the basal state was attenuated by CME (Fig. 4f-h). During the day, the postprandial levels of TG were instead lower following CME intake, which may reflect a somewhat reduced capacity for absorption of dietary fat in that situation. The apparent lowering of hepatic cholesterol availability and hence diminished capacity to increase BA synthesis seen with CME+STAT did not result in a more pronounced increase in TGs. Based on the present work, an interesting new hypothesis may be proposed regarding the interaction between BA EHC and hepatic TG synthesis, related to the increased nightly influx of unconjugated BAs challenging the hepatic capacity to conjugate BAs. As a first step in that process, returning unconjugated BAs are linked to CoA by the enzyme fatty acid transport protein 5 (FATP5; SLC27A5), which also is involved in promoting uptake and CoA-ligation of unconjugated fatty acids [52, 53]. A relative deficiency of this enzyme at late night may thus result in reduced hepatic TG synthesis and increased insulin sensitivity, in agreement with what is observed in FATP5 knockout mice [52, 53]. Further studies in suitable animal and human models, including genetic diseases of BA conjugation [54] and hypertriglyceridemia with BA overproduction [55] will be important for better understanding the metabolic links between BA, glucose and TG metabolism.

In summary (Fig. 5c), in normal humans: (i) the temporal pattern of EHC is different for conjugated and unconjugated BAs, with postprandial fluxes of conjugated BAs during the day, and a marked input of unconjugated BAs late at night, presumably due to altered microbial activity in the small intestine; (ii) the transintestinal flux of conjugated BAs, particularly GCDCA and GDCA, regulates the concentration of circulating FGF19, presumably due to intestinal FXR activation; (iii) the diurnal variation of BA synthesis is influenced by the EHC of conjugated BAs, directly and/or via FGF19; (iv) serum TG or glucose/insulin are not generally linked to serum BAs or FGF19 levels, although increased influx of unconjugated BAs to the liver may contribute to reduced TG levels late at night; (v) by interrupting the EHC of both conjugated and unconjugated BAs, CME treatment attenuates the normal diurnal rhythms of BA and cholesterol synthesis, as well as the normal fasting suppression of TGs and glucose; and (vi) CME+STAT treatment modulates these responses. Thus, the metabolic response to interruption of the EHC of BAs in humans is highly dynamic and includes stimulation of BA, cholesterol and TG production, as well as compensatory changes in lipoprotein metabolism. Further studies on the interaction between diurnal rhythms of the BA EHC and the small intestinal microflora should provide important information.

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Conflict of interests

Chanchal Kumar is an employee of, and the work of Veronika Voronova, Dina Chernikova and Victor Sokolov is supported by AstraZeneca, which also provides funding of the ICMC at Karolinska Institutet. Bo Angelin is a minor shareholder in KaroPharma AB.

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Supporting Information
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Table S1. Basal measurements of all participants.

Table S2. Composition of meals served to the study participants.

Figure S1. Interindividual variation in serum levels among the 8 subjects under basal and treatments conditions.

Figure S2. Suppression of diurnal variation of conjugated and Unconjugated forms of individual BAs in response to treatments with cholestyramine only (CME) and combined with statin (CME+Statin).

Figure S3. Cross correlation analysis of BAs and FGF19 serum levels.

Figure S4. Interindividual variation in serum levels among the 8 subjects under basal and treatments conditions.