RESEARCH

FGF21 acts as a negative regulator of bile acid synthesis

Michelle M Chen*, Clarence Hale*, Shanaka Stanislaus, Jing Xu and Murielle M Véniant

Department of Cardiometabolic Disorders, Amgen Inc., Thousand Oaks, California, USA

Correspondence should be addressed to M M Véniant: mveniant@amgen.com

*(M M Chen and C Hale contributed equally to this work)

Abstract

Fibroblast growth factor 21 (FGF21) is a potent regulator of glucose and lipid homeostasis *in vivo*; its most closely related subfamily member, FGF19, is known to be a critical negative regulator of bile acid synthesis. To delineate whether FGF21 also plays a functional role in bile acid metabolism, we evaluated the effects of short- and long-term exposure to native FGF21 and long-acting FGF21 analogs on hepatic signal transduction, gene expression and enterohepatic bile acid levels in primary hepatocytes and in rodent and monkey models. FGF21 acutely induced ERK phosphorylation and inhibited *Cyp7A1* mRNA expression in primary hepatocytes and in different rodent models, although less potently than recombinant human FGF19. Long-term administration of FGF21 in mice fed a standard chow diet resulted in a 50–60% decrease in bile acid levels in the liver and small intestines and consequently a 60% reduction of bile acid pool size. In parallel, colonic and fecal bile acid was decreased, whereas fecal cholesterol and fatty acid excretions were elevated. The long-acting FGF21 analog showed superiority to recombinant human FGF21 and FGF19 in decreasing bile acid levels with long duration of effect action in mice. Long-term administration of the long-acting FGF21 analogs in obese cynomolgus monkeys suppressed plasma total bile acid and 7 α -hydroxy-4-cholesten-3-one levels, a biomarker for bile acid synthesis. Collectively, these data reveal a previously unidentified role of FGF21 in bile acid metabolism as a negative regulator of bile acid synthesis.

Key Words

- ▶ FGF21
- ▶ Fc-fusion protein
- ▶ β -klotho binding
- ▶ bile acid

Journal of Endocrinology
(2018) **237**, 139–152

Introduction

Among the 22 fibroblast growth factors (FGFs) identified in humans, FGF19, FGF21 and FGF23, which belong to the FGF19 subfamily, are vastly different from other FGFs and are primarily involved in the regulation of metabolic processes rather than cell proliferation, differentiation and growth (Itoh 2010). These 3 FGFs require the klotho family of proteins as an obligate co-receptor to activate the FGF receptors (FGFRs) and elicit the downstream signaling cascade and functional activity (Tomiya *et al.* 2010). Although FGF19 and FGF21 share the same co-receptor

β -klotho (Kurosu *et al.* 2007), they have different FGFR selectivity (Goetz *et al.* 2007). FGF19 preferentially binds to and is more potent toward FGFR4 (Xie *et al.* 1999), while FGF21 preferentially binds to and is more potent toward FGFR1c (Ogawa *et al.* 2007). *Fgfr4* is mainly expressed in the liver, whereas *Fgfr1c* is expressed in the adipose tissue (Kurosu *et al.* 2007). The different receptor selectivity and tissue expression pattern give rise to the functional specificity of FGF19 and FGF21 (Yang *et al.* 2012). Based on these findings, many investigators have concluded that

FGF19 is primarily involved in the regulation of bile acid metabolism in the liver (Inagaki *et al.* 2005), while FGF21 is solely involved in glucose, lipid and energy metabolism in the adipose tissue (Kharitonov *et al.* 2005).

Evidence suggests that FGF19 and FGF21 display some overlapping functions, particularly evident at supra-physiologic concentrations when administered at pharmacologic doses, overexpressed in transgenic mice (Tomlinson *et al.* 2002, Inagaki *et al.* 2007) or delivered by adeno-associated virus (AAV). Transgenic mice overexpressing *Fgf19* showed reduced blood glucose, lipid and insulin levels, improved insulin sensitivity, dramatically reduced body weight and improved energy metabolism with increased metabolic rate and energy expenditure (Tomlinson *et al.* 2002). These metabolic phenotypes are reminiscent of those observed in FGF21 transgenic mice or mice treated with FGF21 (Inagaki *et al.* 2007, Xu *et al.* 2009). These phenotypes do not appear to be solely mediated by changes in bile acid metabolism or to be a liver/intestinal-specific phenomenon, but rather raises the possibility that other metabolic tissues, such as the adipose tissue, may be involved. This overlapping functionality could be accomplished through receptor promiscuity and crossover receptor activity at pharmacologic concentrations. FGF19 may activate receptors in the adipose tissue and elicit many functional consequences similar to FGF21 when administered at supra-physiologic concentrations.

Bile acids are synthesized from cholesterol in the liver and are critical for generating bile flow and for biliary cholesterol excretion. In the intestine, they also function as amphipathic molecules required to form micelles with dietary cholesterol and fat to facilitate dietary lipid digestion and absorption (Monte *et al.* 2009). A growing body of evidence suggests that bile acids are important in the regulation of cholesterol metabolism (Staels & Fonseca 2009). However, bile acids are toxic due to their hydrophobic chemical matter (Attili *et al.* 1986). Accumulation of bile acids damages the cell membrane leading to inflammation, fibrosis and necrosis of the cells (Zakharia *et al.* 2017), which is the underlying pathology of many liver and bile duct diseases (Bidot-Lopez *et al.* 1979, Boberg *et al.* 1994, Pan & Perumalswami 2011, Xie *et al.* 2016). Thus, it is essential that bile acid levels are tightly regulated to facilitate post-prandial lipid breakdown while minimizing hepatobiliary tissue breakdown. Several mechanisms have been explored in the feedback regulation of bile acid synthesis, including the farnesoid X receptor/small heterodimer partner (FXR/SHP) axis and FGF19 (Chiang 2015). Elevated bile

acid levels activate the FXR/SHP axis in the liver, repressing bile acid synthesis (Ding *et al.* 2015), and in the intestine, inducing intestinal expression of *Fgf19*, which suppresses bile acid synthesis in the liver through activation of hepatic FGFR4 (Song *et al.* 2009). Our objective was to explore the functional activity of FGF21 in the liver and examine whether FGF21 is involved in the regulation of bile acid metabolism. We compared the functional potency and efficacy of FGF21 and long-acting analogs of FGF21 with FGF19 and examined whether FGF21 and FGF21 analogs may have therapeutic potential to treat cholestatic diseases.

Materials and methods

Reagents

Studies were conducted with full-length recombinant human FGF19 (rhFGF19), rhFGF21 and FGF21 analogs with N-terminal Fc-fusion proteins designed to reduce aggregation and proteolytic degradation. Fc-fusion FGF21 analogs included 2 amino acid (AA) substitutions Fc-RG (Hecht *et al.* 2012) and 3 AA substitutions Fc-RGE (Stanislaus *et al.* 2017). All proteins were expressed in *Escherichia coli* as previously described (Yie *et al.* 2009, Hecht *et al.* 2012, Veniant *et al.* 2012). Constructs were folded in solubilized inclusion bodies and purified by ion exchange and hydrophobic interaction chromatography to obtain >90% purity.

Rodents, diet and housing

All rodent studies were conducted following guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of Amgen Inc. Mice were allowed to acclimate to a 12:12-h light:darkness cycle, housing humidity and temperature, and routine handling at least 2 weeks prior to initiation of the study. C57BL6 mice were acquired from Harlan Laboratories (Hayward, CA, USA) at 8–10 weeks of age. Mice were single-housed and maintained on a standard rodent diet (Harlan-Teklad 2020x). Diet-induced obesity (DIO) mice were prepared at Amgen Inc. as previously described (Stanislaus *et al.* 2017). DIO mice were maintained on the high-fat diet (D12492, Research Diets Inc., New Brunswick, NJ, USA) for the duration of each study. All animals were provided free access to drinking water. Blood was collected from the retro-orbital sinus and measured on an AlphaTRAK glucometer (Zoetis, Parsippany, NJ, USA).

Hepatocyte isolation

Cryopreserved human hepatocytes were obtained from CellzDirect (Durham, NC, USA) or Life Technologies and cultured according to the vendor's suggested protocol. Murine hepatocytes were isolated from 5- to 7-week-old C57BL6 mice, and rat hepatocytes were isolated from 5- to 7-week-old Sprague–Dawley rats as described by Shen and Li (Li *et al.* 2010, Shen *et al.* 2012). Briefly, liver perfusion medium (Gibco) was perfused through the liver followed by collagenase treatment via retrograde perfusion. Liver cells were gently shaken in a liver wash media (Gibco), and hepatocytes were purified through gentle centrifugation and washing. Hepatocytes were plated in 6-well or 96-well plates for subsequent treatment and assays.

Hepatocyte culture and treatment

Cryopreserved primary human hepatocytes (Life Technologies) from a single donor were suspended in 50 mL of cryopreserved hepatocyte recovery medium (Life Technologies) and pelleted by centrifugation at 100g for 10 min at room temperature. The pellets were suspended in DMEM supplemented with 1× penicillin/streptomycin/L-glutamine (PSG; Life Technologies), 1× insulin–transferrin–selenium (ITS; Life Technologies), 100 nM dexamethasone (Sigma Aldrich) and 10% fetal bovine serum (Gibco). The hepatocytes were then seeded at a density of 70,000 viable cells per well in 96-well collagen-I-coated cell culture plates (BD Biosciences, San Jose, CA, USA) and placed in a 37°C incubator with 5% CO₂ and 90% relative humidity. When the cells were fully attached (~4–6 h post-seeding), the medium was replaced with Williams E media (Gibco) supplemented with 1× PSG, 1× ITS, 100 nM dexamethasone and 0.275 mg/mL Matrigel (BD Biosciences) and cultured overnight. On the following morning, the medium was replaced with the same Williams E media without Matrigel, and the plates were returned to the incubator for an additional 24 h. The cells were then treated with test articles in the Williams E media without Matrigel for 6 h. At the end of the treatment period, cells were harvested for RNA extraction and gene expression analysis.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from treated primary hepatocytes or frozen tissues, such as the liver, gallbladder, small intestine and colon using RNeasy kits (Qiagen). qPCR was

carried out with 50–100 ng of total RNA in 20 µL volume in 384-well plates as previously described (Xu *et al.* 2009). *Cyclophilin B* or *18S* was used as the reference gene. Relative gene expression was determined by using the comparative CT method. Reactions with CT greater than 35 were regarded as below the limit of detection.

Western blot analysis of phosphorylated ERK and total ERK

Primary hepatocytes were plated in 6-well collagen-coated plates in DMEM and 10% fetal bovine serum and incubated at 37°C for 2–4 h before changing to Williams E media with 1× glutamine for overnight incubation. Cells were treated with the indicated doses of FGF1, rhFGF21 and rhFGF19 for 10 min, washed twice in cold Dulbecco's phosphate buffered saline (PBS; Life Technologies) and lysed in 300 µL/well of lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.25% sodium deoxycholate, 1 mM NaF, complete protease inhibitor cocktail (Roche), 0.7 µg/mL Pepstatin and 1 mM Na₃VO₄). Liver samples were homogenized in the lysis buffer described earlier. Lysates were quantitated with the DC protein assay (Bio-Rad #500-0116) according to the manufacturer's instructions. Lysates were subjected to Western blot analysis as described previously (Xu *et al.* 2009). Briefly, samples were resolved on a 4–12% Bis-Tris gel and transferred onto PVDF membranes. Proteins were detected using the following antibodies – anti-ERK 1&2 (pTpY185/187) phosphospecific ab (Thermo Fisher scientific, 44-680G), anti-total ERK1/2 (Cell Signaling Technology 9102), anti-CYP7A1 (Millipore Sigma MABD42) and anti-β-actin (Sigma Aldrich, A2228). Images were captured on AlphaImager (Protein Simple, San Jose, CA, USA) according to the manufacturer's instructions.

Acute studies with recombinant rhFGF19 and rhFGF21 in mice

Mice were stratified into treatment groups based on body weight and *ad libitum* fed plasma glucose levels. To measure acute changes in *Cyp7A1* expression, a single intra-peritoneal injection (i.p.) of rhFGF19 or rhFGF21 was administered at increasing doses, and tissue samples were collected following a 3-h fast post injection. In a separate study, rhFGF21 was administered at 0.3, 3 and 6 mg/kg (i.p.) and an LXR-agonist (T0901317, Cayman Chemical, CAS 293754-55-9) was administered by a single oral gavage at 50 mg/kg. Terminal blood and tissue samples were collected under *ad libitum* fed conditions 3 h

post injection. Tissues were snap-frozen in liquid nitrogen for gene expression analysis.

Sub-chronic study with rhFGF19, rhFGF21 and Fc-RGE in mice

A 9-day study was conducted in 18-week-old C57BL6 mice. Mice were administered i.p. twice a day (BID) with vehicle, rhFGF19, or rhFGF21 at 0.3 and 3 mg/kg. A long-acting FGF21 analog, Fc-RGE, was administered i.p. at 1 and 10 mg/kg every 3 days (Q3D). To ensure that all mice were consistently handled and underwent the same number of injections, Fc-RGE-treated groups were administered with saline (i.p.) in between their scheduled Q3D doses of Fc-RGE. Food intake and 3-day total feces were collected during the treatment period from day 0 to 3 and from day 6 to 9. In order to minimize stress, all mice were maintained in standard housing conditions throughout the study. On day 9, mice received the last drug dose and were placed into new cages without food. Terminal blood and tissues samples were collected 3 h after the last drug dose for bile acid analysis. The liver was snap-frozen in liquid nitrogen, and the gallbladder was ligated and weighed using a pre-weighed Eppendorf tube. An incision was made to the gallbladder, and bile was collected following centrifugation. The empty gallbladder was weighed, and the difference between the filled and empty gallbladder was recorded as the bile volume. The small intestine and colon were collected with contents left intact.

Long-term study with long-acting FGF21 analogs in obese cynomolgus monkeys

A dose-escalation study with long-acting FGF21 analogs was conducted in cynomolgus monkeys at Yunnan Laboratory Primate Inc. (Kunming, Yunnan, China) (Stanislaus *et al.* 2017). The research protocol and animal housing were approved by the IACUC of Yunnan Laboratory Primate Inc., China. Briefly, animals were individually housed in a controlled environment with 12:12-h light:darkness cycle, controlled humidity range of 60–80% and temperature maintained in the range of 18–26°C. Animals were fed BID with a snack in between meals and had free access to drinking water. Animals were acclimated to all experimental procedures prior to study initiation. Vehicle, Fc-RG or Fc-RGE was administered weekly by subcutaneous injection for 9 consecutive weeks as previously described (Stanislaus *et al.* 2017). The dose was escalated every 3 weeks

(0.3 mg/kg for the first 3 weeks, followed by 1 mg/kg for the next 3 weeks and 3 mg/kg for the last 3 weeks). Blood samples were collected after an overnight fast at pre-dose day 14, and on days 5, 12, 19, 26, 33, 40, 47, 54 and 61 (approximately 117 h after each weekly dose). During the drug-washout phase of the study, blood samples were collected on days 70, 77, 84, 91, 98, 105 and 133. All fasting samples were subsequently analyzed for plasma total bile acids. In addition, fasting samples from pre-dose day –14, and days 19, 40 and 61 were used to measure 7 α -hydroxy-4-cholesten-3-one (C4) levels by LC–MS/MS.

Measurement of plasma, tissue and fecal bile acids

Plasma cholesterol and total bile acids were measured using an Olympus AU400e Chemistry Analyzer (Olympus America). Total bile acid was extracted from tissues and feces as described by Yu *et al.*, with a few modifications (Yu *et al.* 2000). Briefly, the frozen liver, gallbladder, small intestine and colon (all in whole with contents) were each individually extracted with 75% ethanol in a volume that was 5–8 times the tissue weight depending on the bile acid contents in each tissue. Tissue was homogenized using the TissueLyser (Qiagen). Tissue homogenate was incubated in a 50°C shaker for 2 h to extract the total bile acid. Extracts were cleared by centrifugation and diluted in 25% PBS solution. The dilution factor for each tissue extract was estimated to ensure that bile acid measurements fell in the linear range of the standard curve using mouse bile acid kit (Crystal Chem, Downers Grove, IL, USA, cat# 80370). The bile acid pool size was determined as sum of the amount of total bile acids in the liver, gallbladder and small intestine and its contents.

Feces from individually housed mice were collected over a 72-h period (days 0–3 and 6–9) and allowed to dry at room temperature for 72 h. Feces were weighed and powdered before extraction. Total bile acids were extracted from powdered fecal sample in 75% ethanol in a volume that was 10 times the fecal weight, and the subsequent steps were followed as described earlier.

Measurement of liver and fecal cholesterol and fatty acids

Fecal and liver total lipids were extracted from dried powdered feces using chloroform:methanol (2:1) according to the classic Folch method. Extracted lipids were dried under nitrogen gas and suspended in 90% isopropanol and 10% Triton-X-100 solution. Cholesterol

and non-esterified fatty acid levels were determined using commercial kits from Wako Diagnostics (Richmond, VA, USA).

Determination of C4 in cynomolgus monkey plasma

C4 measurements were performed on monkey plasma samples by Metabolon Inc., in accordance with Metabolon's standard operating procedure for sample analysis. Briefly, sample analysis was performed in a 96-well format containing 2 calibration curves and 6 replicates of quality control samples. Plasma was spiked with internal standard, C4-D7 and subjected to protein precipitation with acetonitrile followed by liquid-liquid extraction with a cyclohexane/ethyl acetate mixture. After centrifugation, the organic supernatant was removed, evaporated and reconstituted in acetonitrile and diluted in water. An aliquot of the reconstituted extract was injected onto an Agilent 1290/AB Sciex QTrap 5500 LC-MS/MS system equipped with a C18 reversed-phase UHPLC column. The mass spectrometer was operated in positive mode using atmospheric pressure chemical ionization.

The peak area of the m/z 401→177 product ion of C4 was measured against the peak area of the C4-D7 product ion of m/z 408→97. Quantitation was performed using a weighted ($1/\times 2$) linear least-squares regression analysis generated from fortified calibration standards prepared immediately prior to each run.

Statistical analysis

Data are presented as means±standard error of the mean (S.E.M.). Statistical comparison of the means among the groups was made using 1-way analysis of variance. Differences between the means of individual groups were analyzed by the *post-hoc* Fisher's test using StatView software (SAS Institute, Cary, NC, USA).

Results

rhFGF19, FGF21, Fc-RG and Fc-RGE signaling in hepatocytes

A dose-dependent induction of ERK1/2 phosphorylation by FGF21 was observed in mouse hepatocytes (Fig. 1A), by rhFGF19 and FGF21 in rat hepatocytes (Fig. 1B), and by rhFGF19 and FGF21 in human hepatocytes (Fig. 1C and D). The long-acting forms of FGF21, Fc-RG and Fc-RGE, induced ERK1/2 phosphorylation in mice and rats (data not shown) and human hepatocytes (Fig. 1D).

Effect of rhFGF19 and FGF21 on *Cyp7A1* mRNA expression levels

rhFGF19 and FGF21 acutely inhibited expression of *Cyp7A1* in the liver of C57BL6 mice. In this model, rhFGF19 at the 0.01 mg/kg dose decreased *Cyp7A1* expression

Figure 1

rhFGF19, rhFGF21 and Fc-RGE signaling in hepatocytes. Western blot analysis of mouse primary hepatocytes treated with 0.5 pM to 1 μ M of rhFGF21 for 10 min. rhFGF21 dose dependently induced ERK1/2 phosphorylation in mouse hepatocytes (A). Similar patterns were observed with rat (B) and human (C) primary hepatocytes treated with 0.32 nM to 1 μ M of rhFGF21. (D) rhFGF19, rhFGF21 and recombinant human Fc-FGF21 analogs induced ERK signaling in human primary hepatocytes. Fc-RGE, Fc-fusion FGF21 analog with 3 amino acid substitutions; rhFGF, recombinant human fibroblast growth factor.

levels by 25% relative to vehicle, and the maximal effect was reached at 10mg/kg dose (88% inhibition relative to vehicle Fig. 2A). Although FGF21 administration in the same animal model resulted in *Cyp7A1* inhibition, there was no clear dose response. Inhibition of *Cyp7A1* expression with FGF21 was evident at the 0.001mg/kg dose (33% inhibition relative to vehicle) and reached maximal effect at the 1mg/kg dose (69% inhibition relative to vehicle, Fig. 2A).

In DIO mice, acute administration of rhFGF19 dose-dependently inhibited hepatic *Cyp7A1* expression (Fig. 2B). However, the DIO mice appeared to be less responsive to rhFGF19 than C57BL6 mice. In DIO mice, the effect of acute administration of FGF21 was similar to that observed in C57BL6 mice (Fig. 2B).

In human primary hepatocytes, Fc-control showed no effect on *Cyp7A1* expression levels. FGF19 repressed *Cyp7A1* expression in a dose-dependent manner and fully inhibited *Cyp7A1* expression by 1nM. Both FGF21 and

Fc-RGE decreased *Cyp7A1* expression levels (58% and 34% at 6µM) but to a lesser extent than FGF19 (Fig. 2C).

Effect of FGF21 on gene expression levels for relevant genes of bile acid metabolism

Acute administration of FGF21 dose dependently inhibited hepatic expression of *Cyp7A1* and *Cyp8b1*, both key genes involved in the bile acid synthesis pathway (Fig. 3A). *Cyp27A1*, a key gene in the alternative bile acid synthesis pathway, was also suppressed by 45% in mice treated with the highest dose of FGF21. FGF21 administration increased the expression of bile acid, phospholipids and sterol transporters genes (*Bsep*, *Mrp2*, *Abcg5* and *Abcg8*) in the gallbladder of DIO mice (Fig. 3B). In addition, FGF21 also acutely affected intestinal bile acid absorption in the ileum of DIO mice as evidenced by the decreased apical sodium-dependent bile acid transporter (*Asbt*) and *Osta* expression (Fig. 3C). The LXR-agonist T0901317

Figure 2

rhFGF21 acutely inhibited expression of *Cyp7A1* in the liver of C57BL6 and DIO mice. (A and B) Male C57BL6 and DIO mice treated with rhFGF19 or rhFGF21 at indicated doses (from 0.001 to 10mg/kg) to determine the treatment effect on *Cyp7A1* expression levels. (C) Human primary hepatocytes treated with FGF21, Fc-control, or Fc-RGE with 1:3 titration starting at 6µM, or 10nM of FGF19 to measure relative dose-dependent inhibition of *Cyp7A1* expression levels. All data represent mean $\pm$ s.e.m. ($n=3-5$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs vehicle control. DIO, diet-induced obesity; Fc-RGE, Fc-fusion FGF21 analog with 3 amino acid substitutions; rhFGF, recombinant human fibroblast growth factor; s.e.m., standard error of the mean.

Figure 3

Acute effect of rhFGF21 on *Cyp7A1* mRNA and protein levels and expression of other bile acid metabolism genes. Expression analysis of genes involved in hepatic bile acid synthesis and metabolism from the liver (A), gallbladder (B), ileum (C) and hepatic CYP7A1 Western blot analysis (D) of DIO mice treated with either vehicle (white), rhFGF21 (gray), or a LXR-agonist T0901317 (black) for 3 h. Data represent mean $\pm$ s.e.m. ($n=5$, (A) *Cyp7A1*), and pooled samples run in duplicates (all other figures). Statistical analysis of FGF21-treated groups to vehicle-treated group was performed separately from the LXR-T-treated group to vehicle-treated group (** $P<0.01$; *** $P<0.001$ vs vehicle control). DIO, diet-induced obesity; Fc-RGE, Fc-fusion FGF21 analog with 3 amino acid substitutions; LXR, liver X receptor; rhFGF, recombinant human fibroblast growth factor; s.e.m., standard error of the mean.

(50 mg/kg) induced *Cyp7A1*, *Abcg5*, *Abcg8* and *Bsep* expression levels and suppressed *Cyp8B1* expression levels (Fig. 3A, B and C). *Cyp7A1* hepatic protein levels were decreased when mice were treated with 6 mg/kg of FGF21 and increased when mice were treated with 50 mg/kg of T0901317.

Effects of rhFGF19, FGF21 and Fc-RGE on bile acid levels in C57BL6 mice

A longer-term study was conducted over 9 days to investigate the effects of rhFGF19, FGF21 and Fc-RGE administration on bile acid levels in C57BL6 mice. Based on previous pharmacokinetic characterization, rhFGF19 and FGF21 were administered BID at 0.3 and 3 mg/kg, while Fc-RGE was administered Q3D at an equimolar dose of 1 and 10 mg/kg (Fig. 4A). A statistically significant reduction in total bile acids was observed in the liver (Fig. 4B) and small intestine (Fig. 4C) in mice treated with high dose of rhFGF19 ($P<0.01$ and $P<0.001$, respectively) and rhFGF21 (3 mg/kg, $P<0.05$) as well as in mice treated

with the low and high doses of Fc-RGE (1 and 10 mg/kg, $P<0.01$ and $P<0.001$). The reduction of bile acid in the liver and intestine with Fc-RGE at 1 mg/kg treatment was greater than that with rhFGF19 or rhFGF21 at every dose (Fig. 4B and C). A significant reduction ($P<0.001$) in biliary bile acid concentration was observed at a high dose of rhFGF19 and both doses of Fc-RGE. Both doses of rhFGF21 were similar to levels seen in vehicle-treated mice (Fig. 4D). The empty gall bladder weight was nearly identical in mice across all treatment groups (data not shown). However, an increased calculated bile weight, indicative of increased bile volume, was seen in mice treated with both doses of rhFGF21 (Fig. 4E). Calculated total bile acids in the gall bladder, a product of bile concentration (Fig. 4D) and volume (Fig. 4E), were significantly elevated ($P>0.05$) in rhFGF21-treated mice (Fig. 4F). Mice treated with a high dose of rhFGF19 (trend of reduction) and both doses of Fc-RGE show significantly ($P<0.05$) reduced gall bladder total bile acid, with reduction in both biliary bile acid concentration and bile volume. This cumulative decrease in the liver, small intestine and gall bladder bile

Figure 4

Effect of long-term rhFGF19, rhFGF21, and Fc-RGE administration in C57BL6 mice on total bile acids and cholesterol levels. Male C57BL6 mice treated at indicated doses and frequency for 9 days (A). Blood was collected at the termination for plasma cholesterol measurement and tissues were harvested for bile acid analysis following a 3-h fasting and post-injection of vehicle (white bar), and at indicated low and high doses of rhFGF19 (light gray bar), rhFGF21 (medium gray bar), or Fc-RGE (dark gray bar). All data represent mean $\pm$ S.E.M. ($n=7-8$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs vehicle control. Fc-RGE, Fc-fusion FGF21 analog with 3 amino acid substitutions; rhFGF, recombinant human fibroblast growth factor; S.E.M., standard error of the mean.

acids resulted in a significantly reduced total bile acid pool size in mice treated with rhFGF19 at 3 mg/kg (53%, $P<0.001$) and Fc-RGE at 1 and 10 mg/kg (76% and 66%, respectively, $P<0.001$; Fig. 4G).

Mice treated with rhFGF19 demonstrated significantly increased plasma cholesterol levels (at 0.3 mg/kg with $P<0.001$ and 3 mg/kg with $P<0.01$, Fig. 4H). Mice treated with both doses of rhFGF21 and Fc-RGE showed significantly reduced plasma cholesterol levels ($P<0.001$; Fig. 4H). All mice with FGF19, FGF21 and Fc-RGE demonstrated a modest increase in hepatic cholesterol concentration (Fig. 4I).

Effects of rhFGF19, rhFGF21 and Fc-RGE on total bile acid in the colon and feces

Feces were collected in the same study for 3 cumulative days from day 0 to 3 and again on days 6–9. Total bile

acids in the colon and feces as well as fecal lipids were measured (Fig. 5). Interestingly, total bile acids measured in the colon and feces (Fig. 5A and B) showed a similar profile as the total bile acids of the liver (Fig. 4B), small intestine (Fig. 4C) and total pool size (Fig. 4G). The early fecal collection gathered between days 0 and 3 provided a measurement for acute changes in bile acids levels following treatment (Fig. 5B). Between days 0 and 3, mice in all dosed groups demonstrated a reduction in total bile acid levels by 14–18% with rhFGF19, 28–30% with rhFGF21 and 28–30% with Fc-RGE (Fig. 5B). Interestingly, between days 6 and 9, mice treated with Fc-RGE and the high dose of rhFGF19 showed a further reduction in fecal bile acid excretion of 58% and 38%, respectively, whereas the effect appeared refractory in mice treated with the low dose of rhFGF19 (2%) and both doses of rhFGF21 (9% and 24% at 0.3 and 3 mg/kg, respectively). Bile acids are required for intestinal absorption of cholesterol and fecal

Figure 5

Effect of long-term rhFGF19, rhFGF21, and Fc-RGE administration in C57BL6 mice on colon and fecal total bile acids and fecal lipids. Male C57BL6 mice treated at indicated doses and frequency for 9 days (Fig. 4A). The colon with contents intact were harvested at the termination for bile acid analysis following a 3-h fasting and post injection of vehicle (white bar), and at indicated low and high doses of rhFGF19 (light gray bar), rhFGF21 (medium gray bar) or Fc-RGE (dark gray bar). All data represent mean $\pm$ S.E.M. ($n=7-8$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs vehicle control. Fc-RGE, Fc-fusion FGF21 analog with 3 amino acid substitutions; rhFGF, recombinant human fibroblast growth factor; S.E.M., standard error of the mean.

fatty acids. Between days 6 and 9, fecal cholesterol was significantly increased in mice treated with FGF19 3 mg/kg ($P<0.01$), both doses of FGF21 ($P<0.05$ and $P<0.01$) and Fc-RGE ($P<0.001$) (Fig. 5C), while a significant increase in fecal fatty acids was observed in mice treated with high dose of rhFGF19 ($P<0.05$) or both doses of Fc-RGE ($P<0.001$, Fig. 5D). This effect correlates with the reduction in fecal bile acids (Fig. 5B).

Hepatic gene expression profiles of mice treated with rhFGF19, rhFGF21 and Fc-RGE after 9 days of treatment

β -Klotho expression levels were significantly increased in mice treated with both doses of rhFGF21 ($P<0.05$ and $P<0.001$) and Fc-RGE ($P<0.01$, Fig. 6A). A similar trend was observed for *Fgfr2c* expression levels (Fig. 6A). *Fgfr3c* expression levels were significantly decreased in mice treated with 3 mg/kg of rhFGF19 ($P<0.01$), and *Fgfr1c* expression was significantly increased in mice treated with Fc-RGE 10 mg/kg ($P<0.05$, Fig. 6A). rhFGF19-treated mice demonstrated a general trend of decreased gene expression in bile acid synthesis pathways (*Cyp7A1*, *Cyp8b1*, *Cyp27A1* and *Cyp7b1*, Fig. 6B). In contrast to when rhFGF21 was administered as a single dose (Figs 2 and 3), rhFGF21 did not inhibit *Cyp7A1* and other bile acid synthesis genes when administered for 9 days (Fig. 6B). A trend towards elevated hepatic *Cyp7A1* and *Cyp27A1* gene expression was detected at the 3 mg/kg dose of rhFGF21 suggesting a potential compensatory feedback mechanism. Unlike with rhFGF21, mice treated with Fc-RGE demonstrated robust inhibition of gene expression for *Cyp7A1* and *Cyp8b1* (Fig. 6B), whereas minimal changes in gene

expression for *Cyp27A1* and *Cyp7b1* were observed. *Bsep* expression was significantly decreased in mice treated with 3 mg/kg of rhFGF19 ($P<0.05$, Fig. 6C). Interestingly, unlike *Cyp7A1*, *Bsep* expression levels were significantly increased in mice treated with rhFGF21 at both doses ($P<0.05$ and $P<0.01$, Fig. 6C), retaining the same effect as seen when a single dose of rhFGF21 was administered (Fig. 3B). Na⁺-taurocholate co-transporting polypeptide (*Ntcp*) expression levels were significantly increased in mice treated with rhFGF19 at the 0.3 mg/kg dose ($P<0.001$) but significantly decreased in mice treated with both doses of Fc-RGE ($P<0.001$). *Abcb4*, *Abcg5* and *Abcg8* expression levels were significantly increased in mice treated with rhFGF21 at 3 mg/kg ($P<0.01$) and with both doses of Fc-RGE ($P<0.05$ and $P<0.001$, Fig. 6C). Most of the expression levels of genes involved in the cholesterol and triglyceride synthesis pathway were significantly decreased in mice treated with rhFGF19, rhFGF21 and Fc-RGE (*Srebp2*, *Srebp1*, *Fasn* and *Scd1*; $P<0.05$, $P<0.01$ and $P<0.001$, Fig. 6D). The expression level of *Hmgcs1* was unchanged by any treatment; only rhFGF19 treatment significantly increased *Hmgcr* expression ($P<0.01$, Fig. 6D).

Effects of Fc-RG and Fc-RGE on total bile acid and C4 levels in cynomolgus monkeys

Monkeys treated with Fc-RGE demonstrated lower total bile acid levels across the entire 9 weeks of dosing (Fig. 7A). Following a 3-week treatment washout period, total bile acid levels in Fc-RGE-treated monkeys returned rapidly to the bile acid levels observed in the vehicle-treated monkeys. Similarly, monkeys treated with Fc-RG showed a lower trend of total bile acid levels than monkeys treated

Figure 6

Effect of long-term rhFGF19, rhFGF21, and Fc-RGE administration in C57BL6 mice on hepatic gene expression of rhFGF21, bile acid, and lipid pathways. Male C57BL6 mice treated at indicated doses and frequency for 9 days (Fig. 4A). The livers were harvested at the termination for gene expression analysis following a 3-h fasting and post-injection of vehicle (white bar), and low and high doses of rhFGF19 (light gray bar), rhFGF21 (medium gray bar) or Fc-RGE (dark gray bar). Genes were classified into *Fgf21* (A), bile acid synthesis (B), bile acid transport (C) and cholesterol and triglyceride synthesis pathways (D). All data represent mean $\pm$ S.E.M. ($n=7-8$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs vehicle control. Fc-RGE, Fc-fusion FGF21 analog with 3 amino acid substitutions; rhFGF, recombinant human fibroblast growth factor; S.E.M., standard error of the mean.

Figure 7

Effect of long-term Fc-RG and Fc-RGE treatment on plasma total bile acids and C4 levels in obese cynomolgus monkeys. Plasma samples from overnight fasting monkeys were analyzed for plasma total bile acids (A) and C4 (B) levels. All data represent mean $\pm$ S.E.M. ($n=9-13$). ** $P<0.01$; *** $P<0.001$ vs vehicle control. C4, 7 α -hydroxy-4-cholesten-3-one; Fc-RG, Fc-fusion FGF21 analog with 2 amino acid substitutions; Fc-RGE, Fc-fusion FGF21 analog with 3 amino acid substitutions; rhFGF, recombinant human fibroblast growth factor; S.E.M., standard error of the mean.

with vehicle. Interestingly, the levels rapidly increased within 1 week of Fc-RG washout, potentially due to the less beneficial pharmacokinetic profile of Fc-RG over Fc-RGE. C4 levels measured from fasting plasma samples collected following the third injection of each dose level (Fig. 7B) showed that monkeys treated with both Fc-RG and Fc-RGE demonstrated significant inhibition of C4 at each dose level ($P<0.01$ and $P<0.001$). The C4 levels returned to those seen in monkeys treated with vehicle by 10 weeks of treatment washout (Fig. 7B).

Discussion

Bile is an important regulator of numerous processes, including the emulsification of dietary lipids (Russell 2003, Chiang 2004), cholesterol catabolism (Russell 2003, Chiang 2004), as well as enabling the intestinal absorption of fat-soluble vitamins (Russell 2003, Chiang 2004). Bile acids are produced by the liver, then transported and stored in the gallbladder, and eventually deposited in the intestinal tract (Chiang 2013). Several transporters are present in the intestinal tract that initiates the re-uptake via the portal circulation (Dawson & Karpen 2015) and re-delivery back to the liver (Chiang 2013). This feedback loop is critical for the regulation of bile acid homeostasis (Chiang 2013). Regulation via FXR and the subsequent upregulated expression and enzymatic activity of *Cyp7A1* results in maintenance of a constant bile acid pool (Chiang *et al.* 2000, Rizzo *et al.* 2005). Bile acids, as well as several of their metabolites, are toxic to hepatocytes and thus, are tightly regulated. FGF21 and FGF15/19 have

previously been associated with enterohepatic production and circulation of bile (Inagaki *et al.* 2005, Luo *et al.* 2014, Zhang *et al.* 2017). Here, we present evidence that exogenously administered rhFGF21 may have a role in bile acid metabolism that is independent of the FGF15/19 pathway. This new finding indicates that FGF21, as well as FGF19, may contribute to the maintenance of bile acid homeostasis.

It was previously demonstrated that overexpression of *Fgf21*, using AAV, affected bile acid metabolism (Zhang *et al.* 2017). Zhang *et al.* proposed that FGF21 changes the bile acid levels by antagonizing FGF15/19 function on the β -klotho/FGFR4 receptor complex in the liver, thus inhibiting FGF15/19-mediated suppression of *Cyp7A1* expression (Zhang *et al.* 2017). We took a different approach, administering FGF21 at pharmacologic doses in different animal models. Our study is the first to show the potential effect that systemically administered FGF21 therapy could have on bile and its metabolism.

The use of FGF21 to treat conditions such as bile acid accumulation in the liver as well as the gallbladder and bile duct could result in decreased cholestasis. Cholestasis is defined as impaired secretion of bile from the liver and obstructed flow of bile through bile ducts (Zakharia *et al.* 2017). Cholestatic liver diseases can result in localized tissue injury (Zakharia *et al.* 2017), tissue death and ultimately scarring and fibrotic conditions (Zakharia *et al.* 2017). Whether these conditions originate from genetic abnormalities (Harris *et al.* 2005), occur during pregnancy (Pan & Perumalswami 2011) or are induced by the use of certain drugs (Zimmerman & Lewis 1987), FGF21 may represent an important new avenue toward

therapeutic benefits. Acute administration of rhFGF21 to C57BL6 mice and their DIO counterparts reduced expression of *Cyp7A1*, a key enzyme in converting liver cholesterol into bile acids. We also observed a decrease in CYP7A1 protein in animals treated with 6 mg/kg FGF21 acutely (Fig. 3D). However, the vehicle was highly variable similarly to what had been observed previously in Golden Syrian Hamsters (Mast *et al.* 2010). We confirmed CYP7A1 mRNA and protein levels increases in mice treated with LXR, agonist T0901317 (Fig. 3D) similarly to what has been published previously (Gupta *et al.* 2002).

Interestingly, mice treated with a long-acting form of FGF21, Fc-RGE, showed lower total bile acid in both the liver and small intestine compared with mice treated with rhFGF21, most likely due to the increased half-life of the engineered Fc-RGE molecule. In mice and monkeys, Stanislaus *et al.* reported Fc-RGE to be longer-acting and more stable and demonstrated enhanced efficacy compared to rhFGF21 (Stanislaus *et al.* 2017). The reduction of circulating bile acid was accompanied by a decrease of bile acids in feces and an increase in fecal free cholesterol and fatty acids. The increase in free cholesterol was not correlated to *de novo* cholesterol synthesis, as there was no change in the expression levels of *Hmgcr* and *Srebp2* expression levels, suggesting that the reduction of bile acids by Fc-RGE occurred solely through the decrease of the expression levels of *Cyp7A1* and *Cyp8B1*. Interestingly, the changes seen with *Cyp8B1* expression levels may indicate a switch in bile acid type toward less toxic, hydrophilic bile acids such as β -muricholic acid. Like FGF19, Fc-RGE appears to regulate the classic pathway of bile acid synthesis as demonstrated by the specific and pronounced effect on *Cyp7A1* expression.

Another indication of the potential protective effect of Fc-RGE on the hepatobiliary system is the reduced expression of *Ntcp*. It has previously been shown that a reduction in expression of this gene results in reduction of uptake of bile acids and protects the liver from the toxic effects of excessive bile acid accumulation (Dawson *et al.* 2003, Anwer 2004). Also, co-transporters as adenosine triphosphate-binding cassette (*Abc*) half-transporters (*Abcg5/Abcg8*) are upregulated after 9 days of treatment with Fc-RGE. Both transporters are known to be important regulators of the absorption and excretion of phytosterols and cholesterol (Berge *et al.* 2000, Lee *et al.* 2001). Their increased expression levels in the presence of Fc-RGE may reflect the reduced bile acid pool and increased plasma and fecal cholesterol. Transgenic overexpression of *Abcg5* and

Abcg8 in mice manifests with lower intestinal cholesterol absorption (Yu *et al.* 2002). A key gene of bile reabsorption in the ileum, *Asbt*, was downregulated after rhFGF21 treatment. Alterations in the levels of this transporter would have effects on bile acid enterohepatic circulation and compartmentalization. Administration of SC-435, a lumenally restricted inhibitor of ileal ASBT, resulted in improved insulin sensitivity, reduction of hepatic lipid profiles and reduced atherosclerosis in mice and guinea pigs (West *et al.* 2003, Rao *et al.* 2016). Similar metabolic improvements were observed with FGF21 administration. These findings suggest that some of the beneficial effects on metabolic profiles observed in animal models treated with FGF21 (Xu *et al.* 2009, Veniant *et al.* 2012) may occur through changes in the levels or profiles of circulating bile acids.

The results of the dose-escalation study of Fc-RGE in cynomolgus monkeys suggest that *Cyp7A1* liver expression or activity is reduced after treatment with this long-acting form of FGF21. Talukdar *et al.* (2016) also reported administration of a long-acting analog of FGF21 to both non-human primates and humans. However, no data describing bile acid regulation were reported (Talukdar *et al.* 2016). A previous study using non-human primates treated with a neutralizing antibody to FGF19 showed increases in bile acid synthesis and alterations in bile acid transporter expression (Pai *et al.* 2012). Their findings suggest that inhibition of FGF19 and a resulting alteration of bile acid transporters and bile acid synthesis was responsible for causing diarrhea in nonhuman primates (Pai *et al.* 2012). Implications from our study and others are that FGF19 and FGF21 may represent an overlapping system of bile acid regulation.

In conclusion, our data demonstrate a previously unidentified role of FGF21 in bile acid metabolism as a negative regulator of bile acid synthesis. Unmet medical need for pharmacologic agents with a potential to decrease bile acid accumulation and to reduce bile acid hydrophobicity has been identified. FGF21 and its analogs hold a promise to treat a range of cholestatic liver diseases and may offer therapeutic benefits to patients with bile acid dysregulation.

Declaration of interest

Michelle M Chen stockholder and employee of Amgen, Inc., Clarence Hale stockholder and employee of Amgen, Inc., Shanaka Stanislaus stockholder and employee of Amgen, Inc., Jing Xu stockholder and employee of Amgen, Inc., and Murielle M Véniant stockholder and employee of Amgen, Inc.

Funding

This work was supported by Amgen Inc.

Author contribution statement

Rodent studies were designed by M M V, J X and S S and executed by S S. Subsequent bile acid and lipid analysis executed by S S and M C. Western blotting and gene expression studies were designed by M M C, J X and C H and executed by M M C. Manuscript was written and revised by M M C, C H, S S and M M V.

Acknowledgments

The authors thank Michael Hayashi for isolation of fresh murine and rat hepatocytes. They thank Jim Busby, Jennifer Patel and Anna Solonina for *in vitro* assay technical assistance. They thank Amy Foreman-Wykert, PhD, Certified Medical Publication Professional (Amgen, Inc.) and Gurpreet Kaur (Cactus on behalf of Amgen, Inc.) for providing editorial and formatting support.

References

- Anwer MS 2004 Cellular regulation of hepatic bile acid transport in health and cholestasis. *Hepatology* **39** 581–590. (<https://doi.org/10.1002/hep.20090>)
- Attili AF, Angelico M, Cantafora A, Alvaro D & Capocaccia L 1986 Bile acid-induced liver toxicity: relation to the hydrophobic-hydrophilic balance of bile acids. *Medical Hypotheses* **19** 57–69. ([https://doi.org/10.1016/0306-9877\(86\)90137-4](https://doi.org/10.1016/0306-9877(86)90137-4))
- Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R & Hobbs HH 2000 Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* **290** 1771–1775. (<https://doi.org/10.1126/science.290.5497.1771>)
- Bidot-Lopez P, Labrecque DR, Hsia YE & Riely CA 1979 A study of inheritance in progressive intrahepatic cholestasis: hepatic excretory function in unaffected family members. *Pediatric Research* **13** 1002–1005. (<https://doi.org/10.1203/00006450-197909000-00010>)
- Boberg KM, Lundin KE & Schruppf E 1994 Etiology and pathogenesis in primary sclerosing cholangitis. *Scandinavian Journal of Gastroenterology Supplement* **204** 47–58. (<https://doi.org/10.3109/00365529409103625>)
- Chiang JY 2004 Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms. *Journal of Hepatology* **40** 539–551. (<https://doi.org/10.1016/j.jhep.2003.11.006>)
- Chiang JY 2013 Bile acid metabolism and signaling. *Comprehensive Physiology* **3** 1191–1212.
- Chiang JY 2015 Negative feedback regulation of bile acid metabolism: impact on liver metabolism and diseases. *Hepatology* **62** 1315–1317. (<https://doi.org/10.1002/hep.27964>)
- Chiang JY, Kimmel R, Weinberger C & Stroup D 2000 Farnesoid X receptor responds to bile acids and represses cholesterol 7 α -hydroxylase gene (CYP7A1) transcription. *Journal of Biological Chemistry* **275** 10918–10924. (<https://doi.org/10.1074/jbc.275.15.10918>)
- Dawson PA & Karpen SJ 2015 Intestinal transport and metabolism of bile acids. *Journal of Lipid Research* **56** 1085–1099. (<https://doi.org/10.1194/jlr.R054114>)
- Dawson PA, Haywood J, Caddock AL, Wilson M, Tietjen M, Kluckman K, Maeda N & Parks JS 2003 Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice. *Journal of Biological Chemistry* **278** 33920–33927. (<https://doi.org/10.1074/jbc.M306370200>)
- Ding L, Yang L, Wang Z & Huang W 2015 Bile acid nuclear receptor FXR and digestive system diseases. *Acta Pharmaceutica Sinica B* **5** 135–144. (<https://doi.org/10.1016/j.apsb.2015.01.004>)
- Goetz R, Beenken A, Ibrahim OA, Kalinina J, Olsen SK, Eliseenkova AV, Xu C, Neubert TA, Zhang F, Linhardt RJ, *et al.* 2007 Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. *Molecular and Cellular Biology* **27** 3417–3428. (<https://doi.org/10.1128/MCB.02249-06>)
- Gupta S, Pandak WM & Hylemon PB 2002 LXR alpha is the dominant regulator of CYP7A1 transcription. *Biochemical and Biophysical Research Communications* **293** 338–343. ([https://doi.org/10.1016/S0006-291X\(02\)00229-2](https://doi.org/10.1016/S0006-291X(02)00229-2))
- Harris MJ, Le Couteur DG & Arias IM 2005 Progressive familial intrahepatic cholestasis: genetic disorders of biliary transporters. *Journal of Gastroenterology and Hepatology* **20** 807–817. (<https://doi.org/10.1111/j.1440-1746.2005.03743.x>)
- Hecht R, Li YS, Sun J, Belouski E, Hall M, Hager T, Yie J, Wang W, Winters D, Smith S, *et al.* 2012 Rationale-based engineering of a potent long-acting FGF21 analog for the treatment of type 2 diabetes. *PLoS ONE* **7** e49345. (<https://doi.org/10.1371/journal.pone.0049345>)
- Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, Jones SA, Goodwin B, Richardson JA, *et al.* 2005 Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metabolism* **2** 217–225. (<https://doi.org/10.1016/j.cmet.2005.09.001>)
- Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esser V, *et al.* 2007 Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metabolism* **5** 415–425. (<https://doi.org/10.1016/j.cmet.2007.05.003>)
- Itoh N 2010 Hormone-like (endocrine) Fgfs: their evolutionary history and roles in development, metabolism, and disease. *Cell and Tissue Research* **342** 1–11. (<https://doi.org/10.1007/s00441-010-1024-2>)
- Kharitononkov A, Shiyanova TL, Koester A, Ford AM, Micanovic R, Galbreath EJ, Sandusky GE, Hammond LJ, Moyers JS, Owens RA, *et al.* 2005 FGF-21 as a novel metabolic regulator. *Journal of Clinical Investigation* **115** 1627–1635. (<https://doi.org/10.1172/JCI23606>)
- Kurosu H, Choi M, Ogawa Y, Dickson AS, Goetz R, Eliseenkova AV, Mohammadi M, Rosenblatt KP, Klier SA & Kuro-o M 2007 Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *Journal of Biological Chemistry* **282** 26687–26695. (<https://doi.org/10.1074/jbc.M704165200>)
- Lee MH, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, Kojima H, Allikmets R, Sakuma N, Pegoraro R, *et al.* 2001 Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nature Genetics* **27** 79–83. (<https://doi.org/10.1038/83799>)
- Li H, Fang Q, Gao F, Fan J, Zhou J, Wang X, Zhang H, Pan X, Bao Y, Xiang K, *et al.* 2010 Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. *Journal of Hepatology* **53** 934–940. (<https://doi.org/10.1016/j.jhep.2010.05.018>)
- Luo J, Ko B, Elliott M, Zhou M, Lindhout DA, Phung V, To C, Learned RM, Tian H, DePaoli AM, *et al.* 2014 A nontumorigenic variant of FGF19 treats cholestatic liver diseases. *Science Translational Medicine* **6** 247ra100. (<https://doi.org/10.1126/scitranslmed.3009098>)
- Mast N, Shafaati M, Zaman W, Zheng W, Prusak D, Wood T, Ansari GA, Lovgren-Sandblom A, Olin M, Bjorkhem I, *et al.* 2010 Marked variability in hepatic expression of cytochromes CYP7A1 and CYP27A1 as compared to cerebral CYP46A1. Lessons from a dietary study with omega 3 fatty acids in hamsters. *Biochimica et Biophysica Acta* **1801** 674–681. (<https://doi.org/10.1016/j.bbailip.2010.03.005>)

- Monte MJ, Marin JJ, Antelo A & Vazquez-Tato J 2009 Bile acids: chemistry, physiology, and pathophysiology. *World Journal of Gastroenterology* **15** 804–816. (<https://doi.org/10.3748/wjg.15.804>)
- Ogawa Y, Kurosu H, Yamamoto M, Nandi A, Rosenblatt KP, Goetz R, Eliseenkova AV, Mohammadi M & Kuro-o M 2007 BetaKlotho is required for metabolic activity of fibroblast growth factor 21. *PNAS* **104** 7432–7437. (<https://doi.org/10.1073/pnas.0701600104>)
- Pai R, French D, Ma N, Hotzel K, Plise E, Salphati L, Setchell KD, Ware J, Lauriault V, Schutt L, *et al.* 2012 Antibody-mediated inhibition of fibroblast growth factor 19 results in increased bile acids synthesis and ileal malabsorption of bile acids in cynomolgus monkeys. *Toxicological Sciences* **126** 446–456. (<https://doi.org/10.1093/toxsci/kfs011>)
- Pan C & Perumalswami PV 2011 Pregnancy-related liver diseases. *Clinical Liver Disease* **15** 199–208. (<https://doi.org/10.1016/j.cld.2010.09.007>)
- Rao A, Koters A, Mells JE, Zhang W, Setchell KD, Amanso AM, Wynn GM, Xu T, Keller BT, Yin H, *et al.* 2016 Inhibition of ileal bile acid uptake protects against nonalcoholic fatty liver disease in high-fat diet-fed mice. *Science Translational Medicine* **8** 357ra122. (<https://doi.org/10.1126/scitranslmed.aaf4823>)
- Rizzo G, Renga B, Mencarelli A, Pellicciari R & Fiorucci S 2005 Role of FXR in regulating bile acid homeostasis and relevance for human diseases. *Current Drug Targets. Immune, Endocrine and Metabolic Disorders* **5** 289–303. (<https://doi.org/10.2174/1568008054863781>)
- Russell DW 2003 The enzymes, regulation, and genetics of bile acid synthesis. *Annual Review of Biochemistry* **72** 137–174. (<https://doi.org/10.1146/annurev.biochem.72.121801.161712>)
- Shen J, Chan HL, Wong GL, Choi PC, Chan AW, Chan HY, Chim AM, Yeung DK, Chan FK, Woo J, *et al.* 2012 Non-invasive diagnosis of non-alcoholic steatohepatitis by combined serum biomarkers. *Journal of Hepatology* **56** 1363–1370. (<https://doi.org/10.1016/j.jhep.2011.12.025>)
- Song KH, Li T, Owsley E, Strom S & Chiang JY 2009 Bile acids activate fibroblast growth factor 19 signaling in human hepatocytes to inhibit cholesterol 7 α -hydroxylase gene expression. *Hepatology* **49** 297–305. (<https://doi.org/10.1002/hep.22627>)
- Staels B & Fonseca VA 2009 Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. *Diabetes Care* **32** (Supplement 2) S237–S245. (<https://doi.org/10.2337/dc09-S355>)
- Stanislaus S, Hecht R, Yie J, Hager T, Hall M, Spahr C, Wang W, Weizmann J, Li Y, Deng L, *et al.* 2017 A novel Fc-FGF21 with improved resistance to proteolysis, increased affinity toward beta-klotho, and enhanced efficacy in mice and cynomolgus monkeys. *Endocrinology* **158** 1314–1327. (<https://doi.org/10.1210/en.2016-1917>)
- Talukdar S, Zhou Y, Li D, Rossulek M, Dong J, Somayaji V, Weng Y, Clark R, Lanba A, Owen BM, *et al.* 2016 A long-acting FGF21 molecule, PF-05231023, decreases body weight and improves lipid profile in non-human primates and type 2 diabetic subjects. *Cell Metabolism* **23** 427–440. (<https://doi.org/10.1016/j.cmet.2016.02.001>)
- Tomiyama K, Maeda R, Urakawa I, Yamazaki Y, Tanaka T, Ito S, Nabeshima Y, Tomita T, Odori S, Hosoda K, *et al.* 2010 Relevant use of Klotho in FGF19 subfamily signaling system in vivo. *PNAS* **107** 1666–1671. (<https://doi.org/10.1073/pnas.0913986107>)
- Tomlinson E, Fu L, John L, Hultgren B, Huang X, Renz M, Stephan JP, Tsai SP, Powell-Braxton L, French D, *et al.* 2002 Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology* **143** 1741–1747. (<https://doi.org/10.1210/endo.143.5.8850>)
- Veniant MM, Komorowski R, Chen P, Stanislaus S, Winters K, Hager T, Zhou L, Wada R, Hecht R & Xu J 2012 Long-acting FGF21 has enhanced efficacy in diet-induced obese mice and in obese rhesus monkeys. *Endocrinology* **153** 4192–4203. (<https://doi.org/10.1210/en.2012-1211>)
- West KL, Zern TL, Butteiger DN, Keller BT & Fernandez ML 2003 SC-435, an ileal apical sodium co-dependent bile acid transporter (ASBT) inhibitor lowers plasma cholesterol and reduces atherosclerosis in guinea pigs. *Atherosclerosis* **171** 201–210. (<https://doi.org/10.1016/j.atherosclerosis.2003.08.019>)
- Xie MH, Holcomb I, Deuel B, Dowd P, Huang A, Vagts A, Foster J, Liang J, Brush J, Gu Q, *et al.* 1999 FGF-19, a novel fibroblast growth factor with unique specificity for FGFR4. *Cytokine* **11** 729–735. (<https://doi.org/10.1006/cyto.1999.0485>)
- Xie YQ, Ma HD & Lian ZX 2016 Epigenetics and primary biliary cirrhosis: a comprehensive review and implications for autoimmunity. *Clinical Reviews in Allergy and Immunology* **50** 390–403. (<https://doi.org/10.1007/s12016-015-8502-y>)
- Xu J, Lloyd DJ, Hale C, Stanislaus S, Chen M, Sivits G, Vonderfecht S, Hecht R, Li YS, Lindberg RA, *et al.* 2009 Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. *Diabetes* **58** 250–259. (<https://doi.org/10.2337/db08-0392>)
- Yang C, Jin C, Li X, Wang F, McKeehan WL & Luo Y 2012 Differential specificity of endocrine FGF19 and FGF21 to FGFR1 and FGFR4 in complex with KLB. *PLoS One* **7** e33870.
- Yie J, Hecht R, Patel J, Stevens J, Wang W, Hawkins N, Steavenson S, Smith S, Winters D, Fisher S, *et al.* 2009 FGF21 N- and C-termini play different roles in receptor interaction and activation. *FEBS Letters* **583** 19–24. (<https://doi.org/10.1016/j.febslet.2008.11.023>)
- Yu C, Wang F, Kan M, Jin C, Jones RB, Weinstein M, Deng CX & McKeehan WL 2000 Elevated cholesterol metabolism and bile acid synthesis in mice lacking membrane tyrosine kinase receptor FGFR4. *Journal of Biological Chemistry* **275** 15482–15489. (<https://doi.org/10.1074/jbc.275.20.15482>)
- Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC & Hobbs HH 2002 Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *Journal of Clinical Investigation* **110** 671–680. (<https://doi.org/10.1172/JCI0216001>)
- Zakharia K, Tabibian A, Lindor KD & Tabibian JH 2017 Complications, symptoms, quality of life and pregnancy in cholestatic liver disease. *Liver International* **38** 399–411. (<https://doi.org/10.1111/liv.13591>)
- Zhang J, Gupte J, Gong Y, Weizmann J, Zhang Y, Lee KJ, Richards WG & Li Y 2017 Chronic over-expression of fibroblast growth factor 21 increases bile acid biosynthesis by opposing FGF15/19 action. *EBioMedicine* **15** 173–183. (<https://doi.org/10.1016/j.ebiom.2016.12.016>)
- Zimmerman HJ & Lewis JH 1987 Drug-induced cholestasis. *Medical Toxicology* **2** 112–160. (<https://doi.org/10.1007/BF03260010>)

Received in final form 21 February 2018

Accepted 26 February 2018