Human Bile Acid Metabolism
A Postprandial Perspective

Samuel van Nierop

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Colofon

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Part I

Bile acids

1

Introduction and thesis outline

Bile acid biology and the enterohepatic cycle

Since the 1928 Nobel Prize was awarded to Adolf Windaus for his research into the chemical structure of bile acids, bile acids have been recognized mainly for their function as enteral detergents, facilitating the uptake of fat and fat-soluble vitamins in the gut. Bile acids are synthesized from a cholesterol backbone in hepatocytes, forming the primary bile acids cholate (CA) and chenodeoxycholate (CDCA) (Lefebvre, Cariou, & Lien, 2009). After synthesis, bile acids are stored in the gallbladder and mixed with bicarbonate-rich bile fluid to be secreted as bile into the lumen of the duodenum after ingestion of a meal containing fats and carbohydrates. There, their amphipathic nature aids digestion and absorption by forming micelles with dietary lipids.

Subsequently, bile acids are absorbed in the distal ileum and transported back to the liver via the portal circulation for reuse, forming a closed loop known as the enterohepatic circulation. Generally, bile acids do not pass the enterocyte membrane by passive diffusion, but, rather, are taken up by dedicated molecular transport in the distal part of the small intestine (Hofmann & Hagey, 2008). A fraction of primary bile acids that escape uptake in the terminal ileum is deconjugated and dehydroxylated by the bacterial microbiome in the colon to form deoxycholate (DCA) and lithocholate (LCA), the so-called secondary bile acids. CA and CDCA are converted into DCA and LCA, respectively. Because human hepatocytes lack the molecular pathway to rehydroxylate DCA (in contrast to rodents) this secondary bile acid accumulates to form a considerable part of the circulating bile acid mix in humans, usually around 35%. These secondary bile acids are predominantly taken up by colonic mucosa, allowing for efficient recycling of sterols. The fraction of the bile acid pool that is not reabsorbed and thus lost in the feces, ~5% per cycle of the enterohepatic circulation, is compensated for by de novo bile acid synthesis. This process refuels the enterohepatic cycle. It is worth mentioning that the enterohepatic cycle is highly dynamic. The flow of bile acids through the different compartments is not only dependent on the chemical structure and conjugation status of the bile acid mix, but also on factors such as intestinal motility (Sips et al., 2018).

Because of their soap-like nature, bile acids are reported to be cytotoxic in high concentrations, although evidence for in vivo toxic effects is sparse. However, conjugation to a glycine or taurine amino-acid group decreases cytotoxicity and

increases solubility, aiding secretion into bile fluid. Though intestinal bacteria deconjugate bile acids, the majority of the human bile acid pool (around 80%) is in its conjugated form throughout the enterohepatic cycle (Hofmann & Hagey, 2008). Bile acids are preferentially conjugated to taurine by bile acid coenzyme A:amino acid N-acyltransferase (BAAT), but as taurine is less prevalent in the human diet, the human bile acid pool is predominantly glycine-conjugated (Hardison & Grundy, 1983).

Taking into account the steps of synthesis, conjugation to amino-acids, dehydroxylation by bacteria to secondary bile acids, a certain degree of rehydroxylation in hepatocytes and loss in the feces, it is easy to understand that the resulting mixture of bile acids, which we call the bile acid pool, is a variable entity, both within and between individuals and species (Lefebvre, Cariou, Lien, et al., 2009). To compound these differences, different bile acid species and conjugation states impart differences in hydrophilicity and affinity for the various bile acid receptors (Parks et al., 1999; Sato et al., 2008). This may have consequences for their proposed effects on biological processes and translation of preclinical findings to therapeutic settings, as described in this thesis.

Bile acids as metabolic hormones

The traditional view of bile acids as enteral detergents was upended when, in 1999, it became evident that bile acids are ligands for the (until then) orphan nuclear receptor farnesoid X receptor (FXR), exerting negative feedback on their own biosynthesis (Parks et al., 1999). Activation of FXR was shown to suppress transcription of the CYP7A1 gene and thereby expression of cholesterol 7α-hydroxylase, which is the rate-limiting enzyme of bile acid synthesis (Lefebvre, Cariou, & Lien, 2009). In the distal ileum, activation of FXR in enteroendocrine L-cells induces increased expression and secretion of ileal Fibroblast Growth Factor 19 (FGF19). FGF19 is an enterokine that in turn represses hepatic bile acid production through the FGF-receptor-4 (FGFR4) by inhibiting CYP7A1, creating a negative feedback loop (Schaap et al., 2014). Besides this established pathway, FGF19 has also been implicated in regulation of metabolism. Transgenic mice overexpressing FGF19 or treated with an FXR ligand are resistant to diet-induced obesity and insulin resistance (Tomlinson et al., 2002)(Renga et al., 2010). In mice, FGF19 represses genes involved in gluconeogenesis and lipogenesis, instead stimulating fatty acid oxidation and glycogen synthesis (Kir et al., 2011; Potthoff et al., 2011; Schaap et al., 2014).

FXR is also found in white adipose tissue (WAT), although its physiological role there is unknown. In vitro, activation of FXR by a specific and potent FXR ligand (INT747) in conjunction with insulin stimulates adipocyte differentiation, suggesting a role in enhancing insulin signaling (Rizzo et al., 2006). In pancreatic beta cells, which express FXR in vivo, glucose-dependent insulin transcription and secretion were increased by activation of FXR in vitro (Renga et al., 2010).

In addition to FXR, bile acids are ligands for the transmembrane receptor Takeda G-coupled protein receptor 5 (TGR5), sometimes referred to as GPBAR1 (Kawamata et al., 2003; Maruyama et al., 2002; Sato et al., 2007; Watanabe et al., 2006), which is involved in metabolic signaling. Bile acids stimulate TGR5 on the luminal membrane of enteroendocrine L-cells to secrete Glucagon-like peptide-1 (GLP-1), a so-called incretin hormone that in turn stimulates glucose-dependent insulin secretion in the pancreatic beta cell (Katsuma et al., 2005). Outside of the enterohepatic circulation, TGR5 is mainly expressed in muscle and brown adipose tissue, though it has not yet been determined whether is has a relevant function in humans. In vitro and in mice, stimulation of TGR5 with bile acids increases resting energy expenditure through increased activation of iodothyronine deiodinase 2, which in turn activates thyroid hormone (Watanabe et al., 2006). In these tissues, insulin sensitivity is increased after TGR5 activation (Thomas et al., 2008; Watanabe et al., 2006). Bile acids may also stimulate insulin release independently from GLP-1, as TGR5 is expressed in pancreatic beta cells (as well as FXR, as mentioned previously) (Kumar et al., 2012; Renga et al., 2010). Activation of TGR5 on human pancreatic beta cells leads to rapid basal and glucose-dependent insulin secretion in vitro (Kumar et al., 2012).

In addition to these metabolically active tissues, TGR5 is also expressed in macrophages and Kupffer cells, were TGR5 appears to suppress markers of inflammation. In a mouse model of atherosclerosis, activation of TGR5 was protective (Pols et al., 2011), an effect that was lost in TGR5 -/- mice. This effect has been supported by animal and in vitro studies(Keitel et al., 2008)(Ichikawa et al., 2012). This points to a role for bile acid in regulating the postprandial pro-inflammatory state (Meessen et al., 2019).

Besides the two specific bile acid receptors, a number of other nuclear receptors have been described to be activated by bile acid metabolites, notably the

Constitutive Androstane receptor (CAR), the Pregnane X receptor (PXR), the Liver X receptor (LXR) and the vitamin D receptor (VDR) (Li & Chiang, 2012). These are xenobiotic/endobiotic sensing receptors known to be involved in regulation of hepatic drug oxidation, conjugation and transport, which has considerable overlap with bile acid metabolism. The interaction of bile acids with these receptors falls outside of the scope of this thesis.

Most of the metabolically active tissues expressing bile acid receptors are not exposed to their ligands either through bile in the gut lumen or via the portal circulation. So how would they be activated in a physiological setting? As described in the previous section, concentrated bile from the liver and gallbladder flows into the intestinal lumen. After reuptake, they enter the portal circulation, where the liver efficiently extracts bile acids from the portal vein for rehydroxylation, conjugation and resecretion. Notably, a fraction bypasses the liver to enter the systemic circulation, where they can be measured in a postprandial peak, reaching a concentration of approximately 1-20 μmol/L 30 to 90 minutes after a mixed meal (N. F. LaRusso et al., 1978). As a consequence, extra-intestinal tissues are exposed to bile acid concentrations that might be high enough to exert biological effects through their receptors, although peripheral concentrations are much lower compared to portal vein concentrations (N. LaRusso et al., 1974). This is of critical importance in the assessment of the physiological role of bile acids in human energy metabolism, as most of the aforementioned pathways and effects have only been described in the context of supraphysiological stimulation or knock-out rodent models.

Bile acids in obesity and type 2 diabetes mellitus

As discussed in the previous sections, bile acid levels in the enterohepatic and systemic circulation are responsive to nutrient intake, making bile acid receptors ideally positioned in the digestive system to function as postprandial metabolic integrators by sensing and conveying information about nutrient status (Angelin & Bjorkhem, 1977; Sonne et al., 2016). Indeed, impaired bile acid signaling has been implicated in the deranged glucose metabolism of patients with obesity and type 2 diabetes mellitus (DM2). This is illustrated by the altered plasma bile acid concentrations and bile acid pool composition in patients with insulin resistance and DM2 (Brufau et al., 2010; Haeusler et al., 2013). In these patients, plasma bile acid levels are increased compared to healthy controls, in particular cholic

acid and deoxycholic acid (Haeusler et al., 2013; Brufau et al., 2010). While this relationship is poorly understood, there is some evidence that insulin normally regulates production of cholic acid and deoxycholic acid through the transcription factor Forkhead box protein 1 (FoxO1), which represses Cyp8b1, one of the enzymes responsible for bile acid synthesis (Haeusler et al., 2012). The impaired insulin signaling in DM2 is then thought to lead to increased generation of bile acids.

In contrast, obesity is characterized by decreased postprandial concentrations of bile acids in plasma, while markers of bile acid synthesis were shown to be increased (Glicksman et al., 2010; Haeusler et al., 2016). The blunted postprandial peaks could be attributed to slower intestinal transit in obese subjects, a theory that is underlined by the quick restoration seen in these patients after undergoing Roux-en-Y gastric bypass (RYGB) (Ahmad et al., 2013).

Bariatric surgery on the other hand, RYGB in particular, increases fasting and postprandial plasma bile acid levels, resulting in a complete cure of DM2 in many cases, although if and how these changes are related is presently unclear (Jørgensen et al., 2015; Schauer et al., 2012).

This data from human models of DM2 and obesity suggests that bile acids play a role in modulating the postprandial metabolic response (changes in glucose and lipid metabolism in response to a sudden influx of nutrients) (Lefebvre, Cariou, & Lien, 2009). There seems to be a signaling role in liver, white and brown adipose tissue, pancreas, and in muscle, where the two bile acid receptors are expressed in varying levels of abundance. As discussed above, postprandial plasma bile acid peak concentrations may be high enough to activate these receptors in vivo. Recently, trying to emulate the success of GLP-1 receptor-targeted therapies, TGR5 has been proposed as a pharmacological target to combat obesity and DM2 (Maruyama et al., 2002; Sato et al., 2008; Thomas et al., 2009; Watanabe et al., 2004, 2006). As of the writing of this thesis, this has not yielded any safe and effective compounds.

Conclusion

Many preclinical studies have been performed since the initial discovery TGR5 to elucidate its physiology as a bile acid receptor and possible value as a therapeutic target in DM2, obesity and non-alcoholic steatohepatitis (NASH). However, the question remains whether the discovered pathways function similarly in human physiology, and if they do, whether they their effects are large enough to be relevant. In particular, profound interspecies differences in the biology of bile acids and bile acid receptors in multiple cells and tissues hamper translation to human pathophysiology. Therefore, we aimed to perform translational studies to uncover the relevance and biological importance of the postprandial bile acid response.

Outline

Part II – Variations in bile acid levels: Since it has been proposed that bile acids regulate lipid and carbohydrate metabolism, we set out in ***chapter 2*** to describe postprandial plasma concentrations of 12 individual bile acid species in patients with DM2 and healthy controls using a 75-g oral glucose tolerance test and three isocaloric and isovolemic liquid meals with low, medium, and high fat contents, respectively.

Because variation within, and between, subjects turned out to be considerable, we subsequently investigated portal bile acid concentrations and their increase after a meal, and compared these to peripheral plasma concentrations in a transorgan porcine model. In ***chapter 3*** we describe this model that we used to investigate postprandial plasma concentrations in multiple compartments as well as transorgan fluxes of bile acids, glucose and insulin using the para-aminohippuric acid dilution method. Eleven pigs with multiple intravascular catheters received a standard mixed-meal. Additionally, we sampled fasted venous and portal blood from non-diabetic obese patients during gastric bypass surgery as a human reference point.

Part III - Intervention: To further examine regulating mechanisms in postprandial bile acid metabolism, we devised several interventions aiming to mimic the effect of physiological stimuli on insulin sensitivity. In ***chapter 4*** we used a 2-week very-

low-calorie diet (VLCD) to investigate the effects of rapid weight loss on bile acid pool composition and postprandial dynamics. We performed mixed meal tests in insulin resistant obese subjects before and after the diet. We measured postprandial plasma levels of glucose, insulin, bile acids and the enterokines GLP-1 and FGF19.

In ***chapter 5*** we describe the effect of starvation-induced insulin resistance on bile acid metabolism. We investigated the postprandial bile acid response in relation to insulin, GLP-1 and FGF19 after 40 hours of starvation. After finding a correlation between postprandial levels of insulin and glycine-conjugated deoxycholic acid (gDCA), we performed a follow-up experiment in which we administered gDCA with the meal to try to modulate postprandial glucose, insulin and GLP-1 levels.

After these dietary and pharmacological interventions, we performed bile acid measurements in a bariatric model in ***chapter 6***. The duodenal-jejunal bypass liner (DJBL) is an intraduodenal device that leads to rapid weight loss and restoration of insulin sensitivity in a similar fashion to Roux-en-Y gastric bypass (RYGB) surgery. Analogous to findings after RYGB, increased systemic bile acid levels are candidate effectors for the beneficial effects on metabolism. We performed mixed meal testing in 17 obese patients with DM2 directly before, one week after and 6 months after DJBL placement.

Part IV - Central nervous system: Since autonomic innervation is involved in the regulation of many endocrine and metabolic processes, we speculated that bile acids metabolism could be regulated in a similar fashion. In ***chapter 7***, we examine whether circadian rhythms influence postprandial bile acid responses. Obese patients with DM2 show a disturbed diurnal rhythm in plasma glucose tolerance. Since circulating bile acids affect glucose metabolism and bile acid synthesis is known to be regulated by the circadian clock, we hypothesized that obese patients with DM2 show an altered diurnal rhythm of postprandial bile acid plasma excursions. We studied 6 obese patients with DM2 and 6 matched controls who were provided with three equicaloric equidistant meals per day for three days. Extensive blood sampling was performed throughout the study day.

Part V - Bile acids in perspective: Finally, we reflect on the differences between animal and human studies and their consequences for the future use of bile acid targeted therapy in clinical practice. ***Chapter 8*** details the profound interspecies differences

in the biology of bile acids and their receptors in different cells and tissues. While preclinical studies show promising effects, clinical studies are scarce. We give a summary of key concepts in bile acid metabolism; outline different downstream effects of TGR5 activation; and review available data on TGR5 activation, with a focus on the translation of preclinical studies into clinically applicable findings.

References

Ahmad, N. N., Pfalzer, A., & Kaplan, L. M. (2013). Roux-en-Y gastric bypass normalizes the blunted postprandial bile acid excursion associated with obesity. *International Journal of Obesity*, *37*(12), 1553–1559.

Angelin, B., & Bjorkhem, I. (1977). Postprandial serum bile acids in healthy man: evidence for differences in absorptive pattern between individual bile acids. *Gut*, *18*(8), 606–609.

Brufau, G., Stellaard, F., Prado, K., Bloks, Jonkers, E., Boverhof, R., Kuipers, F., & Murphy, E. J. E. J. (2010). Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism. *Hepatology*, *52*(4), 1455–1464.

Haeusler, R. A., Astiarraga, B., Camastra, S., Accili, D., & Ferrannini, E. (2013). Human insulin resistance is associated with increased plasma levels of 12α-hydroxylated bile acids. *Diabetes*, *62*(12), 4184–4191.

Haeusler, R. A., Camastra, S., Nannipieri, M., Astiarraga, B., Castro-Perez, J., Xie, D., Wang, L., Chakravarthy, M., & Ferrannini, E. (2016). Increased bile acid synthesis and impaired bile acid transport in human obesity. *The Journal of Clinical Endocrinology & Metabolism*, *101*(5), 1935–1944.

Haeusler, R. A., Pratt-Hyatt, M., Welch, C. L., Klaassen, C. D., & Accili, D. (2012). Impaired generation of 12-hydroxylated bile acids links hepatic insulin signaling with dyslipidemia. *Cell Metabolism*, *15*(1), 65–74.

Hardison, W. G. M., & Grundy, S. M. (1983). Effect of bile acid conjugation pattern on bile acid metabolism in normal humans. *Gastroenterology*, *84*(3), 617–620.

Hofmann, A. F., & Hagey, L. R. (2008). Bile acids: Chemistry, pathochemistry, biology, pathobiology, and therapeutics. In *Cellular and Molecular Life Sciences* (Vol. 65, Issue 16, pp. 2461–2483).

Ichikawa, R., Takayama, T., Yoneno, K., Kamada, N., Kitazume, M. T., Higuchi, H., Matsuoka, K., Watanabe, M., Itoh, H., Kanai, T., Hisamatsu, T., & Hibi, T. (2012). Bile acids induce monocyte differentiation toward interleukin-12 hypo-producing dendritic cells via a TGR5-dependent pathway. *Immunology*, *136*(2), 153–162.

Jørgensen, N. B., Dirksen, C., Bojsen-Møller, K. N., Kristiansen, V. B., Wulff, B. S., Rainteau, D., Humbert, L., Rehfeld, J. F., Holst, J. J., Madsbad, S., & Clausen, T. R. (2015). Improvements in glucose metabolism early after gastric bypass surgery are not explained by increases in total bile acids and fibroblast growth factor 19 concentrations. *Journal of Clinical Endocrinology and Metabolism*, *100*(3), E396–E406.

Katsuma, S., Hirasawa, A., & Tsujimoto, G. (2005). Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochemical and Biophysical Research Communications*, *329*(1), 386–390.

Kawamata, Y., Fujii, R., Hosoya, M., Harada, M., Yoshida, H., Miwa, M., Fukusumi, S., Habata, Y., Itoh, T., Shintani, Y., Hinuma, S., Fujisawa, Y., & Fujino, M. (2003). A G protein-coupled receptor responsive to bile acids. *The Journal of Biological Chemistry*, *278*(11), 9435–9440.

Keitel, V., Donner, M., Winandy, S., Kubitz, R., & Häussinger, D. (2008). Expression and function of the bile acid receptor TGR5 in Kupffer cells. *Biochemical and Biophysical Research Communications*, *372*(1), 78–84.

Kir, S., Beddow, S. A., Samuel, V. T., Miller, P., Previs, S. F., Suino-Powell, K., Xu, H. E., Shulman, G. I., Kliewer, S. A., & Mangelsdorf, D. J. (2011). FGF19 as a Postprandial, Insulin-independent Activator of Hepatic Protein and Glycogen Synthesis. *Science*, *331*(6024), 1621–1624.

Kumar, D. P., Rajagopal, S., Mahavadi, S., Mirshahi, F., Grider, J. R., Murthy, K. S., & Sanyal, A. J. (2012). Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic β cells. *Biochemical and Biophysical Research Communications*, *427*(3), 600–605.

LaRusso, N. F., Hoffman, N. E., Korman, M. G., Hofmann, A. F., & Cowen, A. E. (1978). Determinants of fasting and postprandial serum bile acid levels in healthy man. *The American Journal of Digestive Diseases*, *23*(5), 385–391.

LaRusso, N., Korman, M., Hoffman, N., & Hofmann, A. F. (1974). Dynamics of the enterohepatic circulation of bile acids. Postprandial serum concentrations of conjugates of cholic acid in health, cholecystectomized patients, and patients with bile acid malabsorption. *The New England Journal of Medicine*, *291*(14), 689–692.

Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., & Staels, B. (2009). Role of bile acids and bile acid receptors in metabolic regulation. *Physiological Reviews*, *89*(1), 147–191.

Li, T., & Chiang, J. Y. L. (2012). Bile Acid Signaling in Liver Metabolism and Diseases. *Journal of Lipids*, *2012*, 1–9.

Maruyama, T., Miyamoto, Y., Nakamura, T., Tamai, Y., Okada, H., Sugiyama, E., Nakamura, T., Itadani, H., & Tanaka, K. (2002). Identification of membrane-type receptor for bile acids (M-BAR). *Biochemical and Biophysical Research Communications*, *298*(5), 714–719.

Meessen, E. C. E., Warmbrunn, M. V., Nieuwdorp, M., & Soeters, M. R. (2019). Human postprandial nutrient metabolism and low-grade inflammation: A narrative review. *Nutrients*, *11*(12), 3000.

Parks, D. J., Blanchard, S. G., Bledsoe, R. K., Chandra, G., Consler, T. G., Kliewer, S. A., Stimmel, J. B., Willson, T. M., Zavacki, A. M., Moore, D. D., & Lehmann, J. M. (1999). Bile Acids: Natural Ligands for an Orphan Nuclear Receptor . *Science* , *284*(5418), 1365–1368.

Pols, T. W. H., Nomura, M., Harach, T., Lo Sasso, G., Oosterveer, M. H., Thomas, C., Rizzo, G., Gioiello, A., Adorini, L., Pellicciari, R., Auwerx, J., & Schoonjans, K. (2011). TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. *Cell Metabolism*, *14*(6), 747–757.

Potthoff, M. J., Boney-Montoya, J., Choi, M., He, T., Sunny, N. E., Satapati, S., Suino-Powell, K., Xu, H. E., Gerard, R. D., Finck, B. N., Burgess, S. C., Mangelsdorf, D. J., & Kliewer, S. A. (2011). FGF15/19 regulates hepatic glucose metabolism by inhibiting the CREB-PGC-1α pathway. *Cell Metabolism*, *13*(6), 729–738.

Renga, B., Mencarelli, A., Vavassori, P., Brancaleone, V., & Fiorucci, S. (2010). The bile acid sensor FXR regulates insulin transcription and secretion. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1802*(3), 363–372.

Rizzo, G., Disante, M., Mencarelli, A., Renga, B., Gioiello, A., Pellicciari, R., & Fiorucci, S. (2006). The farnesoid X receptor promotes adipocyte differentiation and regulates adipose cell function in vivo. *Molecular Pharmacology*, *70*(4), 1164–1173.

Sato, H., Genet, C., Strehle, A., Thomas, C., Lobstein, A., Wagner, A., Mioskowski, C., Auwerx, J., & Saladin, R. (2007). Anti-hyperglycemic activity of a TGR5 agonist isolated from Olea europaea. *Biochemical and Biophysical Research Communications*, *362*(4), 793–798.

Sato, H., Macchiarulo, A., Thomas, C., Gioiello, A., Une, M., Hofmann, A. F., Saladin, R., Schoonjans, K., Pellicciari, R., & Auwerx, J. (2008). Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. *Journal of Medicinal Chemistry*, *51*(6), 1831–1841.

Schaap, F. G., Trauner, M., & Jansen, P. L. M. (2014). Bile acid receptors as targets for drug development. *Nature Reviews Gastroenterology and Hepatology*, *11*(1), 55–67.

Schauer, P. R., Kashyap, S. R., Wolski, K., Brethauer, S. A., Kirwan, J. P., Pothier, C. E., Thomas, S., Abood, B., Nissen, S. E., & Bhatt, D. L. (2012). Bariatric surgery versus intensive medical therapy in obese patients with diabetes. *New England Journal of Medicine*, *366*(17), 1567–1576.

Sips, F. L. P., Eggink, H. M., Hilbers, P. A. J., Soeters, M. R., Groen, A. K., & van Riel, N. A. W. (2018). In silico analysis identifies intestinal transit as a key determinant of systemic bile acid metabolism. *Frontiers in Physiology*, *9*(JUN), 631.

Sonne, D. P., van Nierop, F. S., Kulik, W., Soeters, M. R., Vilsbøll, T., & Knop, F. K. (2016). Postprandial Plasma Concentrations of Individual Bile Acids and FGF-19 in Patients with Type 2 Diabetes. *The Journal of Clinical Endocrinology & Metabolism*, *101*(August), jc.2016-1607.

Thomas, C., Auwerx, J., & Schoonjans, K. (2008). Bile acids and the membrane bile acid receptor TGR5-connecting nutrition and metabolism. *Thyroid*, *18*(2).

Thomas, C., Gioiello, A., Noriega, L., Strehle, A., Oury, J., Rizzo, G., Macchiarulo, A., Yamamoto, H., Mataki, C., Pruzanski, M., Pellicciari, R., Auwerx, J., & Schoonjans, K. (2009). TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metabolism*, *10*(3), 167–177.

Tomlinson, E., Fu, L., John, L., Hultgren, B., Huang, X., Renz, M., Stephan, J. P., Tsai, S. P., Powell-braxton, L. Y. N., French, D., & Stewart, T. A. (2002). Transgenic Mice Expressing Human Fibroblast Growth Factor-19 Display Increased Metabolic Rate and Decreased Adiposity. *Endocrinology*, *143*, 1741–1747.

Watanabe, M., Houten, S. M., Mataki, C., Christoffolete, M. a, Kim, B. W., Sato, H., Messaddeq, N., Harney, J. W., Ezaki, O., Kodama, T., Schoonjans, K., Bianco, A. C., & Auwerx, J. (2006). Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature*, *439*(7075), 484–489.

Watanabe, M., Houten, S. M., Wang, L., Moschetta, A., Mangelsdorf, D. J., Heyman, R. A., Moore, D. D., & Auwerx, J. (2004). Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *Journal of Clinical Investigation*, *113*(10), 1408–1418.

Part II

Variation

2

Postprandial Plasma Concentrations of Individual Bile Acids and FGF19 in Patients With Type 2 Diabetes

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Abstract

Context: Bile acids regulate lipid and carbohydrate metabolism by interaction with membrane or intracellular proteins including the nuclear farnesoid X receptor (FXR). Postprandial activation of ileal FXR leads to secretion of fibroblast growth factor 19 (FGF19), a gut hormone that may be implicated in postprandial glucose metabolism.

Objective: To describe postprandial plasma concentrations of 12 individual bile acids and FGF19 in patients with type 2 diabetes (T2D) and healthy controls.

Design and Setting: Descriptive study, performed at the Center for Diabetes Research, Gentofte Hospital, Hellerup, Denmark.

Participants: Fifteen patients with T2D and 15 healthy matched controls with normal glucose tolerance.

Interventions: A 75-g oral glucose tolerance test and three isocaloric and isovolemic liquid meals with low, medium, and high fat content, respectively.

Main Outcome Measures: Bile acid and FGF19 concentrations.

Results: Postprandial total bile acid concentrations increased with increasing meal fat content ($P < .05$), peaked after 1–2 hours, and were higher in T2D patients vs controls (oral glucose tolerance test, low and medium fat meals, $P < .05$; high fat meal, $P = .30$). Differences reflected mainly unconjugated and glycine-conjugated forms of deoxycholic acid (DCA) and to a lesser extent cholic acid (CA) and ursodeoxycholic acid (UDCA), whereas chenodeoxycholic acid (CDCA) concentrations were comparable in the two groups. FGF19 concentrations tended to be lower in T2D patients vs controls, but differences were not statistically significant due to considerable variation.

Conclusion: Postprandial plasma patterns of bile acids with FXR agonistic properties (CDCA, DCA, and CA) and FXR antagonistic properties (UDCA) in T2D patients support the notion of a "T2D-bile acid-FGF19" phenotype with possible pathophysiological implications. *(J Clin Endocrinol Metab 101: 3002–3009, 2016)*

Abbreviations: AUC, area under the curve; BMI, body mass index; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; EGP, endogenous glucose production; FGF19, fibroblast growth factor 19; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; HbA1c, hemoglobin A1c; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; TBA, total bile acid; T2D, type 2 diabetes; TGR5, Takeda G-coupled protein receptor 5; UDCA, ursodeoxycholic acid.

Introduction

Since the discovery that bile acids are ligands for the nuclear farnesoid X receptor (FXR) and the Takeda G-coupled protein receptor 5 (TGR5), studies in both animals and humans have established that bile acids are metabolic integrators involved in glucose, lipid, and energy metabolism, particularly in the postprandial state (1). Several recent reviews have addressed conditions in which TGR5 and FXR have emerged as new targets for pharmacological agents (2, 3). TGR5 activation has been linked to increased energy expenditure in muscle and brown adipose tissue, immunosuppressive effects in immune cells, and–perhaps most importantly in the present context-secretion of intestinal L-cell satiety products such as peptide YY and the incretin hormone glucagonlike peptide-1 (GLP-1) (2, 4). FXR activation by bile acids affects the expression of multiple key regulatory genes encoding components of the bile acid synthesis cascade, as well as genes acting in numerous metabolic pathways (5). Interestingly, a recent study demonstrated that FXR activation in L cells decreases GLP-1 secretion in response to glucose (6). Most of the effects of FXR and TGR5 activation have been corroborated in animal models and to a lesser degree in human studies. Alteration of the bile acid pool has been shown to induce changes of parameters that are part of the metabolic syndrome (ie, insulin resistance,hyperglycemia, hepatic steatosis, low high-density lipoprotein cholesterol levels, and cardiovascular risk) (7).

Intriguingly, drugs modulating the enterohepatic circulation of bile acids by binding bile acids in or inhibiting their absorption from the small intestine have been shown to improve glycemic control in patients with type 2 diabetes (T2D) (3, 8). The metabolic effects of bile acids may involve the gut hormone fibroblast growth factor 19 (FGF19), which is released from the terminal ileum upon bile acid-induced FXR activation. FGF19 exerts pleiotropic effects on hepatic bile acid metabolism as well as lipid, protein, and glucose metabolism (9). In light of the predominance of postprandial glycemia in determining overall glycemic control in T2D patients (10), as well as the accumulating evidence that bile acids play an important part in the whole-body response to nutrient ingestion (1), we hypothesized that postprandial bile acid concentrations in T2D patients could reveal a "T2D-bile acidFGF19" phenotype with possible pathophysiological implications. Thus, we performed a characterization of postprandial concentrations of 12 plasma bile acids and FGF19 after various meal stimuli in patients with T2D

and healthy age-, gender-, and body mass index (BMI)matched control subjects with normal glucose tolerance (NGT).

Subjects and Methods

Participants

A detailed description of the experimental procedures and subjects was provided previously (11). In short, plasma was obtained from 15 patients with T2D (mean duration of T2D, 7.5 years [range, 6 –20]; age, 59.4 ± 9.6 years [mean ± SD]; BMI, 28.0 ± 2.2 kg/m^2; hemoglobin A1c [HbA1c], 7.5 ± 1.4%) and 15 healthy, age-, gender-, and BMI-matched control subjects (age, 59.7 ± 10.0 years; BMI, 27.9 ± 2.0 kg/m^2; HbA1c, 5.2 ± 0.2%). None of the T2D patients had overt diabetic complications. Eight patients were treated with metformin, three with sulfonylurea, and four with diet only. Patients were instructed to abstain from taking blood glucose-lowering drugs for at least 1 week before the first study day.

Study design

Patients underwent four separate "meal" tests (visits were separated by 2– 4 days) as follows: 75-g oral glucose tolerance test (OGTT; 75 g of water-free glucose dissolved in 300 mL water), and three isocaloric (500 kcal) and isovolemic (350 mL) liquid meals (low fat, 2.5 g fat, 107 g carbohydrate, and 13 g protein; medium fat, 10 g fat, 93 g carbohydrate, 11 g protein; and high fat, 40 g fat, 32 g carbohydrate, and 3 g protein). Written informed consent was obtained from all participants. Results on postprandial glucose metabolism, gallbladder emptying, and gut hormone secretion have been reported previously (11).

Sample collection

Arterialized blood samples were drawn 20, 10, and 0 minutes before and 15, 30, 45, 60, 90, 120, 180, and 240 minutes after ingestion of the OGTT or meals. Blood was collected into chilled tubes containing EDTA and a specific dipeptidyl peptidase 4 inhibitor (valine-pyrrolidide, final concentration of 0.01 mmol/L; a gift from Novo Nordisk) for plasma analyses of individual bile acids and FGF19. Tubes were kept on ice, centrifuged for 20 minutes at 1200 × *g* and 4°C, and stored at –20°C until analysis. Samples were analyzed for unconjugated cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and

ursodeoxycholic acid (UDCA), as well as their corresponding glycine and taurine amidates (conjugates). Total bile acid (TBA) concentrations were determined by calculating the molar sum of all 12 bile acids. Bile acids were measured using an ultra-performance liquid chromatography ionization tandem mass spectrometry method (12) (Supplemental Data). A sandwich ELISA kit was used for colorimetric determination of FGF19 in plasma (FGF19 Quantikine ELISA kit, catalog no. DF1900; R&D Systems), following the manufacturer´s instructions. Intra- and interassay coefficients of variation were 6.0 and 7.5%, respectively.

Presentation of bile acid data

Bile acid concentrations are presented according to biological groupings (TBA, primary bile acids [CA, CDCA], and secondary bile acids [DCA, UDCA]) in Table 1 and Figures 1 and 2. Postprandial area under the curve (AUC) calculations for bile acids and FGF19 concentrations are shown in Table 1, and postprandial profiles and AUCs are displayed graphically in Figures 1 and 2. Time courses for individual bile acids are found in mental Figures 1–4. Fasting concentrations of bile acids and FGF19 are presented in Supplemental Table 1. Bile acids conjugated with glycine or taurine are termed amidates in tables and figures.

Statistical analysis

Results are reported as medians and interquartile ranges (25th– 75th percentiles). Baseline values are defined as the mean of the three values obtained before consumption of the meals, and AUC values were calculated using the trapezoidal rule. The effect of group and meal type was analyzed by repeated measures ANOVA in a linear mixed-effect model using group and meal as fixed effects and subjects as random effect. The assumptions of a Gaussian distribution of residuals and homogeneity of variances were assessed visually by drawing histograms, residual plots, and probability plots. If assumptions could not be met, continuous variables were transformed using Box-Cox transformations. We chose to calculate type III sums of squares for the fixed effects. Between-group differences were tested using Student's *t* test and Holm-Sidak's adjustment for multiple comparisons. Correlations were assessed with the Spearman rank correlation test. A two-sided *P* value of .05 was used to indicate significant differences. Statistical analyses were performed using GraphPad Prism version 6.0b for Windows/Mac (GraphPad Software) and R (3.2.1) for Windows/Mac (http://cran.r-project.org).

Table 1. Postprandial Bile Acids and FGF19 Concentrations (AUC)

Bile Acids, min × µmol/L	NGT				T2D				rmANOVA		
	OGTT	Low Fat	Medium Fat	High Fat	OGTT	Low Fat	Medium Fat	High Fat	Meal	Group	Interaction
TBA	289 [202– 447]	451 [432–563]	665 [367–1471]	1245 [746–1471]	562 [236–667]	776 [522–1058]	948 [640–994]	1241 [965–1589]	<0.001	<0.001	0.14
Unconjugated	48 [24 –162]	77 [37–107]	76 [39 –202]	137 [76–209]	105 [45–173]	205 [70 –332]	170 [102–250]	216 [105–300]	<0.001	0.49	0.73
Glycine amidates	200 [140 –239]	322 [285–412]	421 [243–518]	829 [591–994]	366 [157–507]	506 [370 –588]	596 [358–738]	955 [714–1427]	<0.001	<0.001	0.28
Taurine amidates	30 [17–39]	52 [39 –79]	64 [43–95]	140 [96 –262]	60 [31–115]	89 [43–115]	95 [68–129]	152 [114–267]	<0.001	0.03	0.10
CA											
Total	58 [38 – 85]	93 [57–115]	119 [64 –208]	239 [120–378]	109 [31–151]	123 [67–206]	164 [83–247]	181 [135–411]	<0.001	0.27	0.19
Unconjugated	18 [0 – 47]	12 [0 –39]	11 [0 –112]	18 [4 –30]	9 [2–16]	9 [3–75]	19 [0 – 48]	21 [8 –27]	0.22	0.46	0.37
Glycine amidates	33 [25– 40]	58 [45– 68]	71 [44 – 82]	162 [96 –260]	67 [23–113]	84 [59 –130]	106 [53–156]	152 [110 –338]	<0.001	0.03	0.44
Taurine amidates	7 [4 –12]	11 [7–17]	15 [6 –28]	31 [17–59]	14 [5–25]	15 [8 –28]	21 [10 –29]	31 [16 –53]	<0.001	0.09	0.39
CDCA											
Total	138 [103–232]	237 [183–351]	250 [176–413]	595 [425–803]	200 [101–288]	283 [205–358]	334 [228–450]	548 [413–660]	<0.001	0.16	0.19
Unconjugated	10 [0 –31]	10 [0 –39]	18 [4 –29]	33 [8 – 65]	10 [0 –23]	20 [0 – 48]	28 [1– 43]	28 [15–52]	0.004	0.87	0.64
Glycine amidates	113 [77–159]	185 [148–277]	187 [143–345]	473 [343–595]	148 [84 –215]	203 [171–256]	246 [175–355]	446 [326 –596]	<0.001	0.14	0.24
Taurine amidates	17 [8 –26]	30 [23–33]	38 [27–53]	89 [41–120]	21 [13– 48]	37 [20 – 45]	39 [31–55]	69 [52–95]	<0.001	0.08	0.12
DCA											
Total	67 [49 –94]	122 [60 –152]	103 [72–205]	243 [168 –336]	202 [71–313]	263 [126 –371]	293 [166–461]	513 [276 –702]	<0.001	<0.001	0.27
Unconjugated	26 [7–39]	29 [17–56]	24 [12– 42]	53 [38 –113]	81 [17–119]	92 [26 –134]	104 [23–126]	120 [56 –167]	<0.001	<0.001	0.43
Glycine amidates	29 [20 – 48]	57 [27– 84]	64 [35–96]	114 [98 –174]	92 [58 –171]	130 [78 –184]	153 [74 –238]	298 [176–487]	<0.001	<0.001	0.31
Taurine amidates	6 [0 –14]	11 [6 –27]	8 [0 –29]	29 [11– 62]	23 [11–34]	27 [12– 45]	33 [21– 48]	51 [38 –95]	<0.001	0.006	0.63

Table 1. Continued

Bile Acids, min × µmol/L	NGT				T2D				rmANOVA		
	OGTT	Low Fat	Medium Fat	High Fat	OGTT	Low Fat	Medium Fat	High Fat	Meal	Group	Interaction
UDCA											
Total	5 [0 –13]	9 [5–31]	11 [6 –28]	24 [14 – 42]	20 [5– 47]	38 [21–73]	43 [16 –71]	43 [16–71]	<0.001	0.01	0.42
Unconjugated	2 [0 –7]	1 [0 –15]	0 [0 –5]	5 [0 –12]	5 [0 –24]	13 [0 –34]	16 [0 –31]	19 [0–38]	0.38	<0.001	0.87
Glycine amidates	3 [0 – 8]	5 [0 –14]	7 [0 –19]	17 [5–32]	16 [5–25]	28 [9 –37]	29 [13– 40]	43 [16–56]	<0.001	0.002	0.57
Taurine amidates	NA	NA	NA	0 [0 –1]	0 [0 –2]	0 [0 –2]	0 [0 –1]	0 [0–2]	NA	NA	NA
FGF19, min × ng/mL	43 [29–55]	59 [38–96]	65 [37–78]	82 [53–97]	46 [23– 65]	49 [37– 60]	57 [44 –92]	55 [40–126]	<0.001	0.51	0.42

Abbreviations: NA, not available; rmANOVA, repeated measures ANOVA. Data are expressed as median [interquartile range]. Differences betweengroups were compared using two-way rmANOVA. AUC is shown for TBA, CA, CDCA, DCA, UDCA, their corresponding glycine and taurine amidates, and FGF19 during a 75-g OGTT and three isocaloric (500 kcal) and isovolemic (350 mL) liquid meals (low fat: 2.5 g fat, 107 g carbohydrate, and 13 g protein; medium fat: 10 g fat, 93 g carbohydrate, 11 g protein; and high fat: 40 g fat, 32 g carbohydrate, and 3 g protein)in T2D patients and NGT subjects.

Results

Postprandial concentrations of TBA

Postprandial TBA concentrations increased with increasing meal fat content (post-test for linear trend, $P < .001$), peaked after 1–2 hours, and were higher in T2D patients vs NGT subjects (OGTT, low and medium fat meals, $P < .05$; high fat meal, $P = .30$) (Figure 1 and Table 1). Fasting concentrations of total, unconjugated, and amidated bile acids were marginally increased in T2D patients vs NGT subjects (Supplemental Table 1). A subgroup analysis revealed no differences in TBA and FGF19 concentrations in metformin-treated patients vs patients treated with sulfonylureas and diet only (data not shown).

Postprandial concentrations of primary bile acids (CA and CDCA)

Postprandial total CA concentrations were dominated by glycine conjugates, which were higher in T2D patients vs NGT subjects (Table 1 and Figure 2) after OGTT and low and medium fat meals, but not after the high fat meal. Other CA fractions did not differ between the two groups. In comparison with the other bile acids measured, glycine-conjugated CDCA was the dominating bile acid found postprandially (Figure 2). In both groups, a clear and positive effect of meal fat (post-test for linear trend, $P < .001$) was demonstrated, but no between-group differences were present. There was a tendency to higher taurine conjugates in NGT subjects, but this difference was not statistically significant (Supplemental Figure 4). Fasting concentrations of total and unconjugated CA and CDCA were comparable between groups, whereas glycine-conjugated CA and taurine-conjugated CA and CDCA were marginally higher in T2D patients vs NGT subjects (Table 1).

Postprandial concentrations of secondary bileacids (DCA and UDCA)

Postprandial DCA concentrations were markedly higher in T2D patients after all meal stimuli. These differences reflected unconjugated and glycine-conjugated bile acids and, to a lesser extent, taurine conjugates (Table 1 and Figure 2). Although postprandial UDCA concentrations were 5- to 10-fold lower relative to the other bile acids measured, a clear increase was demonstrated in T2D patients vs NGT subjects. As for DCA concentrations, this difference reflected increased unconjugated and glycine-conjugated UDCA. However, in both groups, taurine conjugates were very low. Again, meal fat content associated positively with bile acid concentrations (post-test for linear trend, $P < .001$). Fasting concentration of

total, unconjugated, and glycine and taurine conjugates of DCA and UDCA were also higher in T2D patients vs NGT subjects (Supplemental Table 1).

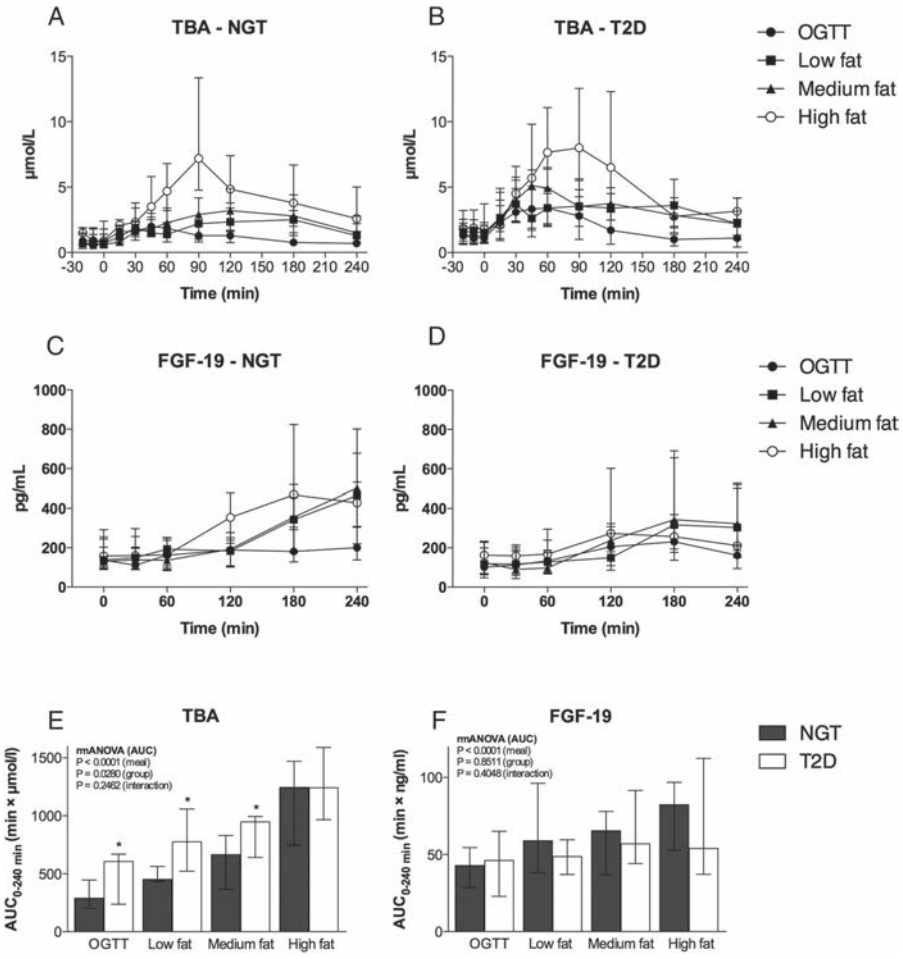

Figure 1. TBAs and FGF19. Postprandial plasma concentrations of TBA and FGF19 are shown during a 75-g OGTT and three isocaloric (500 kcal) and isovolemic (350 mL) liquid meals (low fat, 2.5 g fat, 107 g carbohydrate, and 13 g protein; medium fat, 10 g fat, 93 g carbohydrate, 11 g protein; and high fat: 40 g fat, 32 g carbohydrate, and 3 g protein) in NGT subjects (A and C, n = 15) and T2D patients (B and D, n = 15). AUC is shown for TBA (E) and FGF19 (F) in NGT subjects and T2D patients. Median and interquartile range values are shown. *, Significant differences ($P < .05$) vs NGT.

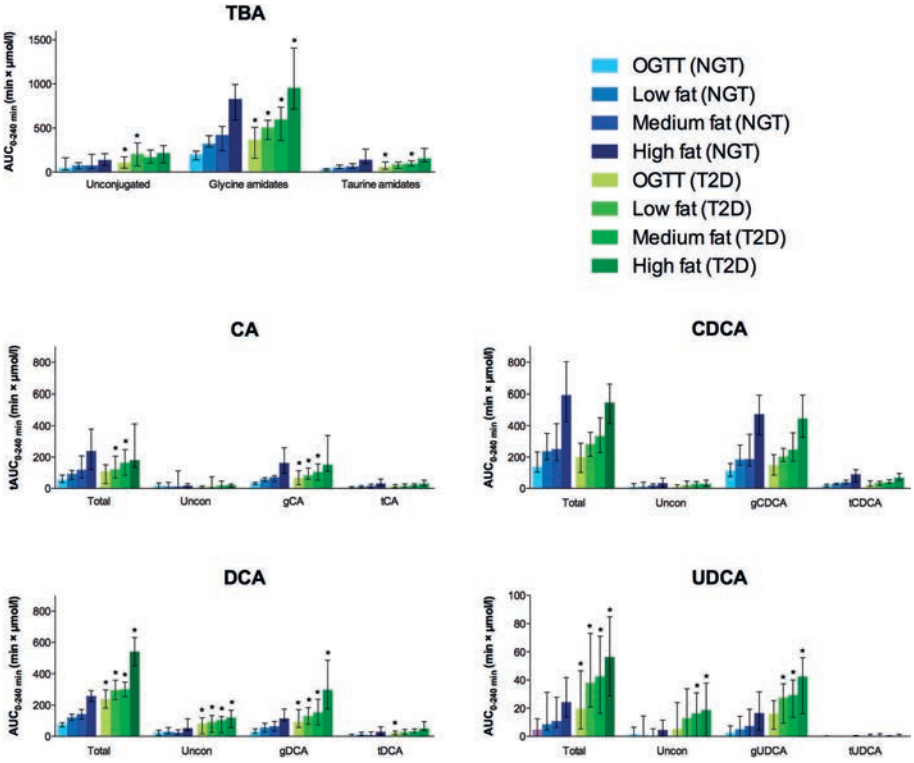

Figure 2. Individual bile acids. AUC is shown for TBA, unconjugated (uncon) CA, CDCA, DCA, and UDCA, and their corresponding glycine (g) and taurine (t) amidates during a 75-g OGTT and three isocaloric (500 kcal) and isovolemic (350 mL) liquid meals (low fat, 2.5 g fat, 107 g carbohydrate, and 13 g protein; medium fat, 10 g fat, 93 g carbohydrate, 11 g protein; and high fat, 40 g fat, 32 g carbohydrate, and 3 g protein) in T2D patients and NGT subjects. Median and interquartile range values are shown. *, Significant differences ($P < .05$) vs NGT.

Postprandial concentrations of FGF19

Both fasting and postprandial concentrations of FGF19 varied considerably in both groups. Hence, although NGT subjects tended to have higher postprandial FGF19 concentrations, differences were not statistically significant (Figure 1, Table 1, and Supplemental Table 1).

In both groups, postprandial peak concentrations occurred late (~3–4 hours after meal ingestion) and were more pronounced with increasing meal fat content (post-test for linear trend, P < .001). By grouping the four meal responses together, correlation analyses showed a weak, but significant, positive correlation between TBA and FGF19 concentrations (AUCs) in both NGT subjects (r = 0.411; P < .001) and T2D patients (r = 0.519; P < .001). The association between FGF19

and the individual bile acids (total concentrations) showed similar patterns (NGT vs T2D: CA, r = 0.426 and P < .001 vs r = 0.471 and P < .001; CDCA, r = 0.384 and P = .003 vs r = 0.520 and P < .001; DCA, r = 0.361 and P = .005 vs r = 0.322 and P < .017; UDCA, r = 0.188 and P = .15 vs r = 0.107 and P = .44). Postprandial concentrations of FGF19 and C-peptide were inversely associated in both NGT subjects (r = –0.266; P = .04) and T2D patients (r = –0.383; P = .003), whereas the association between FGF19 and glucose was negatively associated only in NGT subjects (r = –0.310; P = .02) vs T2D patients (r = 0.061; P = .66).

Discussion

The major findings in this study are: 1) T2D patients exhibited increased fasting and postprandial TBA concentrations after a wide range of nutritional stimuli in comparison with NGT subjects; 2) differences reflected mainly unconjugated and glycine-conjugated forms of the secondary bile acids DCA and UDCA and to a lesser extent CA, whereas postprandial CDCA concentrations were comparable among the two groups; and 3) postprandial concentrations of taurine-conjugated bile acids were also slightly higher in T2D patients (DCA). Furthermore, both fasting and postprandial FGF19 concentrations tended to be lower in T2D patients vs NGT subjects, but due to large variability, these differences were not statistically significant. Lastly, in both groups, a positive correlation between postprandial TBA and FGF19 concentrations was demonstrated.

The aim of the present study was to assess postprandial bile acid concentrations in T2D patients in order to shed more light on the possible mechanisms underlying the role of bile acids in metabolic regulation and T2D pathophysiology. Recent years have seen extensive research on bile acid metabolism in T2D, but no previous study has addressed postprandial bile acid concentrations of individual bile acids together with FGF19 after a wide range of different meals. The importance of this focus is highlighted by recent data showing that bile acids are not just luminal signaling molecules that activate TGR5 (leading to GLP-1 secretion) and FXR (leading to FGF19 secretion) in the intestine (3). FXR and TGR5 receptors are widely expressed and may even be found on pancreatic β-cells (6, 13–15). In fact, systemic bile acids seem to stimulate TGR5 and FXR, which positions postprandial plasma bile acids suitable for the regulation of overall glucose

homeostasis (1, 6, 16, 17). Such a concept could fit with the established notion that in T2D patients with a relatively low HbA1c (~7.5% [58 mmol/mol] or less), postprandial glycemia, as opposed to preprandial blood glucose, makes the predominant contribution to overall glycemic control (10). Indeed, postprandial hyperglycemia—an early defect seen in impaired glucose tolerance and T2D—is a major contributor to HbA1c and is even recognized as an independent risk factor for cardiovascular disease (18, 19)—alink that may also prove important considering the emerging evidence suggesting a role of bile acids in cardioprotection (20).

Substantial rearrangements of bile acid metabolism in T2D patients are well established, including changes in pool size, pool composition, synthesis rate, and postprandial plasma concentrations (21–27). We show that postprandial concentrations of secondary bile acids were higher in T2D patients vs NGT subjects. Indeed, on most study days, even fasting concentrations of total and secondary bile acids were higher in T2D patients vs NGT subjects. Moreover, we found increased postprandial CA concentrations (mainly glycine amidates), whereas CDCA concentrations (the predominant bile acid) were comparable among the groups. The design of the isovolemic and isocaloric meals allowed us to dissect in more detail how postprandial bile acid concentrations are affected by the fat content in the meal. Clearly, in both groups the high fat meal resulted in much higher plasma bile acid concentrations compared to the other meals—in fact, the CA and CDCA concentrations in T2D patients were equaled in the NGT group after the high fat meal (Figure 2). All four stimuli led to augmented DCA and UDCA concentrations in the T2D patients vs NGT subjects. Hence, postprandial concentrations of bile acids were clearly influenced acutely by meal composition. Most likely, this reflected increased gallbladder emptying after a larger fat stimulus (11) and increased enterohepatic circulation of all bile acid species, but it could also point toward the notion of acute effects of macronutrient composition constituting an important regulator of postprandial bile acid pool composition similar to changes seen after more extensive diet changes (28). Indeed, the meals also differed with regard to carbohydrate and protein content. Interestingly, even oral glucose resulted in increased TBA plasma concentrations in T2D patients, whereas concentrations in NGT subjects were almost unaffected. This could fit with our finding of increased gallbladder emptying in the T2D group (~30 vs ~20% in the NGT group) after the OGTT (11). Indeed, oral glucose is a minor stimulus for gallbladder contraction but—perhaps counterintuitively—a rather good stimulus for FGF19 secretion,

as recently demonstrated by Morton et al (29). However, in contrast to the data by Morton et al (29) showing that FGF19 concentrations increase preferentially in response to carbohydrates as opposed to protein and fat, we found that FGF19 concentrations increased with increasing fat and decreasing carbohydrate content in the meals. Notably, despite some gallbladder contraction after the OGTT (11), FGF19 concentrations remained more or less at the basal concentration in the NGTgroup and increased slightly in the T2D patients (Figure 1). Thus, although the data by Morton et al (29) indicate that the increase in postprandial FGF19 concentrations involves mechanisms additional to bile acid-induced FXR activation, our data fit with the notion of FXR dependency. However, in our T2D patients, there was a clear dissociation between postprandial concentrations of bile acids and FGF19 (but positive correlations were still demonstrated) compared to NGT subjects, and the "dose-response" relationship between fat (gallbladder contraction) and FGF19 was clearly reduced. This may indicate that bile acid-induced FXR activation is impaired in T2D, leading to decreased secretion of FGF19, which could explain the higher bile acid concentrations because FGF19 inhibits bile acid synthesis (30). Indeed, reduced FGF19 concentrations in T2D patients have been reported in recent clinical studies (31–33). Although mechanisms independent of FXR could be at work in T2D (ie, altered synthesis, secretion, and degradation of FGF19, activation of the pregnane X and vitamin D receptors by bile acids) (7), the enterohepatic bile acid composition is a likely determinant of the degree of FXR activation. However, in our study, CDCA concentrations, the most potent natural ligand of FXR, were unaltered in T2D patients vs NGT subjects, whereas DCA concentrations (a much weaker FXR agonist) were higher along with CA (not an FXR ligand). Such bile acid milieu should, theoretically, favor FXR activation and subsequent FGF19 secretion. However, postprandial UDCA concentrations were slightly higher in T2D patients vs NGT subjects. Although plasma UDCA concentrations were very low, this finding is of interest because UDCA is considered an FXR antagonist (5, 34).

Interestingly, using the murine GLUTag L-cell line, human intestinal biopsies, and different mouse models, Trabelsi et al (6) demonstrated that FXR activation inhibited glycolysis and ATP production, which in turn decreased proglucagon transcription and GLP-1 secretion in response to glucose. In contrast, FXR deficiency or FXR deactivation (using bile acid sequestering agents) promoted GLP-1 production and secretion (6). Thus, this newly identified FXR/GLP-1 pathway

suggests a positive effect of FXR antagonism on glucose homeostasis, which is also evidenced by the success of bile acid sequestering therapy for the treatment of T2D (3, 6). However, reduced FGF19 concentrations, achieved with bile acid sequestration (FXR inactivation), is not likely to explain the beneficial effect of bile acid sequestering agents. Indeed, animal studies have shown that FGF19 has insulin-like effects in the liver (9). Thus, FGF19 promotes protein and glycogen synthesis in the liver without promoting lipogenesis—in fact, FGF19 may even reduce triglycerides and cholesterol through currently unknown mechanisms (35, 36). A physiologically important difference is the temporal relationship between postprandial insulin and FGF19 secretion. As has been shown in other human studies and is confirmed in our study, FGF19 peak concentrations are segregated from insulin (~3 hours vs ~1 hour). Mice studies suggest that FGF19 —similar to insulin—is responsible for curbing postprandial endogenous glucose production, which is augmented in T2D (9). Specifically, FGF19 may be responsible for a delayed repression of gluconeogenesis, whereas postprandial insulin works in the early postprandial phase (37). Because gluconeogenesis accounts for approximately half of the endogenous glucose production (EGP), being low for up to approximately 4 hours after a meal (38), such a temporal relationship of postprandial insulin and FGF19 could physiologically make sense. However, our finding of very small FGF19 perturbations (in both T2D patients and NGT subjects) after a 75-g OGTT prompts questions about the idea that FGF19 is an important inhibitor of postprandial EGP by suppression of hepatic gluconeogenesis. However, EGP could be stimulated via bile acid-induced glucagon release from the intestine (39).

In summary, we find that T2D patients exhibit marked changes in fasting and postprandial bile acid concentrations compared to matched NGT subjects. These differences were dominated by increased unconjugated and glycine-conjugated secondary bile acids in T2D patients compared to NGT subjects, whereas primary bile acids were comparable among the two groups. In contrast, FGF19 concentrations tended to be lower in T2D patients vs NGT subjects. Most likely, these changes arise secondary to the T2D disease, as suggested by recent studies. Theoretically, this "T2D-bile acid-FGF19" phenotype results in altered FXR/FGF19 signaling in the small intestine and the liver, which could potentially add to the deterioration of postprandial glycemic homeostasis in T2D.

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References

1. Kuipers F, Bloks VW, Groen AK. Beyond intestinal soap– bile acids in metabolic control. *Nat Rev Endocrinol*. 2014;10(8):488 – 498.
2. Duboc H, Taché Y, Hofmann AF. The bile acid TGR5 membrane receptor: from basic research to clinical application. *Dig Liver Dis*. 2014;46(4):302–312.
3. Sonne DP, Hansen M, Knop FK. Bile acid sequestrants in type 2 diabetes: potential effects on GLP1 secretion. *Eur J Endocrinol*. 2014;171(2):R47–R65.
4. Adrian TE, Gariballa S, Parekh KA, et al. Rectal taurocholate increases L cell and insulin secretion, and decreases blood glucose and food intake in obese type 2 diabetic volunteers. *Diabetologia*. 2012; 55(9):2343–23437.
5. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev*. 2009; 89(1):147–91.
6. Trabelsi MS, Daoudi M, Prawitt J, et al. Farnesoid X receptor inhibits glucagon-like peptide-1 production by enteroendocrine L cells. *Nat Commun*. 2015;6:7629.
7. Schaap FG, Trauner M, Jansen PL. Bile acid receptors as targets for drug development. *Nat Rev Gastroenterol Hepatol*. 2014; 11(1):55– 67.
8. Rudling M, Camilleri M, Graffner H, Holst JJ, Rikner L. Specific inhibition of bile acid transport alters plasma lipids and GLP-1. *BMC Cardiovasc Disord*. 2015;15:75.
9. Kir S, Beddow SA, Samuel VT, et al. FGF19 as a postprandial, insulin-independent activator of hepatic protein and glycogen synthesis. *Science*. 2011;331(6024):1621–1624.
10. Monnier L, Lapinski H, Colette C. Contributions of fasting and postprandial plasma glucose increments to the overall diurnal hyperglycemia of type 2 diabetic patients: variations with increasing levels of HbA(1c). *Diabetes Care*. 2003;26(3):881– 885.
11. Sonne DP, Rehfeld JF, Holst JJ, Vilsbøll T, Knop FK. Postprandial gallbladder emptying in patients with type 2 diabetes: potential implications for bile-induced secretion of glucagon-like peptide 1. *Eur J Endocrinol*. 2014;171(4):407– 419.
12. Bootsma AH. Rapid analysis of conjugated bile acids in plasma using electrospray tandem mass spectrometry: application for selective screening of peroxisomal disorders. *J Inherit Metab Dis*. 1999;22(3):307–310.
13. Düfer M, Hörth K, Wagner R, et al. Bile acids acutely stimulate insulin secretion of mouse β-cells via farnesoid X receptor activation and K(ATP) channel inhibition. *Diabetes*. 2012;61(6):1479 –1489.
14. Kumar DP, Rajagopal S, Mahavadi S, et al. Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic β cells. *Biochem Biophys Res Commun*. 2012;427(3): 600 – 605.
15. Popescu IR, Helleboid-Chapman A, Lucas A, et al. The nuclear receptor FXR is expressed in pancreatic β-cells and protects human islets from lipotoxicity. *FEBS Lett*. 2010;584(13):2845–2851.
16. Ullmer C, Alvarez Sanchez R, Sprecher U, et al. Systemic bile acid sensing by G protein-coupled bile acid receptor 1 (GPBAR1) promotes PYY and GLP-1 release. *Br J Pharmacol*. 2013;169(3):671–684.
17. Brighton CA, Rievaj J, Kuhre RE, et al. Bile acids trigger GLP-1 release predominantly by accessing basolaterally located G proteincoupled bile acid receptors. *Endocrinology*. 2015;156(11):3961– 3970.
18. Donahue RP, Abbott RD, Reed DM, Yano K. Postchallenge glucose concentration and coronary heart disease in men of Japanese ancestry. Honolulu Heart Program. *Diabetes*. 1987;36(6):689 – 692.
19. Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative Analysis Of Diagnostic Criteria in Europe. *Lancet*. 1999;354(9179):617– 621.
20. Duboc H, Aelion H, Rainteau D, et al. Crosstalk between the hepatologist and the cardiologist: a future place for the lithocholic acid as a coronary atheroma risk factor? *Hepatology*. 2012;56(6):2426.
21. Bennion LJ, Grundy SM. Effects of diabetes mellitus on cholesterolmetabolism in man. *N Engl J Med*. 1977;296(24):1365–1371.
22. Brufau G, Stellaard F, Prado K, et al. Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism. *Hepatology*. 2010;52(4):1455–1464.

23. Suhre K, Meisinger C, Döring A, et al. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. *PLoS One*. 2010;5(11):e13953.
24. Taylor DR, Alaghband-Zadeh J, Cross GF, Omar S, le Roux CW, Vincent RP. Urine bile acids relate to glucose control in patients with type 2 diabetes mellitus and a body mass index below 30 kg/m^2. *PLoS One*. 2014;9(4):e93540.
25. Vincent RP, Omar S, Ghozlan S, et al. Higher circulating bile acid concentrations in obese patients with type 2 diabetes. *Ann Clin Biochem.* 2013;50(Pt 4):360 –364.
26. Haeusler RA, Astiarraga B, Camastra S, Accili D, Ferrannini E. Human insulin resistance is associated with increased plasma levels of 12α-hydroxylated bile acids. *Diabetes*. 2013;62(12):4184 –4191.
27. Jørgensen NB, Dirksen C, Bojsen-Møller KN, et al. Improvements in glucose metabolism early after gastric bypass surgery are not explained by increases in total bile acids and fibroblast growth factor 19 concentrations. *J Clin Endocrinol Metab*. 2015;100(3):E396 –E406.
28. Bisschop PH, Bandsma RH, Stellaard F, et al. Low-fat, high-carbohydrate and high-fat, low-carbohydrate diets decrease primary bile acid synthesis in humans. *Am J Clin Nutr*. 2004;79(4):570 –576.
29. Morton GJ, Kaiyala KJ, Foster-Schubert KE, Cummings DE, Schwartz MW. Carbohydrate feeding dissociates the postprandial FGF19 response from circulating bile acid levels in humans. *J Clin Endocrinol Metab*. 2014;99(2):E241–E245.
30. Inagaki T, Choi M, Moschetta A, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab*. 2005;2(4):217–225.
31. Barutcuoglu B, Basol G, Cakir Y, et al. Fibroblast growth factor-19 levels in type 2 diabetic patients with metabolic syndrome. *Ann Clin Lab Sci*. 2011;41(4):390 –396.
32. Fang Q, Li H, Song Q, et al. Serum fibroblast growth factor 19 levels are decreased in Chinese subjects with impaired fasting glucose and inversely associated with fasting plasma glucose levels. *Diabetes Care*. 2013;36(9):2810 –2814.
33. Gerhard GS, Styer AM, Wood GC, et al. A role for fibroblast growth factor 19 and bile acids in diabetes remission after Roux-en-Y gastric bypass. *Diabetes Care*. 2013;36(7):1859 –1864.
34. Mueller M, Thorell A, Claudel T, et al. Ursodeoxycholic acid exerts farnesoid X receptor-antagonistic effects on bile acid and lipid metabolism in morbid obesity. *J Hepatol*. 2015;62(6):1398 –1404.
35. Fu L, John LM, Adams SH, et al. Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes. *Endocrinology*. 2004;145(6):2594 –2603.
36. Tomlinson E, Fu L, John L, et al. Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology*. 2002;143(5):1741–1747.
37. Potthoff MJ, Boney-Montoya J, Choi M, et al. FGF15/19 regulates hepatic glucose metabolism by inhibiting the CREB-PGC-1α pathway. *Cell Metab*. 2011;13(6):729 –738.
38. Woerle HJ, Meyer C, Dostou JM, et al. Pathways for glucose disposal after meal ingestion in humans. *Am J Physiol Endocrinol Metab*. 2003;284(4):E716 –E725.
39. Hansen M, Scheltema MJ, Sonne DP, et al. Effect of chenodeoxycholic acid and the bile acid sequestrant colesevelam on glucagonlike peptide-1 secretion. *Diabetes Obes Metab.* 2016;18(6):571– 580.

3

Transhepatic bile acid kinetics in pigs and humans

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Abstract

Background & *aims:* Bile acids (BAs) play a key role in lipid uptake and metabolic signalling in different organs including gut, liver, muscle and brown adipose tissue. Portal and peripheral plasma BA concentrations increase after a meal. However, the exact kinetics of postprandial BA metabolism have never been described in great detail. We used a conscious porcine model to investigate postprandial plasma concentrations and transorgan fluxes of BAs, glucose and insulin using the *para*-aminohippuric acid dilution method.

Methods: Eleven pigs with intravascular catheters received a standard mixed-meal while blood was sampled from different veins such as the portal vein, abdominal aorta and hepatic vein. To translate the data to humans, fasted venous and portal blood was sampled from non-diabetic obese patients during gastric by-pass surgery.

Results: The majority of the plasma bile acid pool and postprandial response consisted of glycine-conjugated forms of primary bile acids. Conjugated bile acids were more efficiently cleared by the liver than unconjugated forms. The timing and size of the postprandial response showed large interindividual variability for bile acids compared to glucose and insulin.

Conclusions: The liver selectively extracts most BAs and BAs with highest affinity for the most important metabolic BA receptor, TGR5, are typically low in both porcine and human peripheral circulation. Our findings raise questions about the magnitude of a peripheral TGR5 signal and its ultimate clinical application.

Abbreviations: BAs, bile acids; ASBT, apical sodium-dependent bile acid transporter; OST, organic solute transporter; NTCP, taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; mEH, microsomal epoxide hydroxylase; BSEP, bile salt export pump; FXR, farnesoid X receptor; TGR5, takeda G protein-coupled receptor 5; FGF, fibroblast growth factor; GLP-1, glucagon-like peptide 1; PAH, para-aminohippuric acid; TCA, trichloroacetic acid; A, abdominal aorta; V, caval vein; R, renal vein; P, portal vein; H, hepatic vein; HQ, hindquarter muscle; PDV, portal drained viscera; SPL, splanchnic compartment; K, kidneys; LC/MS/MS, liquid chromatography tandem mass spectrometry; UDCA, ursodeoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; HDCA, hyodeoxycholic acid; g, glycine-conjugated; t, taurine-conjugated; LOQ, lower limit of quantification; BMI, body mass index; ANOVA, analysis of variance; AUC, area under the curve; CYP7A1, cytochrome P450 7A1; KO, knock-out; SHP, short heterodimer partner; EC50, half maximal effective concentration.

Introduction

Bile acids (BAs) are intestinal lipid solubilizers, facilitating uptake of fats and fat-soluble vitamins. BAs are synthesised in the liver from cholesterol, conjugated to either glycine or taurine, and stored in the gallbladder. After a meal, BAs are released into the intestine and are taken back to the liver via the superior mesenteric and the portal veins, a very efficient BA transporter system (reviewed in [1]). In the intestine, unconjugated BAs can diffuse passively over the intestinal border whereas conjugated forms require active transport to be taken up from the intestinal lumen. Active transport from the intestinal lumen is mediated by the apical sodium-dependent BA transporter (ASBT) in the distal ileum. Subsequently, BAs are transported over the basolateral side of the enterocyte via the organic solute transporter (OST_a/OST_b) and enter the liver via the portal vein. The liver clears most BAs from the portal blood via the Na^+ taurocholate cotransporting polypeptide (NTCP) which is highly expressed in the liver and has a high affinity for all conjugated BA. NTCP is aided by transporters of the organic anion transporting polypeptide (OATP) family that can transport unconjugated and sulphated BAs. In addition, it is hypothesized that the enzyme microsomal epoxide hydrolase (mEH) can also function as a sodium-dependent BA transporter on the sinusoidal plasma membrane [2]. Hepatic BAs are excreted into the biliary tract via the bile salt export pump (BSEP) on the canalicular membrane.

Most of the BAs recycle in the enterohepatic circulation and only a small amount of BAs appears in the peripheral circulation. This may have important consequences for the presumed metabolic effects of BAs that are mediated by the nuclear farnesoid X receptor (FXR) and the transmembrane Takeda G protein-coupled receptor 5 (TGR5) [3]. FXR regulates liver BA synthesis and metabolism, but can also contribute to glucose and lipid metabolism by stimulating the release of fibroblast growth factor (FGF) 15/19. TGR5 is not only proposed to mediate the effects of BAs on glucagon-like peptide-1 (GLP-1) and insulin release in the gut and pancreas, but also to affect energy expenditure and possibly insulin sensitivity via organs such as muscle or brown adipose tissue [3]. Portal vein and peripheral plasma BA concentrations peak after a meal [4-7]. These observations suggest that BAs are potentially important postprandial signals to modulate metabolic and endocrine regulation in the gut, liver, muscle and fat.

Since the discovery of FXR and TGR5, it has been known that different BAs show a hierarchy in receptor activation [8-11]. Additionally, transmembrane transport and conjugation may modulate activation patterns. Hence, porto-peripheral differences of different BA forms and their conjugation and hydrophobicity profiles may predict FXR and TGR5 activation in gut, liver and peripheral organs. In an observational study, we investigated postprandial transhepatic BA fluxes in conscious pigs before and after a mixed meal (Fig. 1). The pig model has been used for diabetes and metabolic research because of its resemblance to human physiology [12]. The aim of this study was to gain more insight in the postprandial transhepatic BA fluxes and plasma profiles of the different BAs and to predict the potential relevance of BAs for FXR and TGR5 activation. In addition, we analysed human portal and peripheral vein BA profiles to translate the experimental results to relevant human data. These data illustrate that the liver selectively extracts most BAs with high TGR5 affinity, which consequently are typically low in the peripheral circulation.

Materials and methods

Animals

Eleven female cross-bred pigs (20–25 kg, 8–12 weeks old) from a commercial breeder (Rosenbaum Farms, Brenham, TX) were individually housed in galvanized bar runs (2 × 3 m) enriched with straw and toys to acclimatize for 2 weeks before surgical catheter placement. They were kept on a 12 h light–dark cycle (lights on at 7 AM) with a radio turned on during the light period. Environmental temperature was 21–25 °C. The pigs were fed 1 kg/day, Harlan-Teklad Vegetarian Pig/Sow Grower (Harlan laboratories, Indianapolis, IN). Water was available *ad libitum*. Data on acylcarnitines from this study have been published previously [13]. All animal experiments were approved by the Institutional Animal Care and Use Committee of Texas A & M University, USA.

Surgery

We placed the intravascular catheters one week before the experiment using the surgical techniques described earlier [14,15]. In short, we fasted the animals for 16 h before surgery and sedated them using an intramuscular injection of tiletamine-zolazepam (3.3 mg/kg) (Telazol; Zoetis, Kalamazoo, MI). Animals were intubated

and the anaesthesia continued with isoflurane (2%). In total, we implanted 7 intravascular catheters (Fig. 1) as well as a feeding tube inserted percutaneously into the stomach.

In order to measure organ fluxes, we placed two catheters upstream of the organs for *para*-aminohippuric acid (PAH) infusion. For muscle flux measurements, we implanted the PAH infusion catheter into the abdominal aorta with its tip 5 cm above the bifurcation and the other PAH infusion catheter into the splenic vein. We inserted sampling catheters into the abdominal aorta above the right renal artery (A) for pre-organ compartment arterial plasma concentrations; into the inferior caval vein (iliac circumflex profunda vein) (V) with its tip 5 cm above the bifurcation for muscle flux measurements and venous concentrations; into the left renal vein (R) for kidney flux measurements; into the portal vein with its tip in the liver hilus (P), and into the hepatic vein (H) by direct puncture of the liver for the splanchnic measurements. To reassure that the catheter H was correctly placed in the hepatic vein, we punctured the external side of the liver and pushed in the catheter for 20 cm, then we retracted it to 5 cm to ensure we were not in the inferior caval vein. We secured all catheters in place and tunnelled them through the left abdominal wall. After abdominal closure the pigs were dressed with a canvas harness to protect the catheters. To keep the catheters patent they were filled with 0.5 mL of gentamycine (20 mg/mL) and α-chymotrypsin solution (225 U/mL).

After surgery we checked the animals twice daily for four days on body temperature, catheter patency and overall behaviour. Also, animals received i.v. injections with antibiotics (6.25 mg/kg lincomycin and 12.5 mg/kg spectinomycin) and analgetics (2 mg/kg flunixin meglumine) and the animals were allowed to recover for 7-10 days. During this period they were also habituated to the experimental cage ($0.9 \times 0.5 \times 0.3$ m on wheels).

Experimental procedure

The animals were conscious during the whole experimental procedure. We removed all food at 16:00 h the day before the experiment. On the day of the experiment all animals were first weighed and at 08:00 h ($t = -60$ min), an hour before the meal, we started the continuous infusion of 25 mM PAH at 60 mL/h and reached steady state before the sampling started. At $t = -10, -5$ and 0 min, we took three baseline blood samples from all catheters. Samples were always taken

in the same order during the experiment: A, P, H, V, R. At 09:00 h, t = 0, the pigs received their test meal via the gastric feeding tube. For a pig of 25 kg, the test meal consisted a 600 mL mixture of 78 g of crude whey protein isolate (100% Premium Whey Protein; Body Fortress, Bohemia, NY) and 110 g of carbohydrates (Malto dextrin; Muscle Feast, Hebron, OH) in water, and 22 g of olive oil (30% of daily energy intake; Korger, Cincinnati, OH). We administered the complete test meal within approximately 5 min and took postprandial blood samples at t = 0, 10, 20, 30, 45, 60, 90, 120, 180 and 240 min. After the experiment, pigs were returned to their normal cages with their normal food and water available. Malfunctioning catheters accounted for missing data (two hepatic vein catheters and one renal vein catheter).

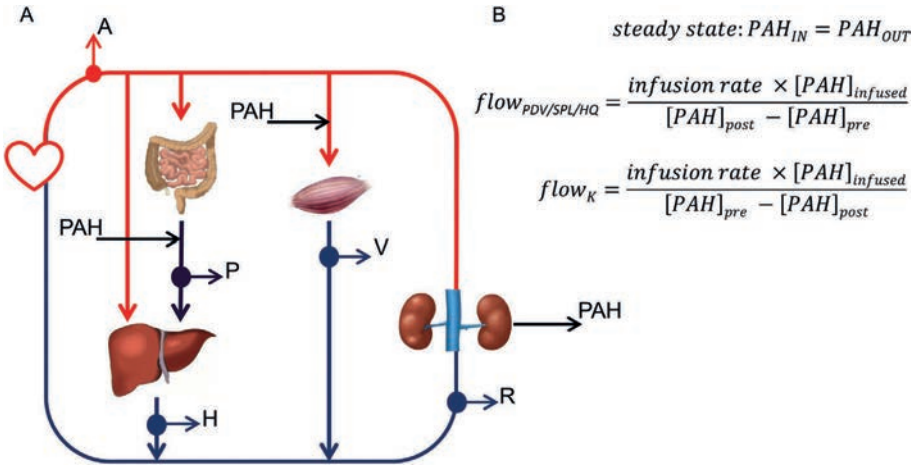

Fig. 1. Intravascular sampling catheters and transorgan flux measurements. (A) Schematic overview of placement of intravascular catheters. The blood stream is going in a clockwise direction from the heart with arterial blood in red and venous blood in blue. Circles with an arrow indicate sampling catheters and black arrows indicate the two infusion catheters for *para*-aminohippuric acid (PAH) and urinary loss via the kidneys. A: arterial line, sampling catheter in the abdominal aorta above the right renal artery to measure pre-organ concentrations; V: venous line, sampling catheter in the iliac circumflex profunda vein with its tip 5 cm above the bifurcation; R: renal line, sampling catheter in the left renal vein; P: portal line, sampling catheter in the portal vein with its tip in the liver hilus; H: hepatic line, sampling catheter in the hepatic vein by direct puncture of the liver. (B) Formulas to calculate the plasma flow through the organ compartments. Blood sampling started when PAH concentrations reached steady state (60 min). Plasma flow through the hindquarter muscle (HQ), the intestine (portal drained viscera, PDV) and the splanchnic compartment (SPL, intestine and liver) can be calculated with the upper flow formula. For HQ the PAH infusion site is the catheter in the abdominal aorta. For PDV and SPL the infusion site is the catheter into the splenic vein. $[PAH]_{pre}$ is the PAH concentration in the main bloodstream, sampled through line A. $[PAH]_{post}$ is the PAH concentration in the efferent vein of the organ: line P for PDV, line H for SPL and line V for HQ. Plasma flow through the kidneys (K) can be calculated with the lower formula. Note that for K the extracted PAH is the infused [PAH] from both infusion sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sample preparation

After withdrawal, we immediately placed the blood samples on ice. For the PAH concentration measurements, we pipetted 250 mL of blood into a tube containing 25 mL of trichloroacetic acid (TCA), thoroughly vortexed, and subsequently, together with the remaining blood samples, centrifuged at 8000 g for 5 min at 4 °C. After spinning, we transferred the plasma samples into clean tubes and snap froze them in liquid nitrogen and stored them at 80 °C until further analysis. For measurements, samples were defrosted and thoroughly homogenized using a multivortex and subsequently centrifuged for 3 min at 1780 or 1800 g and aliquoted for BA, insulin and glucose concentration measurements.

PAH concentrations and flow calculations

The PAH concentrations of the TCA plasma samples were compared to PAH standards and read out using a microplate spectrophotometer (Spectramax; Molecular Devices, Sunnyvale, CA) and SoftmaxPro software (Molecular Devices) [16]. We calculated plasma flow through the organs using the dilution of PAH over the organ compartment [14,15]. Blood sampling (Fig. 1) started when PAH concentrations reached steady state (60 min). So PAH_{IN} PAH_{OUT}. We calculated the plasma flow through the hindquarter muscle (HQ), the intestine (portal drained viscera, PDV) and the splanchnic compartment (PDV and liver, SPL) with the formula:

$$flow_{PDV/SPL/HQ} = \frac{infusion\ rate\ \times [PAH]_{infused}}{[PAH]_{post} - [PAH]_{pre}}$$

For HQ the PAH infusion site is the catheter in the abdominal aorta. For PDV and SPL the infusion site is the catheter into the splenic vein. $[PAH]_{pre}$ is the PAH concentration in the main bloodstream, sampled through line A. $[PAH]_{post}$ is the PAH concentration in the efferent vein of the organ: line P for PDV, line H for SPL and line V for HQ. Plasma flow through the kidneys (K) can be calculated with the formula:

$$flow_K = \frac{infusion\ rate\ \times [PAH]_{infused}}{[PAH]_{pre} - [PAH]_{post}}$$

Note that for K the extracted PAH is the infused [PAH] from both infusion sites. We interpolated missing data points and used the mean plasma flow of all pigs for further flux calculations.

Glucose and insulin concentrations

We measured glucose concentrations in duplicate with a glucose oxidase method using the Biosen C-line plus glucose analyser (EKF Diagnostics, Barleben/Magendeburg, Germany) and insulin concentrations in duplicate with a porcine Insulin ELISA (version 4.0, Mercodia, Uppsala, Sweden) according to the manufacturer protocol. Plates were read with a spectrophotometer (Varioskan Flash version 2.4.3, Thermo Scientific) running matching SkanIt software. The calibration curve was a cubic polynomial extrapolated to concentrations of 0.01–2.0 ng/L. Samples outside this range were set to ≤ 0.01 or ≥ 2.0 ng/L, respectively. Insulin concentrations were not measured in renal vein samples.

BA concentrations

We measured BA concentrations by liquid chromatographytandem mass spectrometry (LC/MS/MS, Supplemental Methods). Pigs have an abundant BA profile [17,18]. Here, we focused mainly on BAs that are prevalent in humans to enable translation: ursodeoxycholic acid (UDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) and hyodeoxycholic acid (HDCA, non-human), and their glycine- (g) and taurine- (t) conjugated forms. In the humans, 15 BAs were measured: UDCA, CA, CDCA, DCA and LCA and their g- and t- conjugated forms. For sample preparation, after homogenizing and spinning, 25 μL plasma was aliquoted into a clean tube for BA analysis. For every 10 samples prepared, one quality control standard plasma was included. To each sample, we added 250 μL internal standard and vortexed for 60 s. Samples were centrifuged at 15,800 ×g and the supernatant poured into a clean glass tube.

The fluid was evaporated under nitrogen at 40 °C. Before measuring samples were reconstituted in 100 mL 50% methanol in water, vortexed for 60 s and centrifuged for 3 min at 1800 ×*g*. We transferred the supernatant into a 0.2 mm spin-filter and centrifuged at 2000 ×*g* for 10 min. After filtering, the samples were transferred into LC/MS vials and analysed (10 mL injection volume). The lower limit of quantification (LOQ) was 0.05 μM for all BA forms.

In pigs, all measured concentrations of CA, tCA, gCA, DCA, gDCA and tDCA were below the LOQ. Unconjugated UDCA concentration was never calculated due to interfering peaks in the chromatogram. Therefore, we excluded the

conjugated and unconjugated forms of CA and DCA, and unconjugated UDCA from analysis. In humans, UDCA, tUDCA and tDCA were excluded from analysis due to interfering peaks in the chromatogram. For all the other BAs, when a concentration was below the lower LOQ, its value was set to 50% of the detection limit i.e., 0.025 μM.

Organ flux calculations

In the present model we measured organ flux through the hindquarter muscle compartment (muscle flux), the kidney compartment (renal flux), the intestine and pancreas compartment (portal drained viscera (PDV) flux) and the splanchnic compartment: the PDV plus the liver (SPL flux). We calculated liver flux as SPL flux minus PDV flux. Flux through a compartment was calculated as the mean plasma flow through that compartment multiplied with the arterio-venous difference in concentration of glucose, insulin or BA. A positive flux value is interpreted as appearance or production by the compartment, while a negative flux is interpreted as disappearance or uptake in the compartment.

Human study

We took blood samples from the cubital vein and portal vein from consecutive subjects during elective gastric by-pass surgery. Eleven healthy non-diabetic patients participated in this study (1 male, 10 females; age 46.0 ± 12.7 yrs; body mass index 41.4 ± 2.8 kg/m^2; mean ± SD). All participants gave informed consent before the surgery and the protocol was approved by the Medical Ethical Committee of the Academic Medical Centre and Slotervaart Hospital, Amsterdam. None of the patients used BA sequestrants. Subjects were fasted overnight prior to the surgery. First, a preoperative peripheral venous sample was taken. During the surgery, the portal vein puncture was performed as previously described [19]. Glucose, insulin and BA concentrations were measured in these samples.

Statistics

Calculations were made using Microsoft Excel 2010 version 14.0 and area-under-the-curve analysis and statistics were done in Graphpad Prism version 7. For porcine data analysis paired t-test or repeated measure ANOVA with Tukey post-hoc analysis were used where appropriate. For human data we used non-parametric tests.

Detected numeric outliers were excluded based on Grubbs' test. Data are presented as mean ± standard error of the mean (SEM).

Results

Glucose and insulin concentrations and transorgan fluxes validate the porcine model

In addition to the BAs, we measured glucose and insulin concentrations and transorgan fluxes. These data confirm the reproducibility of our postprandial model. The postprandial responses of glucose and insulin concentrations showed similar responses in all pigs with low interindividual variability (Supplemental Figs. S1-4). Plasma glucose and insulin concentrations increased after the meal in all blood vessels as shown in Fig. 2A, B. Postprandial concentrations of both

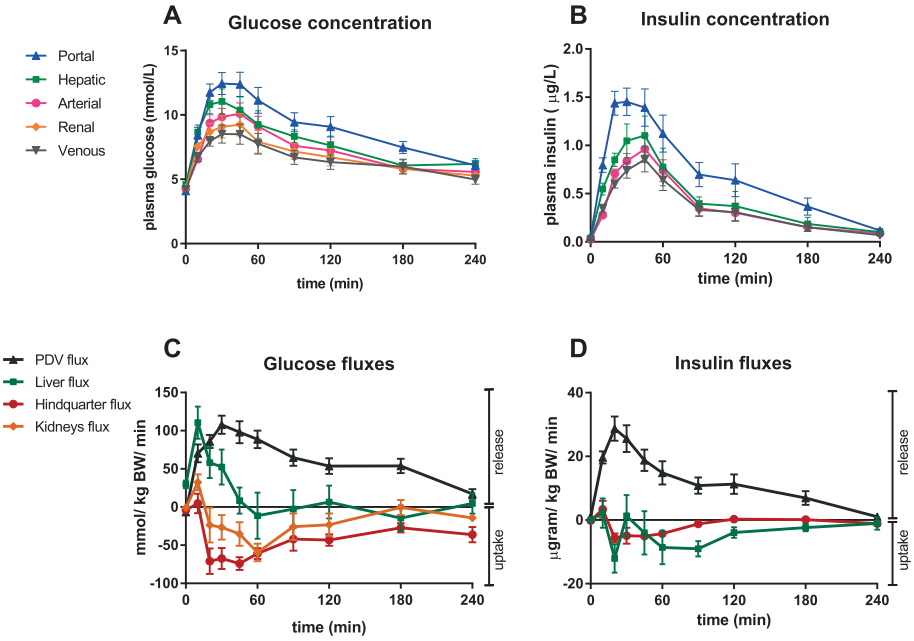

Fig. 2. The postprandial glucose and insulin response throughout the body. Postprandial plasma concentrations (A-B) and transorgan fluxes (C-D) for glucose (A, C) and insulin (B, D). Fasted state before the meal is averaged at t = 0. A positive flux denotes net production by the organ(s). A negative flux reflects net uptake by the organ(s). Please note that in the fasted state liver glucose flux is positive (30 mmol/kg BW/ min). Data represented as mean ± SEM. PDV: portal drained viscera (intestine and pancreas). N = 11, except for the hepatic line (N = 9) and renal line (N = 10) and appurtenant analysis.

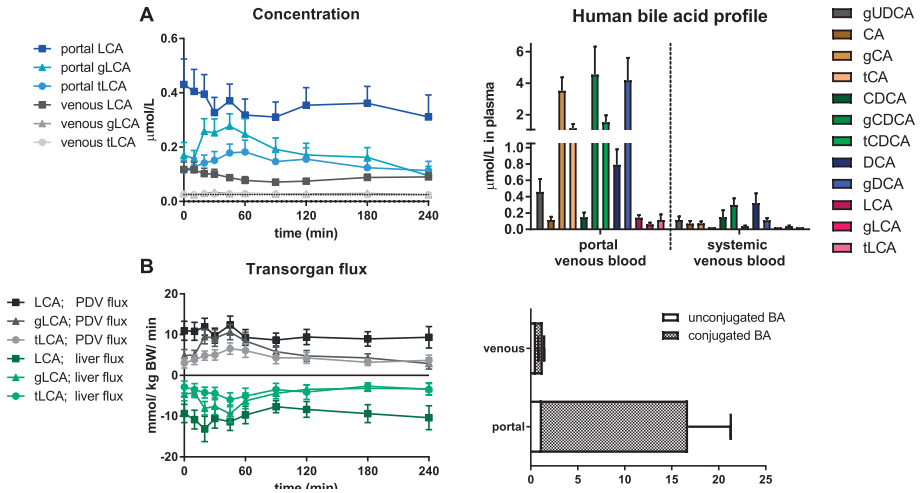

Fig. 3. Postprandial released BAs are efficiently cleared by the liver. (A) During fasting enterohepatic BA concentrations (P) are ~6 times higher than peripherally (V). (B-E) BA concentration curves show an increase after food intake, time 0 represents the fasted state. CDCA: chenodeoxycholic acid (green), HDCA: hyodeoxycholic acid (pink), g: glycine (triangle), t: taurine (circle). (F) The individually depicted postprandial area under the curve (AUC) of CDCA and HDCA and their conjugates shows that the main difference between the enterohepatic and peripheral BA profile is the ratio between gCDCA and gHDCA. Peripheral BA profiles are very similar. R: renal line. (G) Hepatic uptake of conjugated BA is higher than for unconjugated forms and especially conjugated CDCA is efficiently cleared. BA clearance was calculated as $[BA]_{portal\ vein\ aorta} - [BA]_{hepatic\ vein} = [BA]_{portal\ vein+aorta}$. Data represented as mean ± SEM. N = 11; hepatic outcomes (N = 9). ***p < 0.001, two-tailed paired t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glucose and insulin peaked within the first hour and, subsequently, returned to baseline in 4 h. As expected, the concentrations of both glucose and insulin were highest in the portal vein, since glucose and insulin directly appear here postprandial from the intestine and pancreas, respectively (AUC glucose mmol/L one-way ANOVA: portal 2143 ± 90 vs hepatic 1744 ± 119, arterial 1727 ± 85, renal 1629 ± 92 and venous 1571 ± 80; p < 0.0001. AUC insulin mmol/L one-way ANOVA: portal 162 ± 14 vs hepatic 104 ± 12, arterial 88 ± 8 and venous: 81 ± 9; p < 0.0001). Fig. 2C,D show that glucose and insulin transorgan fluxes in the fasted state (i.e., t = 0) were low, except for the positive liver flux, which reflects hepatic glucose output. Transhepatic glucose flux increased initially, but subsequently decreased to zero within 1 h. Therefore, after administration of the meal, hepatic glucose production may have continued for a brief period. Together with glucose from the portal drained viscera (PDV, i.e., intestine and pancreas), this resulted in a substantial postprandial glucose peak in the peripheral circulation. Insulin flux across the liver was negative which demonstrates hepatic insulin clearance.

As a consequence, plasma insulin concentrations in the hepatic vein and beyond were substantially lower than in the portal vein. The postprandial negative muscle flux indicates uptake of glucose by the muscle. The negative kidney glucose flux indicates uptake of glucose by the kidneys.

BAs continuously cycle in the enterohepatic circulation of pigs regardless of food status

Total BA concentrations in the portal vein were at least 6 times higher than in the other blood vessels, reflecting that most BAs are contained within the enterohepatic cycle (fasted state total BA concentration in portal vein: 25.01 ± 2.8 μM and caval vein: 4.29 ± 1.0 μM, $p < 0.0001$). In addition, the baseline fluxof portal vein BAs indicates that BAs also circulate in the enterohepatic circulation in the postabsorptive state, while total BA concentrations between the hepatic, renal or caval veins and aorta were not different (Fig. 3B-E).

Postprandial BA concentrations rise with large variability between animals

After the meal, BA concentrations increased in the portal vein, and, to a much lesser extent, in the peripheral circulation, with a broad peak around 1.5-2 h after the meal (Fig. 3B-E). In contrast to the relatively uniform postprandial glucose and insulin responses, these postprandial BA responses revealed relatively large interanimal variabilities (Supplemental Figs. S5-9). When aligning the individual postprandial concentration curves at their peak a significant postprandial rise in BA concentration becomes apparent, for example for glycine-conjugated hyodeoxycholic acid (gHDCA) (Supplemental Fig. S10). The most prominent BAs present in the portal vein were glycine-conjugated (g) chenodeoxycholic acid (CDCA) and gHDCA, followed by their taurine-conjugated (t) and unconjugated forms (Fig. 3E,F). Conjugated and unconjugated forms of lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) were well detected in the portal vein but their concentrations were very low elsewhere (Fig. 4).

Porcine hepatic clearance is most efficient for conjugated CDCA forms

The BA profile in the peripheral circulation was similar to the portal vein, albeit with much lower concentrations except for gCDCA (Figs. 3 and 4). Even though gCDCA and gHDCA were the most prominent BAs in the portal vein, hepatic vein concentrations of gCDCA were much lower than gHDCA concentrations (Fig. 3F; ratio gHDCA:gCDCA in P: 0.95 ± 0.1 vs in V: 3.04 ± 0.3; $p < 0.0001$).

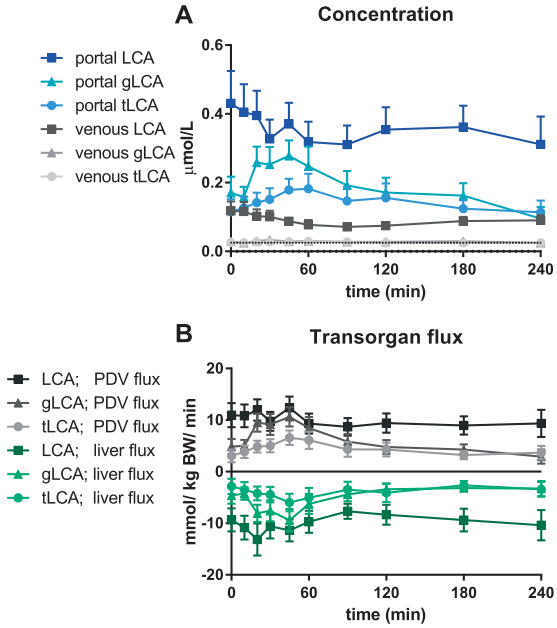

Fig. 4. Lithocholic acid forms, BAs with high TGR5 affinity, are mainly found in the enterohepatic circulation and not peripherally. (A) Lithocholic acid (LCA) and its glycine- (g) and taurine- (t) conjugated forms are present in pig plasma in stable low concentrations. gLCA shows a postprandial increase in the portal vein but not in the peripheral circulation. The other forms do not show a postprandial response and conjugated forms of LCA are not even detected outside the enterohepatic circulation. Fasted state: x = 0, PDV: portal drained viscera, dotted line indicates the 50% detection limit, undetected concentrations were set to this number (0.025 mmol/L). N = 11. (B) Transorgan fluxes of LCA forms. Like the other BAs LCA and conjugates are released from the intestine and very efficiently taken up by the liver.

To quantify hepatic clearance rates, we calculated the differences between BA concentrations proximal and distal of the liver:

$$[BA]_{\text{portal vein aorta}} - [BA]_{\text{hepatic vein}} = [BA]_{\text{portal vein aorta.}}$$

Indeed, the hepatic clearance rate of gCDCA was significantly higher than that of gHDCA (t = 60 min, $p < 0.0001$, Fig. 3G). In general, the hepatic clearance rates of conjugated BA were significantly higher than the clearance rates of their unconjugated forms.

Postprandial BA exposure is high in the enterohepatic cycle compared to the peripheral circulation

Transorgan flux is different from clearance, since it is calculated by multiplying concentration difference by plasma flow. In the fasted state, BA flux in the

portal vein was considerable (i.e., enterohepatic cycle). However, after the meal transorgan fluxes doubled, in particular of the glycine-conjugated BA (Fig. 5A). Thus, the postprandial exposure to BA in the enterohepatic circulation was high. The postprandial peak in portal BA concentrations was also clearly visible in the BA fluxes, as shown in Fig. 5. For all BAs the PDV flux was always positive with a clear postprandial peak, while the liver flux was negative and mirrored the curve of the PDV flux. These fluxes show the appearance of BA from the gut into the portal vein and the efficient absorption of BA by the liver. There was no net transorgan BAs flux over the kidneys and hindquarter (Supplemental Fig. S11).

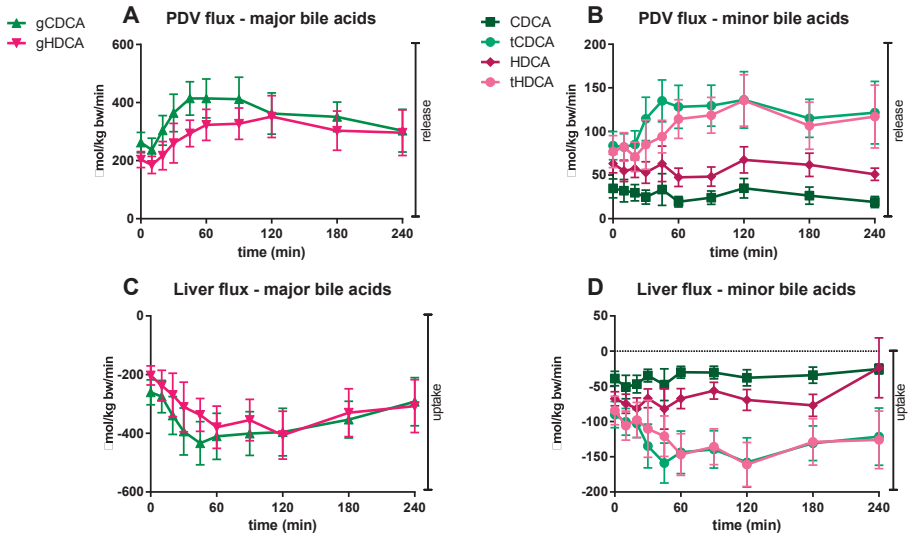

Fig. 5. BAs are released from the intestine and subsequently very efficiently absorbed by the liver. (A and B) After meal intake the BA flux over the portal drained viscera (PDV) steeply increases. A positive flux denotes net production by the organs. Data represent mean ± SEM with N = 11. (C and D) Postprandially, the liver takes up most released BAs. A negative flux reflects net uptake by the organ. CDCA: chenodeoxycholic acid, HDCA: hyodeoxycholic acid, g: glycine-conjugated form, t: taurine-conjugated form. Data represent mean ± SEM with N = 9.

Peripheral exposure to the secondary BA with the highest TGR5 affinity is low

Lithocholic acid (LCA) is a secondary BA that has a high affinity for TGR5. Therefore, LCA had our special interest. In the fasted state unconjugated LCA concentration in the portal vein was approximately two times higher than gLCA and tLCA (Fig. 4A). In general, LCA and its conjugates were detected in all portal

samples, but not in all samples from other sampling sites. After the meal, portal gLCA concentration showed a robust postprandial peak, in contrast to LCA and tLCA concentrations that did not change compared to the fasted state (Fig. 4A). PDV and liver flux curves resembled the postprandial concentration curves, although, because of the biliary secretion, the liver flux was negative (Fig. 4B). Therefore, analogous to primary BAs, the glycine-conjugated forms increase after the ingestion of a meal. In the peripheral circulation LCA concentration was five times lower compared to the portal vein (Fig. 4A, $p < 0.01$). Conjugated forms of LCA were undetectable.

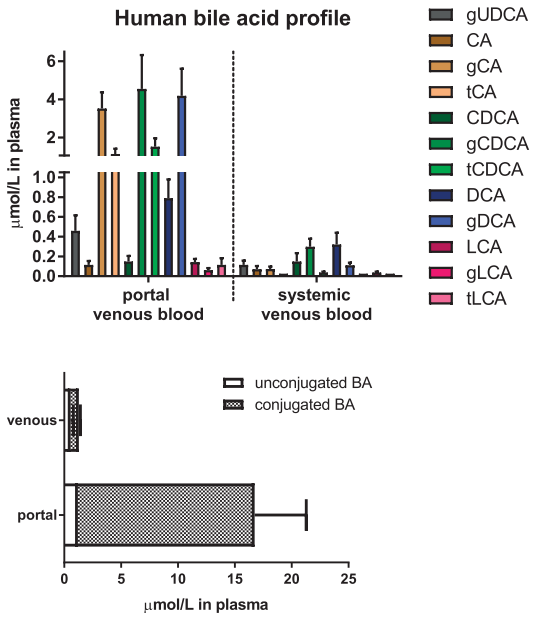

Fig. 6. Portal and peripheral plasma BA profile of nondiabetic obese humans. Portal BA concentrations are much higher than peripheral plasma concentrations. In humans most of the BAs in plasma are conjugated to glycine. UDCA: ursodeoxycholic acid, CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, LCA: lithocholic acid, g: glycine-conjugated, t: taurine-conjugated. Data represented as mean ± SEM. N = 11.

Translating the porcine BA profile to humans

We translated our porcine findings to humans using portal and peripheral blood samples of patients during bariatric surgery. All subjects were obese, but none of the subjects had type 2 diabetes mellitus (mean ± standard deviation: fasting plasma glucose: 5.6 ± 0.5 mmol/L; fasting plasma insulin: 73 ± 31 pmol/L).

Total BA concentrations were significantly higher in the portal vein compared to the peripheral circulation: 7.43 ± 7.2 vs 0.68 ± 0.5 mmol/ L, respectively (p 0.01). In peripheral veins BA concentrations were low and consisted of conjugated and unconjugated forms (Fig. 6). Portal BA concentrations were high and consisted mainly of conjugated forms, whereas the concentrations of unconjugated BAs were similar to those in the periphery (Fig. 6). Similar to pigs, LCA and conjugates were only detected in the portal vein. However, the human BA profile contained another secondary BA, i.e., deoxycholic acid (DCA), that circulated in the portal vein and the periphery and has been associated to insulin sensitivity [20-22].

Discussion

The aim of this study was to quantify porto-peripheral postprandial plasma BA profiles and transorgan flux. Fasting and postprandial peripheral and portal BA concentrations and fluxes are substantial which is supported by the enterohepatic cycle turnover of 12 times per day [23,45]. Physiological cycling of the BA pool may have advantages. In general, metabolic or endocrine cycles enable rapid physiological adaptations when demanded. In the case of the enterohepatic cycle, continuous cycling permits a swift increase of BAs (i.e., concentration) when needed postprandially. Continuous cycling of BAs may also prevent unlimited BA synthesis via FGF15/19 effects on CYP7A1, which is the rate-limiting enzyme of the major classical pathway in BA biosynthesis [24,25]. Liver and intestinal FXR KO murine models have shown that Cyp7a1 repression depends mainly on intestinal FXR activation via FGF15 [26,27]. Additionally, BAs inhibit Cyp7a1 via FXR and small heterodimer partner (SHP) in the liver [3,28,29].

The postprandial BA curves showed a large variability. La Russo et al. found that in humans peripheral conjugated CA forms show modest intra-individual variability with respect to the time of peak, however, peak height in these subjects showed up to ~30% variation [5]. Steiner et al. describe in detail the intra- and inter-individual variability in daily peripheral plasma BA concentrations of 4 healthy humans [30]. Intra-individual variability of postprandial BA curves and its determinants (e.g., gut luminal BA appearance, gut microbioma characteristics, BA transporter genotypes, BA synthetic capacity, gut motility and others) need to be investigated in future studies.

We focused on human BAs and the porcine BA profile mainly consists of CDCA and HDCA forms. CDCA is a primary BA, whereas HDCA may be both primary (since it was detected in germ free pigs [31]) and secondary. Notably, the concentration of the unconjugated forms was unaffected by the ingestion of a meal. So the postprandial response was mainly explained by the glycine-conjugated forms of CDCA and HDCA. Differences in the postprandial response between conjugated and unconjugated BAs may be due to microbiota and the affinity of the BA forms for their transporters [1]. We found that conjugated BAs are more efficiently cleared than their unconjugated forms, showing the high effectiveness and expression of NTCP in the liver.

HDCA cannot diffuse passively and is only a substrate for OATP1, but not for NTCP, which might explain the relative lower HDCA clearance compared to CDCA forms [32]. Despite the large increase in conjugated BA flux through the enterohepatic pathway after food intake, hepatic clearance of the BAs remained stable. The unconjugated BAs flow through the enterohepatic cycle passively regardless of food status and do not appear to have an additional role in postprandial fat digestion [33].

LCA forms have the highest TGR5 affinity, but are often ignored in humans because of their low peripheral plasma concentrations. In the pigs, we showed that unconjugated LCA outnumbered conjugated LCA forms in contrast to HDCA and CDCA forms. The unconjugated LCA can either be derived from deconjugation and transformation of CDCA, or deconjugation of gLCA or tLCA by gut bacteria [34]. LCA was already high in fasted samples and did not show a postprandial change, again indicating ongoing cycling and deconjugation without food intake. However, gLCA showed an increase after the meal, which is likely to rely on early re-uptake of gLCA that has been excreted by the liver. The human LCA conjugation profile was very much comparable to the porcine profile enabling translation. In the enterohepatic circulation the LCA forms and concentrations are likely sufficient to signal TGR5 in intestinal L-cells or liver Kupffer cells.

The porcine BA forms, i.e., HDCA, are not found in humans, whereas pigs do not have DCA which has a relatively high TGR5 affinity [11]. To speculate on the potential of the plasma bile acid pool to activate TGR5 we used a simplified method to calculate the hypothetical TGR5 activating capacity for which we used the plasma BA concentration and the published EC_{50} of the BAs for TGR5

(Fig. 7) [11]. This superficial approach does not take into account the shape and range of linearity of the concentrationeactivation curve and the resulting measure overestimates TGR5 activity since BA concentrations were below their EC_{50} value. However, it shows that TGR5 activation potential may be much higher in the portal vein than elsewhere, most likely because concentrations are also much higher in the portal vein. Interestingly, calculated human TGR5 activation capacities were not different from pigs, whereas the pool composition is very different. This could be explained by the fact that pigs had higher concentrations of BAs, including LCA, and thus similar TGR5 signalling.

Postprandial BAs have been studied to a limited extend, however this is the most sizable study so far in terms of postprandial time points, number of animals/humans and number of individual LC/MS/MS BAs analyses [7]. We did not perform activating calculations for FXR since the necessary data are not uniformly published for this receptor [8,35]. Alternatively, a more uniform hydrophobicity index has been used to qualify BAs, but these data are not available for all BAs [36-38]. In addition, FXR is located in the nucleus, which complicates the calculation of potential FXR activation with plasma BA concentrations even more.

Our study has strengths and weaknesses. The porcine model is suitable for diabetes and metabolic research because it mimics human physiology and pathophysiology in many respects [12]. More importantly, the pig has a dayenight rhythm comparable to humans, is omnivorous and its intestinal transit time and efficiency of digestion resemble that of humans [39]. In an effort to translate the porcine data to humans, we included healthy obese subjects undergoing bariatric surgery. Fasting plasma BA concentrations are not different in obese subjects despite lower postprandial concentrations [40,41]. BMI is positively correlated to total BA concentrations and markers for hepatic BA synthesis in plasma [41,42]. So the human data presented in this study may alter from (postprandial) BA metabolism in lean subjects. Our speculation that TGR5 activation is greater within the entero-hepatic cycle would greatly benefit from additional measurements in the pigs such as plasma GLP-1 concentrations since intestinal TGR5 activation triggers release of GLP-1 [43]. In addition, FGF19 concentrations would be interesting to indicate activation of the FXR-FGF19 axis and its beneficial effects on liver metabolism [43]. Unfortunately, the numerous blood samples limited the amount of blood per sample. Feeding pigs via a gastric tube may have prevented a normal postprandial hedonic response, but the amount and composition of the meal still elicited a robust physiological

postprandial response. Finally, animals received antimicrobial therapy peri-operatively and this may have affected BA pool composition although this has not been reported previously for the antibiotics that we used.

In conclusion, the liver selectively extracts most BAs and BAs with high TGR5 affinity are typically low in the porcine and human peripheral circulation. Our data do not preclude a role for BAs to activate TGR5 in the periphery postprandially. However, their low peripheral plasma concentrations and high interindividual variability raise questions about the magnitude of such a signal and the ultimate clinical application of TGR5 agonists if the liver is not passed [44].

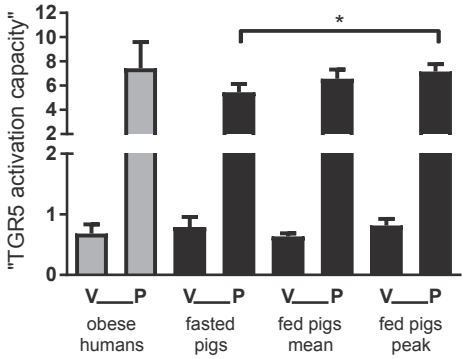

Fig. 7. Hypothetical TGR5 activating capacity is higher for the portal vein compared to the periphery, independently of food intake. This measure expresses in one number the potential amount of TGR5 activation by the total bile acid pool and is calculated for individual BAs in the pool by dividing the average concentration by their EC_{50}, whereafter all are totalled. The value is lower in a peripheral vein (V) compared to the portal vein (P) in healthy obese humans (grey) and in pigs (black). There are no differences between the fasted and the postprandial state (fed pigs vs fasted pigs). However, when comparing the fasted state to the peak of the postprandial concentration curve (fed pigs peak) food intake caused a significant increase in the TGR5 activating potential of over 30%. Data represent mean ± SEM. Porcine outcomes fasted vs fed staged were tested with a one-way ANOVA and Tukey post-hoc test, *$p < 0.05$.

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References

1. Dawson PA, Lan T, Rao A. Bile acid transporters. J Lipid Res 2009;50(12): 2340-57.
2. Kullak-Ublick GA, Stieger B, Hagenbuch B, Meier PJ. Hepatic transport of bile salts. Semin Liver Dis 2000;20(3):273-92.
3. Kuipers F, Bloks VW, Groen AK. Beyond intestinal soapebile acids in metabolic control. Nat Rev Endocrinol 2014;10(8):488-98.
4. Angelin B, Bjorkhem I. Postprandial serum bile acids in healthy man. Evidence for differences in absorptive pattern between individual bile acids. Gut 1977;18(8):606-9.
5. LaRusso NF, Hoffman NE, Korman MG, Hofmann AF, Cowen AE. Determinants of fasting and postprandial serum bile acid levels in healthy man. Am J Dig Dis 1978;23(5):385-91.
6. Schalm SW, LaRusso NF, Hofmann AF, Hoffman NE, van Berge-Henegouwen GP, Korman MG. Diurnal serum levels of primary conjugated bile acids. Assessment by specific radioimmunoassays for conjugates of cholic and chenodeoxycholic acid. Gut 1978;19(11):1006-14.
7. Angelin B, Bjorkhem I, Einarsson K, Ewerth S. Hepatic uptake of bile acids in man. Fasting and postprandial concentrations of individual bile acids in portal venous and systemic blood serum. J Clin Invest 1982;70(4):724-31.
8. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284(5418):1365-8.
9. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, et al. Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun 2002;298(5):714-9.
10. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, et al. A G protein-coupled receptor responsive to bile acids. J Biol Chem 2003;278(11): 9435-40.
11. Sato H, Macchiarulo A, Thomas C, Gioiello A, Une M, Hofmann AF, et al. Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. J Med Chem 2008;51(6):1831-41.
12. Swindle MM, Makin A, Herron AJ, Clubb Jr FJ, Frazier KS. Swine as models in biomedical research and toxicology testing. Vet Pathol 2012;49(2):344-56.
13. Schooneman MG, Ten Have GA, van Vlies N, Houten SM, Deutz NE, Soeters MR. Transorgan fluxes in a porcine model reveal a central role for liver in acylcarnitine metabolism. Am J Physiol Endocrinol Metab 2015;309(3):E256-64.
14. Deutz NE, Ten Have GA, Soeters PB, Moughan PJ. Increased intestinal aminoacid retention from the addition of carbohydrates to a meal. Clin Nutr 1995;14(6):354-64.
15. Ten Have GA, Bost MC, Suyk-Wierts JC, van den Bogaard AE, Deutz NE. Simultaneous measurement of metabolic flux in portally-drained viscera, liver, spleen, kidney and hindquarter in the conscious pig. Lab Anim 1996;30(4):347-58.
16. Agarwal R. Rapid microplate method for PAH estimation. Am J Physiol Renal Physiol 2002;283(2):F236-41.
17. Legrand-Defretin V, Juste C, Henry R, Corring T. Ion-pair high-performance liquid chromatography of bile salt conjugates: application to pig bile. Lipids 1991;26(8):578-83.
18. Ferezou J, Riottot M, Serougne C, Cohen-Solal C, Catala I, Alquier C, et al. Hypocholesterolemic action of beta-cyclodextrin and its effects on cholesterol metabolism in pigs fed a cholesterol-enriched diet. J Lipid Res 1997;38(1):86-100.
19. Fontana L, Eagon JC, Trujillo ME, Scherer PE, Klein S. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. Diabetes 2007;56(4):1010-3.
20. Brufau G, Bahr MJ, Staels B, Claudel T, Ockenga J, Boker KH, et al. Plasma bile acids are not associated with energy metabolism in humans. Nutr Metab (Lond) 2010;7:73.
21. Cariou B, Chetiveaux M, Zair Y, Pouteau E, Disse E, Guyomarc'h-Delasalle B, et al. Fasting plasma chenodeoxycholic acid and cholic acid concentrations are inversely correlated with insulin sensitivity in adults. Nutr Metab (Lond) 2011;8(1):48.
22. Haeusler RA, Astiarraga B, Camastra S, Accili D, Ferrannini E. Human insulin resistance is associated with increased plasma levels of 12alpha-hydroxylated bile acids. Diabetes 2013;62(12):4184-91.

23. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev 2009;89(1):147-91.
24. Holt JA, Luo G, Billin AN, Bisi J, McNeill YY, Kozarsky KF, et al. Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. Genes Dev 2003;17(13):1581-91.
25. Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab 2005;2(4):217-25.
26. Kim I, Ahn SH, Inagaki T, Choi M, Ito S, Guo GL, et al. Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. J Lipid Res 2007;48(12):2664-72.
27. Kong B, Wang L, Chiang JY, Zhang Y, Klaassen CD, Guo GL. Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. Hepatology 2012;56(3):1034-43.
28. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, et al. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell 2000;6(3):517-26.
29. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, et al. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell 2000;6(3):507-15.
30. Steiner C, Othman A, Saely CH, Rein P, Drexel H, von Eckardstein A, et al. Bile acid metabolites in serum: intraindividual variation and associations with coronary heart disease, metabolic syndrome and diabetes mellitus. PLoS One 2011;6(11):e25006.
31. Haslewood GA. Bile salts of germ-free domestic fowl and pigs. Biochem J 1971;123(1):15-8.
32. Hata S, Wang P, Eftychiou N, Ananthanarayanan M, Batta A, Salen G, et al. Substrate specificities of rat oatp1 and ntcp: implications for hepatic organic anion uptake. Am J Physiol Gastrointest Liver Physiol 2003;285(5):G829-39.
33. Hofmann AF. The continuing importance of bile acids in liver and intestinal disease. Arch Intern Med 1999;159(22):2647-58.
34. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci 2008;65(16):2461-83.
35. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, et al. Identification of a nuclear receptor for bile acids. Science 1999;284(5418):1362-5.
36. Heuman DM. Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. J Lipid Res 1989;30(5):719-30.
37. Roda A, Minutello A, Angellotti MA, Fini A. Bile acid structure-activity relationship: evaluation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC. J Lipid Res 1990;31(8):1433-43.
38. Twisk J, Hoekman MF, Muller LM, Iida T, Tamaru T, Ijzerman A, et al. Structural aspects of bile acids involved in the regulation of cholesterol 7 alphahydroxylase and sterol 27-hydroxylase. Eur J Biochem 1995;228(3):596-604.
39. Miller ER, Ullrey DE. The pig as a model for human nutrition. Annu Rev Nutr 1987;7:361-82.
40. Glicksman C, Pournaras DJ, Wright M, Roberts R, Mahon D, Welbourn R, et al. Postprandial plasma bile acid responses in normal weight and obese subjects. Ann Clin Biochem 2010;47(Pt 5):482-4.
41. Haeusler RA, Camastra S, Nannipieri M, Astiarraga B, Castro-Perez J, Xie D, et al. Increased bile acid synthesis and impaired bile acid transport in human obesity. J Clin Endocrinol Metab 2016;101(5):1935-44.
42. Prinz P, Hofmann T, Ahnis A, Elbelt U, Goebel-Stengel M, Klapp BF, et al. Plasma bile acids show a positive correlation with body mass index and are negatively associated with cognitive restraint of eating in obese patients. Front Neurosci 2015;9:199.
43. Jain AK, Stoll B, Burrin DG, Holst JJ, Moore DD. Enteral bile acid treatment improves parenteral nutrition-related liver disease and intestinal mucosal atrophy in neonatal pigs. Am J Physiol Gastrointest Liver Physiol 2012;302(2): G218-24.
44. van Nierop FS, Scheltema MJ, Eggink HM, Pols TW, Sonne DP, Knop FK, et al. Clinical relevance of the bile acid receptor TGR5 in metabolism. Lancet Diabetes Endocrinol 2017 Mar;5(3):224-33.
45. Mok HY, Von Bergmann K, Grundy SM. Regulation of pool size of bile acids in man. Gastroenterology 1977;73(4 Pt 1):684-90.

Part III

Intervention

4

Effects of acute dietary weight loss on postprandial plasma bile acid responses in obese insulin resistant subjects

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Abstract

Background & aims: Bile acids (BA) are pleiotropic hormones affecting glucose and lipid metabolism. The physiochemical properties of different BA species affect their enterohepatic dynamics and their affinity for bile acid receptors. The BA pool composition is altered in patients with type 2 diabetes and obesity. In this study we used a 2-week very-low-calorie diet (VLCD) to investigate the effects of weight loss on BA pool composition and postprandial dynamics.

Methods: We performed mixed meal tests in obese, insulin resistant subjects before and after the VLCD. We measured postprandial plasma levels of glucose, insulin, BA and the BA-induced enterokine fibroblast growth factor 19 (FGF19).

Results: The VLCD decreased weight by 4.5 ± 2.3 kg ($p < 0.0001$) within 14 days. Weight loss increased peak postprandial deoxycholate (DCA) levels (median [IQR]: 0.90 [0.90] vs. 1.25 [1.35] μmol/L; $p = 0.045$*). Other BA species, glucose, insulin and FGF19 levels and prandial excursions were not significantly affected. The VLCD decreased resting and postprandial energy expenditure by 7 and 11% respectively.

Conclusions: VLCD induced weight loss increased postprandial DCA peak levels and decreased resting energy expenditure in obese insulin resistant subjects.

Abbreviations: BA, bile acid; CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodexocholic acid.

Introduction

BA are increasingly recognized as pleiotropic hormones with effects on glucose and lipid metabolism, in addition to their role as intestinal emulgators [1]. This has led to multiple observations that BA metabolism is altered in metabolic disease such as obesity and type 2 diabetes mellitus [2,3]. Patients with type 2 diabetes mellitus show increased fasting levels of deoxycholic acid (DCA), a secondary BA formed by microbial conversion of cholic acid (CA) [4].

Recently, it was also shown that insulin resistance in healthy human subjects is positively correlated to levels of a certain subset of BA that are hydroxylated at the 12α position (12α-OH BA, i.e. CA and DCA) [5]. It is presently unclear whether these changes are causally involved in insulin resistance. Roux-en-Y gastric bypass surgery also increase plasma BA levels. Therefore, BA are putative mediators for the beneficial effects on metabolism and body weight present after bariatric surgery [6].

Primary BA are synthesized from cholesterol in the hepatocyte and subsequently conjugated with glycine (G) or taurine (T), yielding glyco- and tauro-conjugates of CA and chenodeoxycholate (CDCA). Exposure to microbiota in the distal ileum and colon leads to deconjugation and dehydroxylation of CA and CDCA yielding the secondary BA DCA and lithocholic acid (LCA), respectively. Intestinal reabsorption from the intestinal lumen and hepatic extraction from the portal circulation occur through passive and active transport. Subsequently, hepatic return completes the enterohepatic cycle. BA enter the systemic circulation in a pattern of postprandial peaks followed by lows, periodically exposing peripheral tissues to concentrations that may be high enough to activate the Farnesoid X Receptor (FXR) and G-protein-coupled transmembrane BA receptor (GPBAR1/TGR5) [7-10].

These BA receptors are present on tissues inside and outside the trajectory of the enterohepatic circulation. FXR is a nuclear receptor that regulates BA homeostasis in the liver, amongst others by exerting negative feedback control of bile salt synthesis. In contrast, TGR5 is a transmembrane receptor that is abundantly expressed in the gut, brown adipose tissue (BAT) and skeletal muscle [7,8]. Activation of FXR and TGR5 induces secretion of fibroblast growth factor 19 (FGF19) from enterocytes

and glucagon-like peptide-1 (GLP-1) from enteroendocrine L-cells, respectively [11-13]. In turn, FGF19 suppresses hepatic BA synthesis and gluconeogenesis via the FGF receptor 4 (FGFR4). GLP-1 increases the amount of insulin that is released by the pancreatic beta cell in response to glucose [14] making it useful in diabetes therapy. Possibly, BA can also directly stimulate beta cell insulin release via both FXR and TGR5 [15,16].

Is had been shown that postprandial circulating glycine-conjugated BAs are lower in obese subjects, but it is unknown whether this is reversible [3]. We assessed the effects of a twoweek very low calorie diet (VLCD) weight loss intervention on fasting and postprandial plasma BA profiles.

Materials and methods

Subjects

We recruited a total of 12 male and female insulin resistant obese subjects (HOMA-IR >2.7, BMI >30 kg/m^2, age 18-55 years). Exclusion criteria were any previous surgery or current diseases of the liver, biliary or gastrointestinal tract; ethanol abuse; weight loss or weight gain in excess of 10% of body weight in the 6 months prior to start of the study; use of any medication or herbal supplement; fasting plasma glucose >7.0 mmol/L, HbA1c > 53 mmol/mol, creatinine >120 mM, or abnormal renal, liver or thyroid function defined as >2 times the upper limit of the reference interval. Written informed consent was obtained from all subjects before start of the study. The study was approved by the AMC Medical Ethics Committee. The study was conducted in accordance with the principles of the Declaration of Helsinki (sixth revision, 2008).

Hypocaloric VLCD diet

Subjects were instructed to follow a 450 kcal/day diet for 14 days. The diet consisted of 3 sachets of a liquid meal replacement formula (Modifast Intensive®, Nutrition et Sante´, France). Modifast Intensive® is a commercially available diet product, supplying 51% of energy as carbohydrates, 20% as protein and 29% as fat. Subjects were encouraged to complement the diet with low-caloric raw vegetables. Daily phone calls were conducted by the investigators to ensure diet compliance.

In order to counteract the insulin-desensitizing effects of prolonged fasting on glucose and lipid metabolism, the diet period was followed by three days on a eucaloric free diet [17]. Subjects were instructed to eat their estimated daily caloric need (estimated using the Harris Benedict equation).

Study design

The study was performed between May 2012 and February 2014 at the Department of Endocrinology and Metabolism of the Academic Medical Center Amsterdam. On study days, subjects were admitted at 07:30 h to the Metabolic Unit after an overnight fast. A cannula was inserted into an antecubital vein for blood sampling. This hand was kept in a heated hand box throughout the test to arterialize venous blood. At 09:30 h, 3 blood samples were taken at 10-minute intervals for the determination of basal plasma glucose and insulin concentrations. At 10:00 h, subjects consumed a standard meal consisting of 50 g of parmesan cheese, 60 g of boiled egg and 75 g of glucose dissolved in 200 ml water (559 kcal; 30% from fat, 16% from protein, 54% from carbohydrates), after which blood samples were obtained at 0, 15, 30, 45, 60, 75, 90, 120, 150, 180 and 240 min after the meal. Blood was collected into chilled tubes containing either EDTA or Heparin as anticoagulant on ice and immediately centrifuged, and plasma was subsequently stored at 20 °C until analysis. For GLP-1 assays, a dipeptidyl peptidase inhibitor (Ile-Pro-Ile, Sigma-Aldrich, St. Louis, MO, USA) was added at 0.01 mg/ml and plasma was stored at 80 °C. Resting energy expenditure was measured for a 10 min-interval at baseline, 90 and 240 min after the meal by indirect calorimetry using a ventilated hood system (Vmax Encore 29; SensorMedics, Anaheim, CA). Energy expenditure was calculated as described by Frayn [18]. The abbreviated Weir equation was used to calculate 24-hour energy expenditure.

Laboratory analysis

Plasma glucose concentrations were analyzed bedside using the glucose oxidation method (EKF Diagnostics, Barleben/Magdeburg, Germany). Insulin was determined on an IMMULITE 2000 system (Siemens Healthcare Diagnostics, Breda, the Netherlands). GLP-1 concentrations were measured by ELISA using a commercially available assay (EMD Millipore, Billerica, MA, USA). BA were determined using a UPLC-tandem MS method to detect CA, CDCA, DCA and UDCA in their conjugated and unconjugated forms [19]. FGF19 was measured using an in-house developed ELISA as published previously [20].

Calculations and statistical analysis

HOMA-IR was calculated from fasting plasma glucose (FPG) and fasting plasma insulin (FPI) as described by Wallace and Matthews [21]. Fractions of a particular BA species were calculated as the sum of individual measurements of nonconjugated and glycine- and taurine-conjugated forms. Total BA were calculated by adding up all individual BA measurements. Area-under-the-curve (AUC) and incremental AUC (iAUC) were calculated using the trapezoidal method. Statistical analysis was performed using IBM SPSS Statistics 22 (IBM, Armonk, NY, USA). Data was visually and statistically assessed for normality and logarithmically transformed were appropriate. Comparisons between 2 test conditions were made using either paired t-testing for normally distributed data or Wilcoxon matched-pairs signed rank testing for nonparametric data. Correlations were assessed using Pearson's correlation for normally distributed populations or Spearman's Rho for nonparametric data. Data presented are mean and standard deviation (m ± s) for normally distributed variables or median and interquartile range (m [IQR]) for other variables. Graphs were made using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Baseline

We included 12 obese, insulin resistant subjects. Subject characteristics and clinical chemistry pre- and post-VLCD are summarized in Table 1.

Table 1: Study participants.

	Pre-VLCD	Post-VLCD	p
N	12	12	-
Sex (f/m)	4/8	-	-
Age (y)	43.5 ± 14.2	-	-
Body weight (kg)	126 ± 7	121 ± 7	**0.0001**
BMI (kg/m^2)	39.8 ± 2.0	38.4 ± 2.0	**0.0001**
FPG (mmol/L)	4.5 ± 0.2	4.7 ± 0.1	0.78
FPI (pmol/L)	119 ± 11	122 ± 18	0.93
HOMA-IR	3.5 ± 0.4	3.7 ± 0.5	0.67
HbA1c (mmol/mol)	39 ± 1	-	-

Values are presented as m ± SE.

All subjects completed the entire VLCD period in full compliance with the diet. Mean weight loss was 4.5 ± 2.3 kg ($p < 0.0001$). There were no significant effects of the diet on fasting plasma glucose (4.6 ± 0.6 vs. 4.7 ± 0.4 mmol/L; $p = 0.78$) or insulin (119 ± 36 vs. 122 ± 58 pmol/L; $p = 0.94$), which was reflected in unaltered HOMA-IR (3.5 ± 1.2 vs. 3.7 ± 1.7 mmol/L; $p = 0.67$).

Meal test

Postprandial glucose and insulin excursions were unaffected by the VLCD (Fig. 1). Neither peak levels (insulin: 1313 [892] vs. 1570 [964] pmol/L; $p = 0.75$; glucose: 7.8 [1.9] vs. 7.8 [0.9] mmol/L; $p = 0.90$) nor AUC (insulin: 158100 [88793] vs. 180273 [131372] pmol min L^{-1}; $p = 0.70$; glucose: 1368 [190] vs. 1310 [130] mmol min L^{-1}; $p = 0.39$) showed any significant effect of the diet for either plasma glucose or insulin.

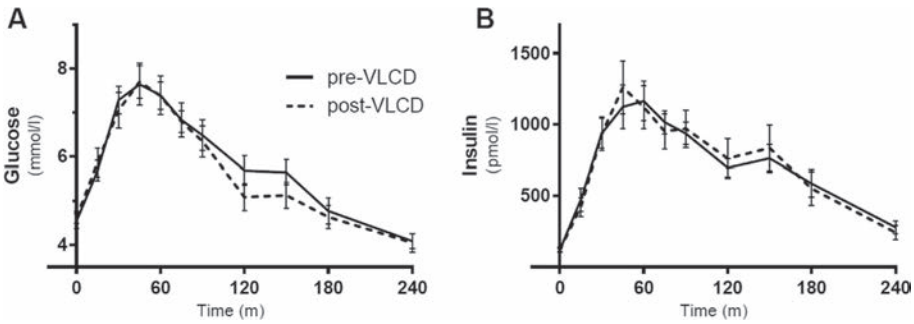

Fig. 1. Postprandial glucose (A) and insulin (B) concentrations were not affected by the VLCD. A mixed meal was consumed at T = 0 min. Values are mean ± SEM.

BA and FGF19

Postprandial BA curves are shown in Fig. 2. Several derivative measures of postprandial plasma BA curves are listed as a 'bilogram' in Table 2. The VLCD increased peak levels of total DCA (0.90 [0.90] vs. 1.25 [1.35] µmol/L; $p = 0.045$*). However, the VLCD did not alter the iAUC of total DCA and baseline, peak, AUC or iAUC of other BA fractions (total BA, total CA, total CDCA, glycine-conjugated BA, taurine-conjugated BA, unconjugated BA and 12α-OH BA). Additional BA species concentrations are listed in Supplementary Table S1. FGF19 levels in plasma were identical at baseline and increased postprandially on both test days (Fig. 3). There was no significant increase in AUC, iAUC or postprandial peak height after the intervention (see Table 2, iAUC not shown).

Table 2: Bilogram and FGF 19.

Bile acid fraction		Pre-VLCD	Post-VLCD	p
Total bile acids (mmol/L)	baseline	0.80 (1.25)	1.05 (1.08)	0.87
	peak	3.80 (3.32)	4.05 (2.95)	0.46
	AUC	517 (400)	517 (227)	0.72
Total CA (mmol/L)	baseline	0.10 (0.18)	0.15 (0.28)	0.75
	peak	0.60 (0.90)	0.85 (0.88)	0.82
	AUC	92 (81)	65 (129)	0.94
Total CDCA (mmol/L)	baseline	0.40 (0.38)	0.40 (0.55)	0.54
	peak	2.00 (1.80)	2.00 (1.53)	0.70
	AUC	257 (226)	231 (79)	1.00
Total DCA (mmol/L)	baseline	0.25 (0.28)	0.30 (0.53)	0.12
	peak	0.90 (0.90)	1.25 (1.35)	**0.045***
	AUC	146 (139)	162 (149)	0.23
Total Glycine-conjugated BA (mmol/L)	baseline	0.30 (0.40)	0.55 (0.55)	0.53
	peak	2.90 (2.68)	2.60 (2.68)	0.67
	AUC	357 (314)	313 (87)	0.75
Total Taurine-conjugated BA (mmol/L)	baseline	0.00 (0.15)	0.00 (0.10)	0.75
	peak	0.35 (0.53)	0.40 (0.80)	0.51
	AUC	64 (49)	53 (48)	0.79
Total unconjugated BA (mmol/L)	baseline	0.30 (0.58)	0.50 (0.55)	0.64
	peak	0.65 (0.98)	1.05 (1.83)	0.72
	AUC	91 (195)	146 (188)	0.70
Total 6 BA (mmol/L)	baseline	0.40 (0.80)	0.45 (1.53)	0.58
	peak	1.65 (1.53)	2.50 (2.30)	0.39
	AUC	242 (185)	237 (269)	0.43
FGF19 (ng/mL)	baseline	0.09 (0.06)	0.10 (0.11)	0.26
	peak	0.29 (0.30)	0.32 (0.28)	0.91
	AUC	56.5 (46.0)	40.8 (34.2)	0.31

Values are presented as median (IQR). The unit of bile acid peak and baseline values is mmol/L, the unit of AUC is mmol min L^{-1}. P-values were calculated using the related-samples Wilcoxon Signed Rank test. * denotes $p < 0.05$.

Table 3: Indirect calorimetry.

Pre-VLCD	Post-VLCD	p		
REE/kg	fasting	17.9 (7.2)	18.0 (4.3)	**0.04**
kcal	1.5 h postprandial	22.8 (6.1)	20.2 (2.8)	**0.02**
	4 h postprandial	19.1 (6.4)	18.6 (4.0)	**0.03**
REE total	fasting	1938 (736)	1810 (356)	**0.03**
kcal	1.5 h postprandial	2210 (594)	1959 (273)	**0.02**
	4 h postprandial	2047 (638)	1859 (377)	**0.03**
RQ	fasting	0.84 (0.08)	0.79 (0.06)	0.12
	1.5 h postprandial	0.83 (0.06)	0.79 (0.07)	0.65
	4 h postprandial	0.82 (0.04)	0.81 (0.04)	0.35

Values are presented as median (IQR). P-values were calculated using the related samples Wilcoxon Signed Rank test.

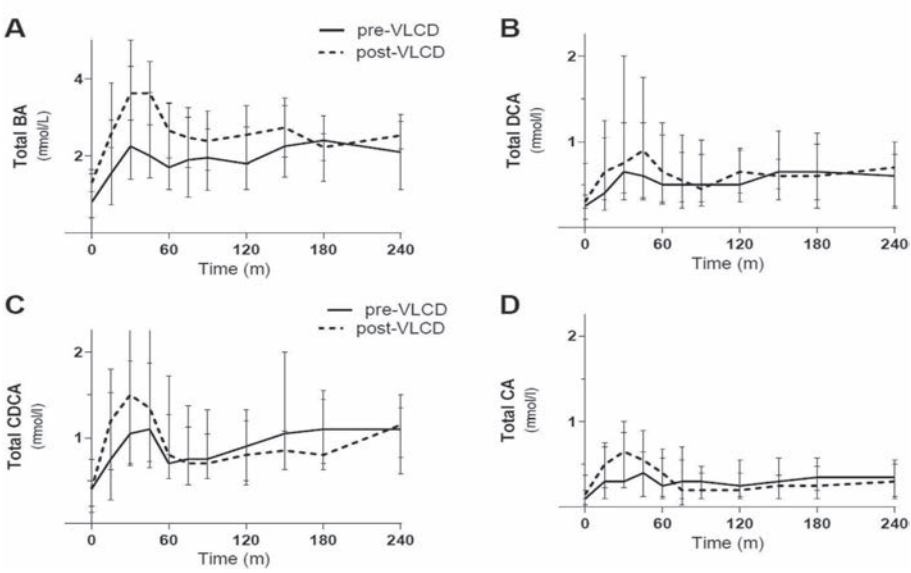

Fig. 2. Postprandial concentrations of total BA (A), total DCA (B), total CDCA (C) and total CA. A mixed meal was consumed at T = 0 min. Values reported are median [IQR]. Totals reflect grouped unconjugated and glycine- and taurine conjugates of the respective BA species.

Energy expenditure

The VLCD decreased both total resting energy expenditure (REE) and REE per kg body weight (Table 3). Respiratory quotient (RQ) did not change during the course of the meal test, and baseline values were similar before and after the VLCD.

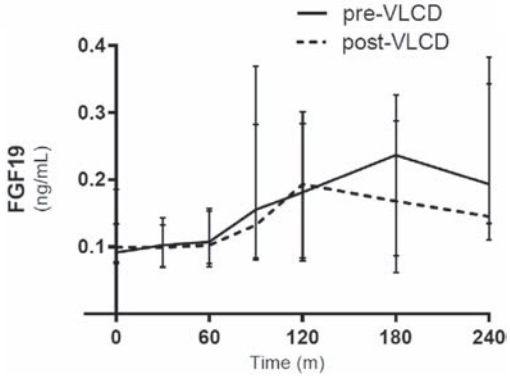

Fig. 3. Postprandial FGF19 concentrations at individual timepoints, iAUC and AUC were not affected by the intervention. Values reported are median [IQR].

Discussion

Here, we studied the effects of dietary short-term weight loss on plasma BA composition in obese insulin resistant subjects. The 3-day eucaloric period following the hypocaloric diet phase allowed us to isolate the effects of weight loss from the generally insulin desensitizing effects of prolonged energy deficit [22]. We found that among BA, dietary weight loss by VLCD in this study modestly increased only postprandial peak levels of deoxycholic acid. Figure 2 suggests higher BA concentrations in the first hour after the test meal, but this was not found to be significant (data not shown).

Postprandial BA levels in the systemic circulation are determined by the uptake rate from the gut and hepatic uptake from the portal vein. Uptake from the gut is by passive and active transluminal transport, where the latter depends on the ileal enterocytic BA transporter apical sodium-dependent BA transporter (ASBT) [23]. Hepatic uptake from the portal blood relies on expression of Na^+-taurocholate co-transporting polypeptide (NTCP) and members of the organic anion transport family. Hepatic uptake via NTCP has a substrate preference for trihydroxylated (i.e. CA in humans) over dihydroxylated BA (i.e. CDCA and DCA), and for conjugated over unconjugated BA as reported by Hofmann [24]. A preferential decrease in DCA uptake over other BA therefore seems unlikely; thus the DCA specific increase in our study may be attributed to an increased DCA uptake from the gut.

The modest increase of peak DCA levels that we found is supported by different studies that have shown DCA responsiveness to different interventions [4,6,25]. Increased gut DCA uptake can be explained by a variety of non-mutually exclusive ways. First, the proportion of DCA in the total BA pool could be increased, similar to the BA pool of patients with T2DM. That in turn leads to increased postprandial DCA concentrations in plasma. The changes in the circulating BA pool could be driven by changes in the microbiome under the influence of caloric restriction, leading to increased rates of conversion of CA into DCA. That would also explain why the increase in DCA was mainly composed of unconjugated DCA (Supplementary Table S1). The early phase of the postprandial bile acid response reflects the influx of bile acids that are stored in the intestine in the fasting state. We showed previously that vancomycin treatment selectively modulated the gut

microbiome and subsequently decreases postprandial plasma DCA levels and peripheral insulin sensitivity [25]. Alternatively, increased activity of the sterol 12α-hydroxylase CYP8B1 would shift de novo BA synthesis towards production of CA, the precursor of microbiota-generated DCA [26]. CYP8B1 is under control of various signals amongst which insulin is a potent suppressor [26]. We cannot exclude the possibility that during the hypocaloric diet insulin sensitivity may have been lower thereby repressing CYP8B1 to a lesser degree.

Interestingly, several studies indicate that obese, insulin resistant subject have increased fasting levels of DCA [2,4], with one group finding decreased total BA in obese subjects [3]. Brufau and colleagues showed by using stable isotope tracers that obese patients with type 2 diabetes mellitus exhibit increased CA synthesis, increased rates of DCA input and consequentially an enlarged DCA pool [4]. Haeusler et al. expanded this observation to healthy human subjects. In a large cohort of nondiabetic subjects, insulin resistance assessed by euglycemic-hyperinsulinemic clamp studies was associated with an increase in fasting levels of 12αOH-BA, to which DCA was the largest contributor [5]. In a follow-up study, they showed that 12αOH-BA synthesis was preferentially increased in obese, insulin resistant subjects, leading to higher fasting and peak levels of total BA [2]. They associated these changes to decreased expression of bile acid transporters in liver biopsies from patients undergoing Roux-en-Y gastric bypass, with these bile salt transporters hypothesized to be under acute control of insulin signaling. In light of these studies, it is counterintuitive that plasma DCA levels are high in obese and diabetic subjects and increase further after weight loss. Although we cannot explain this finding, it is likely that activity of BA transporters, expression of CYP8B1 and the gut microbiome all play a role.

Energy expenditure was decreased by the diet, which is consistent with the large body of literature describing decreased basal metabolism during calorie restriction [22]. BA are thought to be able to increase energy expenditure through activation of TGR5-signaling in myocytes and brown adipocytes. In these cell types, TGR5-activation has been shown to lead to increased expression of type 2 deiodinase, which converts thyroid hormone into its active form [27]. This in turn leads to an increase in thermogenesis, resulting in weight loss. Of the circulating BA, DCA is the most effective ligand for TGR5 with an EC50 of 0.79-1.25 mmol/L depending on conjugation state [12]. In our study, despite DCA levels reaching higher

peak levels, energy expenditure went down. Additionally, BA levels and energy expenditure were not correlated (data not shown), demonstrating that the change in BA in our study did not modulate energy expenditure directly or later after the meal. The timing of the energy expenditure measurements did not coincide with the peak in bile acid levels in our studies, which may explain why there was no discernible effect. This does mean that postprandial BA concentrations are unlikely to play a large role in physiological control of total daily energy expenditure. Supra-physiological stimuli might be capable of enhancing energy expenditure, as proposed recently by Broeders and colleagues [28]. Strengths of this study include the paired design, allowing for a relative small sample size and the use of more sensitive statistical methods, the longer timeframe of postprandial plasma sampling compared to previous studies using mixed-meal testing, and the highly sensitive HPLC-MSMS method for measuring all physiologically relevant BA. Limitations of the study include the fact that we did not perform clamp studies to assess insulin sensitivity since we were interested in the postprandial BA curve. Additionally, the gut microbiome may have been altered by the dietary intervention. Finally, our intervention may not have been long enough to change BA levels profoundly. DCA constitutes a large part of the BA pool. Although we have previously seen acute meal effects on postprandial BA curves (unpublished data), slow changes in the composition of the BA pool and a presumably intact negative feedback mechanism via FXR may explain only a modest change after ~11 days of hypocaloric feeding in our subjects.

In conclusion, a short-duration very low calorie diet modestly increased the DCA component of the postprandial BA response in obese subjects while decreasing energy expenditure. Changes in activity of BA transporters, CYP8B1 and the gut microbiome may be involved in this response.

Statement of authorship

FSvN designed the study, performed clinical experiments, laboratory and statistical analyses, and wrote and edited the manuscript. MRS designed the study, reviewed and edited the manuscript. FGS and WK performed laboratory analyses and reviewed the manuscript. JAR and SWOD reviewed the manuscript.

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References

1. Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bileacid signalling for metabolic diseases. Nat Rev Drug Discov 2008;7(8):678-93.
2. Haeusler RA, Camastra S, Nannipieri M, Astiarraga B, Castro-Perez J, Xie D, et al. Increased bile acid synthesis and impaired bile acid transport in human obesity. J Clin Endocrinol Metabolism 2015.
3. Glicksman C, Pournaras DJ, Wright M, Roberts R, Mahon D, Welbourn R, et al. Postprandial plasma bile acid responses in normal weight and obese subjects. Ann Clin Biochem 2010;47(Pt 5):482-4.
4. Brufau G, Stellaard F, Prado K, Bloks V, Jonkers E, Boverhof R, et al. Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism. Hepatology 2010;52(4):1455-64.
5. Haeusler Ra, Astiarraga B, Camastra S, Accili D, Ferrannini E. Human insulin resistance is associated with increased plasma levels of 12α-hydroxylated bile acids. Diabetes 2013;62(12):4184-91.
6. Dutia R, Embrey M, O'Brien S, Haeusler RA, Age´nor KK, Homel P, et al. Temporal changes in bile acid levels and 12α-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes. Int J Obes 2015;39:806-13.
7. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, et al. Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophysical Res Commun 2002;298(5):714-9.
8. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, et al. AG protein-coupled receptor responsive to bile acids. J Biol Chem 2003;278(11): 9435-40.
9. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, et al. Identification of a nuclear receptor for bile acids. Science 1999;284(5418):1362-5.
11. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284(5418):1365-8.
11. Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, et al. TGR5mediated bile acid sensing controls glucose homeostasis. Cell Metab 2009;10(3):167-77.
12. Sato H, Genet C, Strehl A, Thomas C, Lobstein A, Wagner A, et al. Anti-hyperglycemic activity of a TGR5 agonist isolated from Olea europaea. Biochem Biophysical Res Commun 2007;362(4):793-8.
13. Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab 2005;2(4):217-25.
14. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev 2007;87(4): 1409-39.
15. Kumar DP, Rajagopal S, Mahavadi S, Mirshahi F, Grider JR, Murthy KS, et al. Activation of ransmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic beta cells. Biochem Biophysical Res Commun 2012;427(3):600-5.
16. Renga B, Mencarelli A, Vavassori P, Brancaleone V, Fiorucci S. The bile acid sensor FXR regulates insulin transcription and secretion. Biochimica Biophysica Acta 2010;1802(3):363-72.
17. Bergman BC, Cornier M-A, Horton TJ, Bessesen DH. Effects of fasting on insulin action and glucose kinetics in lean and obese men and women. Am J Physiol-Endocrinol Metabolism 2007;293(4):E1103-E11.
18. Frayn KN. Calculation of substrate oxidation from gaseous exchange rates in vivo. J Appl Physiol 1983;55(2):628-34.
19. Bootsma AH, Overmars H, van Rooij A, van Lint AE, Wanders RJ, van Gennip AH, et al. Rapid analysis of conjugated bile acids in plasma using electrospray tandem mass spectrometry: application for selective screening of peroxisomal disorders. J Inherit Metabolic Dis 1999;22(3):307-10.
20. Lips MA, de Groot GH, Berends FJ, et al. Calorie restriction and Roux-en-Y gastric bypass have opposing effects on circulating FGF21 in morbidly obese subjects. Clin Endocrinol 2014;81(6):862-70.
21. Wallace TM, Matthews DR. The assessment of insulin resistance in man. Diabet Med 2002;19(7):527-34.
22. Soeters MR, Soeters PB, Schooneman MG, Houten SM, Romijn JA. Adaptive reciprocity of lipid and glucose metabolism in human short-term starvation. Am J Physiol Endocrinol Metabolism 2012;303(12):E1397-407.

23. Wong MH, Oelkers P, Craddock AL, Dawson PA. Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter. J Biol Chem 1994;269:1340-7.
24. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci CMLS 2008;65(16):2461-83.
25. Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, et al. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. J Hepatol 2014;60(4):824-31.
26. Ishida H, Kuruta Y, Gotoh O, Yamashita C, Yoshida Y, Noshiro M. Structure, evolution, and liver-specific expression of sterol 12α-hydroxylase (CYP8B1). J Biochem 1999;126(1):19-25.
27. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 2006;439(7075):484-9.
28. Broeders EP, Nascimento EB, Havekes B, Brans B, Roumans KH, Tailleux A, et al. The bile acid chenodeoxycholic acid increases human brown adipose tissue activity. Cell Metab 2015:1-9.

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Differential effects of a 40-hour fast and bile acid supplementation on human GLP-1 and FGF19 responses

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Abstract

Bile acids, glucagon-like peptide-1 (GLP-1), and fibroblast growth factor 19 (FGF19) play an important role in postprandial metabolism. In this study, we investigated the postprandial bile acid response in plasma and its relation to insulin, GLP-1, and FGF19. First, we investigated the postprandial response to 40-h fast. Then we administered glycine-conjugated deoxycholic acid (gDCA) with the meal. We performed two separate observational randomized crossover studies on healthy, lean men. In *experiment 1,* we tested 4-h mixed meal after an overnight fast and a 40-h fast. In *experiment 2*, we tested a 4-h mixed meal test with and without gDCA supplementation. Both studies measured postprandial glucose, insulin, bile acids, GLP-1, and FGF19. In *experiment 1*, 40 h of fasting induced insulin resistance and increased postprandial GLP-1 and FGF19 concentrations. After an overnight fast, we observed strong correlations between postprandial insulin and gDCA levels at specific time points. In *experiment 2*, administration of gDCA increased GLP-1 levels and lowered late postprandial glucose without effect on FGF19. Energy expenditure was not affected by gDCA administration. Unexpectedly, 40 h of fasting increased both GLP-1 and FGF19, where the former appeared bile acid independent and the latter bile acid dependent. Second, a single dose of gDCA increased postprandial GLP-1. Therefore, our data add complexity to the physiological regulation of the enterokines GLP-1 and FGF19 by bile acids.

Abbreviations: bile acids; FGF19; GLP-1; type 2 diabetes; postprandial metabolism

Introduction

Bile acids (BA) are known for their role in hepatobiliary cholesterol secretion (37) and detergent properties that enable enteral lipid uptake (28). Recently, BAs have gained interest for their role as hormone-like mediators involved in energy metabolism (33). Their effects are mainly mediated by the nuclear Farnesoid X Receptor (FXR) and the transmembrane Takeda G-protein-coupled receptor (TGR5) (24, 33). BA dynamics in the enterohepatic and systemic circulation are responsive to nutrient intake, and BA receptors are ideally positioned in the digestive system to function as postprandial metabolic integrators by sensing and conveying information about nutrient status (2, 43).

The two main primary human BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized from cholesterol in the hepatocyte, conjugated with either glycine or taurine, and subsequently stored in the gallbladder. After a meal, these BAs facilitate the absorption of lipids and lipid soluble vitamins. The microbiota in the gut are involved in BA deconjugation and dehydroxylation, yielding the secondary BAs deoxycholic acid (DCA) and lithocholic acid from CA and CDCA, respectively. BA reabsorption occurs primarily through active transport in the ileum, and BAs reach the liver via the portal vein (26, 28, 44). A fraction of BAs escapes hepatic clearance and enters the systemic circulation in a pattern of postprandial peaks that may be high enough to activate BA receptors in the systemic circulation (9).

TGR5 is expressed by entero-endocrine L-cells in the intestine, and its activation by BAs results in the release of the incretin glucagon-like peptide 1 (GLP-1) (45). GLP-1, in turn, increases glucose-dependent insulin release from the pancreatic beta cells. BAs may also directly stimulate insulin release, as both FXR and TGR5 are expressed by the beta cell (25, 38). During enterohepatic BA cycling, FXR activation induces a negative feedback loop comprising ileal fibroblast growth factor 19 (FGF19) that represses hepatic BA production.

FGF19 has been suggested to inhibit gluconeogenesis and hepatic lipogenesis, whereas it stimulates glycogen synthesis (22, 36). In contrast, it was recently shown in rodents that FXR is an activator of fasting hepatic gluconeogenesis (35). In humans, administration of a FGF19 analog diminished liver fat without effects on plasma glucose levels (7, 18).

In this study, we investigated the postprandial BA response in plasma in relation to insulin, GLP-1, and FGF19 levels. First, we analyzed the effects of mixed meal tests after an overnight and a 40-h fast as model of insulin resistance, because BA metabolism is altered in various models of insulin resistance (5, 14, 47). We subsequently assessed the effects of oral administration of glycine-conjugated DCA (gDCA) on postprandial glucose, insulin, and GLP-1 levels in a second separate experiment.

Materials and methods

Subjects

We performed two experiments with different subjects. We recruited 9 lean young men in *experiment 1* and 10 lean young men in *experiment 2*. Exclusion criteria were body mass index (BMI) > 25 kg/m^2, history of gallstones or biliary surgery, use of medication, substance abuse (nicotine or drugs, alcohol >2 units/day), liver test abnormalities (aspartate aminotransferase, alanine aminotransferase, bilirubin, gamma-glutamyl transferase, alkaline phosphatase), or abnormal fasted levels of plasma glucose or insulin. Written informed consent was obtained from all subjects before the start of the study procedures, and the studies were approved by the Academic Medical Center (AMC) Medical Ethics Committee (Amsterdam, The Netherlands). The experiments were conducted in accordance with the principles of the Declaration of Helsinki (sixth revision, 2008). *Experiment 1* was filed at the ethical committee under ABR number NL4083401812. *Experiment 2* was prospectively registered at www. trialregister.nl (NTR5849).

Study Design

Both separate, but related, experiments were mono center and conducted on the Experimental and Clinical Research Unit of the AMC, Amsterdam, The Netherlands.

Experiment 1. Each subject (n = 9) had two mixed meal tests on separate study days in a randomly assigned crossover design. Prior to each study day, subjects were instructed to either consume their regular diet followed by an overnight fast leading up to the study day (14 h FAST) or remain fasted for a total of 40 h (40 h FAST).

Experiment 2. Each subject (n = 10) had two mixed meal tests after an overnight fast on separate study days in a randomly assigned crossover design. In balanced assignment, subjects underwent the control meal test (-gDCA) or received 750 mg of gDCA in capsules concomitantly with the standard meal (+gDCA). We administered a similar dose as used for ursodeoxycholic acid in earlier studies (11, 15). gDCA capsules were prepared for each subject individually under responsibility of the AMC hospital pharmacy.

Mixed Meal Test

For both experiments, subjects were admitted to the unit after an overnight or 40-h fast. The liquid test meal (Nutridrink Compact, Nutricia, Zoetermeer, The Netherlands) contained 16% protein, 35% fat, and 49% carbohydrates. Subjects ingested the caloric equivalent of 25% of their estimated daily energy expenditure calculated using the Harris-Benedict equation. The test meal was ingested at time point 0 (t = 0). In both experiments, blood was sampled from a catheter inserted in a forearm vein. In experiment 1, blood samples were obtained at 0, 30, 60, 90, 120, 180, and 240 min after meal ingestion and in experiment 2 at -20, -10, 0, 15, 30, 45, 60, 75, 90, 120, 180, and 240 min. Samples were collected into chilled EDTA or heparincoated tubes on ice and immediately centrifuged and subsequently stored at -20°C until analysis. For GLP-1 samples, a dipeptidyl peptidase inhibitor (Ile-Pro-Ile, Sigma-Aldrich, St. Louis, MO) was added to the collection tube at 0.01 mg/mL, and plasma was stored at -80°C.

Laboratory Analysis

Plasma glucose concentrations were analyzed bedside using the glucose oxidation method (EKF Diagnostics, Barleben/Magdeburg, Germany). Insulin was determined on an Immulite 2000 system (Siemens Healthcare Diagnostics, Breda, The Netherlands). Active GLP-1 concentrations were measured by ELISA (EMD Millipore, Billerica, MA). FGF19 was measured at the University of Maastricht using an in-house developed ELISA as published previously (40).

Bile Acid Analysis

Taurine- and glycine-conjugated internal standards [2,2,4,4-$^2H_4$]taurocholic acid (tauro-CA), [2,2,4,4-$^2H_4$] taurochenodeoxycholic acid (tauro-CDCA), [2,2,4,4-$^2H_4$]glycocholic acid (glyco-CA), and [2,2,4,4-$^2H_4$] glycochenodeoxycholic acid (glyco-CDCA) were synthesized as described by Mills et al. (30). Plasma (50 µL)

was diluted with 50 µL internal standard solution (30) and 500 µL acetonitrile was added while mixing. After centrifugation, the sample was dried under N_2 and reconstituted in 100 µL methanol:H_2O (1:3). Subsequently, 10 µL of this solution was injected onto a UPLC column (Waters Acquity BEH C18; length 10 cm, internal diameter 2.1 mm, particle size 1.7 µm). The BAs were separated using a gradient from 98% 5 mM ammoniumformate (pH 8.1):methanol (3:1 v:v) to 98% acetonitrile:H_2O (9:1 v:v) in 5 min at a flow rate of 350 µL/min.

BAs were detected with a Waters Quattro Premier XE tandem mass spectrometer in the negative electrospray ionization mode. For the detection of glycine- and taurine-conjugated BAs, cone voltage was set at 60 V and 90 V, respectively, using 40 eV and 60 eV collision energy, respectively. Overall collision gas pressure was 3×10^{-3} mbar argon, and the source temperature was kept at 120 °C. Glycine-conjugated BAs were detected by multiple reaction monitoring using specific transitions with a mass difference of *m/z* 74, since all glycine conjugates specifically lose the *m/z* 74 fragment from the quasimolecular ion after fragmentation (30). Transitions: glyco-CDCA, glyco-DCA, and glyco-ursodeoxycholic acid (UDCA) (448®74); [$^2H_4$]glyco-CDCA (452®74); glyco-CA (464®74); [$^2H_4$]glyco-CA (468®74). Taurine-conjugated BAs were detected in a similar manner using specific transitions with a mass difference of *m/z* 80 due to the loss of a part of the taurine moiety. Transitions: tauro-CDCA, tauro-DCA, and tauro-UDCA (498®80); [$^2H_4$]tauro-CDCA (502®80); tauro-CA (514®80); [$^2H_4$]tauro-CA (518®80). Unconjugated BAs were detected using selected ion recording (cone voltage 70 V, collision energy 10 eV): CDCA and DCA *m/z* 391, CA *m/z* 407.

Absolute concentrations of either taurine- or glycine-conjugated CA and CDCA were calculated using calibration curves by relating the peak area to the peak area of the respective $^2H_4$ internal standard. For DCA and UDCA conjugates, the [$^2H_4$] CDCA internal standards were used. Calibration curves were labeled linear (r 0.98) from 0.1 to 100 µmol/L.

Energy Expenditure

Energy expenditure (REE) was determined by indirect calorimetry before (at -30 min), 90, and 240 min after the meal using a ventilated hood system (Vmax Encore 29; SensorMedics, Anaheim, CA). Substrate oxidation was calculated as described by Frayn (12). The abbreviated Weir equation was used to calculate 24-h energy expenditure.

Statistical Analysis

Concentrations and area under the curves (AUCs) are presented as median and interquartile range (IQR) unless otherwise stated. For visual purposes, data in graphs are displayed as means ± SE. AUCs and incremental AUCs (iAUCs), by using baseline subtracted values, were calculated with the trapezoidal rule. The total bile acid (TBA) concentration is the sum of the unconjugated and conjugated forms of CA, CDCA, DCA, and UDCA. The lithocholic acid (LCA) concentrations were all below the detection limit and therefore not included in the data analysis. A P value below 0.05 is considered as statistically significant. Comparisons between two conditions were evaluated with either paired t-testing for normally distributed data or the Wilcoxon signed rank test for nonparametric distribution. We used a two-way repeated measurement ANOVA (2-way RM-ANOVA) for the analyses of differences in postprandial curves between the mixed meal tests, including fasted concentrations (t = 0). When the two-way RM-ANOVA was statistically significant, we performed post hoc Bonferroni testing to detect the specific differences in the individual time points. Correlations between individual BA species, insulin, GLP-1, and FGF19 were tested on all time points using Pearson's correlation for normally distributed data or Spearman's Rho for nonparametric data. The Bonferroni test was used to correct for multiple testing and only highly significant results are reported. Statistical analysis was performed using IBM SPSS Statistics 24 (IBM, Armonk, NY) and GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA). Graphs were made using GraphPad Prism 7.02.

Results

Subject Characteristics

We included 9 healthy, lean men (median [IQR] age 23.1 [2.8] years, 79.0 [14.0] kg, height 187.0 [9.5] cm, BMI 23.1 [2.8] kg/m^2) for the *experiment 1* and 10 healthy lean men (age 24.5 [5.0] years, 74.4 [10.2] kg, height 183.0 [7.8] cm, BMI 22.1 [2.6] kg/m^2) for *experiment 2*. In both experiments, all subjects completed the study procedures.

Experiment 1

Plasma glucose and insulin concentrations after 40 h of fasting. Forty hours of fasting lowered premeal plasma glucose concentrations (baseline 14 h FAST: 5.3

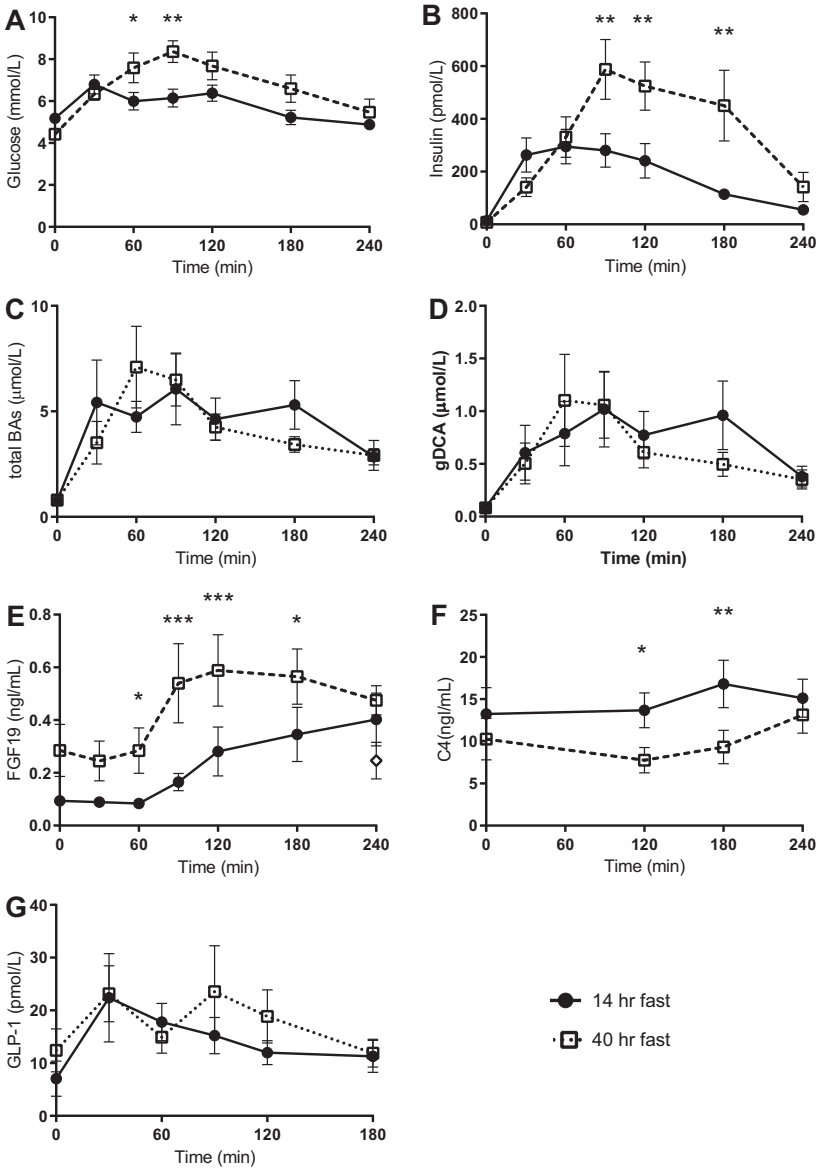

Fig. 1. Postprandial homeostasis in fasting-induced insulin resistance in healthy men. In a crossover design, healthy men (n = 9) consumed a standardized liquid meal test at t = 0 after an overnight (14-h FAST) or 40 h fast (40-h FAST). Postprandial excursion of glucose (*A*), insulin (*B*), total bile acids (BA; *C*), glycine-conjugated deoxycholic acid (gDCA; *D*), fibroblast growth fac tor 19 (FGF19; *E*), C4 (7α-hydroxy-4-cholesten-3-one; *F*). ●, 14 h FAST; □, 40 h FAST. *P < 0.05, **P < 0.01, and ***P < 0.001 after post hoc analysis. Data are means ± SE.

[0.35] mmol/L vs. 40 h FAST: 4.2 [1.0] mmol/L; P < 0.05) but had no effect on premeal insulin concentrations (baseline 14 h FAST: 7.5 [15.0] pmol/L vs. 40 h FAST: 7.5 [0] pmol/L; P > 0.05). Extended fasting increased postprandial glucose and insulin concentrations ($AUC_{0\text{-}240}$ glucose 14 h FAST: 1,401.0 [240.5] mmol/L × min vs. 40 h FAST: 1,579.5 [498.2] mmol/L × min; P < 0.05, 2-way RM-ANOVA. P < 0.01; post hoc analysis time (T) 60 P < 0.05 and T90 P < 0.01 $AUC_{0\text{-}240}$ insulin 14 h FAST: 40,447.0 [8,775.0] pmol/L × min vs. 40 h FAST: 64,807.5 [67320.0] pmol/L × min; P < 0.01, 2-way RM-ANOVA P < 0.05, post hoc analysis T90, T120, and T180 P < 0.01) (Fig. 1, A and B), denoting that 40 h fasting induced glucose intolerance. iAUCs yielded the same results (data not shown).

Plasma BA concentrations after 40 h of fasting. Postprandial TBA concentrations increased in both conditions (Fig. 1*C*). Fasting and postprandial BA levels showed large interindividual variations in BA composition (Table 1). The 40-h fast did not affect baseline (14 h FAST: 0.70 [0.63] μmol/L vs. 40 h FAST: 0.54 [0.60] μmol/L; *P* > 0.05) or postprandial total BA levels in plasma ($AUC_{0\text{-}240}$ 14 h FAST: 989.3 [848.4] μmol/L × min vs. 40 h FAST: 987.5 [516.0] μmol/L × min; *P* > 0.05, 2-way RM-ANOVA *P* > 0.05) (Table 1, Fig. 1*C*).

There was no significant effect of the 40-h fast on baseline levels or postprandial excursion of other individual BA species (Table 1). We did not find any differences in the fasted CA:CDCA ratio (14 h FAST 0.54 ± 0.3 vs. 40 h FAST 0.44 ± 0.1; *P* > 0.05) and the primary (CA and CDCA): secondary bile acid (DCA and UDCA) ratio (14 h FAST 1.52 ± 0.7 vs. 40 h FAST 1.31 ± 0.1; *P* > 0.05). Furthermore, the postprandial CA:CDCA ratio (AUC 14 h FAST 0.39 ± 0.2 vs. 40 h FAST 0.36 ± 0.1; *P* > 0.05) and the primary:secondary bile acid ratio (AUC 14 h FAST 2.6 ± 1.6 vs. 40 h FAST 2.6 ± 1.9; *P* > 0.05) did not differ.

Since we were interested in the correlations between plasma levels of BAs and insulin, we performed correlation analyses at different time points after ingestion of the meal. We observed a strong positive correlation between post absorptive insulin and gDCA levels at *t* = 60 min after an overnight fast (Spearman's Rho 14 h FAST: *r* = +0.88, *P* < 0.01, Fig. 2*A*, 40 h FAST: *r* = +0.42, *P* = 0.27) and *t* = 90 min (14 h FAST: *r* = +0.75, *P* < 0.05; 40 h FAST: *r* = +0.73, *P* < 0.05). The correlation at 60 min was strongest after an overnight fast, where the correlation remained significant after correction for multiple testing. iAUCs showed no differences (data not shown).

Table 1. Effects of short-term fasting and single-dose gDCA administration on plasma bile acid composition in healthy men

		Experiment 1, n = 9 men			*Experiment 2, n* = 10 men		
Bilogram, µmol/L		14 h FAST	40 h FAST	P	—gDCA	+gDCA	P
Total bile acids	baseline	0.70 [0.63]	0.54 [0.60]	0.86	1.10 [2.14]	0.73 [1.13]	0.11
	AUC	987.1 [859.1]	987.5 [515.2]	0.31	1126.7 [621.0]	1104.3 [686.7]	0.88
	2-way RM-ANOVA			0.20			0.63
Unconj. CA	baseline	0.04 [0.04]	0.02 [0.02]	0.14	0.10 [0.20]	0.11 [0.75]	0.25
	AUC	10.13 [3.78]	7.4 [2.69]	0.14	22.1 [106.6]	27.8 [27.5]	0.28
	2-way RM-ANOVA			0.11			0.16
G-conj. CA	baseline	0.05 [0.04]	0.04 [0.07]	0.91	0.13 [0.25]	0.05 [0.09]	0.37
	AUC	170.3 [130.2]	159.3 [158.6]	0.31	155.4 [143.2]	92.7 [112.1]	0.72
	2-way RM-ANOVA			0.55			0.69
T-conj. CA	baseline	0.02 [0.01]	0.01 [0.02]	0.77	0.0 [0.07]	0.0 [0.01]	0.16
	AUC	14.7 [17.6]	16.4 [27.4]	0.77	21.8 [22.5]	15.3 [20.8]	0.33
	2-way RM-ANOVA			0.87			0.63
Unconj. CDCA	baseline	0.04 [0.04]	0.01 [0.03]	0.18	0.10 [0.34]	0.09 [0.29]	0.21
	AUC	21.2 [46.0]	16.7 [10.8]	0.31	26.8 [58.6]	23.6 [30.0]	0.06
	2-way RM-ANOVA			0.46			0.62
G-conj. CDCA	baseline	0.14 [0.26]	0.06 [0.11]	0.86	0.35 [0.28]	0.22 [0.15]	0.37
	AUC	353.5 [399.6]	452.9 [279.6]	0.44	414.1 [285.3]	454.3 [169.1]	0.72
	2-way RM-ANOVA			0.28			0.34
T-conj. CDCA	baseline	0.01 [0.02]	0.02 [0.02]	0.44	0.02 [0.05]	0.0 [0.02]	0.08
	AUC	40.9 [40.0]	35.4 [58.8]	0.86	48.7 [31.8]	54.6 [35.3]	0.39
	2-way RM-ANOVA			0.28			0.50
Unconj. DCA	baseline	0.11 [0.21]	0.09 [0.42]	0.83	0.22 [0.39]	0.23 [0.34]	0.31
	AUC	66.8 [160.5]	108.7 [144.2]	0.26	69.0 [61.9]	48.8 [101.3]	0.58
	2-way RM-ANOVA			0.30			0.84
G-conj. DCA	baseline	0.03 [0.12]	0.28 [0.74]	0.51	0.10 [0.14]	0.07 [0.04]	0.24
	AUC	121.1 [227.1]	108.1 [171.1]	0.44	152.0 [141.4]	231.1 [133.0]	0.02*
	2-way RM-ANOVA			0.28			0.17
T-conj. DCA	baseline	0.0 [0.01]	0.01 [0.03]	0.91	0.01 [0.08]	0.0 [0.0]	0.04*
	AUC	17.1 [45.3]	17.4 [34.4]	0.77	33.4 [47.4]	21.0 [18.2]	0.06
	2-way RM-ANOVA			0.21			0.78

Data are median and [interquartile range]. AUC, area under the curve; TBA, total bile acids; G-conj., glycine conjugated; T-conj., taurine conjugated; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; RM, repeated measures. *$P < 0.05$.

Plasma GLP-1 and FGF19 concentrations after 40 h of fasting. Forty hours of fasting did not change GLP-1 baseline concentrations (baseline 14 h FAST: 4.4 [8.7] pmol/L vs. 40 h FAST: 7.1 [15.0] pmol/L; $P > 0.05$) but increased postprandial AUC of GLP-1 ($AUC_{0\text{-}180}$ 14 h FAST: 1,896.0 [2,463.8] pmol/L × min vs. 40 h FAST: 2,232.0 [2,470.5] pmol/L × min; $P < 0.05$, 2-way RM-ANOVA $P = 0.059$) (Fig. 1*G*). The iAUC analysis for GLP-1 showed no differences (data not shown).

Forty hours of fasting increased FGF19 baseline concentrations (baseline 14 h FAST: 0.10 [0.07] ng/mL vs. 40 h FAST: 0.22 [0.40] ng/mL; $P < 0.05$), thereby tremendously increasing postprandial AUC of FGF19 ($AUC_{0\text{-}240}$ 14 h FAST: 58.6 [44.3] ng/mL × min vs. 40 h FAST: 123.8 [124.1] ng/mL × min; $P < 0.05$, 2-way RM-ANOVA $P < 0.01$, post hoc analysis T60 and T180 ($P < 0.05$), T90 and T120 $P < 0.001$) (Fig. 1*E*). However, postprandial iAUC of FGF19 was not increased after 40 h of fasting ($AUC_{0\text{–}240}$ 14 h FAST: 33.4 [28.2] ng/mL × min vs. 40 h FAST 65.8 [49.8] ng/mL × min; P > 0.05). Additionally, we measured 7α-hydroxy-4-cholesten-3-one (C4) as a marker of bile acid synthesis. In contrast to FGF19, 40 h of fasting did not affect C4 baseline levels (baseline 14 h FAST: 10.0 [12.6] ng/mL vs. 40 h FAST: 7.1 [10] ng/mL; $P > 0.05$). However, postprandial C4 was lower after a 40-h fast ($AUC_{0\text{-}240}$ 14 h FAST 3,419 [2,099] vs. 40 h FAST 2,058 [952] ng/mL × min, $P < 0.05$, 2-way RM-ANOVA $P < 0.01$, post hoc analyses T120 $P < 0.05$ and T180 $P < 0.01$) (Fig. 1*F*)**.** TBA did not correlate with FGF19, but we observed a strong positive correlation between baseline glycochenodeoxycholic acid and FGF19 levels after 40 h of fasting (Spearman's Rho +0.98, $P < 0.01$) and baseline glycocholic acid and FGF19 (Spearman's Rho +0.81, $P < 0.05$) (Fig. 2, *B* and *C*).

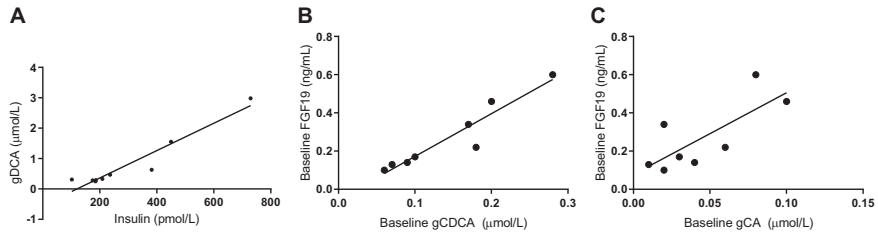

Fig. 2. Correlations of enteroendocrine factors in healthy men. In a crossover design, healthy male subjects ($n = 9$) consumed a standardized liquid meal test at $t = 0$ after an overnight (14-h fast) or 40-h fast (40-h fast). Depicted are the postprandial Spearman's Rho correlation between insulin and glycine-conjugated deoxycholic acid (gDCA) 60-min postprandial after 14 h of fasting ($P < 0.01$) (*A*), the Spearman's Rho correlation between baseline fibroblast growth factor 19 (FGF19) and glycochenodeoxycholic acid (GCDCA) ($P < 0.01$) (*B*), and between baseline FGF19 and glycocholic acid (gCA) ($P < 0.05$) (*C*).

Experiment 2

The changes in GLP-1 and FGF19 and correlation between gDCA and insulin levels in *experiment 1* prompted us to explore the effect of gDCA supplementation on these postprandial parameters.

Plasma glucose and insulin concentrations after gDCA administration. Administration of gDCA had no effect on the total postprandial excursion of glucose ($AUC_{0\text{-}240}$ –gDCA: 1,176.9 [173.0] mmol/L × min vs. +gDCA: 1,145.1 [63.3]; $P > 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 3*A*). Because the postprandial curve showed a glucose lowering effect after gDCA administration between T75 and T180, we calculated the AUC of glucose for these specific time points and we found that gDCA administration decreased the $AUC_{75\text{-}180}$ before glucose levels returned to baseline (-gDCA: 616.5 [76.3] mmol/L × min vs. +gDCA: 544.4 [109.7]) mmol/L × min; $P < 0.05$). However, gDCA administration did not affect postprandial insulin concentrations ($AUC_{0\text{-}240}$ –gDCA: 46,188.8 [30,275.9] vs. +gDCA 45,978.8 [4,4045.6] $P > 0.05$; 2-way RM-ANOVA $P > 0.05$) (Fig. 3*B*). Analyses of the iAUCs of glucose and insulin yielded the same results (data not shown).

Plasma BA concentrations after gDCA administration. TBA excursions after the test meal were not affected by gDCA administration ($AUC_{0\text{-}240}$ -gDCA: 1,126.6 [621.0] μmol/L × min vs. +gDCA: 1,104.3 [686.7] μmol/L × min; $P > 0.05$, 2-way RM-ANOVA $P > 0.05$), although some differences in BA composition were uncovered (Table 1)**.** In line with our expectations, postprandial gDCA concentrations (AUC) were increased after gDCA administration ($AUC_{0\text{-}240}$ -gDCA: 152.0 [141.4] μmol/L × min vs. +gDCA: 231.1 [133.0] μmol/L × min, $P < 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 3*C*). Correlation analyses showed no significant correlations after gDCA administration. Analysis of the iAUC of TBAs yielded the same results (data not shown).

Plasma GLP-1 and FGF19 concentrations after gDCA administration. The AUC of GLP-1 was similar between the study days ($AUC_{0\text{-}240}$ -gDCA: 3,110.6 [3,471.7] pmol/L × min vs. +gDCA: 3,232.5 [2,865.8] pmol/L × min; $P > 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 2*E*), whereas the postprandial incremental AUC of the first phase secretion of GLP-1 was higher after gDCA ($iAUC_{0\text{-}60}$: –gDCA: 56.8 [483.8] pmol/L × min vs. +gDCA: 365.7 [388.8] pmol/L × min; $P < 0.01$) (Fig. 2*E*).

Finally, postprandial excursion of the BA/FXR-induced enterokine FGF19 was not affected by gDCA administration ($AUC_{0\text{-}240}$ –gDCA: 48.8 [16.9] ng/mL × min vs. +gDCA: 46.7 [12.9] ng/mL × min, $P > 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 2*D*). We found no correlations between BAs, GLP-1, and FGF19 (data not shown). The iAUCs of both GLP-1 and FGF19 yielded the same results (data not shown).

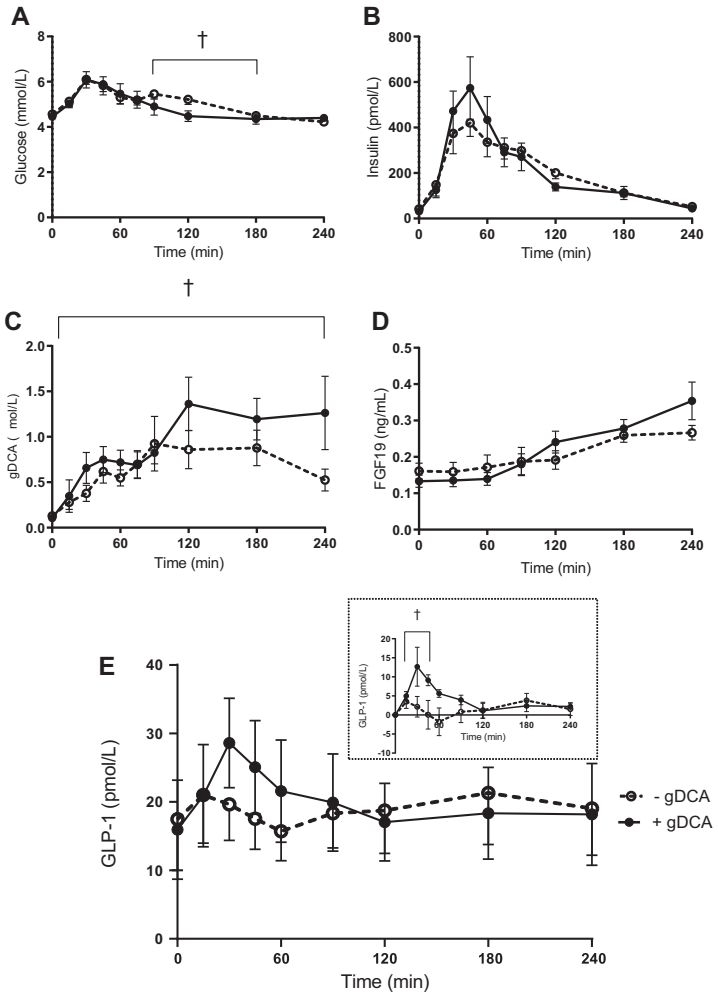

Fig. 3. Postprandial glucose homeostasis after oral glycine-conjugated deoxycholic acid (gDCA) administration in healthy men. In a crossover design, healthy men ($n = 10$) consumed a standardized liquid meal test at $t = 0$ with or without 750 mg gDCA. Postprandial excursion of glucose (*A*), insulin (*B*), gDCA (*C*), fibroblast growth factor 19 (FGF19; *D*), and (incremental) glucagon-like peptide 1 (GLP-1; *F*). ○, –gDCA; ●, +gDCA. † at the curve represents significant effect on area under the curve. Data are means ± SE.

Pre- and postprandial energy expenditure. Baseline REE per kilogram body weight was slightly less in the control setting (–gDCA: 21.0 ± [2.51] vs. +gDCA: 21.7 ± [2.7] kcal·kg^{-1}·day^{-1}; $P < 0.05$), whereas total REE at baseline was not different between the groups (-gDCA: 1655.0 [244.5] vs. +gDCA: 1720.0 [164.0] kcal·kg^{-1}·day^{-1}; $P > 0.05$). Total energy expenditure (REE) and REE per kilogram body weight increased 90 min after meal ingestion (REE –gDCA: 1,899.0 [130.0] vs. + gDCA: 2,057.5 [483.3] kcal·kg^{-1}·day^{-1}; $P > 0.05$; REE per kilogram body weight –gDCA: 24.1 [4.2] vs. +gDCA: 26.1 [4.7] kcal·kg^{-1}·day^{-1}; $P > 0.05$) and leveled off again after 240 min (REE -gDCA: 1605.0 [301.8] kcal·kg^{-1}·day^{-1} vs. +gDCA: 1,750.5 [228.8] kcal·kg^{-1}·day^{-1}; $P > 0.05$; REE per kilogram body weight –gDCA: 20.6 [3.1] kcal·kg^{-1}·day^{-1} vs. +gDCA: 22.0 [3.6] kcal·kg^{-1}·day^{-1}, $P > 0.05$). However, gDCA did not alter the postprandial response of REE. Furthermore, we did not find effects of gDCA supplementation on oxidation of individual macronutrients (data not shown).

Discussion

BAs and their targets GLP-1 and FGF19 have received interest as hormone-like mediators that modulate energy metabolism. Here we explored these axes in response to 40 h fasting-induced insulin resistance and by administering oral gDCA. In the first experiment, we assessed the effects of a 40-h fast on BA metabolism because this induces insulin resistance (42) and BA metabolism is altered in various models of insulin resistance (5, 14, 47). More specifically, CA synthesis is increased and the DCA pool is relatively enlarged in patients with type 2 diabetes (5), most likely leading to the observed increased postprandial BA peaks (43, 47). Postprandial BA concentrations in plasma are suppressed in subjects with obesity (14).

We reproduced the effects of 40 h fasting on insulin sensitivity from Horton and Hill al. (19), but we found no effects of 40 h fasting on postprandial BA concentrations. Obviously, there are important differences between fasting-induced insulin resistance and the metabolic changes seen in obesity and type 2 diabetes (42). It was shown previously that insulin resistance affects the BA pool composition via upregulation of CYP8B1 (12α-hydroxylase, engaged in synthesis of CA) by diminished FoxO1 inhibition (16, 17, 20). Here we found no differences in the ratio of 12α-hydroxylated and non-12α-hydroxylated bile acids, which may

either negate an effect on CYP8B1 or may be due to considerable intraindividual variation. Moreover, only small changes in BA levels may be seen after a diet intervention of 2 wk (32), whereas after bariatric surgery beneficial changes in glucose metabolism appeared after changes in BA pool composition (8).

We found positive correlations between gDCA and insulin levels at multiple postprandial time points in both overnight fasted and 40-h fasted states. TGR5 mediates release of GLP-1 by enteroendocrine L cells in response to stimulation with BAs (45, 46, 49). GLP-1 can in turn lead to increased insulin release. Alternatively, activation of TGR5 on human pancreatic beta cells leads to rapid basal and glucose-dependent insulin secretion in vitro (25). DCA is a secondary BA that makes up around 30% of the total BA pool in humans, where it is mainly found in its glycine-conjugated form (5). In patients with type 2 diabetes, the relative contribution of DCA to the total BA pool is increased (5). This could be interpreted as an adaptive response to counter declining beta cell insulin secretion. Sato and colleagues (39) showed that gDCA is a particularly strong TGR5 agonist, activating 50% of this receptor at a concentration of 1.18 μM.

Forty hours of fasting actually did increase postprandial GLP-1 concentrations. BAs continue to cycle in the enterohepatic cycle during fasting, but it is unclear if increased luminal stimulation by BAs such as gDCA increased the GLP-1 levels after the 40-h fast, because we did not witness increased BA levels in our peripheral samples. Preclinical work has linked an increase of GLP-1 production to an increase of energy status, i.e., AMPK-dependent regulation of GLP1 expression in L-like cells (21).

Likewise, it may be difficult to explain both the increased basal and postprandial FGF19 concentrations after 40 h of fasting. Here, FGF19 may be high to suppress hepatic BA biosynthesis while starving despite absence of stimuli that would normally induce FGF19 release. 7α-Hydroxy-4-cholesten-3-one (C4) reflects hepatic CYP7A1 activity and, indeed, we found suppressed postprandial C4 levels after 40 h of fasting as seen in other studies where high FGF19 levels lower C4 (13, 41). However, increased basal FGF19 after 40 h of fasting did not result in statistically lower basal C4 concentrations. This may be due to lack of power, insufficient basal FGF19 concentrations to inhibit C4, or different circumstances (i.e., glucose, insulin, and bile acid composition) in the fasted state compared

with the postprandial state (9). Alternatively, FGF19 is not obligatory for reduced BA biosynthesis (1). Forty hours of fasting induces profound reciprocal changes in energy metabolism that may have influenced FGF19 biology (42). It is also tempting to speculate that ongoing cycling of BAs such as CDCA, which correlated with FGF19 levels, may have led to increased FGF19 production as shown earlier in other models (29, 50).

In the second experiment, we further explored this relationship and administered a single dose of 750 mg gDCA with the meal. The dose is similar to that recommended for ursodeoxycholic acid, prescribed for treatment of primary biliary cholangitis and used in translational studies (11, 15), but lower than the dose of CDCA (15 mg/kg) used in the study by Broeders et al. (4). Unconjugated DCA has been suggested to exert carcinogenic effects in in vitro studies employing supraphysiological concentrations (31). However, in our study, we administered glycine-conjugated DCA, which is abundantly present in the human BA pool and enters the gut lumen in high concentrations after a meal (5).

We found slightly lower plasma glucose levels in our healthy volunteers after gDCA administration between 75 and 180 min after the meal. Although this could be perceived as a chance finding, lower glucose levels were very reproducible between subjects, showed marginal spread, and reached a mean difference of ~0.5 mM at time points 90 and 120 min. We could not attribute this to a relevant increase in insulin levels. Moreover, gDCA supplementation acutely increased GLP-1 levels. This is likely explained by increased secretion and not diminished clearance, because GLP-1 normally is broken down quickly by dipeptidyl peptidase-4 (3). The key question remains then: how does gDCA increase GLP-1 secretion? Stimulation of TGR5 receptors in the gut by gDCA is most likely responsible for this phenomenon, because the GLP-1 effects were rather acute (after 30 min, Fig. 3*E*) and preceded the increased gDCA plasma levels, which only became evident after ~120 min (Fig. 3*C*). Our data support previous work that shows bile acids as important regulators of appetite- and metabolism-regulating hormones by activation of basolateral intestinal TGR5 (23). However, the combination of a short GLP-1 surge and healthy lean volunteers may have prevented clear effects on glucose levels. The fact that we found no effects on FGF19 levels after gDCA administration may be explained by lower, but not absent, affinity of gDCA for FXR compared with CDCA (34).

Contrary to our expectations, we did not detect an effect of oral gDCA on energy expenditure in this study, despite observing significantly increased gDCA plasma concentrations at the end of the meal. TGR5/DIO2 coexpression has been confirmed to be present in both human skeletal muscle myoblasts and brown adipose tissue in humans, suggesting that this pathway is functionally active (4). Broeders et al. (4) described a 5% increase in early morning basal metabolic rate in response to two consecutive doses of unconjugated CDCA given over the course of 24 h. Despite the fact that unconjugated CDCA is a BA with weaker TGR5 affinity compared with gDCA, the increased brown adipose tissue activity was attributed to TGR5 activation (39).

Our study had a few limitations. First, the experiments were designed to assess acute effects of BAs mediated by TGR5 and not by changes in FXR stimulation or changes to the composition of the circulating BA pool. Plasma BA concentrations are probably not under short-term regulation but instead are a function of their portal concentration and a relatively constant hepatic uptake (27). However, longer duration of BA supplementation may lead to more profound changes in the BA pool and downstream targets, including prolonged TGR5 and FXR activation (10, 48). Another limitation of this study was the high interindividual variability of BA curves, which is a recurring finding in postprandial BA studies (14, 47) and may warrant the inclusion of a larger number of subjects. Also, the results of experiment 1 may be affected by the rate of stomach emptying, which is slower after a 40-h fast (6). Finally, we were not able to measure portal vein concentrations of the substrates and hormones of interest, and peripheral sampling may not provide optimal information about changes within the enterohepatic cycle during our studies (9).

In this study, we show the different effects of 40 h fasting and gDCA administration on BAs and their downstream targets GLP-1 and FGF19. Unexpectedly, 40 h fasting increased both GLP-1 and FGF19, where the former appeared BA independent and the latter BA dependent. Therefore, our data add complexity to the physiological regulation of the enterokines GLP-1 and FGF19 by BAs.

Grants

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References

1. Al-Khaifi A, Rudling M, Angelin B. An FXR agonist reduces bile acid synthesis independently of increases in FGF19 in healthy volunteers. *Gastroenterology* 155: 1012–1016, 2018.
2. Angelin B, Björkhem I. Postprandial serum bile acids in healthy man. Evidence for differences in absorptive pattern between individual bile acids. *Gut* 18: 606–609, 1977.
3. Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. *Gastroenterology* 132: 2131-2157, 2007.
4. Broeders EPM, Nascimento EBM, Havekes B, Brans B, Roumans KHM, Tailleux A, Schaart G, Kouach M, Charton J, Deprez B, Bouvy ND, Mottaghy F, Staels B, van Marken Lichtenbelt WD, Schrauwen P. The bile acid chenodeoxycholic acid increases human brown adipose tissue activity. *Cell Metab* 22: 418-426, 2015.
5. Brufau G, Stellaard F, Prado K, Bloks VWVWVW, Jonkers E, Boverhof R, Kuipers F, Murphy EJEJ. Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism. *Hepatology* 52: 1455–1464, 2010.
6. Corvilain B, Abramowicz M, Féry F, Schoutens A, Verlinden M, Balasse E, Horowitz M. Effect of short-term starvation on gastric emptying in humans: relationship to oral glucose tolerance. *Am J Physiol Gastrointest Liver Physiol* 269: G512–G517, 1995.
7. DePaoli AM, Zhou M, Kaplan DD, Hunt SC, Adams TD, Learned RM, Tian H, Ling L. FGF19 analog as a surgical factor mimetic that contributes to metabolic effects beyond glucose homeostasis. *Diabetes* 68: 1315–1328, 2019.
8. Dutia R, Embrey M, O'Brien CS, Haeusler RA, Agénor KK, Homel P, McGinty J, Vincent RP, Alaghband-Zadeh J, Staels B, le Roux CW, Yu J, Laferrère B. Temporal changes in bile acid levels and 12α-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes. *Int J Obes* 39: 806 –813, 2015 [Erratum in *Int J Obes* 40:554, 2016].
9. Eggink HM, van Nierop FS, Schooneman MG, Boelen A, Kalsbeek A, Koehorst M, ten Have GAM, de Brauw LM, Groen AK, Romijn JA, Deutz NEP, Soeters MR. Transhepatic bile acid kinetics in pigs and humans. *Clin Nutr* 37: 1406–1414, 2018.
10. Einarsson C, Hillebrant CG, Axelson M. Effects of treatment with deoxycholic acid and chenodeoxycholic acid on the hepatic synthesis of cholesterol and bile acids in healthy subjects. *Hepatology* 33: 1189–1193, 2001.
11. Einarsson K, Björkhem I, Eklöf R, Ewerth S, Nilsell K, Blomstrand R. Effect of ursodeoxycholic acid treatment on intestinal absorption of triglycerides in man. *Scand J Gastroenterol* 19: 283–288, 1984.
12. Frayn K. Calculation of substrate oxidation from gaseous exchange rates in vivo. *J Appl Physiol Respir Environ Exerc Physiol* 55: 628–634, 1983.
13. Friedrich D, Marschall H-U, Lammert F. Response of fibroblast growth factor 19 and bile acid synthesis after a body weight-adjusted oral fat tolerance test in overweight and obese NAFLD patients: a non-randomized controlled pilot trial. *BMC Gastroenterol* 18: 76, 2018.
14. Glicksman C, Pournaras DJ, Wright M, Roberts R, Mahon D, Welbourn R, Sherwood R, Alaghband-Zadeh J, le Roux CW. Postprandial plasma bile acid responses in normal weight and obese subjects. *Ann Clin Biochem* 47: 482–484, 2010.
15. von Haehling S, Schefold JC, Jankowska EA, Springer J, Vazir A, Kalra PR, Sandek A, Fauler G, Stojakovic T, Trauner M, Ponikowski P, Volk HD, Doehner W, Coats AJ, Poole-Wilson PA, Anker SD. Ursodeoxycholic acid in patients with chronic heart failure: a doubleblind, randomized, placebo-controlled, crossover trial. *J Am Coll Cardiol* 59: 585–592, 2012.
16. Haeusler RA, Astiarraga B, Camastra S, Accili D, Ferrannini E. Human insulin resistance is associated with increased plasma levels of 12α-hydroxylated bile acids. *Diabetes* 62: 4184–4191, 2013.
17. Haeusler RA, Pratt-Hyatt M, Welch CL, Klaassen CD, Accili D. Impaired generation of 12-hydroxylated bile acids links hepatic insulin signaling with dyslipidemia. *Cell Metab* 15: 65–74, 2012.
18. Harrison SA, Rinella ME, Abdelmalek MF, Trotter JF, Paredes AH, Arnold HL, Kugelmas M, Bashir MR, Jaros MJ, Ling L, Rossi SJ, DePaoli AM, Loomba R. NGM282 for treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet* 391: 1174–1185, 2018.

19. Horton TJ, Hill JO. Prolonged fasting significantly changes nutrient oxidation and glucose tolerance after a normal mixed meal. *J Appl Physiol (1985)* 90: 155-163, 2001.
20. Ishida H, Kuruta Y, Gotoh O, Yamashita C, Yoshida Y, Noshiro M. Structure, evolution, and liver-specific expression of sterol 12alpha-hydroxylase P450 (CYP8B). *J Biochem* 126: 19–25, 1999.
21. Jiang S, Zhai H, Li D, Huang J, Zhang H, Li Z, Zhang W, Xu G. AMPK-dependent regulation of GLP1 expression in L-like cells. *J Mol Endocrinol* 57: 151-160, 2016.
22. Kir S, Beddow SA, Samuel VT, Miller P, Previs SF, Suino-Powell K, Xu HE, Shulman GI, Kliewer SA, Mangelsdorf DJ. FGF19 as a postprandial, insulin-independent activator of hepatic protein and glycogen synthesis. *Science* 331: 1621–1624, 2011.
23. Kuhre RE, Wewer Albrechtsen NJ, Larsen O, Jepsen SL, Balk-Møller E, Andersen DB, Deacon CF, Schoonjans K, Reimann F, Gribble FM, Albrechtsen R, Hartmann B, Rosenkilde MM, Holst JJ. Bile acids are important direct and indirect regulators of the secretion of appetite- and metabolism-regulating hormones from the gut and pancreas. *Mol Metab* 11: 84–95, 2018.
24. Kuipers F, Bloks VW, Groen AK. Beyond intestinal soap– bile acids in metabolic control. *Nat Rev Endocrinol* 10: 488–498, 2014.
25. Kumar DP, Rajagopal S, Mahavadi S, Mirshahi F, Grider JR, Murthy KS, Sanyal AJ. Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic β cells. *Biochem Biophys Res Commun* 427: 600–605, 2012.
26. LaRusso NF, Hoffman NE, Korman MG, Hofmann AF, Cowen AE. Determinants of fasting and postprandial serum bile acid levels in healthy man. *Am J Dig Dis* 23: 385–391, 1978.
27. LaRusso NF, Korman MG, Hoffman NE, Hofmann AF. Dynamics of the enterohepatic circulation of bile acids. Postprandial serum concentrations of conjugates of cholic acid in health, cholecystectomized patients, and patients with bile acid malabsorption. *N Engl J Med* 291: 689–692, 1974.
28. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev* 89: 147–191, 2009.
29. Meyer-Gerspach AC, Steinert RE, Keller S, Malarski A, Schulte FH, Beglinger C. Effects of chenodeoxycholic acid on the secretion of gut peptides and fibroblast growth factors in healthy humans. *J Clin Endocrinol Metab* 98: 3351–3358, 2013.
30. Mills KA, Mushtaq I, Johnson AW, Whitfield PD, Clayton PT. A method for the quantitation of conjugated bile acids in dried blood spots using electrospray ionization-mass spectrometry. *Pediatr Res* 43: 361–368, 1998.
31. Mrestani Y, Bretschneider B, Härtl A, Neubert RHH. In-vitro and in-vivo studies of cefpirom using bile salts as absorption enhancers. *J Pharm Pharmacol* 55: 1601-1606, 2003.
32. van Nierop FS, Kulik W, Endert E, Schaap FG, Olde Damink SW, Romijn JA, Soeters MR. Effects of acute dietary weight loss on postprandial plasma bile acid responses in obese insulin resistant subjects. *Clin Nutr* 36: 1615-1620, 2017.
33. van Nierop FS, Scheltema MJ, Eggink HM, Pols TW, Sonne DP, Knop FK, Soeters MR. Clinical relevance of the bile acid receptor TGR5 in metabolism. *Lancet Diabetes Endocrinol* 5: 224–233, 2017.
34. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 284: 1365–1368, 1999.
35. Ploton M, Mazuy C, Gheeraert C, Dubois V, Berthier A, Dubois-Chevalier J, Maréchal X, Bantubungi K, Diemer H, Cianférani S, Strub J-M, Helleboid-Chapman A, Eeckhoute J, Staels B, Lefebvre P. The nuclear bile acid receptor FXR is a PKA- and FOXA2-sensitive activator of fasting hepatic gluconeogenesis. *J Hepatol* 69: 1099–1109, 2018.
36. Potthoff MJ, Boney-Montoya J, Choi M, He T, Sunny NE, Satapati S, Suino-Powell K, Xu HE, Gerard RD, Finck BN, Burgess SC, Mangelsdorf DJ, Kliewer SA. FGF15/19 regulates hepatic glucose metabolism by inhibiting the CREB-PGC-1α pathway. *Cell Metab* 13: 729–738, 2011.
37. Reeskamp LF, Meessen ECE, Groen AK. Transintestinal cholesterol excretion in humans. *Curr Opin Lipidol* 29: 10–17, 2018.
38. Renga B, Mencarelli A, Vavassori P, Brancaleone V, Fiorucci S. The bile acid sensor FXR regulates insulin transcription and secretion. *Biochim Biophys Acta* 1802: 363–372, 2010.

39. Sato H, Macchiarulo A, Thomas C, Gioiello A, Une M, Hofmann AF, Saladin R, Schoonjans K, Pellicciari R, Auwerx J. Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. *J Med Chem* 51: 1831–1841, 2008.
40. Schaap FG, van der Gaag NA, Gouma DJ, Jansen PLM. High expression of the bile salt-homeostatic hormone fibroblast growth factor 19 in the liver of patients with extrahepatic cholestasis. *Hepatology* 49: 1228–1235, 2009.
41. Schreuder TCMA, Marsman HA, Lenicek M, van Werven JR, Nederveen AJ, Jansen PLM, Schaap FG. The hepatic response to FGF19 is impaired in patients with nonalcoholic fatty liver disease and insulin resistance. *Am J Physiol Gastrointest Liver Physiol* 298: G440–G445, 2010.
42. Soeters MR, Soeters PB, Schooneman MG, Houten SM, Romijn JA. Adaptive reciprocity of lipid and glucose metabolism in human short-term starvation. *Am J Physiol Endocrinol Metab* 303: E1397–E1407, 2012.
43. Sonne DP, van Nierop FS, Kulik W, Soeters MR, Vilsbøll T, Knop FK. Postprandial plasma concentrations of individual bile acids and FGF-19 in patients with type 2 diabetes. *J Clin Endocrinol Metab* 101: 3002–3009, 2016.
44. St-Pierre MV, Kullak-Ublick GA, Hagenbuch B, Meier PJ. Transport of bile acids in hepatic and non-hepatic tissues. *J Exp Biol* 204: 1673–1686, 2001.
45. Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, Macchiarulo A, Yamamoto H, Mataki C, Pruzanski M, Pellicciari R, Auwerx J, Schoonjans K. TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metab* 10: 167–177, 2009.
46. Ullmer C, Alvarez Sanchez R, Sprecher U, Raab S, Mattei P, Dehmlow H, Sewing S, Iglesias A, Beauchamp J, Conde-Knape K. Systemic bile acid sensing by G protein-coupled bile acid receptor 1 (GPBAR1) promotes PYY and GLP-1 release. *Br J Pharmacol* 169: 671–684, 2013.
47. Vincent RP, Omar S, Ghozlan S, Taylor DR, Cross G, Sherwood RA, Fandriks L, Olbers T, Werling M, Alaghband-Zadeh J, le Roux CW. Higher circulating bile acid concentrations in obese patients with type 2 diabetes. *Ann Clin Biochem* 50: 360–364, 2013.
48. Wang Y, Jones PJH, Woollett LA, Buckley DD, Yao L, Granholm NA, Tolley EA, Heubi JE. Effects of chenodeoxycholic acid and deoxycholic acid on cholesterol absorption and metabolism in humans. *Transl Res* 148: 37–45, 2006.
49. Wu T, Bound MJ, Standfield SD, Jones KL, Horowitz M, Rayner CK. Effects of taurocholic acid on glycemic, glucagon-like peptide-1, and insulin responses to small intestinal glucose infusion in healthy humans. *J Clin Endocrinol Metab* 98: E718–E722, 2013.
50. Zweers SJ, de Vries EM, Lenicek M, Tolenaars D, de Waart DR, Koelfat KVK, Groen AK, Olde Damink SWM, Beuers U, Ponsioen C, Jansen PLM, Schaap FG. Prolonged fibroblast growth factor 19 response in patients with primary sclerosing cholangitis after an oral chenodeoxycholic acid challenge. *Hepatol Int* 11: 132–140, 2017.

6

Duodenal-jejunal lining increases postprandial unconjugated bile acid responses and disrupts the bile acid-FXR-FGF19 axis in humans

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Abstract

Background and Aims: Placement of the duodenal-jejunal bypass liner (DJBL) leads to rapid weight loss and restoration of insulin sensitivity in a similar fashion to bariatric surgery. Increased systemic bile acid levels are candidate effectors for these effects through postprandial activation of their receptors TGR5 and FXR. We aimed to quantify postprandial bile acid, GLP-1 and FGF19 responses and assess their temporal relation to the weight loss and metabolic and hormonal changes seen after DJBL placement.

Methods: We performed mixed meal testing in 17 obese patients with type 2 diabetes mellitus (DM2) directly before, one week after and 6 months after DJBL placement.

Results: Both fasting and postprandial bile acid levels were unchanged at 1 week after implantation, and greatly increased 6 months after implantation. The increase consisted of unconjugated bile acid species. 3 hour-postprandial GLP-1 levels increased after 1 week and were sustained, whereas FGF19 levels and postprandial plasma courses were unaffected.

Conclusions: DJBL placement leads to profound increases in unconjugated bile acid levels after 6 months, similar to the effects of bariatric surgery. The temporal dissociation between the changes in bile acids, GLP-1 and FGF19 and other gut hormone responses warrant caution about the beneficial role of bile acids after DJBL placement. This observational uncontrolled study emphasizes the need for future controlled studies.

Introduction

Bile acids have gained attention as hormone-like factors in metabolism exerting effects via the transmembrane receptor Takeda G-coupled protein receptor 5 (TGR5) [1–4]. The feedback repression of hepatic bile acid synthesis is managed through the nuclear farnesoid X receptor (FXR) and involves ileal Fibroblast Growth Factor 19 (FGF19) [4,5]. Intestinal L-cell secretion of glucagon-like peptide 1 (GLP-1) is one of the best-described ways of in-vivo stimulation of TGR5 by bile acids [6].

Bile acids have been implicated in the deranged glucose metabolism of patients with obesity and type 2 diabetes mellitus (DM2) [7,8]. Obesity is characterized by decreased postprandial bile acid concentrations [9] and increased bile acid synthesis [10]. DM2 patients have increased postprandial plasma concentrations of the bile acids cholate (CA), deoxycholate (DCA) and chenodeoxycholic acid (CDCA) [11].

Bariatric surgery, and the Roux-en-Y gastric bypass procedure (RYGB) in particular, increases fasting and postprandial bile acid levels and results in 45% of patients not needing medication after 5 years [12,13]. The Duodenal-Jejunal Bypass Liner (DJBL, GI Dynamics, Lexington, MA) is a 60 cm long impermeable liner, which is delivered and retrieved endoscopically. Its placement results in weight loss and improvement of DM2 with lowering of the use of antidiabetic medication such as sulfonylurea derivatives, metformin and insulin [14–16].

Given the proposed role of bile acids in DM2 and RYGB, we examined the effects of DJBL placement on the postprandial bile acid response at different time points after the procedure.

Material and methods

Subjects

Seventeen subjects with obesity and DM2 were included in the Maastricht University Medical Center, Maastricht, and the Atrium Medical Center Parkstad, Heerlen, the Netherlands between February and July 2010 as reported previously

[17]. Inclusion criteria were: age between 18 and 65 years; body mass index (BMI) between 30 and 50 kg/m^2; duration of DM2 <10 years; and HbA1c between 7.5 and 10.0%. Main exclusion criteria have been depicted earlier [17].

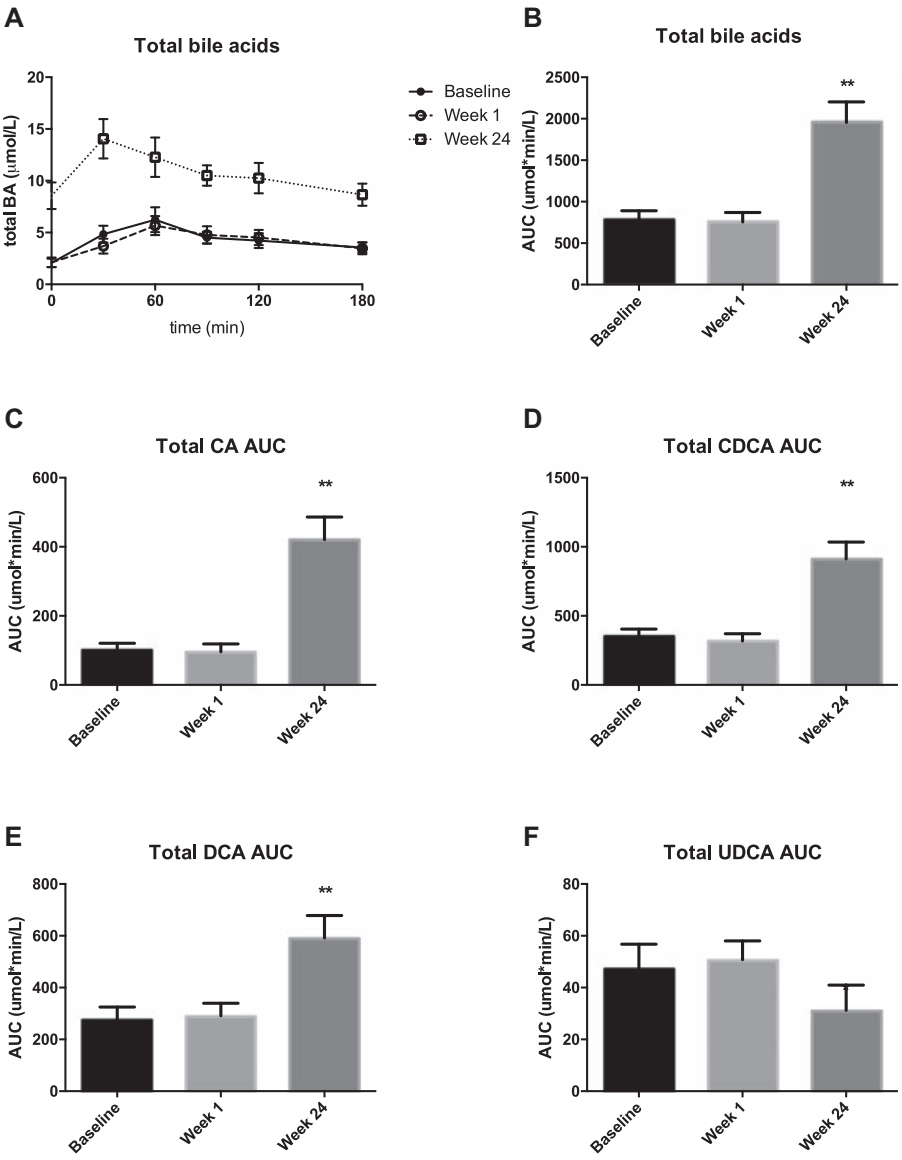

Fig. 1. Postprandial course of bile acids and AUC before DJBL placement and after 1 and 24 weeks of DJBL placement. ** = $p < 0.05$.

Study Design

The DJBL was delivered and retrieved endoscopically where after subjects were studied and followed up upon as described previously [17]. Here, we report on three visits: 1) within one month prior to implantation (baseline), 2) one week after implantation (week 1), and 3) 24 weeks after implantation, just prior to removal (week 24). Standardized meal tolerance tests were performed. An intravenous cannula was placed for blood sampling. The first sample was drawn after an overnight fast; subsequently a standard liquid meal was consumed (Ensure Plus, Abbott Laboratories, IL; 333 mL, 500 kcal, 20.8 g protein, 67.3 g carbohydrates, and 16.4 g fat), followed by collection of blood samples after 30, 60, 90, 120 and 180 min (BD Vacutainer EDTA tube/EDTA aprotinin tube, BD diagnostics, Erembodegem-Aalst, Belgium). Samples were immediately cooled, centrifuged, and stored at −80 °C until analysis.

Laboratory Analysis

Bile acid concentrations were determined using a UPLC-tandem MS method to quantify CA, CDCA, DCA and ursodeoxycholic acid (UDCA) in their conjugated and unconjugated forms [18]. FGF19 was measured using an in-house developed ELISA as published previously [19]. Total bile acids were calculated by adding up all the individual bile acids measurements.

Ethics

The study was approved by the Medical Ethics Committee of both centers and conducted according to the revised version of the Declaration of Helsinki. Written informed consent was obtained from every subject prior to study participation. Clinical Trial Registration Number: NCT00985114.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 22 (IBM, Armonk, NY, USA). Data were visually and statistically assessed for normality and logarithmically transformed where appropriate. Area-under-the-curve (AUC) of postprandial plasma levels was calculated using the trapezoidal method; subsequent correction for baseline values yielded the incremental-area-under-the-curve (iAUC). Peak time was defined as the first time point at which maximal concentration was reached. Comparisons between 3 test conditions were made using Friedman's test to determine significant differences. Individual comparisons

between 2 test conditions were made with Wilcoxon matched pairs signed rank testing. Correlations were assessed using Pearson's correlation for normally distributed populations or Spearman's Rho for other data. A p-value of 0.05 was considered statistically significant after Bonferroni corrections. Data presented are mean and standard deviation ($\mu \pm \sigma$) for normally distributed variables or median and interquartile range (m [IQR]) for other variables. Graphs were made using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Lining of the Proximal Small Intestine by DJBL Reduces Body Weight

The weight loss effects of the DJBL have been published previously [17]. At baseline, subjects (14 males, 3 females) were 51 ± 2 years old and weighed 116.0 ± 5.8 kg with a BMI of 37.0 ± 1.3 kg/m^2. One week after placement of the DJBL, body weight had decreased by 4.3 ± 0.6 kg, corresponding to an excess weight loss of 10.2 ± 1.7% and a BMI reduction of 1.4 ± 0.2 kg/m^2 ($p < 0.01$). After 24 weeks, at the time of device removal, mean body weight had further decreased resulting in a total weight loss of 12.7 ± 1.3 kg, and a BMI reduction of 4.1 ± 0.4 kg/m^2 ($p < 0.01$). All subjects reported lowered caloric intake during the study and 88% of the subjects (15/17) reported increased feelings of satiety.

Lining of the Proximal Small Intestine by DJBL Increases Fasting and Postprandial Unconjugated Bile Acid Levels

All subjects completed the study. AUC, fasting and peak levels of total bile acids and all bile acid subsets except UDCA were markedly increased 24 weeks after placement but not after 1 week (see Fig. 1 and Table 1). In fact, the increase of AUCs relied primarily on the increase in fasting plasma bile acids since incremental AUCs were unaffected by DJBL placement (data not shown). Peak times (time from start of the meal to the peak of the plasma bile acids) were unchanged for all bile acids and bile acid subfractions (data not shown).

The Increase in Plasma Bile Acids by DJBL is Completely Caused by Unconjugated Bile Acids

To gain further insight in the composition of the bile acid pool after overnight fasting, we analysed the different bile acids species as well as the conjugation status.

Table 1 : Bilogram & FGF19.

Bile acid fraction		Baseline	Week 1	Week 24	*p*
Total bile acids	Baseline	1.1 (1.5)	1.6 (1.4)	7.8 (3.9)*†	b0.01
(μmol/L)	Peak	4.8 (9.0)	5.9 (8.4)	13.1 (6.5)*†	b0.01
	AUC	679 (642)	602 (722)	1661 (966)*†	b0.01
Total CA	Baseline	0.1 (0.1)	0.2 (0.3)	1.6 (1.3)*†	b0.01
(μmol/L)	Peak	0.8 (1.3)	0.6 (0.6)	3.0 (2.9)*†	b0.01
	AUC	80 (123)	69 (261)	315 (306)*†	b0.01
Total CDCA	Baseline	0.4 (0.9)	0.5 (0.6)	3.3 (2.8)*†	b0.01
(μmol/L)	Peak	2.5 (3.7)	2.4 (2.7)	6.0 (4.3)*†	b0.01
	AUC	318 (348)	246 (222)	722 (657)*†	b0.01
Total DCA	Baseline	0.5 (0.4)	0.6 (0.6)	2.8 (2.3)*†	b0.01
(μmol/L)	Peak	1.6 (3.0)	2.4 (2.9)	4.0 (2.6)*†	b0.01
	AUC	222 (278)	246 (327)	519 (326)*†	b0.01
Total UDCA	Baseline	0.0 (0.1)	0.2 (0.2)	0.0 (0.2)	0.13
(μmol/L)	Peak	0.5 (0.6)	0.4 (0.2)	0.2 (0.3)*	0.03
	AUC	45 (39)	53 (33)	14 (41)	0.08
Total taurine-conjugated BA	Baseline	0.1 (0.8)	0.1 (0.2)	0.0 (0.1)*†	0.01
(μmol/L)	Peak	0.7 (1.2)	0.5 (0.8)	0.1 (0.1)*†	b0.01
	AUC	50 (126)	54 (56)	6 (24)*†	b0.01
Total glycine-conjugated BA	Baseline	0.5 (0.8)	0.6 (1.0)	1.3 (0.9)	0.39
(μmol/L)	Peak	3.4 (5.4)	3.9 (5.0)	4.2 (3.3)	0.79
	AUC	468 (543)	366 (438)	422 (306)	0.19
Total unconjugated BA	Baseline	0.5 (0.6)	0.8 (0.4)	6.2 (3.7)*†	b0.01
(μmol/L)	Peak	1.3 (1.6)	1.5 (0.6)	8.1 (6.2)*†	b0.01
	AUC	156 (125)	182 (53)	1118 (789)*†	b0.01
Total 12α-OH BA	Baseline	0.6 (0.7)	0.8 (0.7)	4.6 (2.8)*†	b0.01
(μmol/L)	Peak	2.5 (5.0)	3.2 (3.5)	6.4 (4.0)*†	b0.01
	AUC	335 (401)	324 (404)	834 (467)*†	b0.01
FGF19	Baseline	51.9 (37.5)	61.9 (20.4)	43.7 (24.2)	0.09
(pg/mL)	Peak	70.7 (199.7)	111.9 (74)	91.8 (127.0)	0.84
	AUC	6303 (7661)	7986 (2712)	6694 (3272)	0.10

Bilogram Baseline, peak concentration and AUC of bile acid subtypes and FGF19. Values are presented as median (IQR). The unit of bile acid peak and baseline values is μmol/L, the unit of AUC is μmol/L×min. Overall difference between the three study days was assessed using the Friedman test. If significant, *p*-values were calculated using the related-samples Wilcoxon signed-rank test.

***** Denotes significant difference compared to baseline.

† Denotes significant difference compared to week 1.

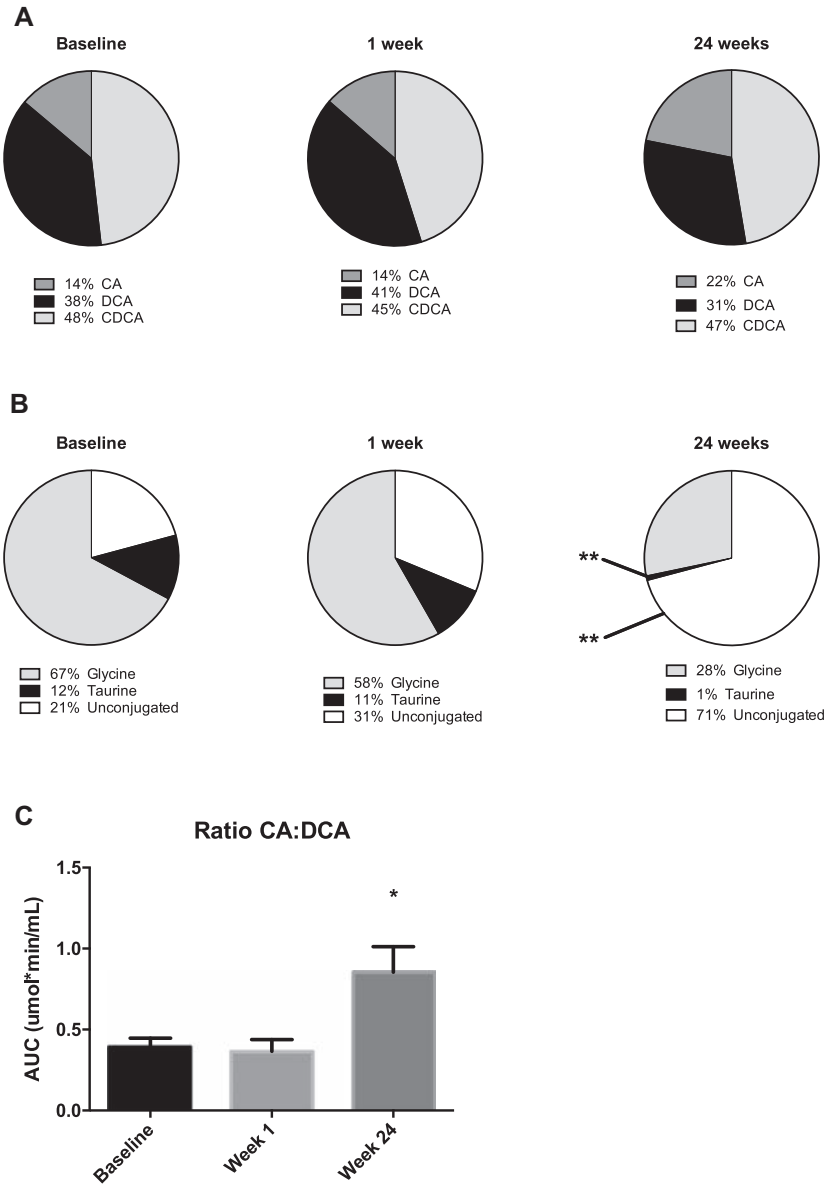

Fig. 2. Composition of the postprandial bile acid pool composition (AUC) for the bile acids CA, CDCA, DCA and UDCA before DJBL placement and after 1 and 24 weeks of DJBL placement (panel A). Composition of the postprandial bile acid pool composition (AUC) for the taurine-, and glycine-conjugated and unconjugated bile acids before DJBL placement and after 1 and 24 weeks of DJBL placement (panel B). ** = $p < 0.05$. Ratio postprandial CA:DCA AUCs before DJBL placement and after 1 and 24 weeks of DJBL placement (panel C). * = $p < 0.05$.

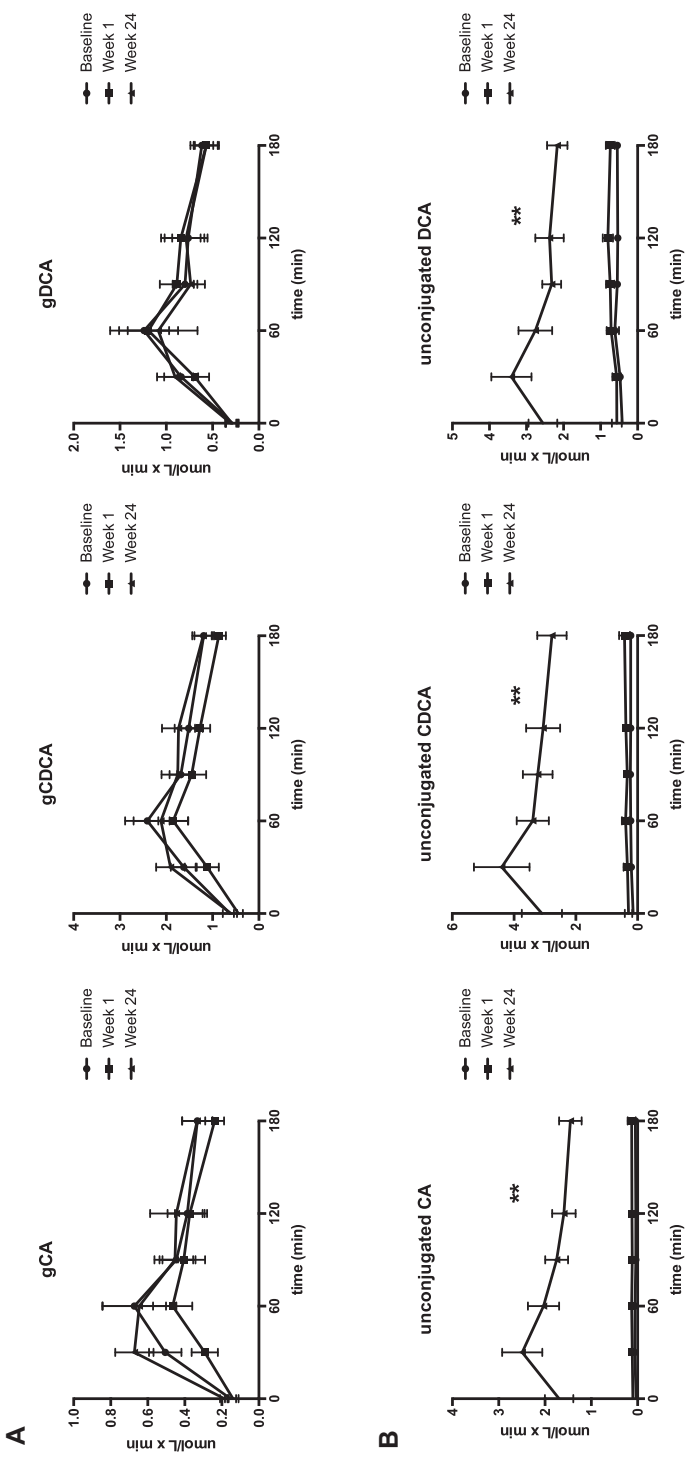

Fig. 3. Postprandial courses of individual glycine-conjugated bile acid species, before and 1 and 24 weeks after DJBL placement (panel A). Postprandial courses of individual unconjugated bile acid species, before and 1 and 24 weeks after DJBL placement (panel A). ** = $p < 0.05$

(Fig. 2, panel A). In general, CDCA was the largest contributor to the pool during the entire study, followed by DCA and CA respectively. Remarkably, the increase in plasma bile acids was completely caused by unconjugated bile acids (Fig. 2, panel B), which reached significance after 24 weeks. Expressed as the fraction of total bile acids, unconjugated bile acid AUC rose from a median of 22% (IQR 13%) at baseline to 26% (IQR 20%, $p < 0.01$**) at week 1 and 76% (IQR 19%, $p < 0.01$**) at week 24 after placement. Total glycine conjugates were unaltered after 24 weeks in contrast to the taurine conjugates that showed a ~90% reduction (Table 1). This pattern was also observed for the individual conjugated forms of CA, CDCA

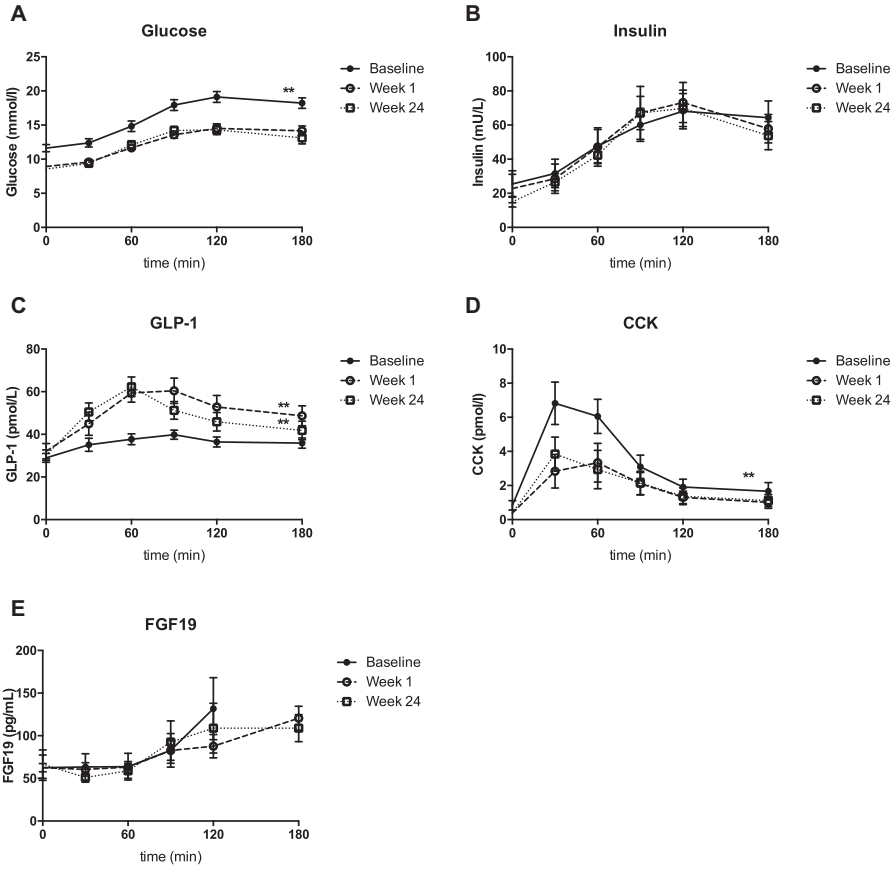

Fig. 4. Postprandial curves of glucose (panel A), insulin (panel B), GLP-1 (panel C), CCK (panel D) and FGF19 (panel E) before DJBL placement and after 1 and 24 weeks of DJBL, ** = $p < 0.05$. $T = 180$ values are missing for FGF19 at baseline.

and DCA as shown in Fig. 3, emphasizing the fact that the increase in unconjugated plasma bile acids after DJBL was not due to one bile acid species. We found no changes in the ratio of 12α-hydroxylated/non12α-hydroxylated bile acids (data not shown).

Postprandial Levels of Glucose, Insulin, GLP-1 Cholecystokinin (CCK) and FGF19

The effects of DJBL on glucose, insulin, GLP-1 and CCK in the studied subjects have been published previously [16,17]. In summary, duodenal lining lowered glucose and CCK within one week and led to increased postprandial AUC of GLP-1 at week 1 and week 24 after DJBL placement (Fig. 4). Insulin levels were not affected. The only bile acid species that consistently correlated with the AUC of GLP-1 on all study days, was fasting DCA: baseline: r = 0.50, p < 0.05*****; week 1 after placement: r = 0.56, p < 0.05*****; week 24 after placement: r = 0.63, p < 0.01******. No other correlations were detected for bile acid species to AUC, fasting or peak levels of either glucose, CCK or GLP-1 (data not shown).

There were no significant differences in either fasting levels, postprandial plasma course, or postprandial levels of FGF19 at baseline, week 1 or week 24 after DJBL placement (see Table 1). However, at baseline, there were strong correlations between peak FGF19 and virtually all bile acids (peak total bile acids $r = 0.79$, $p < 0.001$*******; peak total CA, $r = 0.78$, $p < 0.001$*******; peak total CDCA, $r = 0.79$, $p < 0.001$******* and peak total DCA, $r = 0.63$, $p < 0.007$******* that were lost after DJBL placement. Likewise, postprandial AUC of FGF19 and AUC of total bile acids correlated ($r = 0.84$, $p < 0.001$*******) at baseline but not after treatment.

Individual AUCs of CA, CDCA or DCA did not correlate significantly with FGF19 AUC after Bonferroni correction. FGF19 samples at week 0 for timepoint 180 min were missing due to insufficient sample volume.

Discussion

Duodenal lining markedly increases fasting and postprandial levels of unconjugated bile acids 24 weeks after placement of the DJBL. The TGR5 target GLP-1 was higher after 1 and 24 weeks. Initial strong correlations of bile acids

and FGF19 were lost at 1 week after placement. Bile acid levels were unchanged 1 week after placement, which supports the notion that the bile acid pool size may have increased gradually over time although metabolic improvements occurred immediately after placement [17]. This is in line with several studies performed in patients after RYGB surgery [12,20–23].

The DJBL-induced increase of bile acids was dependent on unconjugated bile acids, and more exaggerated compared to changes seen after RYGB surgery [12,21,23–25]. Dietary weight loss only marginally increases specific bile acids [26]. Hepatic conjugation of *newly* synthesized bile acids to occurs in the peroxisome, whereas re-conjugation of deconjugated bile acids occurs in the cytosol [27]. The reconjugation machinery is able to conjugate a potentially enlarged bile acid pool, as 99% of serum bile acids in patients treated with large doses of unconjugated ursodeoxycholate is conjugated [28]. Changes in the gut microbiome are possibly responsible for our results. Bacterial deconjugation is complete in the cecum and gut biotransformation of bile acids (i.e. ratios of CA: DCA and CDCA:LCA) were altered after 24 weeks [29]. The DJBL may have led to a different interplay between bile acids, gut bacteria and the gut lumen with effects on entero-endocrine hormones that regulate motility, transit and bile salt reabsorption via the apical sodium-dependent bile acid transporter (ASBT). This could result in increased deconjugation and increased colonic uptake not buffered by liver clearance [30]. The initial increase in unconjugated bile acids after DJBL placement may go unnoticed until the amount of unconjugated bile acids reaches a tipping point, exceeding liver clearance.

It has been suggested that the increase in plasma bile acids after RYGB surgery may be due to increased bile acid recirculation and FXR dependent transcriptional upregulation [31,32]. The former could be achieved by adaptive growth and concomitant increases in ASBT. However, previous studies after RYGB were not unequivocal [12,21,23–25].

The increased GLP-1 seems attributable to increased TGR5 signalling in the gut, although insulin secretion remained unchanged. Moreover, GLP-1 levels increased at week 1 whereas bile acid levels increased later which may be explained by an early increase of bile acids in the gut that were not yet noticeable peripherally. Indeed, our findings are in partial agreement with a previous study that examined

the effects of DJBL and also found increased GLP-1 levels [33]. However, this study did not investigate subfractions of bile acids. Interestingly, they were able to observe an increase in FGF19 that did not result in lower plasma bile acids. Most subjects with obesity or DM2 exhibit reduced serum FGF19 levels [34]. Here, FGF19 levels were unchanged throughout the entire study. The mismatch between plasma bile acid and FGF19 levels is difficult to explain FXR sensitivity to its ligand could be decreased, either as a cause of the increased circulating bile acid pool or as a consequence of it. A recent study in healthy subjects revealed that FXR agonist-induced repression of bile salt synthesis occurred without alterations in FGF19 level [35].

Our study has a few limitations. First, we were only able to perform a short mixed-meal test, which may have missed differences in postprandial signalling factors that occur later (such as FGF19 that peaks between 3 and 4 h). Also, we were not able to document the exact moment where changes in bile acid homeostasis became apparent due to the timing of experiments at week 1 and 24. Additionally, this was an observational uncontrolled study emphasizing the need for future controlled studies. Finally, we did not quantify satiety and appetite with visual analogue scores.

In this study, duodenal lining led to beneficial improvements in glucose metabolism and weight in patients with DM2. Surprisingly, unconjugated bile acids showed a marked increase after 24 weeks whereas the increase in GLP-1 was evident after one week. In contrast, correlations of bile acids and FGF19 were abolished immediately. Our data suggest that future studies should be comparing effects of duodenal lining and other means of weight loss on bile acids, GLP-1 and FGF19 taking into account the time course of these changes.

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References

1. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone acti- vation. Nature 2006;439(7075):484–9.
2. Sato H, Genet C, Strehle A, Thomas C, Lobstein A, Wagner A, et al. Anti- hyperglycemic activity of a TGR5 agonist isolated from Olea europaea. Biochem Biophys Res Commun 2007;362(4):793–8.
3. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284(5418):1365–8.
4. Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, et al. Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP- 1c. J Clin Invest 2004;113(10):1408–18.
5. Kliewer SA, Mangelsdorf DJ. Bile acids as hormones: the FXR-FGF15/19 pathway. Dig Dis 2015;33(3):327–31.
6. Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, et al. TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metab 2009;10(3):167–77.
7. Haeusler RA, Astiarraga B, Camastra S, Accili D, Ferrannini E. Human insulin resis- tance is associated with increased plasma levels of 12α-hydroxylated bile acids. Di-abetes 2013;62(12):4184–91.
8. Brufau G, Stellaard F, Prado K, Bloks VW, Jonkers E, Boverhof R, et al. Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly asso- ciated with changes in bile acid metabolism. Hepatology 2010;52(4):1455–64.
9. Glicksman C, Pournaras DJ, Wright M, Roberts R, Mahon D, Welbourn R, et al. Post- prandial plasma bile acid responses in normal weight and obese subjects. Ann Clin Biochem 2010;47(Pt 5):482–4.
10. Haeusler RA, Camastra S, Nannipieri M, Astiarraga B, Castro-Perez J, Xie D, et al. In- creased bile acid synthesis and impaired bile acid transport in human obesity. J Clin Endocrinol Metab 2016;101(5):1935–44.
11. Sonne DP, van Nierop F Samuel, Kulik W, Soeters MR, Vilsbøll T, Knop FK, et al. Post- prandial plasma concentrations of individual bile acids and FGF-19 in patients with type 2 diabetes. J Clin Endocrinol Metab 2016;101(8):jc20161607.
12. Jørgensen NB, Dirksen C, Bojsen-Møller KN, Kristiansen VB, Wulff BS, Rainteau D, et al. Improvements in glucose metabolism early after gastric bypass surgery are not explained by increases in total bile acids and fibroblast growth factor 19 concen- trations. J Clin Endocrinol Metab 2015;100(3):E396–406.
13. Schauer PR, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, Pothier CE, et al. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. N Engl J Med 2012;366(17):1567–76.
14. Escalona A, Pimentel F, Sharp A, Becerra P, Slako M, Turiel D, et al. Weight loss and metabolic improvement in morbidly obese subjects implanted for 1 year with an en- doscopic duodenal-jejunal bypass liner. Ann Surg 2012;255(6):1080–5.
15. Schouten R, Rijs CS, Bouvy ND, Hameeteman W, Koek GH, Janssen IMC, et al. A mul- ticenter, randomized efficacy study of the EndoBarrier Gastrointestinal Liner for presurgical weight loss prior to bariatric surgery. Ann Surg 2010;251(2):236–43.
16. de Jonge C, Rensen SS, Verdam FJ, Vincent RP, Bloom SR, Buurman WA, et al. Impact of duodenal-jejunal exclusion on satiety hormones. Obes Surg 2016;26(3):672–8.
17. de Jonge C, Rensen SS, Verdam FJ, Vincent RP, Bloom SR, Buurman WA, et al. Endo- scopic duodenal–jejunal bypass liner rapidly improves type 2 diabetes. Obes Surg 2013;23(9):1354–60.
18. Bootsma AH, Overmars H, van Rooij A, van Lint AE, Wanders RJ, van Gennip AH, et al. Rapid analysis of conjugated bile acids in plasma using electrospray tandem mass spectrometry: application for selective screening of peroxisomal disorders. J Inherit Metab Dis 1999;22(3):307–10.
19. Lips MA, De Groot GH, Berends FJ, Wiezer R, Van Wagensveld BA, Swank DJ, et al. Calorie restriction and Roux-en-Y gastric bypass have opposing effects on circulating FGF21 in morbidly obese subjects. Clin Endocrinol (Oxf) 2014;81(6):862–70.
20. Patti M-E, Houten SM, Bianco AC, Bernier R, Larsen PR, Holst JJ, et al. Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. Obesity 2009;17(9):1671–7.

21. Kohli R, Bradley D, Setchell KD, Eagon JC, Abumrad N, Klein S. Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding in- creases circulating bile acids. J Clin Endocrinol Metab 2013;98(April):708–12.
22. LaRusso NF, Hoffman NE, Korman MG, Hofmann AF, Cowen AE. Determinants of fasting and postprandial serum bile acid levels in healthy man. Am J Dig Dis 1978; 23(5):385–91.
23. Dutia R, Embrey M, O'Brien CS, Haeusler RA, Agénor KK, Homel P, et al. Temporal changes in bile acid levels and 12α-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes. Int J Obes (Lond) 2015;39(April):806–13.
24. Sachdev S, Wang Q, Billington C, Connett J, Ahmed L, Inabnet W, et al. FGF 19 and bile acids increase following Roux-en-Y gastric bypass but not after medical man- agement in patients with type 2 diabetes. Obes Surg 2016;26(5):957–65.
25. De Giorgi S, Campos V, Egli L, Toepel U, Carrel G, Cariou B, et al. Long-term effects of Roux-en-Y gastric bypass on postprandial plasma lipid and bile acids kinetics in female non diabetic subjects: a cross-sectional pilot study. Clin Nutr 2015;34(5):911–7.
26. van Nierop FS, Kulik W, Endert E, Schaap FG, Olde Damink SW, Romijn JA, et al. Ef- fects of acute dietary weight loss on postprandial plasma bile acid responses in obese insulin resistant subjects. Clin Nutr 2016;13:1–6.
27. Pellicoro A, van den Heuvel FAJ, Geuken M, Moshage H, Jansen PLM, Faber KN. Human and rat bile acid-CoA:amino acid N-acyltransferase are liver-specific peroxisomal enzymes: implications for intracellular bile salt transport. Hepatology 2007;45(2):340–8.
28. Gurantz D, Schteingart CD, Hagey LR, Steinbach JH, Grotmol T, Hofmann AF. Hypercholeresis induced by unconjugated bile acid infusion correlates with recov- ery in bile of unconjugated bile acids. Hepatology 1991;13(3):540–50.
29. Hamilton JP, Xie G, Raufman J-P, Hogan S, Griffin TL, Packard CA, et al. Human cecal bile acids: concentration and spectrum. Am J Physiol Gastrointest Liver Physiol 2007;293(1):G256–63.
30. Eggink HM, van Nierop FS, Schooneman MG, Boelen A, Kalsbeek A, Koehorst M, et al. Transhepatic bile acid kinetics in pigs and humans. Clin Nutr 2017;37(4):1–9.
31. Docherty NG, Le Roux CW. Physiological adaptations following Roux-en-Y gastric bypass and the identification of targets for bariatric mimetic pharmacotherapy. Curr Opin Pharmacol 2015;25(18):23–9.
32. Pournaras DJ, Glicksman C, Vincent RP, Kuganolipava S, Alaghband-Zadeh J, Mahon D, et al. The role of bile after Roux-en-Y gastric bypass in promoting weight loss and improving glycaemic control. Endocrinology 2012;153(August):1–7.
33. Kaválková P, Mráz M, Trachta P, Kloučková J, Cinkajzlová A, Lacinová Z, et al. Endo- crine effects of duodenal–jejunal exclusion in obese patients with type 2 diabetes mellitus. J Endocrinol 2016;231(1):11–22.
34. Zhang F, Yu L, Lin X, Cheng P, He L, Li X, et al. Minireview: roles of fibroblast growth factors 19 and 21 in metabolic regulation and chronic diseases. Mol Endocrinol 2015;29(10):1400–13.
35. Al-khaifi A, Rudling M, Angelin B. An FXR agonist reduces bile acid synthesis inde- pendently of increases in FGF19 in healthy volunteers. Gastroenterology 2018 Oct; 155(4):1012–6.

Part IV

Central nervous system

7

Diurnal analysis of postprandial plasma bile acid excursions in obese patients with type 2 diabetes and healthy control subjects

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Abstract

Patients with type 2 diabetes (T2D) have a disturbed diurnal rhythm of plasma glucose tolerance. We hypothesized that this may be due to altered diurnal regulation of plasma bile acid (BA) levels. We provided six T2D patients and six age-matched healthy controls with three identical meals per day, and measured plasma concentrations of primary and secondary BAs using tandem mass spectrometry. Postprandial BA responses were not affected by time of day.
However, postprandial peak times were earlier for total BAs, chenodeoxycholic acid, and glycine conjugates in T2D, which may be explained by increased intestinal and/or decreased liver BA uptake.
Keywords: Diurnal rhythm, obesity, type 2 diabetes, bile acids, glucose metabolism
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Introduction

Bile acids (BAs) are secreted into the gut after every meal in order to emulsify lipid droplets. After reabsorption into the portal circulation, a fraction of BAs escapes the enterohepatic cycle and enters the systemic circulation in a pattern of postprandial peaks. Experimental and observational data suggest that these circulating BAs affect lipid and glucose metabolism (1). By binding to the nuclear Farnesoid X Receptor (FXR), circulating BAs decrease hepatic glucose production and increase muscle insulin sensitivity (2) and possibly increase pancreatic insulin secretion (3). Additionally, activation of the G-protein-coupled BA receptor (GPBAR1/TGR5) increases energy expenditure in brown adipose tissue (4) and intestinal incretin secretion (5).

In clinical studies, the BA sequestrants cholestyramine (6) and colesevelam (reviewed in (7; 8)) effectively reduce plasma glucose levels in patients with type 2 diabetes, indicating that interventions in the BA pool affect glucose metabolism. In human observational studies, obese patients with type 2 diabetes showed exaggerated postprandial BA responses (9; 10) and patients with type 2 diabetes (11) and insulin resistance (12) show an altered composition of the fasting BA pool. Since individual BA subtypes show different receptor binding affinities *in vitro* (13; 14) and in mice (15), changes in pool composition may affect glucose metabolism.

There is evidence suggesting a circadian rhythm in hepatic BA synthesis (16-19). This rhythm is likely to be driven by the mammalian circadian (~24hr) timing system based on the molecular transcriptional-translational clock cycle (20; 21). However, the question whether postprandial BA excursions change over the day remains to be answered, since most studies investigating the diurnal patterns in postprandial BA excursions used varying meal sizes (22; 23) or varying intervals between meals (24-26). The only study that used identical and equidistant meals stems from the 70s, measured only glycine conjugates using a non-specific radioimmunoassay method, and detected no diurnal rhythm in glycine conjugate excursions (27). Since then, more specific mass spectrometric methods have been developed to detect BA concentrations.

In a recent study we demonstrated that obese patients with type 2 diabetes show attenuated diurnal rhythms of plasma glucose tolerance in response to

three equidistant identical meals, compared to healthy control subjects (28). We hypothesized that an altered diurnal rhythm of postprandial BA excursions in obese patients with type 2 diabetes contributes to the altered diurnal rhythm in glucose metabolism. In the present study we determined the diurnal rhythm of postprandial BA excursions after three equidistant, equicaloric meals in obese subjects with type 2 diabetes and lean healthy control subjects. We used tandem mass spectrometry to detect the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) and the secondary BAs deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) in their conjugated and unconjugated forms.

Materials and methods

Subjects

This study is an ancillary study of a previous study investigating the diurnal rhythms in glucose metabolism and adipose tissue gene expression of obese patients with type 2 diabetes (28). Briefly, we included 6 male obese patients with type 2 diabetes according to the 2010 American Diabetes Association (ADA) criteria (10), BMI 25-40 kg/m2, age 30-75 yr, and 6 healthy age-matched male control subjects with BMI ≤ 25 kg/m2. Exclusion criteria were any acute or chronic disease that impairs metabolism or food digestion or absorption, shift work in the month prior to study participation and crossing more than one time zone in the month prior to study participation. In addition, type 2 diabetes patients were excluded if they used any antidiabetic drug other than metformin. Subjects were recruited by announcements at public locations, in local newspapers and patient magazines.

Study protocol

The study was approved by the Institutional Review Board of the AMC, and performed according to the Declaration of Helsinki of October 2004. The study was registered as NTR3234. The study was performed between February 2012 and March 2013 at the department of Endocrinology and Metabolism of the AMC. During a three-day baseline assessment, participants completed food diaries and a sleep-wake log. Subsequently, on the morning of day 1, the average wake-up time and sleep time were determined for each participant from the sleep-wake log. Individual Zeitgeber Times (ZT) were determined with ZT 0 representing average wake-up time. From the lunch on day 1, participants consumed three

identical liquid meals (Ensure Plus; 1.5 kcal/ml, 54E% carbohydrates, 29E% fat, 17E% protein; Abbott Nutrition, Columbus, Ohio, USA) per day. The total daily amounts of calories was set at 25 kcal/kg bodyweight, except for one healthy control who had very high baseline daily food intake and was provided with 35 kcal/kg bodyweight. The type 2 diabetes patients were instructed to pause metformin use from two days prior to the measurements until study end (in total 5 days).

At the evening of day 2 subjects were admitted to the clinical research unit. Subjects slept undisturbed in darkness (0 lux) during their habitual sleep times. On day 3 at ZT 0, the light was turned on at ~150 lux at eye level. Participants were provided with the three identical liquid meals at ZT 0:30, ZT 6:00 and ZT 11:30. Blood samples for BA measurements were obtained from a cannula in a peripheral arm vein prior to every meal and with 60-min intervals in the postprandial period. Blood samples were centrifuged for 10 minutes at 3000 rpm at 4°C and plasma was stored at -20°C.

Endpoints

Primary endpoints were the effect of mealtime on postprandial BA excursions of total BAs, CA, CDCA, DCA, UDCA, taurine conjugated, glycine conjugated and unconjugated BAs in lean subjects and in obese patients with type 2 diabetes. Secondary endpoints were the differences between lean control subjects and obese patients with type 2 diabetes in fasting total BAs, CA, CDCA, DCA, UDCA, taurine conjugated, glycine conjugated and unconjugated BAs.

Measurement of bile acids

Taurine- and glycine-conjugated internal standards [2,2,4,4-$^2H_4$]taurocholic acid (tauro-CA), [2,2,4,4-$^2H_4$] taurochenodeoxycholic acid (tauro-CDCA), [2,2,4,4-$^2H_4$]glycocholic acid (glyco-CA) and [2,2,4,4-$^2H_4$] glycochenodeoxycholic acid (glyco-CDCA) were synthesized as described by Mills et al (29). Plasma (50μL) was diluted with 50μL internal standard solution (29) and 500μL acetonitrile was added while mixing. After centrifugation, the sample was dried under N_2 and reconstituted in 100μL methanol:H_2O (1:3). Subsequently, 10μL of this solution was injected onto a UPLC column (Waters Acquity BEH C18; length 10cm, i.d. 2.1mm, particle size 1.7μm). The BAs were separated using a gradient from 98% 5mM ammoniumformate (pH8.1):methanol (3:1 v:v) to 98% acetonitrile:H_2O (9:1 v:v) in 5 minutes at a flow rate of 350μl/min.

BAs were detected with a Waters Quattro Premier XE tandem mass spectrometer in the negative electrospray ionization mode. For the detection of glycine- and taurine-conjugated BAs, cone voltage was set at 60V and 90V respectively, using 40eV and 60eV collision energy respectively. Overall collision gas pressure was 3 x 10^{-3}mbar argon and the source temperature was kept at 120°C. Glycine-conjugated BAs were detected by multiple reaction monitoring (MRM) using specific transitions with a mass difference of m/z 74, since all glycine conjugates specifically lose the m/z 74 fragment from the quasimolecular ion after fragmentation (29). Transitions: glyco-CDCA, glyco-DCA and glyco-UDCA (448→74); [$^2H_4$]glyco-CDCA (452→74); glyco-CA (464→74); [$^2H_4$]glyco-CA (468→74). Taurine-conjugated BAs were detected in a similar manner using specific transitions with a mass difference of m/z 80, due to the loss of a part of the taurine moiety. Transitions: tauro-CDCA, tauro-DCA and tauro-UDCA (498→80); [$^2H_4$]tauro-CDCA (502→80); tauro-CA (514→80); [$^2H_4$]tauro-CA (518→80). Unconjugated BAs were detected using selected ion recording (cone voltage 70V, collision energy 10eV); CDCA and DCA *m/z* 391; CA *m/z* 407.

Absolute concentrations of either taurine- or glycine-conjugated CA and CDCA were calculated using calibration curves by relating the peak area to the peak area of the respective $^2H_4$ internal standard. For DCA and UDCA conjugates, the [$^2H_4$] CDCA internal standards were used. Calibration curves were labelled linear (r 0.98) from 0.1 to 100μmol/L.

Power analysis and statistics

This study had sufficient power to detect a *meal time* effect of 180 µmol·min/L on the postprandial area under the curve (AUC) (corresponding to an average change of 1 µmol/L in plasma BA levels) and an effect of *group x meal time* interaction of 360 µmol·min/L on the AUC (corresponding to an average 2 µmol/L difference between the meal effects of two groups). A 1 µmol/L difference was considered a relevant difference based on *in vitro* ligand-receptor interaction between BAs and either FXR (14) or TGR5 (30). The study could detect overall *group* effects on AUC of 1100 µmol·min/L on the AUC (corresponding to a group difference of 6 µmol/L in average plasma BA levels).

Furthermore, the study had sufficient power to detect the following differences in fasting plasma levels: total BAs: 4.7 µmol/L, CA: 0.6 µmol/L, CDCA: 2.7 µmol/L,

DCA: 1.1 µmol/L, UDCA: 1.5 µmol/L, taurine conjugates: 0.5 µmol/L, glycine conjugates: 3.0 µmol/L, unconjugated: 1.4 µmol/L. Power analysis was performed with a significance level of 0.05 a power of 80% and the observed standard deviations, using nQuery Advisor 7.0 (Statistical Solutions Limited).

Data are presented as means with their standard errors of the mean. The postprandial period was defined to extend until 180min after meal onset, and AUCs were calculated with the trapezoid rule with Excel 2010 (Microsoft, Redmond, Washington, USA). Individual postprandial peak times were defined as the first timepoint at which maximal concentration was reached. AUC and peak time data were subsequently analyzed with a full factorial repeated measures general linear model with *meal time* as the within-subjects factor and *group* as the between-subjects factor. Fasting BA levels were assessed with a two-tailed independent-samples Student's t-test. All statistical tests were performed with IBM SPSS Statistics (version 21; SPSS, Inc.).

Results

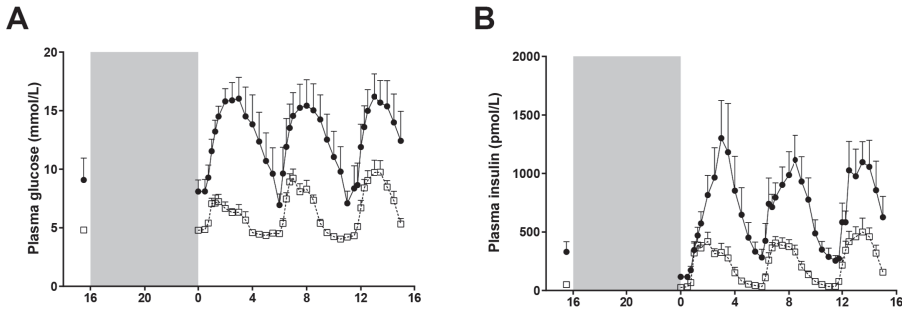
We included 6 obese patients with type 2 diabetes (age 60±3 yr, BMI 30±1 kg/m^2, all metformin users, diabetes duration 9±4 yr) and 6 healthy lean control subjects (age 57±4 yr, BMI 24±1 kg/m^2) (28).

As described previously (28) the diurnal rhythm of plasma glucose iAUC was different between obese patients with type 2 diabetes and healthy controls. The obese patients with type 2 diabetes showed a decrease in plasma glucose iAUC

Table 1. Fasting bile acid levels. Plasma was obtained after an overnight fast on day 3. Student's T-test showed no significance between group differences. Data are means ± SEM.

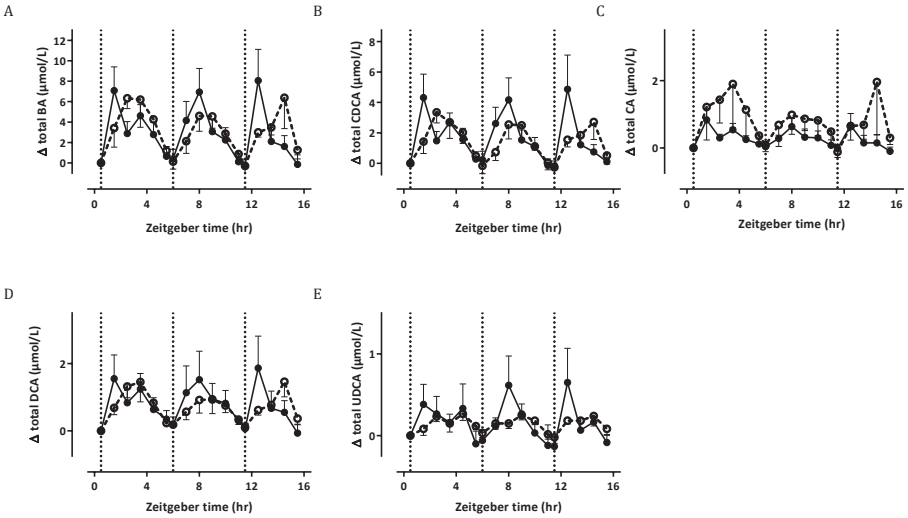
		Control	Type 2 diabetes	P value
Total bile acids	(µmol/L)	2.1 ± 1.0	3.6 ± 1.1	0.327
CDCA	(µmol/L)	1.2 ± 0.7	1.6 ± 0.5	0.705
CA	(µmol/L)	0.4 ± 0.2	0.5 ± 0.1	0.583
DCA	(µmol/L)	0.3 ± 0.0	0.8 ± 0.3	0.192
UDCA	(µmol/L)	0.2 ± 0.1	0.8 ± 0.4	0.212
Glycine conjugates	(µmol/L)	1.4 ± 0.7	2.3 ± 0.7	0.393
Taurine conjugates	(µmol/L)	0.1 ± 0.1	0.3 ± 0.1	0.292
Unconjugated	(µmol/L)	0.6 ± 0.2	1.1 ± 0.4	0.270

Figure 1. Patients with type 2 diabetes show a reduced diurnal rhythm in postprandial plasma glucose excursions.



A Patients with type 2 diabetes (closed circles connected with black line) show a decrease of the glucose incremental area under the curve (iAUC) from breakfast to lunch and no change in the iAUC from lunch to dinner, whereas healthy controls (open squares connected with striped line) showed a diurnal rhythm in postprandial glucose excursions with an increase of the glucose iAUC from breakfast to lunch and no change in the iAUC from lunch to dinner. (Linear mixed-effects model, *group* x *meal time* interaction P=0.004). **B** Patients with type 2 diabetes show increased insulin levels compared to healthy subjects, but neither group shows a diurnal rhythm in postprandial insulin excursions. Figure adapted from (28).

Figure 2. Postprandial plasma bile acids: subtypes.



Lean subjects (open symbols) and obese patients with type 2 diabetes (closed symbols) were provided with equicaloric meals at ZT 0:30, 6:00 and 11:30 (dotted lines). **A** Total bile acids **B** CDCA **C** CA **D** DCA and **E** UDCA responses. Data are change versus baseline (ZT 0:15) expressed as means ± SEM. Zeitgeber Time 0 represents the time of lights on.

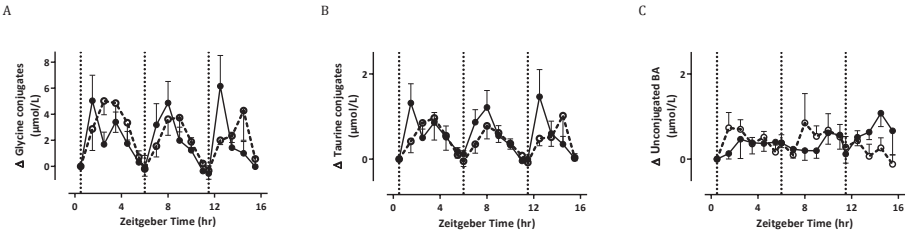
Table 2. Area under the curve of postprandial bile acid levels. Lean healthy control subjects and patients with type 2 diabetes (n=6 per group) were provided with three identical liquid meals at 5.5hr intervals. P values represent the effects of the fixed effects of *meal time* and *group x meal time* interaction as assessed with a Repeated Measures General Linear Model.

		Control			Type 2 diabetes			P values		
		Breakfast	Lunch	Dinner	Breakfast	Lunch	Dinner	*meal time*	*group*	*group x meal time*
Total bile acids	(μmol • min/L)	1146 ± 266	920 ± 220	945 ± 248	1362 ± 204	1434 ± 292	1304 ± 246	0,357	0,304	0,269
CDCA	(μmol • min/L)	584 ± 167	483 ± 166	491 ± 184	691 ± 145	744 ± 188	661 ± 193	0,427	0,474	0,272
CA	(μmol • min/L)	279 ± 138	192 ± 85	202 ± 105	166 ± 15	152 ± 16	137 ± 15	0,084	0,521	0,285
DCA	(μmol • min/L)	224 ± 35	183 ± 45	186 ± 14	317 ± 93	339 ± 127	315 ± 92	0,262	0,262	0,317
UDCA	(μmol • min/L)	60 ± 21	63 ± 21	65 ± 21	189 ± 104	199 ± 99	191 ± 94	0,574	0,226	0,676
Glycine conjugates	(μmol • min/L)	868 ± 233	665 ± 179	622 ± 172	886 ± 134	958 ± 194	877 ± 175	0,249	0.460	0,159
Taurine conjugates	(μmol • min/L)	129 ± 48	109 ± 40	112 ± 47	185 ± 46	201 ± 61	189 ± 65	0,912	0,316	0,560
Unconjugated	(μmol • min/L)	150 ± 36	146 ± 24	211 ± 57	291 ± 83	276 ± 77	239 ± 49	0,882	0,214	0,120

Table 3. Peak times of postprandial bile acid responses. P values represent the effects of the fixed effects of *meal time* and *group x meal time* interaction as assessed with a Repeated Measures General Linear Model.

		Control			Type 2 diabetes			P values		
		Breakfast	Lunch	Dinner	Breakfast	Lunch	Dinner	*meal time*	*group*	*group x meal time*
Total bile acids	(min)	160 ± 20	190 ± 18	150 ± 26	90 ± 20	100 ± 13	70 ± 10	0,186	0,001	0,747
CDCA	(min)	160 ± 20	150 ± 26	150 ± 26	90 ± 30	100 ± 13	70 ± 10	0,644	0,015	0,709
CA	(min)	130 ± 18	130 ± 24	190 ± 18	90 ± 20	170 ± 36	70 ± 24	0,286	0,069	0,014
DCA	(min)	120 ± 16	190 ± 19	130 ± 29	100 ± 20	144 ± 41	72 ± 12	0,056	0,060	0,687
UDCA	(min)	170 ± 24	160 ± 25	90 ± 20	120 ± 38	120 ± 19	80 ± 13	0,057	0,057	0,916
Glycine conjugates	(min)	180 ± 22	160 ± 25	120 ± 22	90 ± 30	100 ± 13	70 ± 10	0,189	0.001	0,667
Taurine conjugates	(min)	150 ± 26	110 ± 29	110 ± 24	100 ± 25	100 ± 13	70 ± 10	0,337	0,070	0,673
Unconjugated	(min)	140 ± 37	100 ± 55	190 ± 18	130 ± 29	170 ± 45	70 ± 29	0,988	0,521	0,062

Figure 3. Postprandial plasma bile acids: conjugates.



Lean subjects (open symbols) and obese patients with type 2 diabetes (closed symbols) were provided with equicaloric meals at ZT 0:30, 6:00 and 11:30 (dotted lines). **A** glycine conjugated **B** taurine conjugated and **C** unconjugated bile acid excursions. Data are change versus baseline (ZT 0:15) expressed as means ± SEM. Zeitgeber Time 0 represents the time of lights on.

from breakfast to lunch and no change from lunch to dinner, whereas the healthy controls showed an increase in plasma glucose iAUC from breakfast to lunch and a further increase to dinner. Plasma insulin iAUC values showed no significant rhythm over the day in either of the groups (Fig. 1).

Fasting total BA levels and fasting BA subtype levels were not different between healthy subjects and obese patients with type 2 diabetes (Table 1). We observed clear postprandial peaks of total plasma BAs and the individual BA subtypes CA, CDCA, DCA after all three meals. The major constituent of the postprandial BA response was CDCA (Fig. 2). Glycine-conjugated forms peaked in both obese patients with type 2 diabetes and healthy subjects, whereas taurine-conjugated and unconjugated BA peaks were less noticeable. Most BAs appeared in plasma in their glycine-conjugated form (Fig. 3).

Meal time did not affect postprandial AUCs of either total BAs, any individual BA subtype or the distribution of conjugated vs unconjugated BAs (Fig. 2 and 3, Table 2). AUCs did not differ between the obese type 2 diabetes patients and healthy subjects. However, postprandial peak times were earlier for total BAs, CDCA, and glycine conjugates in the obese patients with type 2 diabetes compared to healthy subjects (Fig. 2 and 3, Table 3, *group* effect on peak time $P<0.05$). Meal time did not affect peak times (Table 2).

Discussion

In the present study, we analyzed the diurnal rhythm in postprandial responses of all individual BA subtypes after equidistant equicaloric meals in type 2 diabetes patients and controls. Obese patients with type 2 diabetes show an attenuated diurnal rhythm of postprandial plasma glucose AUCs in response to equidistant identical meals, but no altered diurnal rhythm in postprandial BA AUCs. Therefore, it is unlikely that an altered rhythm in postprandial BA responses contributes to the disturbed diurnal rhythm in glucose metabolism in obese patients with type 2 diabetes.

Despite the demonstration in healthy human volunteers (17; 19) of a circadian rhythm in BA synthesis, we did not observe a diurnal rhythm of postprandial total BA excursions in either obese patients with type 2 diabetes or controls. It is important to distinguish postprandial BA *levels* from BA *biosynthesis*. The former can be measured with different mass spectrometry techniques whereas the latter can be analyzed by different isotopic dilution methods or the measurement of plasma 7α-hydroxy-4-cholesten-3-one (C4) levels (17; 19; 31). C4 strongly relates to the hepatic enzymatic activity of CYP7A1 during diurnal changes. Possibly, the rhythm in BA biosynthesis is buffered by the BA pool in the gallbladder and the intestine, since the majority of postprandial plasma BAs originates from the recirculating pool. Studies from the late 1970's shows that the major determinant of serum BAs is their rate of intestinal absorption (25). Alternatively, the diurnal rhythm in BA biosynthesis may be counteracted by a rhythm in hepatic BA reuptake from the portal circulation, since in healthy humans hepatic cholesterol synthesis peaks during the night (32; 33), whereas BA biosynthesis peaks during the daytime (17).

Since individual BA subtypes have different receptor binding affinities (13-15), we analyzed the diurnal rhythms in the plasma excursions of cholic acid (CA) and chenodeoxycholic acid (CDCA) and the secondary BAs deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) in their conjugated and unconjugated forms. CDCA was the major constituent of postprandial BA excursions, and most BAs appeared in the glycine-conjugated form, both in control subjects and in obese patients with type 2 diabetes. This is consistent with previous studies on postprandial BA excursions in healthy subjects (12; 22; 26; 27; 34). However, none

of the individual BA subtypes or conjugates showed a diurnal rhythm in plasma excursions.

Postprandial BA peaks occurred significantly earlier after all meals in obese patients with type 2 diabetes. Previous publications comparing postprandial BA concentrations between healthy and pathological states have not addressed this (9-11). The earlier peaks may be explained by increased intestinal uptake and/or decreased liver sinusoidal uptake of BAs (25). Specifically, increased intestinal absorption can be facilitated by hypertrophic changes of the gut mucosa in patients with type 2 diabetes (35; 36). In addition, the expression of hepatic BA transporters is negatively correlated with BMI (37) and reduced hepatic BA uptake may contribute to the earlier peak levels in patients with type 2 diabetes. The difference in peak time did not extend to differences in BA peak concentrations or AUCs, which are mostly determined by meal size and composition (10).

This study was specifically designed to detect diurnal rhythms in postprandial glucose and BA excursions, but not to detect small differences in fasting plasma BA levels or postprandial BA excursions between obese patients with type 2 diabetes and healthy control subjects. Consequently, we cannot confirm previously published studies showing that obese patients with type 2 diabetes have elevated plasma BA levels compared to healthy subjects (9-12). Although we stopped metformin prior to the study, we cannot exclude the possibility that chronic metformin use influenced our results since it increases the bile acid pool within the intestine and modulates the intestinal microbiome (38).

In conclusion, in the present study with equidistant equicaloric meals we did not detect a diurnal rhythm in the postprandial BA responses in either healthy subjects or obese patients with type 2 diabetes. Therefore, the altered diurnal rhythm of glucose tolerance in patients with type 2 diabetes is unlikely to be caused by an altered diurnal rhythm in plasma BA responses.

References

1. Kuipers F, Bloks VW, Groen AK: Beyond intestinal soap--bile acids in metabolic control. Nature reviews Endocrinology 2014;10:488-498.
2. Ma K, Saha PK, Chan L, Moore DD: Farnesoid X receptor is essential for normal glucose homeostasis. The Journal of clinical investigation 2006;116:1102-1109.
3. Renga B, Mencarelli A, Vavassori P, Brancaleone V, Fiorucci S: The bile acid sensor FXR regulates insulin transcription and secretion. Biochimica et biophysica acta 2010;1802:363-372.
4. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J: Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 2006;439:484-489.
5. Katsuma S, Hirasawa A, Tsujimoto G: Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochemical and biophysical research communications 2005;329:386-390.
6. Garg A, Grundy SM: Cholestyramine therapy for dyslipidemia in non-insulindependent diabetes mellitus. A short-term, double-blind, crossover trial. Annals of internal medicine 1994;121:416-422.
7. Ooi CP, Loke SC: Colesevelam for type 2 diabetes mellitus. The Cochrane database of systematic reviews 2012;12:CD009361.
7. Handelsman Y: Role of bile acid sequestrants in the treatment of type 2 diabetes. Diabetes Care 2011;34 Suppl 2:S244-250.
8. Vincent RP, Omar S, Ghozlan S, Taylor DR, Cross G, Sherwood RA, Fandriks L, Olbers T, Werling M, Alaghband-Zadeh J, Roux CW: Higher circulating bile acid concentrations in obese patients with type 2 diabetes. Ann Clin Biochem 2013; Jul;50(Pt 4):360-4.
10. Sonne DP, van Nierop FS, Kulik W, Soeters MR, Vilsboll T, Knop FK: Postprandial Plasma Concentrations of Individual Bile Acids and FGF-19 in Patients With Type 2 Diabetes. J Clin Endocrinol Metab 2016;101:3002-3009.
11. Brufau G, Stellaard F, Prado K, Bloks VW, Jonkers E, Boverhof R, Kuipers F, Murphy EJ: Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism. Hepatology 2010;52:1455-1464.
12. Haeusler RA, Astiarraga B, Camastra S, Accili D, Ferrannini E: Human insulin resistance is associated with increased plasma levels of 12alpha-hydroxylated bile acids. Diabetes 2013;62:4184-4191.
13. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM: Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284:1365-1368.
14. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B: Identification of a nuclear receptor for bile acids. Science 1999;284:1362-1365.
15. Song P, Rockwell CE, Cui JY, Klaassen CD: Individual bile acids have differential effects on bile acid signaling in mice. Toxicology and applied pharmacology 2015;283:57-64.
16. Mitropoulos KA, Balasubramaniam S, Gibbons GF, Reeves BE: Diurnal variation in the activity of cholesterol 7 -hydroxylase in the livers of fed and fasted rats. FEBS letters 1972;27:203-206.
17. Galman C, Angelin B, Rudling M: Bile acid synthesis in humans has a rapid diurnal variation that is asynchronous with cholesterol synthesis. Gastroenterology 2005;129:1445-1453.
18. Ma K, Xiao R, Tseng HT, Shan L, Fu L, Moore DD: Circadian dysregulation disrupts bile acid homeostasis. PLoSOne 2009;4:e6843.
19. Duane WC, Levitt DG, Mueller SM, Behrens JC: Regulation of bile acid synthesis in man. Presence of a diurnal rhythm. The Journal of clinical investigation 1983;72:1930-1936.
20. Stenvers DJ, Jonkers CF, Fliers E, Bisschop PH, Kalsbeek A: Nutrition and the circadian timing system. Progress in brain research 2012;199:359-376.
21. Stenvers DJ, Scheer F, Schrauwen P, la Fleur SE, Kalsbeek A: Circadian clocks and insulin resistance. Nature reviews Endocrinology 2019;15:75-89.
22. Angelin B, Bjorkhem I: Postprandial serum bile acids in healthy man. Evidence for differences in absorptive pattern between individual bile acids. Gut 1977;18:606-609.

23. Setchell KD, Lawson AM, Blackstock EJ, Murphy GM: Diurnal changes in serum unconjugated bile acids in normal man. Gut 1982;23:637-642.

24. LaRusso NF, Korman MG, Hoffman NE, Hofmann AF: Dynamics of the enterohepatic circulation of bile acids. Postprandial serum concentrations of conjugates of cholic acid in health, cholecystectomized patients, and patients with bile acid malabsorption. The New England journal of medicine 1974;291:689-692.

25. LaRusso NF, Hoffman NE, Korman MG, Hofmann AF, Cowen AE: Determinants of fasting and postprandial serum bile acid levels in healthy man. The American journal of digestive diseases 1978;23:385-391.

26. Schalm SW, LaRusso NF, Hofmann AF, Hoffman NE, van Berge-Henegouwen GP, Korman MG: Diurnal serum levels of primary conjugated bile acids. Assessment by specific radioimmunoassays for conjugates of cholic and chenodeoxycholic acid. Gut 1978;19:1006-1014.

27. Hepner GW, Demers LM: Dynamics of the enterohepatic circulation of the glycine conjugates of cholic, chenodeoxycholic, deoxycholic, and sulfolithocholic acid in man. Gastroenterology 1977;72:499-501.

28. Stenvers DJ, Jongejan A, Atiqi S, Vreijling JP, Limonard EJ, Endert E, Baas F, Moerland PD, Fliers E, Kalsbeek A, Bisschop PH: Diurnal rhythms in the white adipose tissue transcriptome are disturbed in obese individuals with type 2 diabetes compared with lean control individuals. Diabetologia 2019;62:704-716.

29. Mills KA, Mushtaq I, Johnson AW, Whitfield PD, Clayton PT: A method for the quantitation of conjugated bile acids in dried blood spots using electrospray ionization-mass spectrometry. Pediatric research 1998;43:361-368. Sato H, Macchiarulo A, Thomas C, Gioiello A, Une M, Hofmann AF, Saladin R, Schoonjans K, Pellicciari R, Auwerx J: Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. J Med Chem 2008;51:1831-1841.

30. Stellaard F, Bloks VW, Burgerhof HG, Scheltema RA, Murphy EJ, Romijn HA, Smelt GH, Brufau G, Kuipers F: Two time-point assessment of bile acid kinetics in humans using stable isotopes. Isotopes Environ Health Stud 2010;46:325-336.

31. Jones PJ, Schoeller DA: Evidence for diurnal periodicity in human cholesterol synthesis. Journal of lipid research 1990;31:667-673.

32. Cella LK, Van Cauter E, Schoeller DA: Diurnal rhythmicity of human cholesterol synthesis: normal pattern and adaptation to simulated "jet lag". The American journal of physiology 1995;269:E489-498.

33. Steiner C, Othman A, Saely CH, Rein P, Drexel H, von Eckardstein A, Rentsch KM: Bile acid metabolites in serum: intraindividual variation and associations with coronary heart disease, metabolic syndrome and diabetes mellitus. PloS one 2011;6:e25006.

34. Verdam FJ, Greve JW, Roosta S, van Eijk H, Bouvy N, Buurman WA, Rensen SS: Small intestinal alterations in severely obese hyperglycemic subjects. J Clin Endocrinol Metab 2011;96:E379-383.

35. Adachi T, Mori C, Sakurai K, Shihara N, Tsuda K, Yasuda K: Morphological changes and increased sucrase and isomaltase activity in small intestines of insulin-deficient and type 2 diabetic rats. Endocrine journal 2003;50:271-279.

36. Haeusler RA, Camastra S, Nannipieri M, Astiarraga B, Castro-Perez J, Xie D, Wang L, Chakravarthy M, Ferrannini E: Increased Bile Acid Synthesis and Impaired Bile Acid Transport in Human Obesity. J Clin Endocrinol Metab 2016;101:1935-1944.

37. McCreight LJ, Bailey CJ, Pearson ER: Metformin and the gastrointestinal tract. Diabetologia 2016;59:426-435.

Part V

Bile acids in perspective

8

Clinical relevance of the bile acid receptor TGR5 in metabolism

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Abstract

The bile acid receptor TGR5 (also known as GPBAR1) is a promising target for the development of pharmacological interventions in metabolic diseases, including type 2 diabetes, obesity, and non-alcoholic steatohepatitis. TGR5 is expressed in many metabolically active tissues, but complex enterohepatic bile acid cycling limits the exposure of some of these tissues to the receptor ligand. Profound interspecies differences in the biology of bile acids and their receptors in different cells and tissues exist. Data from preclinical studies show promising effects of targeting TGR5 on outcomes such as weight loss, glucose metabolism, energy expenditure, and suppression of inflammation. However, clinical studies are scarce. We give a summary of key concepts in bile acid metabolism; outline different downstream effects of TGR5 activation; and review available data on TGR5 activation, with a focus on the translation of preclinical studies into clinically applicable findings. Studies in rodents suggest an important role for TGR5 in GLP-1 secretion, insulin sensitivity, and energy expenditure. However, evidence of effects on these processes from human studies is less convincing. Ultimately, safe and selective human TGR5 agonists are needed to test the therapeutic potential of TGR5.

Introduction

Reducing postprandial hyperglycaemia is one of the main goals of modern diabetes therapy. Strategies that increase the postprandial anabolic response, such as short-acting insulin analogue therapy, have been shown to reduce HbA1c and long-term complications of the disease. Trying to emulate the success of therapies that target the glucagon-like peptide-1 (GLP-1) receptor, other G protein-coupled receptors have been suggested as potential therapeutic targets. Among these receptors, the G protein-coupled bile acid receptor 1 (GPBAR1), which is also known as TGR5, has received much attention in preclinical studies.[1–4] TGR5 and its bile acid ligands have been suggested to be drivers of the metabolic improvements seen after Roux-en-Y gastric bypass (RYGB) surgery.[5,6] As discussed in this Review, bile acid-induced TGR5 activation increases GLP-1 and insulin release, stimulates resting thermogenesis, and decreases inflammation, which are effects that could be beneficial in the treatment of obesity and type 2 diabetes.[1,2]

TGR5 was the first transmembrane G protein-coupled receptor shown to be responsive to bile acids.[7,8] It predominantly transmits its signal by increasing intracellular concentrations of cyclic AMP (cAMP), leading to rapid phosphorylation of downstream kinases. TGR5 is expressed in various cell types involved in bile acid secretion, the anti-inflammatory response, and energy metabolism, including gallbladder epithelial cells (cholangiocytes), gallbladder smooth muscle cells, Kupffer cells, intestinal L cells, pancreatic β cells, skeletal muscle cells, nerve cells, and brown adipocytes[8–10] (panel 1). Considering the meal-dependent rhythm of bile acid secretion—and thereby exposure of TGR5 to its ligand—in metabolically active tissues, this receptor might be regarded as a nutrient sensor, ideally placed to regulate integration of postprandial metabolic processes involved in trafficking, storage, and oxidation of nutrients.[19] However, the interplay of nutrients, different bile acids and their conjugates, GLP-1, insulin, and gastrointestinal transit time is complex and highly variable. TGR5 expression and function have mainly been studied in rodents and in vitro, and clinical studies are scarce. Nevertheless, treatments targeting Tgr5 might be promising for the treatment of several conditions such as type 2 diabetes, obesity, and non-alcoholic steatohepatitis.[20] To establish the clinical and translational impact of current findings, distinct differentiation between human and animal bile acid physiology and TGR5 function is necessary.

Here, we review physiological and pharmacological studies of TGR5 in relation to metabolism, and differences in animal and human bile acid physiology (panel 2). We focus on the translation of preclinical animal studies and in-vitro research to clinically significant improvements in the treatment of disorders such as obesity and type 2 diabetes.

TGR5 in GLP-1 secretion and glucose homoeostasis

Background

The association between human bile acid and glucose metabolism originated from the observation that patients with type 2 diabetes have increased bile acid pools.[27] In a study of bile acid kinetics from 2010,[28] patients with type 2 diabetes were shown to have increased concentrations of deoxycholic acid in the bile acid pool, at the expense of chenodeoxycholic acid. Additionally, cholic acid synthesis was upregulated, whereas the total pool size remained the same, suggesting increased conversion of cholic acid by the gut microbiota. Other studies showed similar increases in deoxycholic acid pool size in patients with type 2 diabetes.[29]

One of the links between bile acids and glucose homoeostasis is TGR5-mediated GLP-1 secretion (figure 1).[2] Subcutaneously injected GLP-1 receptor agonists have been part of the antidiabetic repertoire for the past 10 years. GLP-1 is a peptide hormone secreted by specialised enteroendocrine cells (L cells) in response to meal ingestion.[30] GLP-1 amplifies glucose-dependent insulin secretion, inhibits glucagon secretion, and reduces gastric emptying and appetite.[31] However, clinical application of GLP-1 has not delivered the impact on diabetes care that was predicted on the basis of its effect in animal studies.[32] One theory is that by subcutaneously delivering exogenous GLP-1 receptor agonists, local effects of GLP-1 near its site of secretion in the liver—such as stimulation of local afferent nerve terminals that project to the hypothalamus from the lamina propria, the portal vein, and the liver—are not achieved.[33] Because GLP-1 function is thought to be at least partially dependent on this local neural activation, an attempt to increase endogenous GLP-1 secretion is an attractive strategy for the treatment of type 2 diabetes. One approach is to potentiate stimulation of GLP-1 release with agents that are neither deposited (ie, bile acids or synthetic TGR5 receptor analogues) nor absorbed (ie, bile acid binding sequestrants); this approach might

prove superior in controlling type 2 diabetes and obesity compared with other incretin-based treatment strategies. This new treatment concept has gained support from preclinical and clinical studies.[2,34–36]

Preclinical data

The bile acid–TGR5–GLP-1 pathway was first explored by Thomas and colleagues,[2] who showed that glucose tolerance was improved in transgenic mice overexpressing Tgr5, and this improvement was accompanied by increased GLP-1 and insulin secretion. Adding a selective TGR5 agonist to a high-fat diet improved glucose tolerance, insulin and GLP-1 secretion, and insulin sensitivity in both liver and muscle of Tgr5-Tg mice, but not in Tgr5 knockout ($Tgr5^{-/-}$) mice.[2] The insulin-sensitising effect might be explained by the difference in bodyweight, because GLP-1 is not known to directly affect insulin sensitivity.

Enteral administration of several specific TGR5 agonists increases GLP-1 secretion and glucose tolerance.[3,37–40] Stimulation of TGR5 on the basolateral side of the L cell has been suggested as an important mediator of bile acid-induced GLP-1 secretion.[11,37] Rectal administration of taurocholic acid increased Glp-1 concentrations in the portal vein of wild-type mice but not in $Tgr5^{-/-}$ mice, showing that luminal bile acid administration can induce TGR5-mediated GLP-1 secretion.[41] However, studies now show that TGR5 activation occurs basolaterally after transport across the enterocyte. Ullmer and colleagues[37] used a potent non-absorbable TGR5 agonist in monkeys and rodents to show that incretin release only occurred after intravenous administration and not after enteral administration. Another TGR5-dependent pathway that might explain increased insulin secretion and improved glucose tolerance in mice after oral administration of bile acids is the direct effects on pancreatic β cells, independent of Glp-1. Tgr5 is expressed in mouse and human β cells, and activation by lithocholic acid increased insulin secretion to a similar extent under both basal and glucose-stimulated conditions, suggesting that the effect is not glucose dependent.[12] The data from these preclinical trials show that bile acids increase both GLP-1 and insulin secretion, which might have a valuable effect in patients with type 2 diabetes.

Panel 1: Tissues and cell types in which TGR5 mRNA is expressed in human beings[7,8]

Adipose tissue
- Brown adipocytes: increase transcription of deiodinase-2 and subsequent thyroid hormone-induced respiration[1]

Skeletal muscle
- Myocytes: increase transcription of deiodinase-2 and subsequent thyroid hormone-induced respiration[1]

Intestine
- Enteric neurons: decrease gastrointestinal motility[10]
- L cells: basolateral TGR5 activation stimulates GLP-1 release[11]

Pancreas
- β cells: increase glucose-dependent secretion of insulin[12]

Spleen
- Macrophages: decrease inflammatory cytokine response[13]

Gallbladder
- Cholangiocytes: increase secretion of chloride-rich fluid[14]
- Smooth muscle cells: cause dilatation and increase gallbladder filling[14]

Skin
- Neurons: relay itch signals[15]

Liver
- Kupffer cells: decrease inflammatory response[9]
- Sinusoidal endothelial cells: increase nitric oxide production[16]

Vascular wall
- Smooth muscle cells: increase arterial dilatation[17]
- Endothelial cells: decrease inflammatory response[18]

Stomach
- Enteric neurons: decrease gastrointestinal motility[10]

Bone marrow
- Haemopoietic cells: function unknown

Heart
- Cardiomyocytes: function unknown

Lung
- Macrophages: function unknown

Kidney
- Podocytes: function unknown

Brain
- Neurons: function unknown
- Astrocytes: function unknown

Tissues in which Tgr5 is expressed, but exact cell types and functions are unknown: thyroid, mammary, adrenal, prostate, and pituitary glands, placenta, and uterus.

Panel 2: Enterohepatic circulation of the bile acid pool

The enterohepatic cycle of bile acids through liver, gallbladder, intestine, and portal vein back to the liver (figure 1) is highly dynamic. Its timing is dependent on the chemical structure and conjugation status of the bile acids. Upon meal ingestion, concentrated bile from the liver and gallbladder flows into the intestinal lumen where it aids digestion by emulsifying lipids. Generally, conjugated bile acids do not pass the enterocyte membrane and are taken up by active transport more distally in the small intestine.[21] After absorption, the liver efficiently extracts most bile acids from the portal vein for re-secretion. Bile acids that are not taken up form a postprandial peak in plasma total bile acid concentration of approximately 1–20 μmol/L 30–90 min after a meal.[22] Thus, extraintestinal tissues are exposed to bile acid concentrations that are high enough to activate TGR5 (figure 1).[23] The dynamics of plasma bile acid concentrations are mainly a function of rate of absorption from the gastrointestinal tract.[22] Approximately 5% of the bile acid pool is not reabsorbed and is lost in the faeces. This loss of bile acids is compensated for by de-novo bile acid synthesis from cholesterol in the liver, refuelling the enterohepatic cycle. Synthesis of bile acids and the expression of various intestinal and hepatic transporters are regulated through feedback inhibition via the nuclear bile acid receptor FXR (reviewed elsewhere[24]).

A mixture of bile acids, differing in TGR5 affinity and hydrophilicity, constitutes the bile acid pool (figure 2). A shift in pool composition affects TGR5-mediated pathways.[23] In human beings, the primary bile acids cholic acid and chenodeoxycholic acid are the endpoint of bile acid synthesis. Primary bile acids are dehydroxylated by the gut microbiota in the distal ileum and the colon into their corresponding secondary bile acids: lithocholic acid from chenodeoxycholic acid and deoxycholic acid from cholic acid. The most abundant bile acid species in human beings are cholic acid, deoxycholic acid, and chenodeoxycholic acid, whereas they are cholic acid and muricholic acid in rodents and chenodeoxycholic acid and hyocholic and hyodeoxycholic acid in pigs.[21,23,25]

Because of their soap-like nature, bile acids are cytotoxic in high concentrations. Conjugation decreases cytotoxicity and increases solubility, aiding secretion into bile. Although intestinal bacteria deconjugate bile acids, the majority of the human bile acid pool (~80%) is in its conjugated form throughout the enterohepatic cycle.[21] Bile acids are preferentially conjugated to taurine, which is reflected in the predominance of these conjugates in the murine bile acid pool. Because taurine is less prevalent in the human diet, the human bile acid pool is predominantly glycine conjugated.[26]

Clinical data

Before the discovery of TGR5, a study showed that infusion of deoxycholic acid into the colon of human participants induced a dose-dependent increase in peptide YY (PYY) and glucagon-related compounds.42 Rectal administration of taurocholic acid prompted a dose-dependent GLP-1 response and suppressed appetite in both patients with type 2 diabetes and healthy participants.[43] However, intrajejunal infusion of taurocholic acid alone did not seem to affect plasma glucose concentrations or GLP-1 secretion whereas coadministration of glucose and taurocholic acid increased GLP-1 secretion, suggesting a glucose-dependent effect of bile acids on GLP-1 secretion.[44] A small study in healthy participants[45]

showed that ursodeoxycholic acid (a bile acid found predominantly in bear bile, a synthetic version of which is used to treat gallstones and liver diseases such as primary biliary cirrhosis) mixed with a meal increased postprandial plasma GLP-1 and decreased plasma glucose concentrations. Insulin concentrations remained unchanged, indicating a higher ratio of insulin to glucose, and thus increased insulin secretion. In another study,[36] an intragastric infusion of 1·25 g unconjugated chenodeoxycholic acid slightly increased plasma GLP-1 concentrations without effects on glucose and insulin concentrations. Bile acids are ligands for multiple receptors, so whether these effects are purely TGR5-mediated is unclear.

Only one human trial involving a specific TGR5 agonist has been published to date. The GlaxoSmithKline (Brentford, London) compound SB-756050 is a specific TGR5 agonist.[46] Four overlapping cohorts received either placebo, SB-756050 15 mg, 50 mg, or 100 mg once a day, or 200 mg twice a day for 6 days. However, short-term daily use did not dose-dependently affect plasma glucose, GLP-1, or other endpoints in a consistent fashion. Low, but not higher doses of the compound caused an unexpected increase in glucose excursions in patients with type 2 diabetes after an oral glucose challenge.[46] At intermediate doses the concentration of incretin hormones increased, but no effect on glucose was shown. This phenomenon was not seen at lower and higher doses. The authors attribute the variability to differential activation of proximal and more distant TGR5 targets in the gut. The other known specific agonists include the cholic acid derivative 6α-ethyl-23(S)-methylcholic acid (INT777, a compound discovered in 2009 that has been used in all landmark animal studies[2]) and two groups of non-bile acid-related compounds known as 3-aryl-4-isoxazolecarboxamides[47] and 2-aryl-3-aminomethylquinolines.[39] None of these compounds have yet been tested in clinical trials.

The notion of bile acid-induced GLP-1 secretion via TGR5 has also gained support from studies in patients with obesity and type 2 diabetes undergoing RYGB surgery, in which the stomach is restricted to a small pouch and is diverted to empty more distally on the mid-jejunum, bypassing the duodenum and much of the jejunum. Type 2 diabetes is resolved in 78% of these patients postoperatively, alongside substantial increases in postprandial GLP-1, PYY, and early-phase insulin secretion.[48–51] Postprandial bile acid concentrations, which are lower in patients with obesity than in those who are not obese,[52] are restored after RYGB

surgery.[53] Patti and colleagues[54] showed that fasting bile acid plasma concentrations were negatively correlated to postprandial glucose and positively correlated to postprandial GLP-1 plasma concentrations in patients who had undergone RYGB surgery. The surgery might increase luminal bile acid delivery to the distal bowel and thereby enhance TGR5-mediated GLP-1 secretion with beneficial effects on glucose and insulin concentrations. Alternatively, the increased plasma bile acid concentrations could increase systemic TGR5 activation, leading to increased energy expenditure as described later in this Review. By contrast, Jørgensen and colleagues[55] showed that although diabetes resolution occurs very rapidly (<1 week), postprandial bile acid concentrations only increase slowly in the months following the surgery, questioning an acute effect.

Generally, bile acids might contribute to GLP-1 and insulin secretion via TGR5, but there is no robust evidence that TGR5 activation contributes directly to increased insulin sensitivity in human beings (figure 3). Additionally, possible confounding factors related to food intake exist when comparing rodent biology to human biology, such as day versus night feeding cycles, feeding behaviour (meals vs grazing), and exposure to stress. As such, bile acid-based therapies for patients with type 2 diabetes to increase GLP-1 secretion seem promising, but require further study.

TGR5 and bile acid sequestrants

Preclinical data

Bile acid sequestrants are non-absorbable resins that bind negatively charged bile salts, and other negatively charged molecules, in the intestinal lumen. These complexes are then excreted in the faeces, diverting bile acids from the enterohepatic circulation. The reduced bile acid concentration in the enterohepatic circulation increases bile acid synthesis, which uses cholesterol as its substrate, ultimately lowering plasma cholesterol. In addition to combating dyslipidaemia, bile acid sequestrant therapy lowers HbA_{1c} in patients with type 2 diabetes.[56] In $Tgr5^{-/-}$ enteroendocrine cells from tissue explants and in $Tgr5^{-/-}$ mice, these metabolic improvements (ie, amelioration of dyslipidaemia and lower $HbA_{1c)}$ after bile acid sequestrant treatment have been shown to be dependent on TGR5-mediated GLP-1 release.[35,41] Bile acids bound to the sequestrant colesevelam are still able to activate

TGR5,[41] thus bile acid sequestrants increase the postprandial GLP-1 response by promoting the delivery of bile acids to the distal intestine where L-cell density is higher. Similarly, 2-week oral administration of an inhibitor of the ileal sodium/bile acid cotransporter in diabetic rats decreased intestinal bile acid reabsorption and increased faecal excretion of bile acids.[57] Glucose concentrations during an oral glucose tolerance test were dose-dependently lowered, accompanied by greater GLP-1 and insulin secretion.[57]

Clinical data

In human beings, bile acid sequestering agents have been reported to lower plasma glucose concentrations, although the causal mechanisms probably involve more than TGR5 activation alone.[28,56,58] Two clinical studies in patients with type 2 diabetes given bile acid sequestrant therapy used plasma GLP-1 concentration as an endpoint and both reported an increase in GLP-1 secretion.[59] However, Brufau and colleagues[28] found no correlation between the effects of 6 weeks of sequestrant therapy on glucose metabolism and various parameters of bile acid metabolism in patients with type 2 diabetes, or in healthy controls. Inhibition of the ileal sodium/bile acid cotransporter in human beings actually increases peak GLP-1 concentrations, in accordance with higher luminal bile acid concentrations that activate TGR5.[60]

In conclusion, although rodent studies suggest that bile acid-mediated TGR5 signalling strongly increases GLP-1 secretion and insulin release, corresponding evidence for these mechanisms in human beings does not exist. The distal intestinal L cells, where Tgr5 is expressed abundantly, are the most potent sites of bile acid-induced GLP-1 secretion. However, orally administered bile acids induce minimal GLP-1 secretion, with almost no effect on glucose and insulin concentrations.[36,61] Here, the postprandial mechanisms in humans (interplay of nutrients, bile acids, incretins, and gastrointestinal transit time) are complex and variable. Rates of gastric emptying differ between species and could play a role in the differential effects seen with bile acid sequesterant-based treatments. Other species differences may also contribute to the different effects seen with such treatments between species.

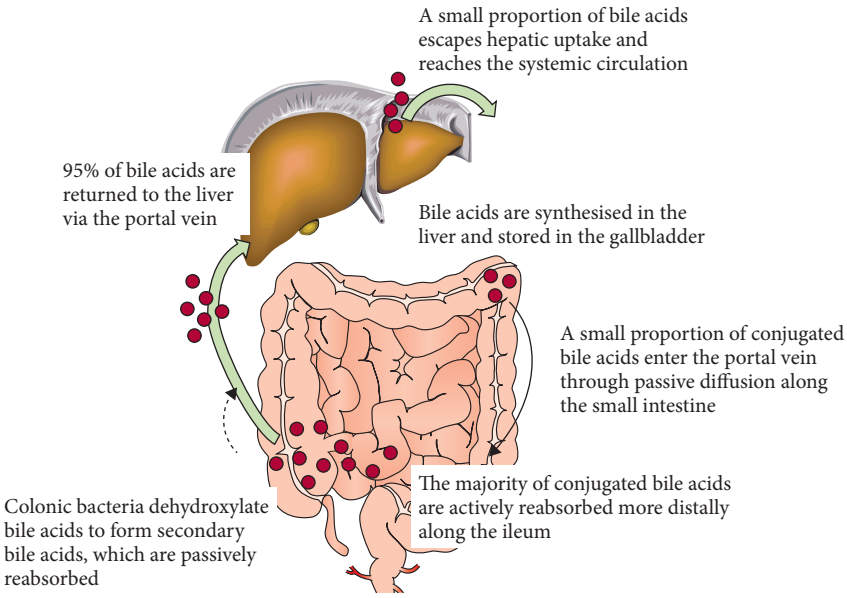
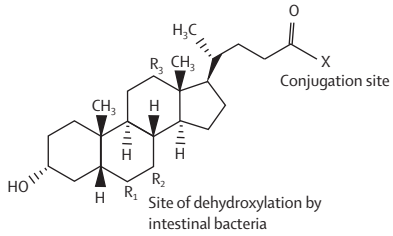


Figure 1: The enterohepatic circulation of bile acids
After a meal, bile fl ows into the intestine. After reabsorption, the liver extracts most but not all bile acids from the portal vein, leading to postprandial peaks in systemic plasma concentration.

A



B

	R_1	R_2	R_3	Affinity to TGR5
Muricholic acid (rodent)	β-OH	H	H	+
Hyodeoxycholic acid (porcine)	α-OH	H	H	–
Ursodeoxycholic acid	H	β-OH	H	–
Cholic acid	H	α-OH	α-OH	–/+
Chenodeoxycholic acid	H	α-OH	H	+
Deoxycholic acid	H	H	α-OH	++
Lithocholic acid	H	H	H	+++

Hydrophobicity

Figure 2: Biochemical properties of the bile acid pool
The basic chemical structure of bile acids (A), and the specific hydroxyl groups that characterise each specific bile acid and their affinity to TGR5 (B). An OH group at R_1, R_2, or R_3, and its spatial orientation determine the type of bile acid. R_2 marks the site of dehydroxylation. X marks the site of conjugation.[21] +=weak affinity. ++=moderate affinity. +++=strong affi nity. –=no affinity. –/+= weak or no affinity.

TGR5 and energy expenditure

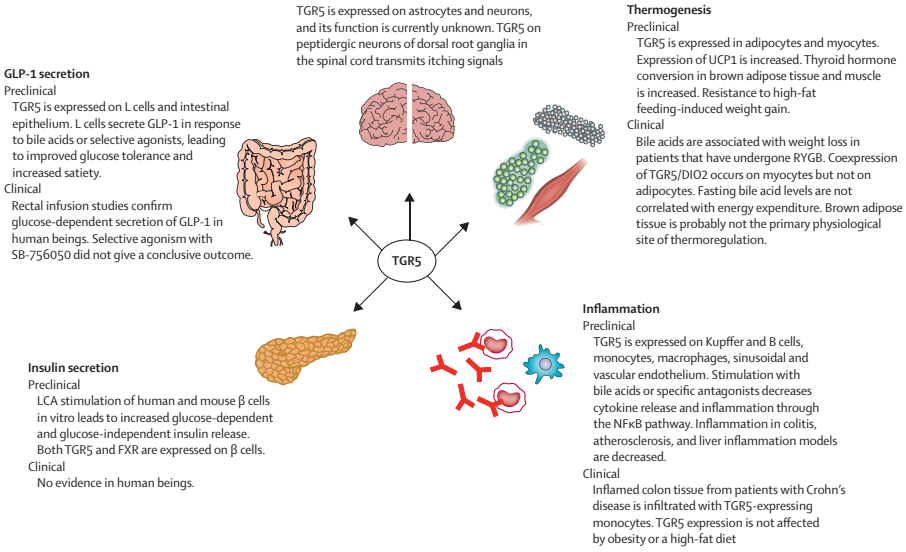
Preclinical data

One of the potential targets of extraintestinal bile acid signalling is the increased energy expenditure leading to reduced weight gain seen in animal models of obesity (figure 3). Watanabe and colleagues1 showed that dietary supplementation of cholic acid in mice prevents and reverses high-fat diet-induced weight gain via Tgr5-dependent energy expenditure in muscle and brown adipose tissue (BAT). Quantitative real-time PCR analysis of BAT in these mice showed upregulation of key genes in energy expenditure such as peroxisome-proliferator-activated receptor γ coactivator 1α (*Pgc1*α) and type II iodothyronine deiodinase (*Dio2*). Increased transcription of these genes induces upregulation of uncoupling protein 1 (Ucp1) in mitochondria, which diverts electron gradients towards thermogenesis instead of ATP production. The effect of cholic acid supplementation on bodyweight was lost in *Dio2*-knockout mice that were fed a high-fat diet.

In-vitro stimulation with either bile acids or a specific TGR5 agonist of BAT cells isolated from mice that were either fed chow or a high-fat diet increased transcription and activity of DIO2 and UCP1 through TGR5 activation.[1] In-vivo studies with other natural and semisynthetic selective TGR5 agonists have confirmed the observed effects on energy expenditure and bodyweight.[2,3] Treatment with colestilan, a second-generation bile acid sequestrant, had opposing effects on parameters of bile acid signalling—for example, decreasing activation of the bile acid receptor FXR—but mimicking the effects of cholic acid supplementation on the bile acid pool and energy expenditure in diet-induced obese mice.[62] By contrast, bodyweight of $Tgr5^{-/-}$ mice fed an isocaloric diet is similar to that of wild-type mice.[2,4,63]

Plasma bile acid concentrations are consistently increased in animal models of RYGB surgery.[64] RYGB leads to weight loss through increased energy expenditure and changes in gastrointestinal physiology, which has been detected by pair-feeding experiments and indirect calorimetry in rodents.[65,66] Both the resting energy expenditure and the postprandial increase in thermogenesis were significantly increased in mice that had RYGB surgery compared with mice that had not undergone surgery; these changes might be due to increased TGR5 activation due to increased plasma bile acid concentrations, but other explanations are possible. [67]

Figure 3: Effects of TGR5 activation in various tissues



LCA=lithocholic acid. RYGB=Roux-en-Y gastric bypass.

Clinical data

Tgr5 is expressed in human BAT, and coexpression of Tgr5 and Dio2 has been shown, suggesting that the bile acid–TGR5–DIO2 pathway is present in human BAT.[68] However, rodents and human beings are fundamentally different when it comes to thermoregulation (figure 3). Rodents depend on thermogenesis in BAT to maintain core body temperature, whereas the physiological role of BAT in human beings is only partially understood. In rodents, BAT is mostly located in a few well-circumscribed compartments, and is reliably activated upon cold exposure. In human beings, these compartments are quite small and scarce; additionally not all human beings show the same thermogenic response to cold.[69]

Human skeletal muscle cells also coexpress Tgr5 and Dio2, and can be activated in vitro by stimulation with bile acids or a specific TGR5 agonist.[1] In vivo, resting energy expenditure has been positively correlated with fasting concentrations of bile acids in lean healthy men; particularly with deoxycholic acid, which is a potent TGR5 stimulator.[8,70] However, Brufau and colleagues[71] found no correlation between fasting bile acid concentrations and resting energy expenditure in patients with cirrhosis, patients with type 2 diabetes, or matched controls. Orally delivered

chenodeoxycholic acid was proposed to increase BAT thermogenesis in lean healthy volunteers via TGR5 stimulation.[68] However, the temporary 5% increase in basal energy expenditure was not readily explained by the marginally increased BAT activity, suggesting that other bile acid signalling pathways such as FXR and its downstream hormone, fibroblast growth factor 19, might be more important than chenodeoxycholic acid.

An increase in both fasting and postprandial bile acid concentrations is well established in patients after they have undergone bariatric surgery.[54,55,72,73] Whether the increase in plasma bile acid concentrations precedes the increase in energy expenditure, however, is controversial. Notably, the postprandial neuroendocrine response, consisting of hormone and bile acid release after a meal, is blunted in patients with obesity but restored after RYGB surgery.[53] Werling and colleagues[74] showed that both postprandial and total energy expenditure were increased in seven women with stable bodyweight 9 years after RYGB surgery, compared with women matched for anthropometric parameters who had undergone vertical sleeve gastrectomy. The effect in women who had RYGB surgery was ascribed to increased postprandial neuroendocrine response. Major limitations in these studies are the heterogeneity of patient populations (eg, sex, body composition, BMI, surgical technique) and the short timeframe in which energy expenditure measurements were done (ie, resting instead of total or postprandial energy expenditure).

In conclusion, Tgr5–Dio2-mediated thermogenesis in murine BAT suggests a promising bile acid signalling-related pharmacological target. However, quantitative and physical properties of human thermogenesis (eg, organ site and size, molecular mechanisms) restrict the clinical translation of animal studies to human beings at present.

TGR5 as a modulator of inflammatory signals

Background

With the presumed function of TGR5 as a nutrient sensor, its role in modulating inflammation is of interest. Additionally, the postprandial state is established to be proinflammatory and atherogenic. TGR5 is abundantly expressed on immune cells of the monocyte lineage, namely Kupffer cells, monocytes, macrophages, and

also on T lymphocytes (figures 2, 3).[7,75] Initially, exposure of macrophages to bile acids was thought to dampen the proinflammatory cytokine response, but TGR5 also modulates inflammatory signals in other ways.[7,75–77] Controlling postprandial inflammatory responses in the intestine and liver might be required for a normal physiological response to food intake.

Preclinical data

In vitro, TGR5 activation in Kupffer cells decreases expression of interleukins and TNFα after stimulation with lipopolysaccharide.[9] This effect was mediated by inhibition of the NfκB pathway.[78] Additionally, TGR5 stimulation reduces lipopolysaccharide-induced liver inflammation in mice.[79] Without the presence of an inflammatory stimulus, TGR5 might actually increase the expression of interleukin 1β and TNFα.[80] This theory is disputed by most other studies, which have found an anti-inflammatory effect.[13] Treatment with the cholic acid derivative INT777 led to increased NfκB-mediated cytokine release in primary cultures of macrophages isolated from wild-type and Tgr5$^{-/-}$ mice, but not in those from Tgr5-overexpressing mice.[13] TGR5 activation also showed antiatherogenic effects in a mouse model of atherogenesis.[13] Similarly, TGR5 activation increased the ratio between the anti-inflammatory interleukin [10] and proinflammatory interleukin [12] in human macrophages,[81,82] and monocyte-derived dendritic cells produced less interleukin [12] and TNFα after contact with bacterial pathogens when cultured with a TGR5 agonist.[83] By contrast, one report showed increased bile acid-induced inflammatory responses in a human monocyte line that could have been species-dependent.[84] Finally, TGR5 activates the Akt-mTOR signalling pathway, which attenuates the inflammatory chemokine response of macrophages.[85]

Clinical data

Cipriani and colleagues[86] and Yoneno and colleagues[87] showed increased Tgr5 expression in lamina propria mononuclear cells in ex-vivo colonic samples from patients with Crohn's disease with colonic inflammation. Here, Tgr5 expression might be upregulated in response to inflammatory cells or luminal antigens. Polymorphisms in the Tgr5 gene are associated with primary sclerosing cholangitis, further suggesting a link with inflammatory conditions.[88]

Food intake has been shown to evoke a postprandial inflammatory response;[89–91] this response depends on the fat content of the meal and is characterised by increased

circulating lipopolysaccharide and chylomicrons that induce mononuclear cell expression of inflammatory proteins such as interleukins, TNFα, and NFκB.[91–94] This phenomenon is exaggerated in patients with obesity and type 2 diabetes.[14,89]

In summary, preclinical evidence shows that TGR5 modulates inflammatory signals predominantly via the monocyte lineage. Whether TGR5 modulates postprandial inflammation under physiological conditions is of clinical interest and remains to be elucidated.

Challenges for clinical application

Although the possible benefits of bile acid therapy could be large, the greatest concern for clinical application will be to restrict unwanted effects, because Tgr5 is widely expressed (panel 1). Several drug development studies done by pharmaceutical companies have led to the discontinuation of TGR5-based trial programmes.

In the gallbladder, where Tgr5 is expressed to a higher degree than in any other tissue, TGR5 activation causes smooth muscle relaxation and secretion of chloride-rich fluid, leading to increased gallbladder filling and potentially increasing the risk of gallstone formation.[95] Findings from a drug development study using small molecule agonist compounds showed that exaggerated gallbladder filling occurred at the same doses needed to induce GLP-1 secretion in rats.[96]

In another pharmacochemical study, Fryer and colleagues[97] reported large reductions in peripheral vascular tone and blood pressure immediately upon infusion of low doses of a TGR5 agonist in dogs. They were not able to separate this unwanted effect from the intended effects on inflammation, leading them to discontinue development of these agonists. Other pharmacochemical reports have also mentioned (but did not actually show) cardiovascular effects such as treatment-dependent changes in heart rate and blood pressure.[17]

Other clear side-effects are nausea or obstipation, mediated by TGR5-expressing neurons in the gastrointestinal tract, and pruritus.[15] Bile acids have long been suspected to be the effectors of the itching sensation reported by patients with cholestatic liver disease. Using $Tgr5^{-/-}$ and Tgr5-Tg mice, Alemi and colleages[15] showed that activation of TGR5 induced the release of itch neuropeptides. If

TGR5-mediated itch is present in human beings as well as mice, which seems likely given the overlap between human and rodent tissue distribution of the receptor, it could prove a major barrier in implementing TGR5-targeted therapy.

Some reports have shown increased cell proliferation in response to TGR5 activation. In in-vitro models of gastric[98] and endometrial cancer,[99] stimulation with high doses of TGR5 agonists promoted cell proliferation. Increased TGR5 expression on tumour cells has also been correlated with worsening clinical prognosis in patients with oesophageal and gastric adenocarcinoma.[100,101] Adverse effects have mainly been reported in cell cultures or animal models under extreme conditions and await further investigation. Regardless, these side-effects raise concern that systemic TGR5 agonists will not be tolerated therapeutically.

Conclusion

Since the discovery of bile acid-mediated activation of TGR5, many preclinical studies have been done to elucidate the physiology of this receptor and its potential as a therapeutic target in type 2 diabetes, obesity, and non-alcoholic steatohepatitis. However, profound interspecies differences in the biology of bile acids and their receptors in different cells and tissues exist, which hampers the translation of preclinical findings to human pathophysiology.

Studies in rodents point to an important role for TGR5 in GLP-1 secretion, insulin sensitivity, and energy expenditure. Targeting the immune system through TGR5 could also prove beneficial in inflammatory conditions. Studies of administration of natural bile acids and bile acid-sequestering agents, and models of bariatric surgery provide only limited mechanistic clues, as a result of the complex dynamics of the multi-compartmental enterohepatic circulation. Although we acknowledge that molecular mechanisms in human beings are harder to understand than those in rodents, future studies should aim to study the equivalent processes in human beings.

Studies in human beings should focus on TGR5 activation within the enterohepatic cycle and its potential role in GLP-1 secretion and modulation of inflammation. TGR5-mediated increases in energy expenditure via BAT and white adipose tissue browning would require systemic administration, which is unlikely to be achievable in human beings because no safe systemic TGR5 agonists exist. Non-absorbable

compounds, such as those being developed for other intestinal receptors, might not deliver the desired effect, because basolateral TGR5 stimulation is required for GLP-1 secretion. Hepatic clearance of novel TGR5 agonists should be very high to prevent side-effects. The question then is how TGR5 ligands can be increased and made to be effective in the enterohepatic cycle only. This specificity might be achieved via changes in bile acid pool composition, either by extended administration of bile acids with high TGR5 affinity or manipulation of bile acid biosynthesis and bile acid transporters. Alternatively, synthetic agonists could be developed that are contained within the enterohepatic circulation, either using dual ligand methods or by optimising first pass clearance. Both academic and pharmaceutical researchers have put much effort into developing clinical TGR5 agonists; however, these agonists have not yet reached clinical application.

The metabolic phenotypes of Tgr5 polymorphisms should be studied in human beings to assess the contribution of TGR5 to clinically relevant outcomes. Ultimately, strategies to increase TGR5 signalling will require more insight into the dynamics of physiological bile acid metabolism and transport.

References

1. Watanabe M, Houten SM, Mataki C, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 2006; 439: 484–89.
2. Thomas C, Gioiello A, Noriega L, et al. TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metab* 2009; 10: 167–77.
3. Sato H, Genet C, Strehle A, et al. Anti-hyperglycemic activity of a TGR5 agonist isolated from *Olea europaea*. *Biochem Biophys Res Commun* 2007; 362: 793–98.
4. Maruyama T, Tanaka K, Suzuki J, et al. Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice. *J Endocrinol* 2006; 191: 197–205.
5. Reimann F, Gribble F. G protein-coupled receptors as new therapeutic targets for type 2 diabetes. *Diabetologia* 2016; 59: 229–33.
6. Hodge R, Nunez D. The Therapeutic potential of TGR5 agonists. Hope or hype? *Diabetes Obes Metab* 2016; 18: 439–43.
7. Kawamata Y, Fujii R, Hosoya M, et al. A G protein-coupled receptor responsive to bile acids. *J Biol Chem* 2003; 278: 9435–40.
8. Maruyama T, Miyamoto Y, Nakamura T, et al. Identification of membrane-type receptor for bile acids (M-BAR). *Biochem Biophys Res Commun* 2002; 298: 714–19.
9. Keitel V, Donner M, Winandy S, Kubitz R, Häussinger D. Expression and function of the bile acid receptor TGR5 in Kupffer cells. *Biochem Biophys Res Commun* 2008; 372: 78–84.
10. Poole DP, Godfrey C, Cattaruzza F, et al. Expression and function of the bile acid receptor GpBAR1 (TGR5) in the murine enteric nervous system. *Neurogastroenterol Motil* 2010; 22: 814–25, e227–28.
11. Brighton CA, Rievaj J, Kuhre RE, et al. Bile acids trigger GLP-1 release predominantly by accessing basolaterally-located G-protein coupled bile acid receptors. *Endocrinology* 2015; 156: 3961–70.
12. Kumar DP, Rajagopal S, Mahavadi S, et al. Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic beta cells. *Biochem Biophys Res Commun* 2012; 427: 600–05.
13. Pols TWH, Nomura M, Harach T, et al. TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. *Cell Metab* 2011; 14: 47–57.
14. Vors C, Pineau G, Drai J, et al. Postprandial endotoxemia linked with chylomicrons and lipopolysaccharides handling in obese versus lean men: a lipid dose-effect trial. *J Clin Endocrinol Metab* 2015; 100: 3427–35.
15. Alemi F, Kwon E, Poole D, Lieu T. The TGR5 receptor mediates bile acid-induced itch and analgesia. *J Clin Invest* 2013; 123: 1513–30.
16. Keitel V, Reinehr R, Gatsios P, et al. The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. *Hepatology* 2007; 45: 695–704.
17. Futatsugi K, Bahnck K, Brenner M, et al. Optimization of triazole-based TGR5 agonists towards orally available agents. *Med Chem Comm* 2013; 4: 205–10.
18. Kida T, Tsubosaka Y, Hori M, et al. Bile acid receptor TGR5 agonism induces NO production and reduces monocyte adhesion in vascular endothelial cells. *Arterioscler Thromb Vasc Biol* 2013; 33: 1663–69.
19. Thomas C, Auwerx J, Schoonjans K. Bile acids and the membrane bile acid receptor TGR5-connecting nutrition and metabolism. *Thyroid* 2008; 18: 167–74.
20. Duboc H, Taché Y, Hofmann AF. The bile acid TGR5 membrane receptor: from basic research to clinical application. *Dig Liver Dis* 2014; 46: 302–12.
21. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci* 2008; 65: 2461–83.
22. LaRusso N, Hoffman N, Korman M, Hofmann AF, Cowen A. Determinants of fasting and postprandial serum bile acid levels in healthy man. *Am J Dig Dis* 1978; 23: 385–91.
23. Sato H, Macchiarulo A, Thomas C, et al. Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. *J Med Chem* 2008; 51: 1831–41.

24. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev* 2009; 89: 147–91.
25. Spinelli V, Lalloyer F, Baud G, et al. Influence of Roux-en-Y gastric bypass on plasma bile acid profiles: a comparative study between rats, pigs and humans. *Int J Obes* 2016; published online Mar 22. DOI:10.1038/ijo.2016.46.
26. Hardison WG, Grundy SM. Effect of bile acid conjugation pattern on bile acid metabolism in normal humans. *Gastroenterology* 1983; 84: 617–20.
27. Bennion LJ, Grundy SM. Effects of diabetes mellitus on cholesterol metabolism in man. *N Engl J Med* 1977; 296: 1365–71.
28. Brufau G, Stellaard F, Prado K, et al. Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism. *Hepatology* 2010; 52: 1455–64.
29. Sonne DP, van Nierop FS, Kulik W, Soeters MR, Vilsbøll T, Knop FK. Postprandial plasma concentrations of individual bile acids and FGF-19 in patients with type 2 diabetes. *J Clin Endocrinol Metab* 2016; 101: 3002–09.
30. Orskov C, Wettergren A, Holst J. Secretion of the incretin hormones glucagon-like peptide-1 and gastric inhibitory polypeptide correlates with insulin secretion in normal man throughout the day. *Scand J Gastroenterol* 1996; 31: 665–70.
31. Holst J. The physiology of glucagon-like peptide 1. *Physiol Rev* 2007; 87: 1409–39.
32. Vedtofte L, Bodvarsdottir TB, Gotfredsen CF, Karlsen AE, Knudsen LB, Heller RS. Liraglutide, but not vildagliptin, restores normoglycaemia and insulin content in the animal model of type 2 diabetes, *Psammomys obesus*. *Regul Pept* 2010; 160: 106–14.
33. Knop F. Bile-induced secretion of glucagon-like peptide-1: pathophysiological implications in type 2 diabetes? *Am J Physiol Endocrinol Metab* 2010; 299: E10–13.
34. Katsuma S, Hirasawa A, Tsujimoto G. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochem Biophys Res Commun* 2005; 329: 386–90.
35. Harach T, Pols TWH, Nomura M, et al. TGR5 potentiates GLP-1 secretion in response to anionic exchange resins. *Sci Rep* 2012; 2: 2–8.
36. Hansen M, Scheltema MJ, Sonne DP, et al. Effect of chenodeoxycholic acid and the bile acid sequestrant colesevelam on glucagon-like peptide-1 secretion. *Diabetes Obes Metab* 2016; 18: 571–80.
37. Ullmer C, Alvarez Sanchez R, Sprecher U, et al. Systemic bile acid sensing by G protein-coupled bile acid receptor 1 (GPBAR1) promotes PYY and GLP-1 release. *Br J Pharmacol* 2013; 169: 671–84.
38. Duan H, Ning M, Chen X, et al. Design, synthesis, and antidiabetic activity of 4-phenoxynicotinamide and 4-phenoxypyrimidine-5-carboxamide derivatives as potent and orally efficacious TGR5 agonists. *J Med Chem* 2012; 55: 10475–89.
39. Herbert MR, Siegel DL, Staszewski L, et al. Synthesis and SAR of 2-aryl-3-aminomethylquinolines as agonists of the bile acid receptor TGR5. *Bioorg Med Chem Lett* 2010; 20: 5718–21.
40. Gomez G, Lluis F, Ishizuka J, et al. Bile enhances release of insulin: an incretin-mediated effect. *Surgery* 1987; 102: 195–99.
41. Potthoff MJ, Potts A, He T, et al. Colesevelam suppresses hepatic glycogenolysis by TGR5-mediated induction of GLP-1 action in DIO mice. *Am J Physiol Gastrointest Liver Physiol* 2013; 304: G37–80.
42. Wu T, Bound MJ, Standfield SD, et al. Effects of rectal administration of taurocholic acid on glucagon-like peptide-1 and peptide YY secretion in healthy humans. *Diabetes Obes Metab* 2013; 15: 474–77.
43. Adrian TE, Gariballa S, Parekh KA, et al. Rectal taurocholate increases L cell and insulin secretion, and decreases blood glucose and food intake in obese type 2 diabetic volunteers. *Diabetologia* 2012; 55: 2343–47.
44. Wu T, Bound MJ, Standfield SD, Jones KL, Horowitz M, Rayner CK. Effects of taurocholic acid on glycemic, glucagon-like peptide-1, and insulin responses to small intestinal glucose infusion in healthy humans. *J Clin Endocrinol Metab* 2013; 98: E718–22.
45. Murakami M, Une N, Nishizawa M, Suzuki S, Ito H, Horiuchi T. Incretin secretion stimulated by ursodeoxycholic acid in healthy subjects. *Springerplus* 2013; 2: 20.

46. Hodge R, Lin J, Vasist L, EP G, Bowers G, Nunez D. Safety, pharmacokinetics, and pharmacodynamic effects of a selective TGR5 agonist, SB–756050, in type 2 diabetes. *Clin Pharmacol Drug Dev* 2013; 2: 213–22.
47. Budzik BW, Evans KA, Wisnoski DD, et al. Synthesis and structure-activity relationships of a series of 3-aryl-4-isoxazolecarboxamides as a new class of TGR5 agonists. *Bioorg Med Chem Lett* 2010; 20: 1363–67.
48. Laferrère B, Heshka S, Wang K, et al. Incretin levels and effect are markedly enhanced 1 month after roux-en-Y gastric bypass surgery in obese patients with type 2 diabetes. *Diabetes Care* 2007; 30: 1709–16.
49. le Roux CW, Welbourn R, Werling M, et al. Gut hormones as mediators of appetite and weight loss after Roux-en-Y gastric bypass. *Ann Surg* 2007; 246: 780–85.
50. Morínigo R, Moizé V, Musri M, et al. Glucagon-like peptide-1, peptide YY, hunger, and satiety after gastric bypass surgery in morbidly obese subjects. *J Clin Endocrinol Metab* 2006; 91: 1735–40.
51. Salehi M, Prigeon RL, D'Alessio DA. Gastric bypass surgery enhances glucagon-like peptide 1-stimulated postprandial insulin secretion in humans. *Diabetes* 2011; 60: 2308–14.
52. Glicksman C, Pournaras DJ, Wright M, et al. Postprandial plasma bile acid responses in normal weight and obese subjects. *Ann Clin Biochem* 2010; 47: 482–84.
53. Ahmad NN, Pfalzer A, Kaplan LM. Roux-en-Y gastric bypass normalizes the blunted postprandial bile acid excursion associated with obesity. *Int J Obes (Lond)* 2013; 37: 1553–59.
54. Patti ME, Houten SM, Bianco AC, et al. Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. *Obesity (Silver Spring)* 2009; 17: 1671–77.
55. Jørgensen NB, Dirksen C, Bojsen-Moller KN, et al. Improvements in glucose metabolism early after gastric bypass surgery are not explained by increases in total bile acids and fibroblast growth factor 19 concentrations. *J Clin Endocrinol Metab* 2015; 100: E396–406.
56. Bays H. Colesevelam HCI added to background metformin therapy in patients with type 2 diabetes mellitus: a pooled analysis from three clinical studies. *Endocr Pract* 2011; 17: 933–38.
57. Chen L, Yao X, Young A, et al. Inhibition of apical sodium-dependent bile acid transporter as a novel treatment for diabetes. *Am J Physiol Endocrinol Metab* 2012; 302: E68–76.
58. Goldberg RB, Fonseca Va, Truitt KE, Jones MR. Efficacy and safety of colesevelam in patients with type 2 diabetes mellitus and inadequate glycemic control receiving insulin-based therapy. *Arch Intern Med* 2008; 168: 1531–40.
59. Beysen C, Murphy EJ, Deines K, et al. Effect of bile acid sequestrants on glucose metabolism, hepatic de novo lipogenesis, and cholesterol and bile acid kinetics in type 2 diabetes: a randomised controlled study. *Diabetologia* 2012; 55: 432–42.
60. Rudling M, Camilleri M, Graffner H, Holst JJ, Rikner L. Specific inhibition of bile acid transport alters plasma lipids and GLP-1. *BMC Cardiovasc Disord* 2015; 15: 75.
61. Meyer-Gerspach AC, Steinert RE, Keller S, Malarski A, Schulte FH, Beglinger C. Effects of chenodeoxycholic acid on the secretion of gut peptides and fibroblast growth factors in healthy humans. *J Clin Endocrinol Metab* 2013; 98: 3351–58.
62. Watanabe M, Morimoto K, Houten SM, et al. Bile acid binding resin improves metabolic control through the induction of energy expenditure. *PLoS One* 2012; 7: e38286.
63. Vassileva G, Hu W, Hoos L, et al. Gender-dependent effect of *Gpbar1* genetic deletion on the metabolic profiles of diet-induced obese mice. *J Endocrinol* 2010; 205: 225–32.
64. Pournaras DJ, Glicksman C, Vincent RP, et al. The role of bile after Roux-en-Y gastric bypass in promoting weight loss and improving glycaemic control. *Endocrinology* 2012; 153: 3613–19.
65. Bueter M, Löwenstein C, Olbers T, et al. Gastric bypass increases energy expenditure in rats. *Gastroenterology* 2010; 138: 1845–53.
66. Stylopoulos N, Hoppin AG, Kaplan LM. Roux-en-Y gastric bypass enhances energy expenditure and extends lifespan in diet-induced obese rats. *Obesity (Silver Spring)* 2009; 17: 1839–47.
67. Nestoridi E, Kvas S, Kucharczyk J, Stylopoulos N. Resting energy expenditure and energetic cost of feeding are augmented after Roux-en-Y gastric bypass in obese mice. *Endocrinology* 2012; 153: 2234–44.

68. Broeders EPM, Nascimento EBM, Havekes B, et al. The bile acid chenodeoxycholic acid increases human brown adipose tissue activity. *Cell Metab* 2015; 22: 418–26.
69. Langeveld M, Tan CY, Soeters M, et al. Mild cold effects on hunger, food intake, satiety and skin temperature in humans. *Endocr Connect* 2016; 5: 65–73.
70. Ockenga J, Valentini L, Schuetz T, et al. Plasma bile acids are associated with energy expenditure and thyroid function in humans. *J Clin Endocrinol Metab* 2012; 97: 535–42.
71. Brufau G, Bahr MJ, Staels B, et al. Plasma bile acids are not associated with energy metabolism in humans. *Nutr Metab (Lond)* 2010; 7: 73.
72. Benedetti G, Mingrone G, Marcoccia S, et al. Body composition and energy expenditure after weight loss following bariatric surgery. *J Am Coll Nutr* 2000; 9: 270–74.
73. Kohli R, Bradley D, Setchell KD, Eagon JC, Abumrad N, Klein S. Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile acids. *J Clin Endocrinol Metab* 2013; 98: E708–12.
74. Werling M, Olbers T, Fandriks L, et al. Increased postprandial energy expenditure may explain superior long term weight loss after Roux-en-Y gastric bypass compared to vertical banded gastroplasty. *PLoS One* 2013; 8: e60280.
75. Eggink HM, Soeters MR, Pols TW. TGR5 ligands as potential therapeutics in inflammatory diseases. *Int J Interfer Cytokine Med Res* 2014; 6: 27–38.
76. Pols TW. TGR5 in inflammation and cardiovascular disease. *Biochem Soc Trans* 2014; 42: 244–49.
77. Fiorucci S, Cipriani S, Mencarelli A, Renga B, Distrutti E, Baldelli F. Counter-regulatory role of bile acid activated receptors in immunity and inflammation. *Curr Mol Med* 2010; 10: 579–95.
78. Wang YD, Chen WD, Yu D, Forman BM, Huang W. The G-protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor kappa light-chain enhancer of activated B cells (NF-kappaB) in mice. *Hepatology* 2011; 54: 1421–32.
79. Péan N, Doignon I, Garcin I, et al. The receptor TGR5 protects the liver from bile acid overload during liver regeneration in mice. *Hepatology* 2013; 58: 1451–60.
80. Lou G, Ma X, Fu X, et al. GPBAR1/TGR5 mediates bile acid-induced cytokine expression in murine Kupffer cells. *PLoS One* 2014; 9: 1–10.
81. Haselow K, Bode JG, Wammers M, et al. Bile acids PKA-dependently induce a switch of the IL-10/IL-12 ratio and reduce proinflammatory capability of human macrophages. *J Leukoc Biol* 2013; 94: 1253–64.
82. Hogenauer K, Arista L, Schmiedeberg N, et al. G-protein-coupled bile acid receptor 1 (GPBAR1, TGR5) agonists reduce the production of proinflammatory cytokines and stabilize the alternative macrophage phenotype. *J Med Chem* 2014; 57: 10343–54.
83. Ichikawa R, Takayama T, Yoneno K, et al. Bile acids induce monocyte differentiation toward interleukin-12 hypo-producing dendritic cells via a TGR5-dependent pathway. *Immunology* 2012; 136: 153–62.
84. Mobraten K, Haugbro T, Karlstrom E, Kleiveland CR, Lea T. Activation of the bile acid receptor TGR5 enhances LPS-induced inflammatory responses in a human monocytic cell line. *J Recept Signal Transduct Res* 2015; 35: 402–09.
85. Perino A, Pols TW, Nomura M, Stein S, Pellicciari R, Schoonjans K. TGR5 reduces macrophage migration through mTOR-induced C/EBPbeta differential translation. *J Clin Invest* 2014; 124: 5424–36.
86. Cipriani S, Mencarelli A, Chini MG, et al. The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis. *PLoS One* 2011; 6: e25637.
87. Yoneno K, Hisamatsu T, Shimamura K, et al. TGR5 signalling inhibits the production of pro-inflammatory cytokines by in vitro differentiated inflammatory and intestinal macrophages in Crohn's disease. *Immunology* 2013; 139: 19–29.
88. Hov JR, Keitel V, Schrumpf E, Häussinger D, Karlsen TH. TGR5 sequence variation in primary sclerosing cholangitis. *Dig Dis* 2011; 29: 78–84.
89. Nappo F, Esposito K, Cioffi M, et al. Postprandial endothelial activation in healthy subjects and in type 2 diabetic patients: role of fat and carbohydrate meals. *J Am Coll Cardiol* 2002; 39: 1145–50.

90. Aljada A, Mohanty P, Ghanim H. Increase in intranuclear nuclear factor κB and decrease in inhibitor κB in mononuclear cells after a mixed meal: evidence for a proinflammatory effect. *Am J Clin Nutr* 2004; 79: 682–90.

91. Dickinson S, Hancock DP, Petocz P, Ceriello A, Brand-Miller J. High-glycemic index carbohydrate increases nuclear factor-kB activation in mononuclear cells of young, lean healthy subjects. *Am J Clin Nutr* 2008; 87: 1188–93.

9

Discussion and future perspectives

The foundation of the present thesis is the relatively underexplored notion that bile acids may act as endocrine hormones through the receptors Takeda G protein-coupled receptor 5 (TGR5) and Farnesoid X Receptor (FXR) in human metabolism. The experimental studies described here address three important topics in the current understanding of human (and porcine) bile acid biology, namely natural variation, response to metabolic challenges and enterohepatic versus systemic effects. We chose to investigate postprandial bile acid metabolism in different metabolic states and to modulate the postprandial bile acid response using interventions aimed at energy metabolism.

Bile acids as systemic postprandial signals

Bile acids and bile alcohols likely arose from the need of early vertebrates to excrete cholesterol in a water-soluble form in a watery environment. They are structurally diverse molecules in vertebrate biology, differing in the number and orientation of hydroxyl subgroups on the sterol backbone, and are instrumental in absorbing lipid-soluble nutrients (Hofmann, 2009). Two specific bile acid receptors have been described, namely FXR and TGR5, with differing affinity for each individual bile acid subtype, as well as the non-specific nuclear receptors Constitutive Androstane Receptor (CAR), Vitamin D Receptor (VDR), Liver X Receptor (LXR) and Pregnane X Receptor (PXR) (Lefebvre et al., 2009). While both FXR and TGR5 have been found to be heavily involved in feedback regulation of bile acid metabolism, their role in human energy metabolism remains underexplored. They are found outside of the enterohepatic tissues, and have been shown to interact with glucose, energy and lipid metabolism in human and rodent models of metabolic disease, but whether they play a relevant part is so far unknown (for a further discussion of the underlying evidence, see chapter 8). While peripheral plasma levels do peak to levels shown to activate these receptors (Sato et al., 2008a), it remains in question whether these are relevant signals in human physiology. In chapter 5 of this thesis, we have described trends and significant correlations at multiple timepoints postprandially between bile acids (in particular the secondary bile acid glycodeoxycholic acid, gDCA) and both GLP-1 and insulin levels. This suggests that the postprandial increase in GLP-1 is at least in part explained by enteral stimulation of TGR5 by bile acids, as previously described by Kuhre and colleagues (Kuhre et al., 2018). In addition, supraphysiological stimulation with gDCA also led to an increase in early postprandial levels of GLP-1, further supporting this notion. This could be a direct effect of gDCA on enteroendocrine

cells through TGR5, but the timing argues against it: GLP-1 levels rapidly increased in the first hour after administration of gDCA. On the whole, it does fit our assumed idea of bile acids as metabolic signals, stimulating downstream GLP-1 effects: increasing pancreatic beta cell mass, glucose-dependent insulin release and sensation of satiety. In our study, the increased GLP-1 levels did not translate into higher insulin or lower glucose levels, which may be explained by well-tuned compensatory mechanisms in these healthy individuals, which are absent in metabolically deranged patients.

In obese insulin resistant patients with type 2 diabetes (DM2), the relative contribution of DCA, a strong TGR5 agonist, to the total bile acid pool is increased (Brufau et al., 2010; Glicksman et al., 2010; Haeusler et al., 2013; Vincent et al., 2013). We speculate that this may be an adaptive response to hyperglycemia, analogous to the increase in insulin seen in these patients when they develop insulin resistance. In this view, decreased insulin signaling increases bile acid synthesis through decreased activation of FOXO1, which normally suppresses CYP8B1 transcription. Increased enteral bile acid signaling through TGR5 could then lead to an increase in circulating GLP-1, which in turn stimulates glucose-dependent insulin release from the pancreatic beta cell.

Natural variation in bile acid pool composition

Recurring themes in this thesis are the large intra- and interindividual variations in both human and porcine postprandial bile acid profiles. We have demonstrated this in healthy subjects (Chapters 2, 3 and 5) as well as in subjects with metabolic disturbances (Chapters 2, 5 and 6). This is in line with data reported by others. Already in 1978, LaRusso et al found intra-individual variations in postprandial peak-time and peak levels (LaRusso et al., 1978; Schalm et al., 1978). This has been repeated in more detail by Steiner et al., who describe considerable interindividual variations in bile acid levels over the course of a 24-hour study day (Steiner et al., 2011).

We speculate that the variability in the data can arise from multiple sources. Firstly, we are measuring peripheral plasma concentrations in a system where there is flow through different compartments, as evidenced by our catheter study in a porcine model (Chapter 3). This means that more robust signals that are contained in the enterohepatic cycle may remain out of sight, bypassing the sampled compartment.

Secondly, timing matters. It has previously been published that postprandial bile acid concentrations in peripheral plasma are crucially dependent on intestinal transit time (Meessen et al., 2020), which may differ both between and within test subjects, dependent on genetics and behavior leading up to study days, leading to signal interference. This is again illustrated by the work of LaRusso and Steiner, where the arrival of bile acids in the sampled compartment, peripheral plasma, appeared to vary widely between and within subjects.

A third factor is the striking interindividual difference in bile acid pool composition and conjugation state in both healthy and diseased subjects. This may be explained by heterogeneity of the enterohepatic enzymatic apparatus tasked with bile acid circulation, arising from not only genetic variation but also anatomic, dietary and behavioral factors as well as the host microbiome. We speculate that the existence of these different bile acid "phenotypes" could be a contributing factor in the development of metabolic disease at a later age. To explore this further, more research is needed into the determinants of bile acid pool composition, i.e. enteral and hepatic transport kinetics, intestinal transit time, and bile acid synthesis.

Ultimately, the wide array of individual bile acid pool compositions found in otherwise healthy subjects may be indicative of the absence of a strong causal link between bile acid pool composition and the pathophysiology of energy metabolism.

Bile acid responses to metabolic challenges

A point of interest of current bile acid research is the suggested involvement of bile acids in the pathophysiology of obesity and DM2. As such, bile acid biology has been shown to be altered in patients with obesity and/or DM2 (Brufau et al., 2010; Glicksman et al., 2010; Haeusler et al., 2013; Sonne et al., 2016; Vincent et al., 2013). We set out to characterize the postprandial bile acid response in a selection of models of metabolic derangement.

We showed that in healthy, lean, male subjects, bile acid levels peak postprandially in unison with insulin and GLP-1. After a 40-h fast, an intervention known to powerfully induce insulin resistance in a short time-span, bile acid responses were unaffected. This suggests that the acutely developed increase in postprandial glucose and insulin levels in these fasting subjects, denoting insulin resistance,

are not the result of an increase in bile acid levels outside of the enterohepatic circulation. Of course, the insulin insensitivity of obesity and DM2 may not arise from the same pathways as fasting-induced insulin resistance (Soeters et al., 2012).

The lack of an effect of short-term fasting on bile acid concentrations also denotes that the relationship between insulin resistance and bile acids as seen in obesity and DM2 likely develops over a longer period of time. This is additionally supported by our own results after placement of the duodenal-jejunal bypass liner (DJBL), where beneficial effects on insulin and glucose levels predate the massive changes in bile acid metabolism, as well as reports by other authors (Dutia et al., 2015; Haeu et al., 2015; Kohli et al., 2013; Patti et al., 2009). This fits with the concept of a bile acid pool that is constantly efficiently being recycled (as we also demonstrated in chapter 3), only showing gradual change after several cycles when the underlying deconjugation and rehydroxylation apparatus is adjusted. Alternatively, changes in the bile acid pool could occur immediately after placement, but not become apparent until hepatic deconjugation and clearance is overwhelmed, leading to increased peripheral plasma bile acid levels. This is supported by the increase in GLP-1 and FGF19 seen immediately after placement of the DJBL, which suggests that bile acid signaling within the enterohepatic circulation is increased, even though peripheral bile acid levels are (still) low (Kaválková et al., 2016).

Recent papers have described increased synthesis of 12α-hydroxylated bile acids in insulin resistant human subjects (Haeusler et al., 2013; Legry et al., 2017). In mice, decreased insulin signaling leads to reduced activity of the transcription factor FoxO1, which normally represses Cyp8B1. The subsequent increase in Cyp8B1 activity then leads to a bile acid pool more skewed to 12α-hydroxylated subtypes, which have less affinity for FXR (Haeusler et al., 2012). The authors of this finding speculate that this leads to a decrease in FXR-activation and a subsequent increase in triglyceride levels, linking bile acid pool changes to the dyslipidemia that is characteristically seen in DM2. We were unable to show any significant changes in the 12α-hydroxylated subfraction of bile acids in our studies. This is most likely explained by the timescale involved for changes in hepatic insulin sensitivity to translate to changes in the bile acid pool measurable in peripheral plasma.

Bile acid signals in the portal and systemic compartments

As we describe in Chapter 3, postprandial bile acid concentrations in portal blood are circa five- to tenfold higher than in venous blood due to efficient hepatic extraction. In particular, conjugated versions of the strong TGR5 agonists lithocholic acid (LCA) and hyodeoxycholic acid (HDCA, a secondary bile acid specific to pigs, comparable to the human deoxycholic acid (DCA)) are cleared more efficiently than unconjugated bile acids and chenodeoxycholic acid (CDCA), which has weak affinity for TGR5 (Sato et al., 2008b). This again raises questions whether the TGR5-activating signal in the systemic compartment is meaningful in normal physiology. It has been suggested that supraphysiological stimulation of TGR5 with 15 mg/kg oral CDCA increases energy expenditure *in vivo*, an effect that is traceable to increased glucose uptake by brown adipose tissue (Broeders et al., 2015). In this study, CDCA concentrations of 12 mmol/L were reached, representing a concentration around five- to tenfold higher than that normally seen after a meal, though this is subject to interindividual variability. In our study with gDCA (Chapter 5), we did not see an increase in resting energy expenditure after a single dose of 750 mg gDCA, which was around 10 mg/kg. Interestingly, gDCA levels in venous blood were only modestly raised, to a peak level of ~1.4 mmol. This suggests that the oral gDCA was hepatically cleared, foregoing direct effects outside of the enterohepatic circulation. This is in line with our finding that unconjugated bile acids pass the liver barrier more easily than conjugated bile acids, making them more suitable to target the systemic compartment.

Future perspectives

Bile acids and their downstream effectors remain a promising area of potential treatment in DM2, obesity and liver disease. However, before potential treatments can be implemented, more research into basic human bile acid metabolism is needed. As referred to in this thesis, the relation between bile acid pool changes in metabolic disease and development of metabolic impairment has so far not been solved. The direction of causality in particular needs to be identified: how is bile acid metabolism affected by metabolic disease, or are bile acid changes a link in the chain of events leading from obesity to hyperlipidemia and insulin resistance and eventually full-blown DM2?

A compounding factor is the efficient negative feedback-inhibition loop of bile acids on their own synthesis via FXR and FGF19, which makes the system

resistant to short-term disturbances. This leads to a slow timeframe in which interventions in bile acid metabolism lead to observable changes in the bile acid pool, and thus to a temporal disconnect between interventions and outcomes, making those outcomes difficult to interpret. We do know that alterations in bile acid synthesis can have large effects on the bile acid pool, as evidenced by the grossly abnormal bile acid pool compositions in patients with inborn errors of bile acid metabolism such as cerebrotendinous xanthomatosis. Unfortunately, these defects are exceedingly rare therefore not available for larger-scale studies. While *in vitro* and rodent models offer the opportunity to isolate molecular mechanisms, there is a need for more translational and physiological studies in human models of metabolic disease. The challenge will be to gain a direct view into the different compartments of the enterohepatic bile acid pool, as sampling the peripheral circulation alone does not capture the majority of bile acid-receptor interactions. Creative solutions involving clinicians, such as peroperative sampling of the portal vein or nuclear imaging techniques, will need to be found to sample the relevant compartments. Another avenue of great interest is the development of specific synthetic TGR5 and FXR agonists. Making these widely available for research would open up new opportunities to dissect human bile acid physiology.

References

Broeders, E. P. M., Nascimento, E. B. M., Havekes, B., Brans, B., Roumans, K. H. M., Tailleux, A., Schaart, G., Kouach, M., Charton, J., Deprez, B., Bouvy, N. D., Mottaghy, F., Staels, B., van Marken Lichtenbelt, W. D., & Schrauwen, P. (2015). The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity. *Cell Metabolism*, 1–9.

Brufau, G., Stellaard, F., Prado, K., Bloks, V. W., Jonkers, E., Boverhof, R., Kuipers, F., & Murphy, E. J. (2010). Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism. *Hepatology (Baltimore, Md.)*, *52*(4), 1455–1464.

Dutia, R., Embrey, M., O'Brien, C. S., Haeusler, R. A., Agénor, K. K., Homel, P., McGinty, J., Vincent, R. P., Alaghband-Zadeh, J., Staels, B., le Roux, C. W., Yu, J., & Laferrère, B. (2015). Temporal changes in bile acid levels and 12α-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes. *International Journal of Obesity*, *39*(5), 806–813.

Glicksman, C., Pournaras, D. J., Wright, M., Roberts, R., Mahon, D., Welbourn, R., Sherwood, R., Alaghband-Zadeh, J., & le Roux, C. (2010). Postprandial plasma bile acid responses in normal weight and obese subjects. *Annals of Clinical Biochemistry*, *47*(Pt 5), 482–484.

Haeu, N. B., Dirksen, C., Bojsen-Møller, K. N., Kristiansen, V. B., Wulff, B. S., Rainteau, D., Humbert, L., Rehfeld, J. F., Holst, J. J., Madsbad, S., & Clausen, T. R. (2015). Improvements in glucose metabolism early after gastric bypass surgery are not explained by increases in total bile acids and fibroblast growth factor 19 concentrations. *Journal of Clinical Endocrinology and Metabolism*, *100*(3), E396–E406.

Haeusler, R. A., Astiarraga, B., Camastra, S., Accili, D., & Ferrannini, E. (2013). Human insulin resistance is associated with increased plasma levels of 12α-hydroxylated bile acids. *Diabetes*, *62*(12), 4184–4191.

Haeusler, R. A., Pratt-Hyatt, M., Welch, C. L., Klaassen, C. D., & Accili, D. (2012). Impaired generation of 12-hydroxylated bile acids links hepatic insulin signaling with dyslipidemia. *Cell Metabolism*, *15*(1), 65–74.

Hofmann, A. F. (2009). Bile acids: Trying to understand their chemistry and biology with the hope of helping patients. *Hepatology*, *49*(5), 1403–1418.

Kaválková, P., Mráz, M., Trachta, P., Kloučková, J., Cinkajzlová, A., Lacinová, Z., Haluzíková, D., Beneš, M., Vlasáková, Z., Burda, V., Novák, D., Petr, T., Vítek, L., Pelikánová, T., & Haluzík, M. (2016). Endocrine effects of duodenal-jejunal exclusion in obese patients with type 2 diabetes mellitus. *Journal of Endocrinology*, *231*(1), 11–22.

Kohli, R., Bradley, D., Setchell, K. D., Eagon, J. C., Abumrad, N., & Klein, S. (2013). Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile acids. *The Journal of Clinical Endocrinology and Metabolism*, *98*(4), E708-12.

Kuhre, R. E., Wewer Albrechtsen, N. J., Larsen, O., Jepsen, S. L., Balk-Møller, E., Andersen, D. B., Deacon, C. F., Schoonjans, K., Reimann, F., Gribble, F. M., Albrechtsen, R., Hartmann, B., Rosenkilde, M. M., & Holst, J. J. (2018). Bile acids are important direct and indirect regulators of the secretion of appetite- and metabolism-regulating hormones from the gut and pancreas. *Molecular Metabolism*, *11*, 84–95.

LaRusso, N. F., Hoffman, N. E., Korman, M. G., Hofmann, A. F., & Cowen, A. E. (1978). Determinants of fasting and postprandial serum bile acid levels in healthy man. *The American Journal of Digestive Diseases*, *23*(5), 385–391.

Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., & Staels, B. (2009). Role of bile acids and bile acid receptors in metabolic regulation. *Physiological Reviews*, *89*(1), 147–191.

Legry, V., Francque, S., Haas, J. T., Verrijken, A., Caron, S., Chávez-Talavera, O., Vallez, E., Vonghia, L., Dirinck, E., Verhaegen, A., Kouach, M., Lestavel, S., Lefebvre, P., Van Gaal, L., Tailleux, A., Paumelle, R., & Staels, B. (2017). Bile acid alterations are associated with insulin resistance, but not with NASH, in obese subjects. *Journal of Clinical Endocrinology and Metabolism*, *102*(10), 3783–3794.

Meessen, E. C. E., Sips, F. L. P., Eggink, H. M., Koehorst, M., Romijn, J. A., Groen, A. K., van Riel, N. A. W., & Soeters, M. R. (2020). Model-based data analysis of individual human postprandial plasma bile acid responses indicates a major role for the gallbladder and intestine. *Physiological Reports*, *8*(5).

Patti, M.-E., Houten, S. M., Bianco, A. C., Bernier, R., Larsen, P. R., Holst, J. J., Badman, M. K., Maratos-Flier, E., Mun, E. C., Pihlajamaki, J., Auwerx, J., & Goldfine, A. B. (2009). Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. *Obesity (Silver Spring, Md.)*, *17*(9), 1671–1677.

Sato, H., Macchiarulo, A., Thomas, C., Gioiello, A., Une, M., Hofmann, A. F., Saladin, R., Schoonjans, K., Pellicciari, R., & Auwerx, J. (2008a). Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. *Journal of Medicinal Chemistry*, *51*(6), 1831–1841.

Sato, H., Macchiarulo, A., Thomas, C., Gioiello, A., Une, M., Hofmann, A. F., Saladin, R., Schoonjans, K., Pellicciari, R., & Auwerx, J. (2008b). Novel potent and selective bile acid derivatives as TGR5 agonists: Biological screening, structure-activity relationships, and molecular modeling studies. *Journal of Medicinal Chemistry*, *51*(6), 1831–1841.

Schalm, S., LaRusso, N., Hofmann, A. F., Hoffman, N., van Berge-Henegouwen, G., & Korman, M. (1978). Diurnal serum levels of primary conjugated bile acids. *Gut*, *19*, 1006–1014.

Soeters, M. R., Soeters, P. B., Schooneman, M. G., Houten, S. M., & Romijn, J. A. (2012). Adaptive reciprocity of lipid and glucose metabolism in human short-term starvation. *Am J Physiol Endocrinol Metab*, *303*(12), E1397-407.

Sonne, D. P., van Nierop, F. S., Kulik, W., Soeters, M. R., Vilsbøll, T., & Knop, F. K. (2016). Postprandial Plasma Concentrations of Individual Bile Acids and FGF-19 in Patients with Type 2 Diabetes. *The Journal of Clinical Endocrinology & Metabolism*, *101*(August), jc.2016-1607.

Steiner, C., Othman, A., Saely, C. H., Rein, P., Drexel, H., von Eckardstein, A., & Rentsch, K. M. (2011). Bile acid metabolites in serum: intraindividual variation and associations with coronary heart disease, metabolic syndrome and diabetes mellitus. *PloS One*, *6*(11), e25006.

Vincent, R. P., Omar, S., Ghozlan, S., Taylor, D. R., Cross, G., Sherwood, R. A., Fandriks, L., Olbers, T., Werling, M., Alaghband-Zadeh, J., & le Roux, C. (2013). Higher circulating bile acid concentrations in obese patients with type 2 diabetes. *Annals of Clinical Biochemistry*, *50*(4), 360–364.

10

Summary

Bile acid metabolism in humans: postprandial perspective

Chapter 1
An introduction in current understanding of bile acid biology. This chapter outlines the enterohepatic cycle of bile acid synthesis, secretion and reabsorption, as well as the presumed role of bile acids in metabolism through their effects on the two main bile acid receptors, Farnesoid X Receptor (FXR) and Takeda G-protein coupled receptor 5 (TGR5). Thereafter, the research questions and experiments that form the basis of this thesis are described.

Chapter 2 (Sonne et al., 2016)
In this chapter, we investigated the effect of diabetes on postprandial bile acid levels and the gut hormone fibroblast growth factor-19 (FGF19). We describe postprandial plasma concentrations of 12 individual bile acid subspecies as well as FGF19 in fifteen patients with type 2 diabetes (DM2), compared to healthy controls with normal glucose tolerance. To this end, we used a 75-g oral glucose tolerance test and three isocaloric and isovolemic liquid meals with low, medium, and high fat content, respectively. We showed that postprandial total bile acid concentrations increased with increasing meal fat content ($p<.05$), peaked after 1–2 hours, and were higher in DM2 patients than in controls (oral glucose tolerance test, low and medium fat meals, $p<.05$; high fat meal, $p=.30$). The differences reflected mainly unconjugated and glycine-conjugated forms of deoxycholic acid (DCA) and to a lesser extent cholic acid (CA) and ursodeoxycholic acid (UDCA), whereas chenodeoxycholic acid (CDCA) concentrations were comparable in the two groups. FGF19 concentrations tended to be lower in patients with DM2 then in controls, but the difference was not statistically significant due to considerable variation.

Chapter 3 (Eggink et al., 2018)
Bile acids play a key role in lipid uptake and possibly in metabolic signalling in different organs including gut, liver, muscle and brown adipose tissue. Portal and peripheral plasma BA concentrations are known to increase after a meal containing fats and carbohydrates. However, the exact kinetics of postprandial bile acid metabolism have never been described in great detail. We used a porcine model to investigate postprandial plasma concentrations and transorgan fluxes of bile acids, glucose and insulin, using the para-aminohippuric acid dilution

method. To this end, eleven pigs with indwelling intravascular catheters received a standardized mixed meal. During and after the meal, venous blood was sampled from different veins such as the portal vein, abdominal aorta and hepatic vein to calculate fluxes of bile acids across different organ compartments. To compare the data to humans, fasted venous and portal blood was obtained from non-diabetic obese patients during gastric bypass surgery.

The majority of the circulating plasma bile acid pool and postprandial response consisted of glycine-conjugated forms of the primary bile acids CA and CDCA. Conjugated bile acids, which have higher affinity for TGR5, were more efficiently cleared by the liver than unconjugated forms. Taking into account the previously published EC50 of individual BA subtypes for TGR5, the potential TGR5-activating signal in the portal vein was ~5-10 times stronger than in the systemic circulation. The timing and size of the postprandial response showed large interindividual variability for bile acids.

Chapter 4 (van Nierop et al., 2017a)
The physiochemical properties of different bile acid subspecies affect their enterohepatic dynamics and their affinity for bile acid receptors. The composition of the bile acid pool is known to be altered in patients with type 2 diabetes and obesity, as we have again observed in Chapter 2, suggesting a causal link between bile acid metabolism and metabolic derangement. Here, we used a 2-week very-low-calorie diet to investigate the effects of acute weight loss through calorie restriction on bile acid pool composition and postprandial bile acid dynamics. We performed mixed meal tests in obese, insulin-resistant subjects before and after the weight loss period. Weight loss increased peak postprandial levels of the secondary bile acid DCA and decreased resting energy expenditure. Other BA species, glucose, insulin and FGF19 levels and postprandial excursions were not significantly affected. We hypothesize that the modestly increased postprandial DCA levels are secondary to a combination of increased intestinal uptake and increased CYP8B1 activity. Additionally, the timeframe of the intervention may have been too short to induce observable changes in the bile acid pool.

Chapter 5 (van Nierop et al., 2019)
In this study, we investigated the postprandial bile acid response in peripheral plasma and its relation to insulin, GLP-1, and FGF19. First, we investigated the postprandial response after a 40-h fast. Then we administered glycine-conjugated deoxycholic acid (gDCA) with the meal. We performed two separate observational randomized crossover studies on healthy, lean men. In experiment 1, we tested a 4-h mixed meal after an overnight fast and a 40-h fast. In experiment 2, we tested a 4-h mixed meal test with and without gDCA supplementation. Both studies measured postprandial glucose, insulin, bile acids, GLP-1, and FGF19. In experiment 1, 40 h of fasting induced insulin resistance and increased postprandial GLP-1 and FGF19 concentrations. After an overnight fast, we observed strong correlations between postprandial insulin and gDCA levels at various time points. Unexpectedly, 40 h of fasting increased both GLP-1 and FGF19. If this is due to increased enteral TGR5 and FXR activation due to exposure to bile acids, that would be counterintuitive, as enteral bile acid levels are usually linked to food intake. Alternatively, FGF19 could be upregulated through some other mechanism in order to suppress bile acid synthesis during starvation. In the second experiment, we observed increased postprandial GLP-1 levels after a meal in the fed state, indicating enteral TGR5 activation by orally administered bile acids. gDCA stimulation did not affect FGF19-levels, suggesting no enteral FXR-activation. Energy expenditure was not affected by gDCA administration. This experiment is a first step to explore the feasibility of bile acid supplementation in humans with metabolic disorders.

Chapter 6 (van Nierop et al., 2019)
Placement of the duodenal-jejunal bypass liner (DJBL), a bariatric device, in obese patients with type 2 diabetes leads to rapid weight loss and restoration of insulin sensitivity in a similar fashion to bariatric surgery. Increased systemic bile acid levels are candidate effectors for these effects through postprandial activation of their receptors TGR5 and FXR. We aimed to quantify postprandial bile acid, GLP-1 and FGF19 responses and assess their temporal relation to the weight loss and metabolic and hormonal changes seen after DJBL placement. To that end, we performed mixed meal testing in 17 obese patients with type 2 diabetes mellitus (DM2) directly before, one week after and 6 months after DJBL placement. A weight loss 4.3 ± 0.6 kg was achieved at 1 week, which increased to 12.7 ± 1.3 kg after 6 months. Both fasting and postprandial bile acid levels were unchanged at 1 week after implantation, and greatly increased 6 months after implantation.

The increase consisted of unconjugated bile acid species. 3 hour-postprandial GLP-1 levels increased after 1 week and were sustained, whereas FGF19 levels and postprandial plasma courses were unaffected. We conclude that DJBL placement leads to profound increases in unconjugated bile acid levels after 6 months, similar to the effects of bariatric surgery. The temporal dissociation between the changes in bile acids, GLP-1 and FGF19 and other gut hormone responses suggest that the improvement in insulin sensitivity is not induced by increased bile acid signaling. This observational uncontrolled study emphasizes the need for future controlled studies.

Chapter 7 (Stenvers et al., unpublished)
Patients with type 2 diabetes have a disturbed diurnal rhythm of plasma glucose tolerance. We hypothesized that this may be due to altered diurnal regulation of plasma bile acid levels. We provided six patients with DM2 and six age-matched healthy controls with three identical meals per day, and measured plasma concentrations of primary and secondary BAs using tandem mass spectrometry. Postprandial BA responses were not affected by time of day. However, postprandial peak times were earlier for total BAs, chenodeoxycholic acid, and glycine conjugates in DM2, which may be explained by increased intestinal and/or decreased liver BA uptake. Our results suggest that while bile acid synthesis is known to vary during the day, bile acid responses to a meal do not, and are therefore not responsible for the altered diurnal rhythm of glucose tolerance seen in DM2.

Chapter 8 (van Nierop et al., 2017b)
In chapter 8 we reviewed the literature on the physiological relevance of the Takeda G protein-coupled receptor 5 (TGR5) with a focus on translating the data to clinical applications in human metabolism. We summarize the key concepts of bile acid signaling and discuss the physiological and pharmacological literature on TGR5 activation in glucagon-like receptor 1 (GLP-1) secretion and glucose homeostasis, energy expenditure and modulation of inflammatory signals. The available preclinical data suggests that TGR5 plays a role in GLP-1 secretion, insulin sensitivity and energy expenditure. However, evidence in humans is ambiguous and clinical application is still far away. Ultimately, safe and selective human TGR5 agonists are needed to further study the therapeutic potential of TGR5.

Part VI

Appendix

11

Appendix

Nederlandse samenvatting

Dit proefschrift gaat over de relatie tussen galzure zouten (in dit proefschrift doorgaans galzouten genoemd) en stofwisseling. In het bijzonder hebben we gekeken naar de rol van galzouten als signaalmoleculen en hun effect op het glucosemetabolisme en de energiehuishouding in de postprandiale periode (d.w.z. na de maaltijd), wanneer het lichaam zich aanpast om binnenkomende voedingsstoffen te verwerken. Daar een groot deel van onze kennis over galzouten tot dan toe uit diermodellen afkomstig is, hebben we onderzoek gedaan in humane modellen.

Hoofdstuk 1

In het eerste hoofdstuk beschrijven we de huidige stand van de wetenschap rondom de biologie van galzouten en wat er tot nu toe bekend is over hun rol als signaalmoleculen in glucosemetabolisme en de energiehuishouding. Galzouten zijn een groep nauw verwante steroïde moleculen die worden geproduceerd in de lever en opgeslagen in de galblaas. Na een vetrijke maaltijd worden ze in de darm uitgescheiden, waar ze vanwege hun amfipathische structuur bijdragen aan het oplossen van vetten en vetoplosbare vitaminen. Aan het eind van de dunne darm worden ze heropgenomen door de darmwand en via de poortader teruggevoerd naar de lever, waar ze grotendeels worden heropgenomen voor hergebruik. Dit wordt de enterohepatische kringloop genoemd. Tijdens deze kringloop ondergaat een deel van de galzouten een transformatie door bacteriën die in de darm leven. De resulterende moleculen worden secundaire galzouten genoemd, en vormen een belangrijk deel van de uiteindelijke galmix.

Naast hun rol als vetoplossers is de afgelopen jaren steeds meer bekend geworden over de rol van galzouten als endocrien hormoon. Er zijn twee specifieke galzoutreceptoren ontdekt, de Farnesoid X Receptor (FXR) en de Takeda G-protein-coupled Receptor 5 (TGR5), die na activatie effect hebben op het galzout-, glucose-, energie- en lipidenmetabolisme. Het grootste deel van het onderzoek hiernaar is in dieren, vooral muizen en ratten, verricht en kan derhalve niet één-opéén op mensen worden toegepast.

Hoofdstuk 2 (Sonne et al., 2016)
In dit hoofdstuk onderzoeken we het effect van type 2 diabetes mellitus op postprandiale galzout- en FGF19-spiegels in het plasma. We vergelijken hiervoor postprandiale spiegels van de 12 belangrijkste galzoutsoorten en FGF19 tussen 15 patiënten met goed-gereguleerde type 2 diabetes mellitus, en 15 leeftijd-, geslacht- en BMI-gematchte gezonde proefpersonen. Er werden 4 verschillende vloeibare maaltijden getest, die elk evenveel calorieën bevatten, maar verschilden in samenstelling van macronutriënten. Patiënten met type 2 diabetes hadden iets hogere galzoutspiegels in de gevaste toestand, en hun galzoutpiek na de maaltijd was hoger. Voor beide groepen gold dat de galzoutpiek hoger werd met een toenemende vethoudendheid van de maaltijd. FGF19-spiegels stegen met de galzouten mee, maar verschilden niet significant tussen de groepen.

Hoofdstuk 3 (Eggink et al., 2018)
Galzouten spelen een belangrijke rol in vetopname en hebben mogelijk een endocriene functie in verschillende organen, zoals darm-, lever-, spier- en bruin vetweefsel. We weten dat galzoutspiegels in zowel de portale circulatie als perifeer plasma stijgen na de maaltijd, maar de exacte kinetiek van deze compartimenten is nooit in detail beschreven. We gebruikten een varkensmodel om postprandiale stromen van individuele galzoutsoorten over verschillende lichaamscompartimenten te beschrijven. Hiervoor brachten we in elf gezonde varkens intravasculaire catheters in, en dienden ze een gestandaardiseerde maaltijd toe. In de postprandiale periode maten we in verschillende compartimenten insuline, glucose, galzouten en para-aminohippuurzuur om de stromen over de verschillende compartimenten te berekenen. Om dit met mensen te proberen te vergelijken verkregen we peroperatief portaal en perifeer plasma van obese patiënten zonder diabetes die een gastric bypass ondergingen.

Het grootste deel van de galzouten buiten het portale systeem bestond uit glycine-geconjugeerde vormen van de primaire galzouten cholaat en chenodeoxycholaat. De lever klaarde geconjugeerde galzouten efficienter dan ongeconjugeerde galzouten. We berekenden uit eerder gepubliceerde gegevens over de affiniteit van verschillende galzout-subtypes voor TGR5 een index voor potentiele TGR5-activatie. Die index was in portaal bloed ongeveer 5-10 keer hoger dan in perifeer bloed. Opvallend was dat het tijdsbeloop en de intensiteit van postprandiale galzoutpieken sterk verschilde tussen individuen.

Hoofdstuk 4 (van Nierop et al., 2017a)
De chemische eigenschappen van de verschillende galzoutsoorten bepalen deels hun kinetiek in de enterohepatische kringloop en hun affiniteit voor de galzoutreceptoren. Het is bekend dat patiënten met obesitas en type 2 diabetes een afwijkende samenstelling van de galmix hebben (zoals we ook in hoofdstuk 2 beschrijven), hetgeen een causaal verband suggereert tussen galzoutmetabolisme en metabole ontregeling. In deze studie gebruikten we een twee weken durend zeer laagcalorisch dieet om het effect van snel gewichtsverlies middels calorierestrictie op de galmix en op postprandiale galzoutspiegels te onderzoeken. Voor en na de dieetperiode voerden we gestandaardiseerde maaltijdproeven uit. Na gewichtsverlies zagen we toegenomen postprandiale pieken van deoxycholaat, een secundair galzout, en afgenomen basaalmetabolisme. Andere galzouten, FGF19-, insuline- en glucosespiegels bleven gelijk. De toegenomen deoxycholaatspiegels kunnen mogelijk worden toegeschreven aan verhoogde opnamecapaciteit in de darm, danwel toegenomen productie door het enzym CYP8B1. Het lijkt erop dat de interventieperiode te kort is geweest om grote effecten op de galmix te induceren, en dat de veranderingen die we zien bij obesitas zich op een langere tijdschaal afspelen.

Hoofdstuk 5 (van Nierop et al., 2019)
In deze studie onderzochten we in gezonde proefpersonen de relatie tussen postprandiale galzout-, GLP1, FGF19- en insulinespiegels. We voerden gestandaardiseerde maaltijdproeven uit na een vastenperiode van 12 en van 40 uur. Na de lange vastenperiode waren de proefpersonen minder gevoelig voor de werking van insuline, en stegen de GLP1- en FGF19-spiegels fors, maar bleven de galzouten gelijk. Het lijkt erop dat de vastenperiode toegenomen enterale activatie van TGR5 en FXR door galzouten teweeg heeft gebracht. We vonden na de korte vastenperiode sterke correlaties tussen postprandiale insulinespiegels en spiegels van glycodeoxycholaat, een secundair galzout.

In een tweede experiment dienden we glycodeoxycholaat toe bij de maaltijd, waarop de postprandiale spiegels van GLP1, maar niet FGF19, toenamen, hetgeen specifiek enterale activatie van TGR5 suggereert. De gestegen GLP1-spiegels hadden geen effect op insuline- of glucosespiegels of op het basaalmetabolisme.

Hoofdstuk 6 (van Nierop et al., 2019)
Het plaatsen van een DJBL (duodenojejunale bypass liner, een medisch hulpmiddel dat opname van voedsel in de dunne darm grotendeels verhindert) bij patiënten met type 2 diabetes bewerkstelligt snel gewichtsverlies en vaak terugkeer naar normale glucosetolerantie, zoals ook vaak gezien wordt na bariatrische chirurgie. Een mogelijke verklaring voor het verbeteren van de glucosetolerantie zijn de fors verhoogde galzoutspiegels die deze patiënten ontwikkelen na de interventie. We lieten 17 obese patiënten met type 2 diabetes gestandaardiseerde maaltijdproeven met galzoutmetingen ondergaan vóór, een week na, en 6 maanden na plaatsing van de DJBL.

Na plaatsing van de DJBL toonden de patiënten snel gewichtsverlies en resolutie van hun diabetes. Gevaste en postprandiale galzoutspiegels waren onveranderd na een week, maar gigantisch toegenomen na 6 maanden, een toename die vooral bestond uit een toegenomen absolute hoeveelheid ongeconjugeerde galzouten in de galmix. GLP1 was reeds na een week toegenomen, terwijl FGF19 de hele periode onvranderd bleef. Gezien de temporele dissociatie tussen het herstel van insulinegevoeligheid en spiegels van galzouten, GLP1 en FGF19 concluderen we dat het effect van de DJBL op insulinegevoeligheid niet wordt verklaard door toegenomen galzoutsignalering.

Hoofdstuk 7 (Stenvers et al., niet gepubliceerd)
Patiënten met type 2 diabetes hebben een verstoord ritme in hun glucosetolerantie. Wij testten de hypothese dat dit secundair was aan een verstoord ritme in het galzoutmetabolisme. Hiertoe voerden wij 3 identieke gestandaardiseerde maaltijdproeven uit over de loop van een etmaal bij 6 patiënten met type 2 diabetes, en 6 gezonde proefpersonen, waarbij we postprandiale insuline-, glucose- en galzoutspiegels maten. De galzoutspiegels verschilden niet over de loop van de dag, terwijl de insulinegevoeligheid wel varieerde. We viel op dat galzouten bij patiënten met type 2 diabetes iets vroeger na de maaltijd pieken, mogelijk door toegenomen opname in de darm. Wij concluderen dat postprandiale galzoutpieken niet varieren over de dag, en dat galzouten dus niet betrokken zijn bij de ritmiciteit van glucosetolerantie.

Hoofdstuk 8 (van Nierop et al., 2017b)
In dit hoofdstuk beschouwen we de literatuur rondom de fysiologische relevantie van TGR5, waarbij we ons richten op het vertalen van de beschikbare gegevens naar klinische toepassingen in metabole ziekten zoals diabetes mellitus en obesitas. We vatten de belangrijkste concepten binnen het galzoutonderzoek samen en bespreken de beschikbare literatuur over de effecten van TGR5-activatie op secretie van GLP1, glucosehomeostase, basaal energiemetabolisme en het reguleren van inflammatie. Preklinisch onderzoek, vooral in muizen en ratten uitgevoerd, suggereert dat TGR5 een belangrijke rol speelt in deze processen, maar de beperkte hoeveelheid data uit menselijk onderzoek is ambigu. Samenvattend is er nog een lange weg te gaan voor TGR5-modulatie klinisch toepasbaar zal zijn. De belangrijkste stap op die weg is het ontwikkelen en beschikbaar maken van veilige en selectieve TGR5-agonisten voor klinisch onderzoek.

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	Year	ECTS
1. PhD training		
General courses		
The AMC World of Science	2012	0.7
Clinical Data Management	2013	1.4
Specific courses		
Practical Biostatistics	2013	1.4
Basiscursus Regelgeving en Organisatie voor Klinische onderzoekers	2013	1.1
Seminars, workshops and masterclasses		
Annual APROVE PhD symposium	2014	0.1
Weekly research meeting Endocrinology and Metabolism	2012-2016	4.4
Tracer Methodology in Metabolism (Karolinska Institutet)	2014	2.0
Presentations		
EASD (poster x 2)	2013	0.5
Annual Dutch Diabetes Research Meeting (oral)	2014	1.0
ESPEN (poster x 2)	2014	0.5
International Bile Acid Meeting (poster x 3)	2014	1.5
Annual Dutch Diabetes Research Meeting (oral)	2015	0.5
ADA (poster)	2016	0.5
International Bile Acid Meeting (poster)	2016	0.5
(Inter)national conferences		
European Society for the Study of Parenteral and Enteral Nutrition, Leipzig, Germany	2014	1.0
European Association for the Study of Diabetes, Barcelona, Spain	2013	1.0
Falk Symposium – International Bile Acid Meeting, Freiburg, Germany	2014	1.0
Falk Symposium – International Bile Acid Meeting, Düsseldorf, Germany	2016	1.0
American Diabetes Association, New Orleans, USA	2016	1.5
Other		
Mountain Medicine	2014	2.0

	Year	ECTS
2. Teaching		
Lecturing		
Endocrinology course	2015	0.2
Tutoring		
Master Student Geneeskunde (wetenschappelijke stage)	2014	2
Master Student Geneeskunde (literature review and publication)	2015	0.6
3. Parameters of esteem		
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Publications

Differential effects of a 40-hour fast and bile acid supplementation on human GLP-1 and FGF19 responses. van Nierop, FS, Meessen, ECE, Nelissen, KGM, Achterbergh, R, Lammers, LA, Vaz, FM, Mathot, RAA, Klupmpen, HJ, Olde Damink, SW, Schaap, FG, Romijn, JA, Kemper, EM, Soeters, MR. *American Journal of Physiology, Endocrinology and Metabolism,* 317: E494–E502, 2019

Single vagus nerve stimulation reduces early postprandial C-peptide levels but not other hormones or postprandial metabolism. Tang MW, van Nierop FS**,** Eggink HM, et al. *Clinical Rheumatology,* 2018 Feb;37(2):505-514.

Transhepatic Bile Acid Kinetics in Pigs and Humans. Eggink, HM, van Nierop FS, Schooneman et al. *Clinical Nutrition,* 2017 Jun 19. pii: S0261-5614(17)30226-1.

Effects of acute dietary weight loss on postprandial plasma bile acid responses in obese insulin resistant subjects. van Nierop, FS, Kulik, W., Endert, E., Schaap, F. G., Olde Damink, S. W., Romijn, J. A., & Soeters, M. R. (2016). *Clinical Nutrition*, 1–6.

Clinical relevance of the bile acid receptor TGR5 in metabolism. van Nierop, F. S., Scheltema, M., Eggink, H. M., Sonne, D. P., Pols, T. W. H., Knop, F. K., & Soeters, M. R. (2016). *Lancet Diabetes and Endocrinology*, *5*(16).

Postprandial Plasma Concentrations of Individual Bile Acids and FGF-19 in Patients with Type 2 Diabetes. Sonne, D. P., van Nierop, FS, Kulik, W., Soeters, M. R., Vilsbøll, T., & Knop, F. K. (2016). *The Journal of Clinical Endocrinology & Metabolism*, 101(August), jc.2016-1607.

A short-term high fat diet increases exposure to midazolam and omeprazole in healthy subjects. Achterbergh R, Lammers LA, van Nierop FS, et al. (2015). *Expert Opinion on Drug Metabolism and Toxicity.* published online.

Short-Term Fasting alters Cytochrome P450 mediated Drug Metabolism in Humans. Lammers, LA, Achterbergh, R, de Vries, EM, van Nierop, FS, et al. (2015). *Drug Metabolism and Disposition* 2015 43(6), 819-828

In the critically ill patient, diabetes predicts mortality independent of statin therapy but is not associated with acute lung injury: A cohort study. Koh GC, Vlaar AP, Hofstra JJ, de Jong HK, van Nierop FS, et al. (2012). *Critical Care Medicine*, *40*(6), 1835.

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Fico Rubado

Dom. Del Pino dis. in Genova nel 7bre 1819 *Aless. Rinaldi incise in Firenze [illegible]*