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Title: Bile acid alterations in non-alcoholic fatty liver disease, obesity, insulin resistance and type 2 diabetes: what do the human studies tell?

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Abstract

Purpose of review: To discuss the influence of obesity, insulin resistance, type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD) on bile acid (BA) metabolism and analyze whether these findings reinforce current beliefs about the role of BAs in the pathophysiology of these diseases.

Recent findings: Discordant results on plasma BA alterations in NAFLD patients have been reported. Obesity, insulin resistance and T2D, common co-morbidities of NAFLD, have been associated with BA changes, but the individual BA species variations differ between studies (summarized in this review), perhaps due to clinico-biological differences between the studied patient populations and the heterogeneity of statistical analyses applied.

Summary: The regulatory role of BAs in metabolic and cellular homeostasis render BAs attractive candidates as players in the pathophysiology of NAFLD. However, considering the complex relationship between NAFLD, obesity, insulin resistance and T2D, it is difficult to establish clear and independent associations between BA alterations and these individual diseases. Though BA alterations may not drive NAFLD progression, signaling pathways activated by BAs remain potent therapeutic targets for its treatment. Further studies with appropriate matching or adjustment for potential confounding factors are necessary to determine which pathophysiological conditions drive the alterations in BA metabolism.

Keywords: Bile acids, NAFLD, NASH, obesity, insulin resistance, type 2 diabetes, metabolic syndrome.

Keypoints:

1. BA changes have been associated with obesity, insulin resistance and T2D, common co-morbidities of NAFLD.
2. Discordant results on plasma BA alterations in NAFLD patients have been reported.
3. The intricate relationship between NAFLD, obesity, insulin resistance and T2D renders difficult the establishment of clear and independent associations between BA alterations and these individual diseases.
4. Further studies with appropriate matching or adjustment for potential confounding factors are necessary to determine which of these diseases drive the alterations in BA metabolism in NAFLD.

5. BA signaling pathways remain attractive candidates for the treatment of NAFLD, but whether BAs are a driving force in the pathogenesis and progression of NAFLD or just innocuous bystanders remains undetermined.

Introduction

The increasing global prevalence of the metabolic syndrome (MetS), which groups obesity, hyperglycemia, dyslipidaemia and hypertension, is a major worldwide public health problem. Non-alcoholic fatty liver disease (NAFLD) is often considered the hepatic manifestation of MetS, and is closely related to many of its features. NAFLD comprises a hepatic spectrum ranging from simple hepatic steatosis or non-alcoholic fatty liver (NAFL), characterized by abnormal triglyceride accumulation in hepatocytes, which can progress to non-alcoholic steatohepatitis (NASH). While NAFL is generally considered benign, NASH combines NAFL with elements of hepatic tissue injury including evidence of inflammation and hepatocyte ballooning with or without fibrosis. NAFLD is an independent risk factor for cirrhosis, hepatocellular carcinoma, cardiovascular disease and mortality. Importantly, NAFLD is strongly and bidirectionally associated with obesity, insulin resistance (IR) and type 2 diabetes (T2D) [1–3].

There are several unmet research needs in the field of NAFLD. The natural history of NAFLD is not entirely understood, and no pharmacological therapies exist for its treatment. For example, NAFL can be diagnosed with non-invasive imaging techniques, but NASH diagnosis currently requires liver biopsy for histological evaluation, an intervention not devoid of risk, and highly susceptible to inter-observer variations [4]. It is thus urgent to find circulating biomarkers to better identify those patients to be submitted to biopsy for NASH screening and monitoring NAFLD progression and staging for assessment of treatment efficacy. Recently, BAs have emerged as potential candidates, and new emerging therapies for NAFLD target BA signaling pathways. Moreover, some evidence suggests that BA alterations could be associated with NAFLD.

Bile acid physiology

BAs are cholesterol-derived molecules synthesized exclusively in hepatocytes *via* two pathways. The classical pathway produces the majority of BAs. It is initiated by cytochrome P450 (CYP) cholesterol 7 α -hydroxylase (CYP7A1), whose activity correlates with plasma levels of 7 α -hydroxy-4-cholesten-3-one (C4). The alternative pathway, initiated by CYP 27 α -hydroxylase (CYP27A1), synthesizes the remaining minor BA fraction. These pathways produce the primary BAs cholic acid (CA), chenodeoxycholic acid (CDCA) and hyocholic acid (HCA) in humans. Notably, CA is a 12 α -hydroxylated-BA, synthesized by the CYP 12 α -hydroxylase (CYP8B1), which is indirectly regulated by

insulin signalling *via* FoxO1. Thus, the 12 α -hydroxylated:non-12 α -hydroxylated BA ratio reflects the activity of CYP8B1, and is a marker of hepatic insulin sensitivity of the BA synthesis pathway. Upon synthesis, BAs are conjugated to glycine or taurine (the proportion being essentially determined by dietary intake), secreted into bile and released in the duodenum post-prandially to facilitate dietary fat solubilization and absorption. In the gut, microbiota deconjugate and transform primary BAs into secondary BAs: CA into DCA (also a 12 α -OH BA), CDCA into UDCA and LCA, and HCA into HDCA. ~95% of BAs are recaptured in the ileum and return to the liver *via* the portal vein to be re-conjugated and re-secreted into bile. A minor fraction of portal BAs escape hepatic reuptake and reach the systemic circulation, hence potentially allowing signaling actions in peripheral organs[5]. However, it should be noted that circulating plasma BAs do not necessarily reflect the composition of the portal, intra-hepatic, biliary or intestinal BA pools.

Besides their role in dietary lipid solubilization, BAs are natural ligands of the Farnesoid X receptor (FXR) and the Takeda G protein coupled receptor (TGR5), through which BAs regulate their own synthesis, as well as glucose, lipid and energy homeostasis, inflammation, hepatic fibrosis and modulate reparative activity (reviewed in [5]). Importantly, not all BAs activate FXR (CDCA>DCA>CA>LCA) and TGR5 (LCA>DCA>CDCA>CA) with the same affinity nor potency. Hence, qualitative alterations of the BA pool composition, in addition to quantitative changes in total BAs, could modulate the activity of their receptors and hence impact metabolism.

BA-FXR signaling in enterocytes stimulates the secretion of Fibroblast Growth Factor 19 (FGF19) into portal venous blood, which then activates FGFR4/ β -Klotho in the liver. Through this mechanism, FGF19 suppresses CYP7A1, thereby decreasing BA synthesis *via* the classical pathway, and also regulates hepatic carbohydrate and lipid metabolism. Interestingly, plasma FGF19 (a biomarker of intestinal FXR activation) is decreased in the metabolic syndrome, suggesting that it could be implicated in the pathogenesis of NAFLD [6,7]. Considering the many interactions between diet, insulin signaling and BA metabolism, it appears logical that alterations in circulating BAs could be markers of, or even actors in, the pathophysiology of the metabolic processes that they modulate.

Bile acids in obesity, insulin resistance and type 2 diabetes

The effect of obesity on BA metabolism is not entirely clear. BMI has been reported to positively correlate with fasting BAs [8], but negatively with postprandial BAs [9]. Other studies have reported

either no changes [10–12] or increased concentrations of CA [13] in fasting plasma BAs, and no changes [10] or decreased glyco-conjugated BAs [11] in postprandial plasma of obese individuals. There is also evidence that the normal meal-induced BA increase in plasma BA is suppressed with obesity [12,14]. Similarly, insulin infusion during an euglycemic hyperinsulinemic clamp decreased serum BAs in lean but not in obese patients [12]. Fasting plasma C4 levels were higher in obese subjects in two different studies [12,15], suggesting increased BA biosynthesis. Expression of the canalicular (BSEP) and sinusoidal (NTCP) BA transporter mRNA levels were negatively correlated with BMI [12]. Although there is a large heterogeneity between the reported results, possibly due to the known large inter- and intra- individual variability in BAs and the diurnal variations of BAs in humans [15], overall fasting plasma BAs appear elevated, but the post-prandial increase is lower in obese individuals.

A parameter confounding these studies may be related to differences in the level of insulin resistance among the studied patient populations. Studies analyzing BA changes in insulin resistant, but not diabetic, patients have reported higher fasting total and 12 α -OH BAs [16]. Mechanistic studies in insulin-resistant mice suggest that impaired insulin-mediated repression of FoxO1 results in higher 12 α -hydroxylase CYP8B1 expression [17]. Moreover, insulin resistance was associated with increased total BA concentrations [18,19], fasting tauro-conjugated BAs [20], CDCA, CA, DCA [13] and GCDCA [21].

Some studies in overtly T2D patients have reported no changes in fasting [9,22] or postprandial [9,23] BAs compared to controls, whereas other studies have reported either increased fasting [7,16,24] and postprandial [24] total BA concentrations, increased fasting DCA [9,13,25], TCDCA, TDCA and TUDCA [20], or decreased CA and HCA [25]. BA levels were not modified by intensive insulin treatment in diabetic patients [20].

The mechanisms underlying the quantitative and qualitative modifications in BAs in the metabolic syndrome are not fully elucidated. Besides the previously described alterations of the insulin receptor-Akt-FoxO1 signaling pathway impacting BA synthesis in insulin resistance, dysbiosis (occurring in metabolic disorders) could modify BA de-conjugation, secondary BA synthesis and modulate the BA pool composition [5]. Another mechanism implies the entero-hepatic crosstalk *via* intestinally FXR-induced FGF19. Indeed, FGF19 levels correlate positively with total BA concentrations [10,24] and negatively with plasma C4 [10,26]. Interestingly, FGF19 is lower in T2D and obese patients and

correlates negatively with BMI [10,27] and HbA1c [27]. However, these changes in FGF19 in T2D patients [9,24] or its correlation with MetS components [20] are not universally observed. Importantly, differences in control populations and the degree of IR or T2D severity are potential confounding factors between these studies and are not always well reported nor well characterized.

In conclusion, although not all reported data are entirely consistent, it is clear that BA metabolism alterations occur in obese and/or insulin resistant humans (summarized in **Table 1**). However, it remains undetermined whether BAs are innocent bystanders or if they participate to the pathophysiology of these diseases.

Bile acids and NAFLD

Given that BA accumulation in hepatocytes is cytotoxic in intrahepatic cholestatic liver diseases; it is conceivable that BAs could be altered in NAFLD also and that their accumulation in NASH patients could favor progression of the disease. Furthermore, hepatic necro-inflammatory lesions in NAFLD may alter the anatomy of liver zonation. Since the liver is the sole site of BA synthesis and since BA synthesis is zoned in physiological conditions, altered BA metabolism could occur in a setting of pathologically altered liver zonation. Indeed, treatment of NASH with the FXR agonist obeticholic acid [28] or with the FGF19 analog NMG282 [29] improved several NAFLD histological features. Therefore, several groups have studied whether NAFLD patients present BA profile alterations (summarized in **Table 2**).

Fasting plasma BAs were reported to be higher in NASH patients due to increased primary [30] and conjugated BA species [30–32]. Similar changes were also observed in NAFL patients [31]. Moreover, histological lesions of NASH have been associated with BA alterations (*i.e.* associations of high plasma GCA [30] or CA [33] concentrations with lobular inflammation, low TUDCA and high TLCA concentrations with portal inflammation [30], high CA, CDCA [33], GCA, TCA, GCDCA, DCA and GLCA [30] concentrations with hepatocyte ballooning have been reported, as well as elevated total BAs [34], CA, CDCA [33], and both increased [30] or decreased [33] conjugated CA concentrations have been associated with high NAFLD activity score (NAS)). Additionally, increased postprandial total BAs have been reported in plasma and in urine [32], as well as increased fecal BA loss, which positively correlated with NAS and associated with decreased proportions of the gut microbiota phylum *Bacteroidetes* [35]. Furthermore, hepatic tissue-extracted BAs were reported to be altered in

NASH and NAFL patients resulting in qualitative changes in hepatic BA content, although inconsistent results were obtained between both studies [36,37]. Such results should be taken with caution, since BAs were extracted from whole hepatic tissue, preventing discrimination between blood, biliary or intrahepatocyte BA concentrations.

The expression of hepatic BA metabolism genes has also been studied in livers from NASH patients. Both increased [38] and decreased [36] CYP27A1 mRNA levels were reported, in addition to increased CYP7B1 gene and protein levels [36]. These results suggest possible modifications in the alternative BA synthesis pathway. Furthermore, increased expression of the gene encoding CYP7A1 [30,38,39] suggests that the classical BA synthesis pathway may also be activated. FGF19 levels, which regulate bile acid synthesis, have been reported as decreased [38] or unchanged [30] in NASH patients. Plasma C4 levels are, however, unchanged in NASH patients vs controls [30,34,39]. A summary of the hepatic gene expression changes reported in NASH patients is provided in Table 2.

We recently compared BA metabolism parameters in NASH patients matched for BMI- and IR with obese individuals without NASH. No qualitative or quantitative differences in fasting plasma BAs was found in NASH patients, with only GCA weakly correlating with steatosis ($r=0.29$, $p=0.03$). However, re-analysis by severity of IR revealed a tendency towards previously reported associations in fasting plasma BAs (Table 1). These findings suggest that BA metabolism correlates with the metabolic co-morbidities of NAFLD, rather than with the histopathological parameters of NASH itself [39]. It will be important in future studies to carefully assess NASH independently of common co-morbidities to determine which changes, if any, in BA metabolism are specifically related to histological changes in NASH.

Are BA changes associated with NAFLD independently from obesity, IR and T2D?

Assessment of the clinico-biological status of the studied populations appears essential to understand discordant results and allow their appropriate interpretation. In studies lacking information on the nutritional status [34,38,40] and biochemical markers of glucose metabolism as fasting plasma glycaemia, HOMA-IR or HbA1c [30,33,34,38,40], it cannot be excluded that differences between the compared groups, other than histological parameters of NAFLD, influence the interpretation of the results. Many studies that provided the clinico-biological characteristics of the study groups are flawed by the comparison of NASH or NAFL patients with control groups presenting significantly lower BMI

[30–32,34,38], Hb1A1c [34], insulin resistance or fasting glycaemia [31,32]. As discussed above, these metabolic co-morbidities of NAFLD are correlated with BA alterations. It is thus difficult to conclude whether BA alterations are associated *per se* with NAFLD, or with the associated insulin resistance. Appropriate statistical adjustment or patient matching could help to determine whether the associations are independent from potential confounding factors.

Conclusion

Though BA signaling targets are attractive candidates for the treatment of NAFLD, it remains undetermined whether BAs are a driving force in the pathogenesis and progression of NAFLD or just innocuous bystanders. The current literature does not clearly support a causal role. Moreover, the large inter-individual variation and diurnal BA changes in peripheral blood suggest that they are likely unsuitable as biomarkers for the diagnosis or leading to the decision of biopsy taking in NAFLD patients. However, this does not exclude that targeting BA signaling remains an attractive therapeutic target for NASH treatment. Further clinical studies assessing plasma BA profiles should carefully consider matching BMI and IR profiles across controls, NAFL and NASH patients in order to more clearly assess the connection between alterations in BA metabolism and these parameters of the metabolic syndrome.

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Conflicts of interest

None

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Table titles and legends.

Table 1.

Title: Clinical studies assessing BAs in obesity, insulin resistance and type 2 diabetes.

Legend: Data extracted from the articles and presented as Means \pm SD, Means \pm SEM, Medians[Q1-Q3], Medians[IQR] or Medians(range). *Statistically different from control, [§]Statistically different from T2D. Abbreviations: 7 α -hydroxy-4-cholesten-3-one (C4), 7 α -12 α -dihydroxy-4-cholesten-3-one (7,12-diHCO), bile acids (BAs), body mass index (BMI), fasting plasma glycaemia (FPG), glycated hemoglobin (HbA1c), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), insulin sensitivity index (M/I), homeostatic model assessment for insulin resistance (HOMA-IR), metabolic syndrome (MetS), minutes (min), no data (ND), oral fat tolerance test (OFTT).

Table 2

Title: Clinical studies assessing BAs in NAFLD

Legend: Data extracted from the articles and presented either as Mean \pm SD, Means \pm SEM or Median(Range). *NASH statistically different from controls. Abbreviations: bile acids (BAs), body mass index (BMI), fasting plasma glycaemia (FPG) -data reported in sources as mmol/L was transformed to mg/dL by multiplying the reported value by 18-, glycated hemoglobin (HbA1c), homeostatic model assessment for insulin resistance (HOMA-IR), magnetic resonance imaging (MRI) no data (ND).