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Bile acid alterations are associated with insulin resistance, but not with NASH in obese subjects

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Abbreviations

AI, activity index;

ALAT, alanine aminotransferase;

ALP, alkaline phosphatase;

ASAT, aspartate aminotransferase;

ASBT, apical sodium-dependent BA transporter;

BA, bile acid;

BMI, body mass index;

CA, cholic acid;

CDCA, chenodeoxycholic acid;

C4, 7alpha-hydroxy-4-cholesten-3-one;

DCA, deoxycholic acid;

FGF19, fibroblast growth factor 19;

FPG, fasting plasma glucose;

FPI, fasting plasma insulin;

FXR, farnesoid X receptor;

GO, gene ontology;

GGT, gamma-glutamyl transpeptidase;

HCA, hyocholic acid;

HDCA, hyodeoxycholic acid.

HDL-C, high-density lipoprotein cholesterol;

HI, hydrophobicity index;

HOMA-IR, homeostasis model assessment of insulin resistance;

IR, insulin resistance;

LCA, lithocholic acid;

LC-MS/MS, liquid chromatography–tandem mass spectrometry;

LDL-C, low-density lipoprotein cholesterol;

NAFLD, non-alcoholic fatty liver disease;

NASH, non-alcoholic steatohepatitis;

NAS, NAFLD activity score;

OCA, obeticholic acid

PXR, pregnane X receptor;

TGR5, Takeda G-protein-coupled receptor 5, also known as G-protein coupled BA receptor 1 (GPBAR1);

T2D, type 2 diabetes;

VDR, vitamin D receptor.

Context: Bile acids (BA) are signalling molecules controlling energy homeostasis which can be both toxic and protective for the liver. BA alterations have been reported in obesity, insulin resistance (IR) and non-alcoholic steatohepatitis (NASH). However whether BA alterations contribute to NASH independently of the metabolic status is unclear.
Objective: To assess BA alterations associated with NASH independently of BMI and IR.
Design & **Setting:** Patients visiting the obesity clinic of the Antwerp University Hospital (a tertiary referral facility) were recruited from 2006 to 2014.
Patients: Obese patients with biopsy-proven NASH (n=32) and healthy livers (n=26) were matched on BMI and HOMA-IR.
Main Outcome Measures: Transcriptomic analyses were performed on liver biopsies. Plasma concentrations of 21 BA species and 7alpha-hydroxy-4-cholesten-3-one, a marker of BA synthesis, were determined by liquid chromatography–tandem mass spectrometry. Plasma fibroblast growth factor 19 (FGF19) was measured by ELISA.
Results: Plasma BA concentrations did not correlate with any hepatic lesions, whereas, as previously reported, primary BA strongly correlated with IR. Transcriptomic analyses showed unaltered hepatic BA metabolism in NASH patients. In line, plasma 7alpha-hydroxy-4-cholesten-3-one was unchanged in NASH. Moreover, no sign of hepatic BA accumulation or activation of BA receptors – farnesoid X (FXR), pregnane X and vitamin D (VDR) receptors – was found. Finally, plasma FGF19, secondary-to-primary BA and free-to-conjugated BA ratios were similar, suggesting unaltered intestinal BA metabolism and signalling.
Conclusions: In obese patients, BA alterations are related to the metabolic phenotype associated with NASH, especially IR, but not liver necro-inflammation.

Transcriptomic & metabolomic analyses of bile acid metabolism/signaling reveals no alteration in NASH patients compared to BMI- and insulin resistance-matched controls, unlike in insulin resistance.

Introduction

Bile acids (BA) are amphipathic molecules that facilitate absorption of dietary fat and lipophilic vitamins in the small intestine. However, due to their detergent properties, BA also are potentially harmful when accumulating, as seen in cholestatic liver diseases. BA overload induces hepatotoxicity by activation of inflammatory, oxidative stress and necrotic cell death pathways (1,2). Therefore, BA pool size and metabolism are under tight negative retro-control *via* activation of nuclear receptors, such as the farnesoid X (FXR), pregnane X (PXR) and vitamin D (VDR) receptors. BA also activate cell surface receptors, such as the G-protein coupled BA receptor-1 (GPBAR1/TGR5) and sphingosine-1-phosphate receptor (S1PR2) (3).

Chenodeoxycholic (CDCA) and cholic (CA) acids are the major primary BA synthesized from cholesterol in the liver (4). Before secretion into bile, BA are conjugated (mainly to glycine, less to taurine in humans), decreasing their hydrophobicity. Primary BA are converted in the intestine into more hydrophobic secondary BA, deoxycholic acid (DCA) and small amounts of lithocholic acid (LCA), hyodeoxycholic acid (HDCA) and ursodeoxycholic acid (UDCA). Most BA are reabsorbed in the distal intestine and transported back to the liver *via* the portal circulation. A small proportion of BA escapes this enterohepatic cycle and reaches peripheral organs *via* the systemic circulation.

BA also act as signalling molecules controlling glucose, lipid and energy homeostasis, notably *via* activating FXR and TGR5 (3). In humans, interruption of enterohepatic BA circulation using BA sequestrants improves both lipid and glucose metabolism through mechanisms involving increased L-cell glucagon-like peptide-1 (GLP-1) production and enhancement of splanchnic glucose utilisation (5,6).

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease and strongly associates with obesity and insulin resistance (IR). NAFLD encompasses a spectrum ranging from isolated steatosis to non-alcoholic steatohepatitis (NASH), characterized by steatosis, necro-inflammatory changes (ballooned hepatocytes and lobular inflammation) and varying degrees of liver fibrosis (7). IR is a common feature in individuals with NAFLD and reciprocally, >70% of type 2 diabetic (T2D) patients have fatty liver and rapidly progress to NASH (8). NASH pathogenesis is still unclear and appears multi-factorial, resulting from several deleterious events occurring in parallel and involving the interaction of multiple organs, with the gut-liver axis playing a crucial role (9). There is also a genetic basis in NASH and interestingly, the phospholipase domain-containing protein 3 (PNPLA3) I148M polymorphism associates with the severity of necro-inflammatory changes independently of metabolic factors (10).

Obesity, T2D and NAFLD are associated with dysbiosis (11). The intestinal microbiota modulates the development of metabolic diseases in part through BA, since microbiota deconjugate and convert primary into secondary BA. Whereas it is unclear whether systemic total BA concentrations are altered in obesity (12,13), they are elevated in T2D patients (3,14). Moreover, the peripheral blood BA pool composition is altered in IR due to impaired insulin-mediated 12alpha-hydroxylase (CYP8B1) downregulation resulting in increased 12alpha-hydroxylated BA (CA, DCA and their conjugated forms) (15). Bariatric surgery, especially Roux-en-Y gastric bypass, which not only reduces body weight but also reverses T2D and NAFLD, increases peripheral blood BA levels and BA changes might be involved in NAFLD reversion after bariatric surgery (3,16).

Yet, whether alterations in BA metabolism play a role in the pathogenesis of NASH is unclear. Recent publications reported increased hepatic BA concentrations accompanied with altered synthesis in NASH patients (17,18) suggesting the existence of a mild cholestatic injury in NASH. However, liver tissue BA composition and concentration analysis does not allow discrimination of intracellular, ductular, or blood origins. Changes in BA profile in plasma and faeces were also described, notably increased total and primary BA in NASH patients (19–23). However, these studies compared NASH patients to controls with lower body weight, fasting plasma glucose (FPG) (19) and/or IR (20–23). Since body mass index (BMI) and the insulin sensitivity status of the patients were not accounted for in these studies, it is unclear whether NASH and its histological components *per se* are associated with alterations in BA metabolism.

Therefore, we investigated BA metabolism in a cohort of drug-naive obese patients extensively phenotyped for metabolic parameters and biopsy-proven NASH. Plasma BA profiling coupled with transcriptomic analysis of BA metabolism and signalling in liver

biopsies were compared between NASH patients and BMI- and IR -matched control (no-NASH) patients.

Materials and Methods

Description of the patients

Overweight (25≥BMI<30 kg/m²) or obese (BMI≥30 kg/m²) patients visiting the obesity clinic of the Antwerp University Hospital (a tertiary referral facility) were recruited from October 2006 to May 2014 (24). Patients were prospectively screened for the presence of NAFLD and when NAFLD was suspected from abnormal blood biochemistry assays (alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma-glutamyl transpeptidase (GGT)) or ultrasound features, a liver biopsy was subsequently proposed. Exclusion criteria were alcohol consumption, previous bariatric surgery, liver diseases other than NAFLD, diagnosed T2D (as diabetic patients constitute another specific risk group for NAFLD and as long term diabetes and its treatments potentially influence the presence and severity of NAFLD, this was considered a potential confounder and therefore excluded; patients who were *de novo* diagnosed with T2D as a result of their work-up were, however, included in the study), anti-diabetic, lipid-lowering or antibiotic treatments. The study protocol is part of the Hepadip protocol (Belgian registration number B30020071389), it was approved by the Ethical Committee of the Antwerp University Hospital (file 6/25/125) and required written informed consent of the patient.

Clinical assessment and biological measurements

Fasting blood was collected in the morning. Plasma glucose, insulin, triglyceride, total, high-density lipoprotein (HDL-C) and low-density lipoprotein (LDL-C) cholesterol concentrations and liver enzymes (ALAT, ASAT, GGT, alkaline phosphatase (ALP)) were measured as described (10). IR was estimated using homeostasis model assessment (HOMA-IR) calculated as [insulin (mU/l) x glucose (mmol/l)]/22.5.

Histological assessment of the liver biopsies

Haematoxylin-eosin, Sirius red, reticulin stain and Perls' iron stains were routinely performed on all liver biopsies and analysed by two experienced pathologists blinded to clinical data. The histological features of NAFLD (steatosis, ballooning, lobular inflammation and fibrosis) were assessed using the NASH Clinical Research Network Scoring System (25). NASH diagnosis was defined according to recent guidelines requiring the combined presence of steatosis, ballooning and lobular inflammation (26,27). The NAFLD Activity Score (NAS) was also calculated. In line with recent insights on the differential role of steatosis and activity of disease, an Activity Index (AI) was also calculated as the sum of ballooning (range 0-2) and lobular inflammation (range 0-3), hence ranging from 0-5 (25,28).

Selection and matching of patients for BA metabolism analysis

Among the whole cohort, 152 patients had liver biopsies and a complete clinical and biological dataset available for further analyses. For the present study, patients with cholecystectomy or cholelithiasis were excluded. Furthermore, to dissect influences of NASH and metabolic features, 2 clearly distinct histological phenotypes were selected for comparison and matched on BMI and HOMA-IR. Selection of the NASH patients was hence based on the unequivocal presence of NASH. For the no-NASH group, patients with normal liver or minor lesions largely insufficient for the diagnosis of NASH, and absence of fibrosis were selected. Cases with borderline lesions (n=4) were not included in the current study. Then, NASH patients were matched with no-NASH patients on quartiles of BMI (Q1<35.6, Q2<39.5, Q3<43.2, Q4≥43.2 kg/m²) and HOMA-IR (Q1<2.38, Q2<3.49, Q3<4.89, Q4≥4.89). Patients that could not be matched were excluded. This resulted in 32 NASH and 26 no-NASH patients, representative of the whole cohort (Sup.Table-1).

Transcriptomic analysis of liver biopsies

Total RNA was prepared and transcriptomic analysis with Affymetrix GeneChip arrays (HuGene 2.0 ST) performed as described (24,29). Signals were normalized by the Robust Multi-array Average (RMA) method, and the baseline was adjusted to the median of all samples. Proprietary.CEL files were imported into Partek Genomics Suite 6.6 for analyses. Using the linear models for microarray data, 31,135 transcripts were tested for differential expression between NASH (n=32) and no-NASH (n=26) patients. Pathway analysis, including KEGG pathway and gene ontology (GO) term enrichment, was performed on transcripts coding for proteins that exhibit a significant >20% differential expression level between NASH and no-NASH patients.

Subsequently, all genes involved in BA metabolism were further analysed based on the KEGG BA synthesis and bile secretion pathways (www.kegg.jp) and literature (4,18,30) resulting in 87 genes. Heatmap was generated using the heatmap.2 function in the gplots package (R project) including these 87 genes. Hierarchical clustering was performed to group patients by Euclidean distance. Clustering was not performed on genes.

Plasma measurements of BA, C4 and FGF19

All measurements were performed on fasting EDTA-plasma collected in the morning. Twenty-one BA species were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (31). BA concentrations are presented in Sup.Table-2, except for UDCA and its conjugated forms that were only detected as trace amounts in these samples.

Plasma 7alpha-hydroxy-4-cholesten-3-one (C4) was determined as described (32). Briefly, 100µl plasma sample was mixed with 500µl purified water, 50µl of the 0.06µM D7-C4 internal standard solution and 60µl of 1M hydrochloric acid. Solid-phase extraction was performed using cartridges SPE (Bound Elut C18 200 mg) pre-conditioned with 2ml methanol followed by 2ml purified water, upon which the sample was loaded and allowed to pass through it by gravity. The cartridge was washed, the analyte was eluted with MeOH and the eluted solution, evaporated. The residue was dissolved in 80µl methanol and mixed with 10µl ammonium acetate buffer 10mM, pH 6.5, upon which the sample was centrifuged at 13400×g and 10°C for 10min. The supernatant was evaporated. The pellet was then dissolved in 100µl of MeOH/water (v/v) and 2µl were injected into the LC-MS/MS system (31). The separation of C4 was carried out on a Kinetex Coreshell C18 column (100x2.1mm, 5µm) from Phenomenex. The oven temperature was 30°C. Solvent A was water containing formic acid 0,1% and solvent B was acetonitrile. Solvents were delivered at a total flow rate of 500µL/min. After a 2min plateau with 75% B, the gradient profile was from 75% B to 95% B linearly in 5min, followed by a 2min plateau with 95% B. Column was re-equilibrated for 3min. The injection cycle was 12min. MS analysis was carried out in positive ionization mode. The ion source parameters were set as follows: ion spray voltage, 5500V; nebulizer gas (air) and curtain gas (nitrogen) flows, 50 and 20 psi, respectively; source temperature, 650°C with auxiliary gas flow (air), 50 psi; declustering potential, 100V; collision cell exit potential, 15V; collision energy, 31V. The mass spectrometer was operated at a unit resolution for both Q1 and Q3 with a dwell time of 100ms in each transition. The methods allow the quantification of C4 within the range of 1–200nM, with inter- and intra-day precisions <15%. All the analytical methods were validated using the SFSTP guidelines.

Plasma FGF19 concentrations were measured using Human FGF19 Quantikine ELISA Kit DF1900 (R&D Systems).

Statistical analyses

Statistical analyses were performed using the SAS statistical software, version 9.4 (SAS Institute Inc., Cary, NC, USA). Inter-group comparisons of quantitative variables were

performed using the general linear model. To obtain normal data distributions, log-transformation of some variables was applied in all samples, as indicated in table legends. For plasma BA, C4 and FGF19 analyses, non-parametric tests were performed. P-values for the comparison of distribution (gender, histological grades) between subject groups were assessed using the Chi^2 test or Fisher's exact test, as indicated in table legends. Multivariable analyses were performed to compare hepatic gene expression between groups, adjusted on confounding factors, including gender and the PNPLA3 I148M (rs738409) genotype (10). Statistical significance was considered when $p<0.05$.

Results

Characteristics of the studied patients

To analyse BA alterations specifically associated with NASH, independent of obesity or IR, patients with well-established NASH and patients with no NASH were matched on BMI and HOMA-IR, resulting in 32 "NASH" and 26 "no-NASH" patients (Table-1, Sup.Table-1). The no-NASH patients had a liver with little or no steatosis and hepatic injury (Fig.1). By contrast, besides steatosis, NASH patients presented clear signs of disease activity with inflammatory infiltrates (mostly grades 1 and 2) and prominent ballooning (grade 2 in 50% of patients). Approximately 50% of NASH patients presented some degree of fibrosis up to F3 (Sup.Table-1).

Overall, all 58 subjects were severely obese (BMI=39.8±5.8kg/m²) and presented several features of the metabolic syndrome, *ie* mild hypertriglyceridemia (157±73mg/dl), and relatively low plasma HDL-C levels (47±13mg/dl). FPG (84±10mg/dl) was in the normal range, whereas 2 hours post-oral glucose levels were slightly elevated (145±35mg/dl), indicative of impaired glucose homeostasis. As could be expected because of the matching on BMI and HOMA-IR, NASH and no-NASH patients displayed no significant difference in terms of metabolic parameters, except a lower plasma HDL-C level in NASH patients (Table-1).

Systemic BA profile is unchanged in NASH patients

First, correlations between each hepatic histological feature and each plasma BA species concentration were analysed in the 58 studied patients. No correlation, except glyco-CA (G-CA) with steatosis ($r=0.29$, $p=0.03$) was found (Fig.2A). Further, comparison of plasma BA species concentrations between NASH and no-NASH patients did not show any significant difference (Fig.2B). Total, primary, secondary, free or conjugated BA were unchanged with NASH (Sup.Table-2). BA profile composition was also similar between NASH and no-NASH patients (Fig.2C). In multivariable analyses, no significant interactions between NASH and HOMA-IR, BMI, gender or PNPLA3 I148M polymorphism was found on plasma BA concentrations (not shown), indicating that these parameters did not mask associations between NASH and BA alterations. Therefore, when matched for BMI and IR, peripheral plasma BA are not affected by NASH.

Hepatic gene expression analysis reveals limited BA metabolism and signalling changes in NASH

Since previous reports described altered hepatic expression of genes involved in BA synthesis and transport in NASH patients (18,19), gene expression profiles were compared by microarray analyses performed on liver biopsies of all NASH and no-NASH patients. Differential expression analysis revealed 713 transcripts dysregulated in NASH. Pathway enrichment analysis showed clear activation of inflammatory response in NASH patients (Sup.Fig.1). However, no BA pathways (defined by KEGG or GO-TERM) were enriched in NASH.

To analyse in more detail whether changes in hepatic BA metabolism genes may exist, a literature search of all genes involved in BA metabolism (synthesis, transport,

conjugation/detoxification, bile secretion, regulation of BA metabolism and BA-activated signalling pathways) resulted in the identification of 87 genes (Fig.3). Unsupervised hierarchical clustering showed that NASH status was not strongly associated with changes in expression of these genes (Fig.3A). Only few BA metabolism genes were significantly dysregulated in NASH (Fig.3B): among them, as reported (24) *PPARA* was lower, along with *CYP3A4* and *ABCB11* (also known as BSEP), whereas *CYP7A1* and *ATP8B1* were higher in NASH patients. Most of these changes were observed when stratifying the patients on steatosis grade (Fig.3C), but not when stratified on activity index (AI), except for *PPARA* (Fig.3D).

Since the rate-limiting enzyme of BA synthesis, 7alpha-hydroxylase (CYP7A1), mRNA levels were increased, we measured plasma 7alpha-hydroxy-4-cholesten-3-one (C4) concentrations, a surrogate marker of hepatic BA synthesis (33). C4 concentrations did not differ between NASH and no-NASH patients (Fig.3E), indicating that the increased *CYP7A1* gene expression did not translate into increased BA synthesis.

As it has been suggested that hydrophobic BA (CDCA, DCA) could accumulate in the liver of NASH patients and participate in liver injury (17,34,35), indirect markers of hepatic BA accumulation and injury were examined. Liver enzymes and metabolites known to increase with cholestatic injury were not elevated in NASH patients (Table-1). Moreover, upon intra-hepatic BA overload, the BA receptors FXR, PXR and VDR initiate protective mechanisms to down-regulate BA uptake and synthesis, and up-regulate BA detoxification and systemic efflux (4). Here, hepatic expression of well-known target genes of FXR, PXR and VDR revealed increased *CYP7A1* in NASH (Sup.Fig.2A-C) while its expression is typically repressed by these BA receptors (4). Also, *CYP3A4* which is induced upon PXR or VDR activation, and the FXR target gene *ABCB11* (BSEP) (4) were down-regulated in NASH *versus* no-NASH patients (Sup.Fig.2A-C). Expression of the membrane BA receptor *GPBAR1* (TGR5), which mediates anti-inflammatory responses upon BA activation in Kupffer cells (36), was also unchanged (not shown). Taken together, these results indicate the absence of major changes in hepatic BA metabolism and intra-hepatic BA accumulation in NASH patients compared to BMI- and HOMA-IR- matched controls.

Intestinal BA metabolism is unchanged in NASH patients

Since alterations in fecal BA content associated with dysbiosis were reported in NAFLD patients (23), we also investigated parameters of intestinal BA metabolism and signalling. Since neither intestinal gene expression nor BA profiles could be measured, plasma levels of FGF19, a marker of intestinal FXR activation (37), were measured. No difference was found between patients with and without NASH (62.0±53.5 *vs*. 54.7±41.1 pg/ml, p=0.37). Moreover, the ratios of free/conjugated-BA (1.11±0.96 *vs*. 1.27±1.14, p=0.56) and secondary/primary-BA (0.80±0.48 *vs*. 0.80±0.78, p=0.99), markers of microbiota deconjugation and conversion activities, respectively, were also unchanged in NASH patients.

BA alterations are associated with glucose homeostasis independently of NASH

Considering the lack of association between BA alterations and NASH, we finally assessed whether differences in metabolic status correlate with BA metabolism considering the whole (NASH and no-NASH) studied population. Therefore, metabolic parameters were correlated with each plasma BA species concentration (Fig.4A). BMI positively correlated with plasma CA (r=0.36, p=0.006) and CDCA (r=0.31, p=0.02). CA and CDCA also strongly correlated with fasting plasma insulin (FPI) (CA: r=0.42, p=0.0009; CDCA: r=0.39, p=0.003) and HOMA-IR (CA: r=0.45, p=0.0004; CDCA: r=0.42, p=0.001). In addition, plasma HCA correlated with FPG (r=0.39, p=0.003). In accordance with Haeusler *et al.* (15), 12alpha-

hydroxylated BA (CA, DCA and their conjugated forms) tended to correlate with FPI (r=0.14, p=0.08).

Given the strong correlations between parameters of glucose homeostasis and plasma BA, the 58 subjects were stratified according to IR and highest *versus* lowest quartile of HOMA-IR were compared (for clinical and liver histology parameters, see Sup.Table-3). Unlike in NASH, several changes in systemic BA profile occurred with IR, with notably increased primary (free) BA concentrations (Fig.4B-C). Also, the total plasma BA concentration tended to increase in IR patients, which was not the case in NASH patients (Sup.Table-2). Overall, these results show that BA metabolism is correlated with metabolic conditions, especially IR, but not with hepatic lesions.

Discussion

BA metabolism is altered in metabolic disorders associated with NAFLD (3). Since BA overload causes hepatotoxicity in some chronic liver diseases (cholestasis), it is tempting to speculate that BA alterations could participate in NASH pathogenesis, both through their impact on metabolic control and their physico-chemical properties potentially causing liver injury. We therefore designed a study to assess BA changes in NASH, independently of confounding metabolic parameters. To our knowledge, this is the first study comparing BA metabolism and associated signalling pathways between patients with and without NASH matched on BMI and IR levels. Another advantage of this cohort is the absence of anti-diabetic or lipid-lowering medications, which can interfere with BA metabolism (38). Our results revealed that neither circulating BA species nor hepatic or intestinal BA metabolism/signalling was altered in NASH. The absence of BA alterations in relation to NASH suggests that alterations in BA metabolism do not participate in NAFL progression to NASH.

Increased plasma total BA has been reported in NASH patients (19,21) as well as in IR or T2D patients (14,39). Notably, plasma primary BA increased in NASH patients (20–22) as well as in T2D patients (15,39). These BA changes could be attributed to increased hepatic BA synthesis in NASH (23) and T2D (40) patients. Importantly, in all these studies (19–23), NASH patients had higher IR than controls, suggesting that these BA changes could be confounded by the metabolic status. In the present study, although glucose homeostasis was merely assessed by HOMA-IR, we also found that plasma primary BA increase with IR (but not with NASH). Given the number of patients and variability of measurements, our study was at least as statistically powerful as the previous reports. Therefore, our study design, comparing NASH patients to BMI- and HOMA-IR- matched controls, suggests that previous results showing increased primary and total BA are due to IR and not NASH (19–23). Our results also indicate that hepatic BA synthesis is not increased in NASH, which is in accordance with the study of Min *et al.* showing cholesterol accumulation in NASH livers without upregulation of its catabolism (41).

In our study, we did not find any sign of cholestatic injury or activation of BA receptors. In line, UDCA, which is widely used for the treatment of cholestatic liver diseases, and its tauro-conjugated form failed to show beneficial effects on NASH (42,43). This suggests that the mechanisms leading to liver injury and inflammation are different between cholestatic liver diseases and NASH. UDCA, which does not activate FXR, is a hydrophilic BA protecting cellular membranes and preventing cytolysis, endoplasmic reticulum stress and apoptosis. UDCA also decreases BA uptake and synthesis, stimulates basolateral export pumps and improves renal excretion (44). Taken together, this suggests that cholestatic injury is not involved in NASH pathogenesis and that the UDCA's mechanisms of protection are inefficacious in NASH.

BA sequestrants are used to treat cholestatic pruritus as well as hypercholesterolemia and T2D. They prevent intestinal BA absorption, interrupt the enterohepatic cycle, and result in enhanced hepatic cholesterol conversion to BA to maintain the BA pool. Colesevelam treatment decreases LDL-C and reduces FPG and haemoglobin-A1c in T2D patients (5). However, colesevelam did not improve NASH, even slightly worsened steatosis (45), again suggesting that interfering with BA metabolism *per se* is not an efficient therapeutic option for NASH.

Interestingly, the FXR agonist obeticholic acid (OCA) is currently in phase 3 clinical development in NASH patients. Phase 2b trial results showed that OCA improves all hepatic histological features including fibrosis in NASH patients (46). OCA reduces hepatic inflammation and fibrosis *via* inhibition of Kupffer cell and hepatic stellate cell activation in experimental models (47). Intriguingly, OCA exerts beneficial hepato-protective effects despite unfavourable metabolic effects, *ie* worsening plasma lipid profile (increased LDL-C, decreased HDL-C) and IR (46). In the liver, FXR is found in 2 distinct genomic transcriptional regulatory modules, one involved in cellular maintenance and hepato-protection, and the other in liver-specific metabolic functions, especially glucose, lipid and BA metabolism (48). Therefore, it is tempting to speculate that the histological improvement of fibrosing-NASH by FXR activation is mediated through the former module of metabolism-independent effects rather than through changes in BA metabolism. Finally, recent studies showed that intestinal FXR activation promotes NAFLD and that its antagonism improves metabolic control and NAFLD in mouse models of obesity (49,50). However, intestinal FXR activation by OCA also prevents gut barrier dysfunction and attenuates intestinal inflammation in mouse models of cholestasis and cirrhosis (51,52). Therefore, whether intestinal FXR mediates OCA's beneficial effects in NASH remains to be determined.

To conclude, our study shows that in obese patients, the hepatic necro-inflammatory lesions observed in NASH are not associated with alterations in BA metabolism and signalling. BA alterations rather reflect the metabolic phenotype associated with NASH.

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Fig.1: Histological characteristics of the studied patients. Pie charts showing steatosis and activity index (AI=ballooning+inflammation) ranges in NASH (n=32) and no-NASH (n=26) patients.

Fig.2: Plasma BA profiles are not associated with NASH. A: Spearman correlations between plasma BA and hepatic histological features in the 58 patients. Colours reflect the Spearman rho values (yellow for positive, blue for inverse correlations) and dot sizes reflect the p-values. **B**: Comparison of each plasma BA species between NASH (n=32) and no-NASH (n=26) patients. All p-values are below the significance level of 0.05. **C**: Comparison of plasma BA pool composition (expressed as % of the total BA pool) between NASH (n=32) and no-NASH (n=26) patients. Only BA species >3% of the pool are depicted.

Fig.3: Expression of hepatic genes involved in BA metabolism is not altered in NASH patients A: Heatmap showing hepatic expression of 87 genes involved in BA metabolism in NASH (black, n=32) and no-NASH (grey, n=26) patients. Colours reflect the Z-score for each gene: yellow and blue correspond to increased and decreased gene expression, respectively. Black colour indicates the median expression. **B-D**: Volcano plots showing the most dysregulated genes involved in BA metabolism between NASH (n=32) and no-NASH (n=26) patients (**B**), between patients with (grade >1, n=18) and without (grade ≤ 1, n=40) steatosis (**C**), between patients with (AI ≥ 2, n=33) and without (AI < 2, n=25) hepatic necro-inflammation (**D**). Red colour indicates the significantly dysregulated genes with an absolute fold-change ≥ 1.2. **E**: Plasma C4 concentrations as a surrogate marker of hepatic 7alpha-hydroxylase activity in NASH (n=32) and no-NASH (n=26) patients.

Fig.4: Plasma BA profile alterations are associated with metabolic parameters A: Spearman correlations between plasma BA and metabolic parameters in the 58 patients. Colours reflect the Spearman rho values (yellow for positive, blue for inverse correlations) and dot sizes reflect the p-values. **B**: Plasma BA differences between IR (highest quartile of HOMA-IR, n=15) and insulin sensitive (lowest quartile of HOMA-IR, n=15) (IR, open dots), and between NASH (n=32) and no-NASH (n=26) patients (black dots), with p-values plotted against estimation of effect size (Cohen's d = ($mean_{IR\ or\ NASH}$ − $mean_{IS\ or\ no\text{-}NASH}$) / SD_{pooled}). **C**: Comparison of plasma BA pool composition (expressed as % of the total BA pool) species between insulin resistant (IR) and insulin sensitive (IS) patients. Only BA species >3% of the pool are depicted.

Table-1: Clinical, biological and histological characteristics of the studied subjects with and without NASH

	No-NASH	NASH	p value
	(n=26)	(n=32)	
Age (years)	40.5 ± 11.7	41.3 ± 11.9	0.80
Gender (n ♂/♀)*	3/23	13/19	0.01
BMI (kg/m²)	39.4 ± 5.9	40.2 ± 5.8	0.60
Plasma glucose (mg/dl)†	82.3 ± 11.8	85.8 ± 8.4	0.13
Plasma insulin (µU/ml)†	15.7 ± 9.1	18.9 ± 11.4	0.16
HOMA-IR†	3.25 ± 2.05	4.05 ± 2.65	0.11
Plasma TG (mg/dl)†	153 ± 68	160 ± 78	0.70
Plasma cholesterol (mg/dl)	212 ± 58	197 ± 35	0.22
Plasma HDL-C (mg/dl)	51.8 ± 14.9	43.6 ± 9.1	0.01
Plasma LDL-C (mg/dl)	130 ± 50	121 ± 31	0.44
ALAT (U/l)†	27.3 ± 15.8	36.5 ± 22.7	0.08
ASAT (U/l)†	21.3 ± 11.9	24.6 ± 17.5	0.32
GGT (U/l)†	40.6 ± 22.0	40.6 ± 26.4	0.86
ALP (U/l)	80.0 ± 23.5	74.9 ± 17.3	0.35
Total bilirubin (mg/dl)	0.49 ± 0.20	0.56 ± 0.17	0.19
NAS ‡	1 [0-2]	5 [3-8]	<0.0001

Data are means ± SD, except for NAS: median [min-max].
*p value from Fisher's exact test
†p values on log-transformed data
‡p value from non-parametric Mann-Whitney's test

Fig.1

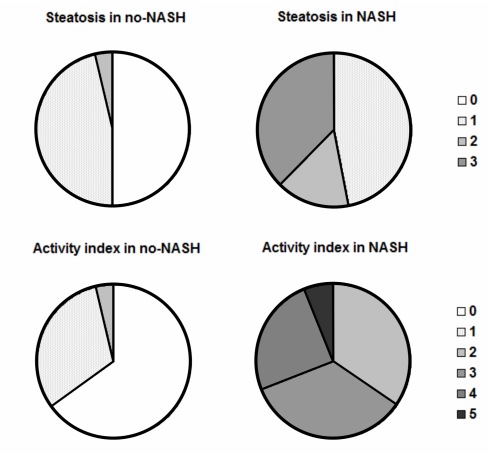

Fig.2

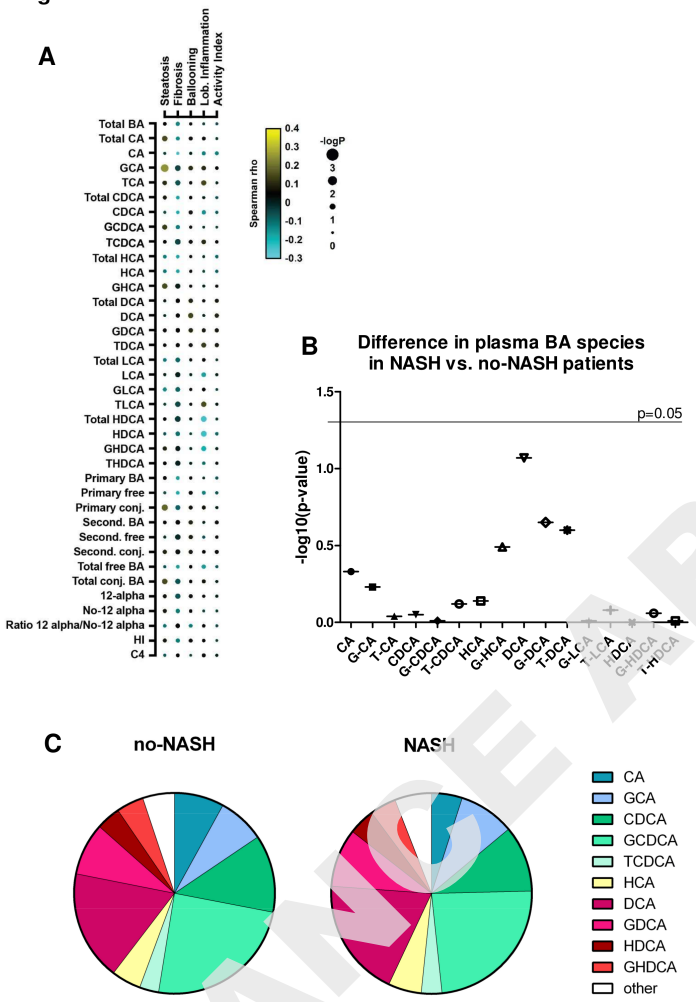

Fig.3

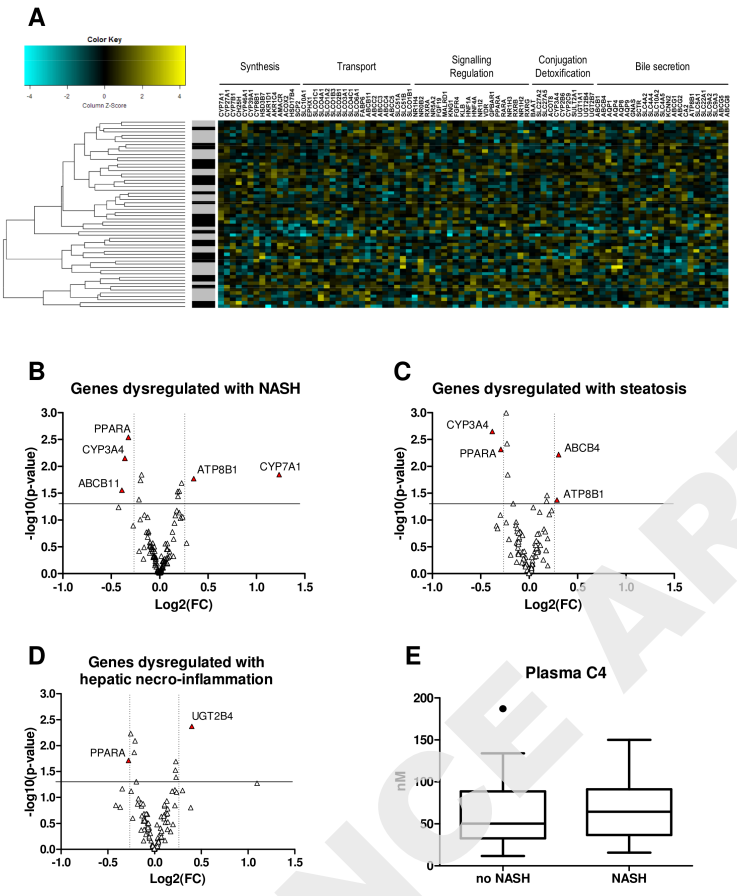

Fig.4