HEPATIC FARNESOID X RECEPTOR PROTEIN LEVEL AND CIRCULATING FIBROBLAST GROWTH FACTOR 19 CONCENTRATION IN CHILDREN WITH NAFLD

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List of Abbreviations:

ALT, alanine-aminotransferases**; AST,** aspartate-aminotransferase**; BA,** bile acid**; BMI,** body mass index **; FGF19,** fibroblast growth factor 19 **; ; FXR,** farnesoid X receptor **; GGT,** gamma-glutamyl-transpeptidase**; HDL,** high-density lipoprotein

HOMA-IR, homeostatic model assessment insulin resistance; **LDL,** low-density lipoprotein; **NAFLD**, non-alcoholic fatty liver disease; **NASH**, non-alcoholic steatohepatitis; **PNHS**, Paediatric NAFLD Histologic Score; **TGs**, triglycerides

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Short Title: Liver FXR in NAFLD children

Abstract

Background & Aims. Treatment with the farnesoid X receptor (FXR) agonist obeticholic acid is ineffective in some patients with non-alcoholic steatohepatitis (NASH) but the explanation is uncertain. We investigated hepatic FXR expression, and measurements of fibroblast growth factor 19 (FGF19) and bile acids (BAs) in children with NAFLD to investigate relationships with NASH.

Methods. 33 children with NAFLD who underwent diagnostic liver biopsy were studied. Hepatic FXR protein levels and circulating FGF19 concentrations were compared with those analyzed in five control subjects with proven normal liver histology. NASH was defined by the Paediatric NAFLD Histologic Score (PNHS). Binary logistic regression with adjustment for covariates and potential confounders was undertaken to test factors independently associated with: a) NASH and b) hepatic FXR protein levels.

Results. Mean ± SD age was 13.7±1.9 years. Nineteen patients had NASH (PNHS≥85) and 14 did not have NASH (PNHS<85). Hepatic FXR level and plasma FGF19 concentration varied ~10 fold and 5 fold respectively between groups, and was highest in control subjects, intermediate in NAFLD without NASH, and lowest in NASH (between group differences p<0.001 and p<0.01, respectively). NASH was independently associated with both FXR protein levels (OR=0.18, 95% CI 0.09,0.38) and FGF19 concentration (OR= 0.55, 95% CI 0.20,0.89).

Conclusions. FXR protein levels vary markedly between normal liver, NAFLD without NASH, and NASH. Low levels of FXR are independently associated with NASH.

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Key points box

- In children with NASH, hepatic FXR protein content and plasma FGF19 concentrations were decreased compared to levels in children with NAFLD but who did not have NASH.
- Hepatic FXR protein level is positively correlated with serum FGF19 concentrations.
- Serum FGF19 levels are inversely associated with NASH.
- Low levels of FXR are independently associated with NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is rapidly becoming the most common cause of chronic pediatric liver disease in Industrialized Countries, consistent with a marked increase in childhood obesity. Recent data show a prevalence of pediatric NAFLD of 3-10%, with a 2:1 male: female ratio, and the prevalence of obesity is up to 70% in children [1]. According to Bellentani et al. [2], the prevalence of NAFLD in obese children living in Western countries, such as Italy, is estimated to be between 40 and 70%. NAFLD represents a spectrum of liver fat-associated disease, ranging from 'simple hepatic steatosis' (abnormal accumulation of fat in more than 5% of the hepatocytes, without evidence of hepatocellular injury or fibrosis) to 'non-alcoholic steatohepatitis' (NASH), which is characterized by the coexistence of hepatic steatosis, inflammation and eventually fibrosis [3].

The precise pathogenesis of NAFLD is uncertain. Recently, abnormal regulation of bile acids (BAs) homeostasis has emerged as an important mechanism of liver injury in NAFLD [4]. Recently, our group demonstrated that serum total BAs levels were decreased in early NAFLD, and were increased with liver fibrosis, suggesting a possible continuous increase in BAs as liver injury advances [5]. The metabolic effects of BAs are mediated by activation of nuclear receptors, such as the farnesoid X receptor (FXR) and to protect against BA-induced cellular toxicity, BAs binds to FXR in enterocytes inducing gene expression of fibroblast growth factor 19 (FGF19) [6]. Secreted FGF19 further inhibits the hepatic synthesis of primary BAs after reaching the liver via the portal circulation [7]. Animal data demonstrate an important role of FXR regulating lipid metabolism, inflammation and liver fibrosis, all of which are important characteristics of NASH. For example, studies with FXR-null mice show increased plasma triglycerides (TGs) and cholesterol levels [8], insulin-resistance and elevated hepatic glucose production [9]. Studies in db/db mice show that treatment with a FXR agonist decreases plasma levels of glucose, fatty acids and triglycerides [9]

and data from a mouse diet-induced model of NAFLD display markedly reduced inflammatory cell infiltration and hepatic fibrosis after treatment with a synthetic FXR agonist [10].

Since FXR activity is a key regulator of various pathways that are affected in NASH [11], and because data from the FLINT trial showed that treatment with the FXR agonist obeticholic acid was effective in only approximately half of treated patients [12], we reasoned that some patients in the FLINT trial may not have responded to treatment because levels of FXR expression were too low for those patients to have responded to treatment with an FXR agonist. To investigate relationships between FXR expression and liver disease in NAFLD, we have assessed levels of circulating BAs, hepatic FXR protein content and circulating FGF19 concentrations, in patients with histologically proven NAFLD. We have also compared hepatic FXR protein content and circulating FGF19 concentrations between patients with NAFLD and control subjects with histologically proven normal liver tissue. We hypothesised that in children with proven NAFLD, levels of circulating BAs, hepatic FXR protein content and circulating FGF19 levels would differ according to the severity of liver disease.

Patients and Methods

In this proof of concept study, 33 obese children and adolescents (age range, 11 to 16.8 years) with biopsy-proven NAFLD were included. A diagnosis of NAFLD was established by excluding: use of drugs known to induce steatosis (e.g. valproate, amiodarone or prednisone); alcohol intake; presence of viral hepatitis markers (A, B, C, Cytomegalovirus and Epstein-Barr virus). Furthermore, patients with autoimmune or metabolic liver diseases, alpha-1-antitrypsin deficiency, Wilson's disease, celiac disease, genetic syndromes, diabetes or genetic dyslipidaemia were also excluded. The patients included in the study were selected from 158 consecutive obese patients with liver steatosis admitted at the Hepato-Metabolic Unit of Bambino Gesù Children's Hospital between October 2013 and January 2015. The inclusion criteria for the study were: a biopsy-proven

diagnosis of NAFLD, subjects were aged between 11 and 17, there was no specific dietary or other therapeutic treatment. 83 patients were excluded from the study because of: age<11 or >17 years, pubertal stage (Tanner stage < 1); presence of comorbidities (OSAS severe, hypertension); medication (such as metformin, ursodeoxycholic acid and steroids); adherence to a diet or to dietary supplements. Of the remaining 75 subjects, only 43 were eligible for liver biopsy as they had NAFLD diagnosis, characterized by the presence at least of 1 features of the metabolic syndrome, ultrasound imaging of the fatty liver, and eventually increased transaminase activity, according to ESPGHAN [13].

In four patients, other liver diseases besides NAFLD were confirmed. Of the remaining 39 patients, six refused to participate in the study.

Clinical and experimental data were acquired at diagnosis in all patients. The study protocol was approved by the Ethical Committee of Bambino Gesù Children's Hospital and written informed consent was obtained from the parents of the children (Prot. N=533/RA).

Anthropometrical and biochemical measurements

Weight, height, waist circumference and body mass index (BMI) were measured as previously reported [14]. CDC percentiles were used to define normal (<85th centile), overweight (>85th and <95th centile) and obese (>95th centile) subjects [14]. Tanner's tables were used to define pubertal stage [15,16]. In plasma, alanine (ALT) and aspartate aminotransferase (AST), gamma-glutamyl-transpeptidase (GGT), total triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) were evaluated using standard laboratory methods. Plasma fasting glucose and insulin were measured in all patients after an overnight 12-h fast. Insulin was measured by radio-immunoassay method (Myria Technogenetics, Milan, Italy). Insulin sensitivity was determined by the homeostatic model assessment insulin resistance (HOMA-IR) [17].

Liver biopsy

Echo-guided liver biopsy was performed using an automatic core biopsy device (Biopince, Amedic, Sweden) with an 18-G needle, under general anaesthesia [18]. Histological analysis was performed by a single pathologist blinded to clinical and laboratory data. The histological features of steatosis (0–3), lobular inflammation (0–3), and hepatocyte ballooning (0–2) were combined and the Pediatric NAFLD Histologic Score (PNHS) formula was applied:

PNHS= 100 x exp (z_{PNHS})/[(1+ exp (z_{PNHS})]; where z_{PNHS} = -8.4 + 2.5* steatosis + 3.5* ballooning + 3.4* lobular inflammation + 0.87* portal inflammation.

A PNHS ≥85 is considered an appropriate cut-off value for the diagnosis of NASH [19].

Bile acid analysis

For BA quantification, blood samples were collected from patients after 12 hours of fasting, with sera immediately separated and stored at -80 C until assays were undertaken [20]. Total BA (tBA) and individual BA levels, including unconjugated and taurine (T)- and glycine (G)- conjugated species, were measured using high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). A full 15-BA profile was determined using 10 µl of serum; it included cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic (DCA), lithocholic (LCA), and ursodeoxycholic (UDCA) acid and their taurine or T-conjugates TCDCA, TLCA, TDCA, TCA and TUDCA and glucuronide or G-conjugates GCA, GCDCA, GLCA, GDCA and GUDCA. Individual BAs were evaluated by HPLC using a reversed-phase C18 column with a methanol and water gradient. Deuterium-labeled internal standards served for quantification. Mass spectrometer Q Exactive™ MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) and high-performance quadrupole precursor selection with high-resolution and accurate-mass (HR/AM) Orbitrap™ detection were used for identification of individual BAs.

Immunofluorescence for FXR

Immunofluorescence to analyse FXR protein content in the liver was performed as previously described [21]. In brief, 2 µm-thick sections obtained from formalin-fixed tissue embedded in paraffin, were subjected to antigen retrieval with EDTA (pH 8). The primary 1:50 goat-polyclonal antibody against FXR (sc-1205), purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was added and the sections were incubated for 2 hours at room temperature. The secondary antibody was 1:800 mouse anti-goat Alexa Fluor® 488 (Life Technologies, Thermo Fisher Scientific Corporation, Foster City, CA, USA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min after extensive washing; sections were mounted with PBS/glycerol (1:1) and covered with a coverslip.

Confocal microscopy imaging was performed on an Olympus Fluoview FV1000 confocal microscope equipped with FV10-ASW version 2.0 software, using 20x objectives. Optical sections were acquired with a format of 1024x1024 pixels, and a sampling speed of 40µs/pixel, and 12 bits/pixel images. Fluorochrome un-mixing was performed by automated-sequential collection of multi-channel images to reduce spectral cross-talk between channels. For imaging analysis, the intensity average of FXR fluorescence (arbitrary units) was calculated from ten measurements of specific regions of interest in at least three randomly selected digital images, by using Image J software. FXR imaging analysis was undertaken by an independent observer who was unaware of the histological diagnosis of NAFLD (NASH vs. Not-NASH). FXR imaging was also performed on liver tissue from control subjects without liver disease. The control subjects were five lean (defined by BMI) children without diabetes (boys, 3; girls, 2; median age, 13 years; range, 12-16 years) who underwent laparotomy or a laparoscopic procedure (for cholecystectomy), or who had confirmed normal liver histology after liver biopsy.

FGF19 assay

Blood samples from patients, collected in the early morning after an overnight fasting, were centrifuged at 2000 g for 12 min and plasma was stored at -80°C pending further analysis. Plasma FGF19 concentrations were analyzed by enzyme-linked immunosorbent assay (ELISA) (Biovendor; Brno; Czech Republic) following the manufacturer's instructions as previously described [22]. The intra-assay CV was ≤ 0.5%.

Statistical Analysis

Data were analysed using STATISTICA software (version 2010, Chicago, IL, USA) unless otherwise stated. The distribution of data was checked for normality using the Kolmogorov-Smirnov test. Normally distributed data are described as means ± standard deviations (SDs) and non-normally distributed data are expressed as medians and IQRs. Differences between NASH and Not-NASH groups were tested by the Student's *t*-test for normally distributed data. Group differences between controls, NASH and Not-NASH groups, for FXR protein content and FGF19 concentrations, were tested using analysis of variance (ANOVA) with a Bonferroni post hoc correction.

Univariate associations between the FXR and FGF19 other anthropometric, laboratory and histological features were investigated with Pearson's correlation. Binary logistic regression was used to test the independence of associations between NASH/Not-NASH as the outcome and anthropometric and biochemical parameters including FXR protein and FGF19 content, as exposures and key potential confounders. A binary indicator variable was generated for NASH, PNHS ≥85 = 1) and Not-NASH, PNHS <85 = 0). Binary logistic regression was also used to test the independence of associations between FXR level (stratified by median FXR measurement into a high and a low group as the outcome, and key anthropometric and biochemical parameters including FGF19 concentration, as exposures.

Results

Characteristics of the study population

Thirty-three obese adolescents (age range 11-17 years) with biopsy-proven NAFLD were included in the study. Based on PNHS assessment, 57.6% (n=19) of patients had NASH (NASH, PNHS≥85) and 42.4% (n=14) did not have NASH (Not-NASH, PNHS<85).

Table 1 shows the anthropometric and biochemical characteristics and the differences between groups for subjects with and without NASH. Subjects belonging to the NASH group had a higher BMI, triglycerides and HOMA-IR than the Not-NASH group ($p<0.05$). Interestingly, the analysis of circulating BA levels showed that children with NASH had lower levels of GCDCA compared to children without NASH. **Table 2** shows the histological pattern stratified by PNHS-defined NASH status. As expected, the NASH group had higher levels of steatosis, portal inflammation and fibrosis than the Not-NASH group.

Assessment of hepatic FXR protein content and plasma levels of FGF19 in control subjects and subjects with NAFLD

Next, we performed the immunofluorescence analysis of FXR protein content in control subjects (CTRL), and in subjects with and without NASH. A representative pattern of staining for FXR hepatic protein content in the three groups is shown in **Supplementary Figure 1 A-D.** All data highlight that FXR protein content was higher in healthy livers than in tissue from Not-NASH and NASH patients (**Figure 1A**). Similarly, FGF19 concentrations were highest in the control group, intermediate in the Not-NASH group and lowest in the NASH group (**Figure 1B**). In **Figure 1C** is shown the heatmap representation of hepatic FXR protein content and FGF19 concentration in each patient.

Associations between NASH, FXR, and FGF19

To test the independence of associations between NASH and anthropometric and biochemical parameters (and specifically to test the independence of associations between NASH and hepatic FXR protein content and NASH and circulating FGF19 concentration), we undertook regression analysis with NASH/Not-NASH as the outcome and key exposure variables that were different in subjects with NASH compared with Not-NASH (e.g. triglycerides, HOMA-IR and BMI). First we included FXR, FGF19, triglyceride and HOMA-IR in the model (**Table 3**). In this model, FXR, FGF19 concentration, HOMA-IR and triglyceride concentration were each independently associated with NASH **(Table 3**). Next, we replaced triglyceride in the model with BMI and repeated the regression modelling (**Supplementary Table 1**). After replacing triglyceride with BMI in the model, the results were very similar to those shown in Table 3, and FXR and FGF-19 were both independently associated with NASH.

Next we investigated factors that were associated with FXR level and FGF19 concentration. **Table 4** shows univariate correlations between anthropometric and biochemical parameters and both hepatic FXR protein content and circulating FGF19 concentrations. We then tested what factors were independently associated with higher FXR levels as the outcome, after stratifying FXR data into two groups according to the median FXR level. The median value of FXR i.e. 50th centile for FXR= 3912720 average intensity/area). In a regression model that included FGF19, HOMA-IR, triglyceride and HDL cholesterol in the model (**Table 5**), FGF19, HOMA-IR and HDL cholesterol were all positively and independently associated with FXR level.

Discussion

Our novel data show that in children and adolescents with NAFLD, hepatic FXR protein content and plasma FGF19 concentrations were both lower in patients with NASH than in patients without NASH. In control subjects without liver disease, both FXR levels and FGF19 concentrations were markedly higher than in patients with NAFLD. Moreover, our results show that both FXR and FGF19 concentration were independently associated with NASH. Furthermore, we show that children with NASH have lower levels of the GCDCA BA, compared to children with NAFLD but without NASH. To the best of our knowledge, ours is the first study in NAFLD which has compared hepatic FXR protein levels and FGF19 concentrations in children and adolescents with and without NASH, and compared levels of FXR and FGF19 in children with and without liver disease.

In our study population of children with NAFLD, we have defined NASH patients by the PNHS [19]. Interestingly, the analysis of circulating BA levels highlighted that children with NASH also showed only decreased GCDCA compared to the Not-NASH group. GCDCA is one of the most toxic BA and the predominant human BA under cholestatic conditions [23,24]. It is plausible that, an increase in GCDCA may promote hepatocyte apoptosis by activating the Fas death receptor or by inducing mitochondrial oxidative damage [24,25]. In patients with NASH, that were the most insulin resistant group, we found a decrease of GCDCA that was associated with increased levels of insulin. In fact, high insulin concentrations may trigger repression of CYP7A1 transcription and consequent inhibition of de novo BA synthesis, leading to impairment of hepatic BA metabolism [26]. Several studies have demonstrated that intestinal BAs activate FXR inducing production and release of FGF19, which, in turn, may be transported to the liver to activate the FGFR4/Klotho- β receptor causing repression of CYP7A1, thus downregulating BA synthesis [27,28]. However, in general, the BA changes that we have observed in NAFLD were not large, and the role of BA levels, and specifically the role of both primary and secondary BAs in promoting more severe liver disease in NAFLD requires further study.

FXR protein content was highest in the livers of patients with normal liver histology, intermediate in the Not-NASH group and lowest in the group with NASH. These results are in line with previous animal and clinical studies that have demonstrated decreased expression of FXR [29,30]. Moreover, our findings suggest an association between levels of FXR and the severity of liver disease. In support of our data, Aguilar-Olivos et al. [31] have shown that protein levels of FXR were decreased in adult patients with NASH compared to those subjects with simple steatosis. We also observed that circulating levels of FGF19 were inversely associated with NASH. Moreover, here, we show that circulating FGF19 concentrations were positively associated with hepatic FXR protein content. We speculate that FGF19 may be an FXR-dependent intestinal signal that regulates BA levels. A feedback loop may exist whereby a BA/FXR interaction in intestinal epithelial cells may influence the production/release of intestinal FGF19, which, in turn modulates FXR levels by its beta-Klotho/FGFR4 receptor complex [32,33]. However, since data regarding the real role of the FXR/FGF-19 axis in liver damage is still controversial, further studies are required to elucidate better the nature of the interaction between FXR and FGF19 in NAFLD [34].

Our study has strengths and limitations that should be mentioned. We have been able to study hepatic FXR protein content in normal liver tissue, and in patients with NAFLD, some of whom had NASH. Furthermore, we have undertaken a detailed analysis of BAs and compared levels in patients with and without NASH. However, one important limitation of our study is the small sample size and it is now important to verify our novel findings in a larger study.

In conclusion, our data show that in children and adolescents with NAFLD, hepatic FXR protein content and plasma FGF19 concentrations were decreased compared to levels in children with NAFLD but without NASH. Hepatic FXR protein level was positively correlated with serum FGF19 levels and both FXR and FGF19 concentration were inversely and independently associated with NASH. The rationale for our research study was based on the observation that more that 50% of subjects with NASH in the FLINT trial failed to derive benefit from treatment with the powerful FXR agonist obeticholic acid [12]. These findings suggested to us that in some patients with

NASH, FXR agonist treatment may fail because of inadequate hepatic FXR protein levels. Our data provide evidence that FXR levels are low in patients with NASH and we suggest that further research is now needed to better understand regulation of FXR and FGF19 in NAFLD.

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References

1. Nobili V, Alisi A, Newton KP, Schwimmer JB. Comparison of the phenotype and approach to pediatric vs adult patients with nonalcoholic fatty liver disease. Gastroenterology 2016;150:1798-1810.
2. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Dig Dis. 2010;28 (1):155-61.
3. Brunt EM, Wong VW, Nobili V, Day CP, Sookoian S, Maher JJ, et al. Nonalcoholic fatty liver disease. Nat Rev Dis Primers 2015;1:15080.
4. Chávez-Talavera O, Tailleux A, Lefebvre P, Staels B. Bile acid control of metabolism and inflammation in obesity, type 2 diabetes, dyslipidemia and NAFLD. Gastroenterology 2017 Feb 15. pii: S0016-5085(17)30157-9. doi: 10.1053/j.gastro.2017.01.055. [Epub ahead of print].
5. Jahnel J, Zöhrer E, Alisi A, Ferrari F, Ceccarelli S, De Vito R, et al. Serum bile acid levels in children with nonalcoholic fatty liver disease. J Pediatr Gastroenterol Nutr 2015;61:85-90.
6. Li T, Chiang JY. Bile acids as metabolic regulators. Curr Opin Gastroenterol 2015;31:159-165.
7. Kim SG, Kim BK, Kim K, Fang S. Bile acid nuclear receptor farnesoid X receptor: therapeutic target for nonalcoholic fatty liver disease. Endocrinol Metab (Seoul) 2016;31:500-504.
8. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell 2000;102:731-744.
9. Zhang Y, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, et al. Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. Proc Natl Acad Sci U S A. 2006;103:1006-1111.
10. Zhang S, Wang J, Liu Q, Harnish DC. Farnesoid X receptor agonist WAY-362450 attenuates

liver inflammation and fibrosis in murine model of non-alcoholic steatohepatitis. J Hepatol 2009;51:380-388.

11. Pozzo L, Vornoli A, Coppola I, Croce CM, Giorgetti L, Gervasi PG, et al. Effect of HFD/STZ on expression of genes involved in lipid, cholesterol and glucose metabolism in rats. Life Sci 2016;166:149-156.
12. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet 2015;385:956-965.
13. Vajro P, Lenta S, Socha P, Dhawan A, McKiernan P, Baumann U, et al. Diagnosis of nonalcoholic fatty liver disease in children and adolescents: position paper of the ESPGHAN Hepatology Committee. J Pediatr Gastroenterol Nutr 2012;54:700-713.
14. CDC center: Clinical Growth Charts; www.cdc.gov/growthcharts/clinical_charts.htm
15. Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. Arch Dis Child 1969;44(235):291-303.
16. Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in boys. Arch Dis Child 1970;45(239):13-23.
17. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-419.
18. Pietrobattista A, Fruwirth R, Natali G, Monti L, Devito R, Nobili V. Is juvenile liver biopsy unsafe? Putting an end to a common misapprehension. Pediatr Radiol 2009;39:959-961.
19. Alkhouri N, De Vito R, Alisi A, Yerian L, Lopez R, Feldstein AE et al. Development and validation of a new histological score for pediatric non-alcoholic fatty liver disease. J Hepatol. 2012;57(6):1312-1318.
20. Amplatz B, Zöhrer E, Haas C, Schäffer M, Stojakovic T, Jahnel J, et al. Bile acid preparation

and comprehensive analysis by high performance liquid chromatography-high-resolution mass spectrometry. Clin Chim Acta 2017;464:85-92.

21. Gnani D, Romito I, Artuso S, Chierici M, De Stefanis C, Panera N, et al. Focal adhesion kinase depletion reduces human hepatocellular carcinoma growth by repressing enhancer of zeste homolog 2. Cell Death Differ 2017 Mar 24. doi: 10.1038/cdd.2017.34
22. Alisi A, Ceccarelli S, Panera N, Prono F, Petrini S, De Stefanis C, et al. Association between serum atypical fibroblast growth factors 21 and 19 and pediatric nonalcoholic fatty liver disease. PLoS One. 2013 Jun 26;8(6):e67160.
23. Guicciardi ME, Malhi H, Mott JL, Gores GJ. Apoptosis and necrosis in the liver. Compr Physiol 2013;3:977-1010.
24. Takikawa Y, Miyoshi H, Rust C, Roberts P, Siegel R, Mandal PK, et al. The bile acid-activated phosphatidylinositol 3-kinase pathway inhibits Fas apoptosis upstream of bid in rodent hepatocytes. Gastroenterology 2001;120:1810-1817.
25. Yerushalmi B, Dahl R, Devereaux MW, Gumpricht E, Sokol RJ. Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. Hepatology 2001;33:616-626.
26. Park WH, Pak YK. Insulin-dependent suppression of cholesterol 7α-hydroxylase is a possible link between glucose and cholesterol metabolisms. Exp Mol Med 2011;43:571-579.
27. Wu X, Ge H, Gupte J, Weiszmann J, Shimamoto G, Stevens J, et al. Co-receptor requirements for fibroblast growth factor-19 signaling. J Biol Chem 2007;282:29069-29072.
28. Lin BC, Wang M, Blackmore C, Desnoyers LR. Liver-specific activities of FGF19 require Klotho beta. J Biol Chem 2007;282:27277-27284.
29. Fiorucci S, Rizzo G, Donini A, Distrutti E, Santucci L. Targeting farnesoid X receptor for liver and metabolic disorders. Trends Mol Med 2007;13:298-309.
30. Yao J, Zhou CS, Ma X, Fu BQ, Tao LS, Chen M, et al. FXR agonist GW4064 alleviates endotoxin-induced hepatic inflammation by repressing macrophage activation. World J

Gastroenterol 2014;20:14430-14441.

31. Aguilar-Olivos NE, Carrillo-Córdova D, Oria-Hernández J, Sánchez-Valle V, Ponciano-Rodríguez G, Ramírez-Jaramillo M, et al. The nuclear receptor FXR, but not LXR, up-regulates bile acid transporter expression in non-alcoholic fatty liver disease. Ann Hepatol 2015;14:487-493.
32. Fu T, Kim YC, Byun S, Kim DH, Seok S, Suino-Powell K et al. FXR Primes the Liver for Intestinal FGF15 Signaling by Transient Induction of β-Klotho. Mol Endocrinol. 2016;30:92-103.
33. Gonzalez FJ, Jiang C, Xie C, Patterson AD. Intestinal Farnesoid X Receptor Signaling Modulates Metabolic Disease. Dig Dis. 2017;35(3):178-184.
34. Schmitt J, Kong B, Stieger B, Tschopp O, Schultze SM, Rau M, et al. Protective effects of farnesoid X receptor (FXR) on hepatic lipid accumulation are mediated by hepatic FXR and independent of intestinal FGF15 signal. Liver Int 2015;35:1133-1144.

Figure legends

Figure 1. A) Histogram reporting FXR protein average intensity in control (CTRL), NASH and Not-NASH patients. Data are expressed as means ± SD. ** = P<0.01; *** = P<0.001. **B)** Histogram reporting FGF19 plasma levels in control (CTRL), NASH and Not-NASH patients. Data are expressed as means ± SD. ** = P<0.01; *** = P<0.001. Test for between group differences with ANOVA. **C)** Heatmap representation of FXR average intensity and FGF19 levels in individual patients (Controls, Not-NASH and NASH).

Supplementary Figure 1. A-D) Representative confocal immunofluorescence of liver FXR protein in healthy subjects (CTRL) (*panel B*), and subjects with NAFLD (Not-NASH –*panel C*, or NASH – *panel D*). The figure shows also control staining from the secondary antibody in healthy control subjects (*panel A*). FXR staining is shown in green and was identified with mouse anti-goat Alexa Fluor® 488 goat anti-rabbit IgG. Nuclei were identified by staining with DAPI (Blue). Original magnification: x60.