Research Article
NAFLD and alcohol-related liver diseases

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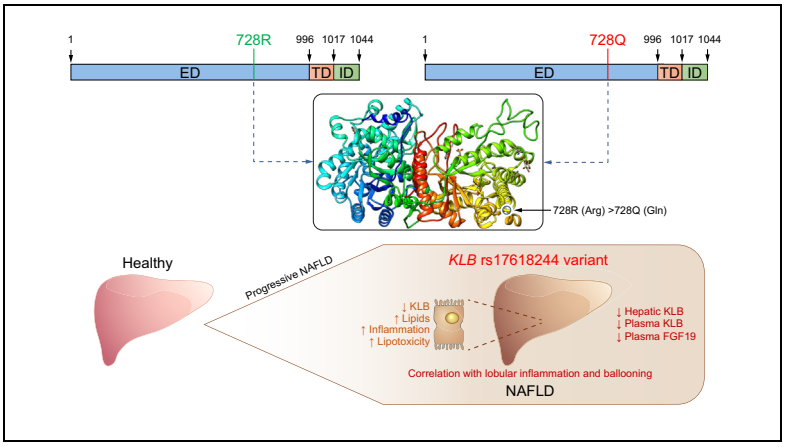
β-Klotho gene variation is associated with liver damage in children with NAFLD

Graphical abstract



Highlights

- The *KLB* rs17618244 variant increases the risk of ballooning and lobular inflammation in children with NAFLD.
- KLB plasma levels are lower in carriers of the rs17618244 minor A allele.
- KLB plasma levels are associated with lobular inflammation, ballooning and fibrosis.
- KLB mutant induces intracellular lipid accumulation in HepG2 and Huh7.
- KLB mutant causes upregulation of lipotoxic and proinflammatory genes.

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Lay summary

Genetic and environmental factors strongly impact on the pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD). The FGF19/FGFR4/KLB pathway plays a pivotal role in the pathogenesis of NAFLD. The aim of the study was to investigate the impact of a genetic variant in the *KLB* gene on the severity of liver disease. Our data suggest that the KLB protein plays a protective role against lipotoxicity and inflammation in hepatocytes.

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β-Klotho gene variation is associated with liver damage in children with NAFLD

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Background & Aim: Non-alcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease in adults and children. Along with obesity, diabetes and insulin resistance, genetic factors strongly impact on NAFLD development and progression. Dysregulated bile acid metabolism and the fibroblast growth factor 19 (FGF19) pathway play a pivotal role in NAFLD pathogenesis. However, the mechanism through which the FGF19 receptor system is associated with liver damage in NAFLD remains to be defined.

Methods: We evaluated the impact of the rs17618244 G>A β-Klotho (*KLB*) variant on liver damage in 249 pediatric patients with biopsy-proven NAFLD and the association of this variant with the expression of hepatic and soluble KLB. *In vitro* models were established to investigate the role of the *KLB* mutant.

Results: The *KLB* rs17618244 variant was associated with an increased risk of ballooning and lobular inflammation. KLB plasma levels were lower in carriers of the rs17618244 minor A allele and were associated with lobular inflammation, ballooning and fibrosis. In HepG2 and Huh7 hepatoma cell lines, exposure to free fatty acids caused a severe reduction of intracellular and secreted KLB. Finally, KLB downregulation obtained by the expression of a *KLB* mutant in HepG2 and Huh7 cells induced intracellular lipid accumulation and upregulation of *p62, ACOX1, ACSL1, IL-1β* and *TNF-α* gene expression.

Conclusion: In conclusion, we showed an association between the rs17618244 *KLB* variant, which leads to reduced KLB expression, and the severity of NAFLD in pediatric patients. We can speculate that the KLB protein may exert a protective role against lipotoxicity and inflammation in hepatocytes.

Lay summary: Genetic and environmental factors strongly impact on the pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD). The FGF19/FGFR4/KLB pathway plays a pivotal role in the pathogenesis of NAFLD. The aim of the study was to investigate the impact of a genetic variant in the *KLB* gene on the severity of liver disease. Our data suggest that the KLB protein plays a protective role against lipotoxicity and inflammation in hepatocytes.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the leading cause of liver damage worldwide.[1] The histologic spectrum of NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and eventually progress to cirrhosis and hepatocellular carcinoma (HCC).[2,3] NAFLD is a multifactorial disease where environmental factors, such as an excessive caloric intake and a sedentary lifestyle, and genetic factors interact with each other, triggering the metabolic and hepatic events that lead to liver fat accumulation and progressive liver disease.[4] In recent years, genome-wide association studies (GWAS) have identified inherited variants in genes involved in hepatic fat uptake, synthesis, storage and mobilization of triglycerides which have been associated with a higher risk of NAFLD in adults.[5–7] Most of these genetic variants, such as the rs738409 C>G in patatin-like phospholipase domain–containing 3 (*PNPLA3*) gene and the rs58542926 C>T in transmembrane 6 superfamily member 2 (*TM6SF2*) gene, also increase the risk of NAFLD in pediatric patients.[8–10] Due to their effect size these polymorphisms explain the genetic susceptibility to NAFLD development and progression in most individuals.[11] However, other genetic variants may contribute to determine the motley pattern of histologic features associated with NAFLD and to explain the missing heritability.[12]

NAFLD development and progression are complex to decipher, and the most innovative pathogenic concept that has been

Keywords: NAFLD; β-Klotho; SNPs; Ballooning; Inflammation.
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proposed involves the crosstalk between the liver, gut and adipose tissue.[13] Recent experimental and clinical evidence has suggested that fibroblast growth factor (FGF) 15/19 and its receptor system represent one of the most important gut-derived signals, which impacts on adipose tissue and liver response during diet-induced NAFLD.[14,15]

FGF19/FGF15 (FGF15 is the mouse orthologue of the human FGF19) belongs to the FGF19 subfamily, together with FGF21 and FGF23. These factors have no affinity for heparan sulfates and are able to freely diffuse from their tissue of origin into the blood circulation. For these reasons, they can act as hormones. In particular, FGF19 regulates bile acid (BA) homeostasis and gallbladder filling/emptying.[16–18] BAs are molecules synthesized in the liver and stored in the gallbladder, essential for solubilization of fatty acids, for digestion and lipid absorption in the small intestine.[19] Liver-derived BA bind to farnesoid X receptor (FXR) in enterocytes thus inducing the expression of FGF19, which is released into the portal circulation. FGF19 is transported to the liver and interacts with fibroblast growth factor receptor-4 (FGFR4) assisted by the β-Klotho (KLB) co-receptor, which is crucial for full activation of the FGF/FGFR complex and for the induction of the intracellular responses, such as downregulation of cholesterol 7a-hydroxylase (CYP7A1) and consequent inhibition of BA synthesis.[20–23]

We previously reported an inverse association between FGF19 plasma levels, hepatic Klotho expression and severity of liver damage in a cohort of obese pediatric patients with NAFLD.[24] These previous data suggested that the decrease of hepatic Klotho protein in pediatric NAFLD could be ascribable to the beta isoform (KLB), which is the main form in the liver.[25–28]

Recently, two functional genetic variants modulating the activity of the KLB/FGFR4 pathway, namely rs17618244 G>A *KLB* and rs1966265 G>A *FGFR4*, encoding for the R728Q and V10I aminoacidic substitutions have been associated with accelerated transit in irritable bowel syndrome,[29] supporting a possible functional impact on the regulation of the gut-liver axis via modulation of FGF19 signaling.[30] Therefore, the aim of this study was to examine the impact of the rs17618244 G>A *KLB* and rs1966265 G>A *FGFR4* variants on liver damage severity in pediatric patients with NAFLD.

Material and methods

Study participants

A cohort of 249 Italian pediatric patients with biopsy-proven NAFLD, evaluated at Bambino Gesù Children's Hospital between September 2011 and May 2016, were enrolled in the study. This study was approved by local ethics committee (Bambino Gesu' Children's Hospital and IRCCS, Rome, Italy – Protocol number 734_OPBG_2014 and 1956_OPBG_2019). Written informed consent was obtained from the parents of each child.

All individuals were of European descent and were consecutively enrolled. Other causes of liver disease including increased alcohol intake (>30/20 g/day in males/females), viral and autoimmune hepatitis, hereditary hemochromatosis, alpha1-antitrypsin deficiency, and history, Wilson disease, and infection with hepatitis B or hepatitis C were excluded.

Body mass index (BMI) and waist circumference were measured using standard procedures. Alanine aminotransferase, aspartate aminotransferase, triglycerides, total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol were measured by standard laboratory methods.

Demographic, anthropometric and clinical features of individuals are shown in Table S1. As a control group, we enrolled 128 pediatric healthy children (age range, 6.67–13.34 years) from the population that adhere to special programs of liver disease screening performed by our hospital each year (Table S2). As an additional control group, 502 healthy European individuals from the 1000 Genomes project for whom the genotypes of interest were available (http://www.internationalgenome.org) were included.

Liver histology

Liver histology was evaluated by 2 experienced pathologists unaware of clinical and genetic data. Briefly, liver biopsies were routinely processed and analyzed by different staining including, H&E, Van Gieson, periodic acid-Schiff diastase, and Prussian blue stain. The main histological features, commonly described in NAFLD, including steatosis, lobular and portal inflammation, hepatocyte ballooning, and fibrosis were scored according to the Scoring System for Non-Alcoholic Fatty Liver Disease developed by the NIH-sponsored NASH Clinical Research Network (CRN).[31] Diagnosis of NASH was based on the presence of steatosis with lobular necroinflammation and ballooning. Among 249 Italian pediatric patients with biopsy-proven NAFLD, 186 (75%) have NASH and 118 (47%) have severe fibrosis (F2-F4). The percentage of patients stratified by the severity of liver damage is reported in Table S3.

Genotyping

The rs17618244 G>A *KLB*, rs1966265 G>A *FGFR4*, rs738409 C>G *PNPLA3* variants were genotyped by TaqMan 5′-nuclease assays (Life Technologies, Carlsbad, CA, USA) in 249 pediatric patients with NAFLD and in 128 pediatric healthy controls. Briefly, genomic DNA was isolated from venous blood using a Blood DNA Extraction Kit (Qiagen, Valencia, CA, USA). The absorbance ratio at 260/280 nm of all the samples ranged from 1.8 to 2 indicating they were all free from contaminants. Real-time PCR was performed using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA).

Results were confirmed in a group of random samples by Sanger sequencing by Applied Biosystems 3500 Genetic Analyzer, using *KLB* forward CGAGCCTCTGTTGCATGC and *KLB* reverse TTGAGCAGCCTCCTTTCGG primers (Sigma-Aldrich, St. Louis, MO, USA), which provided concordant results in all cases. Positive and negative controls were included on each reaction plate, to verify the reproducibility of the results.

ELISA assays

KLB and FGF19 plasma levels were measured by commercially available ELISA kits (LifeSpan BioSciences, Seattle, WA, USA; BioVendor, Prague, Czech Republic) according to the manufacturer's instructions. In detail, KLB levels were measured by commercially available Human KLB/Beta Klotho ELISA Kit (LS-F11894). Specifications of the kit were: detection range, 15.6–1,000 pg/ml; inter-assay precision, coefficient of variability (CV) <7.9%; intra-assay precision, CV <4.4%. Our range data for intra- and inter-assay CV were 1.8–4.3% and 1.7–7.2%, respectively.

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Immunofluorescence

Staining for KLB and Sequestosome 1 (SQSTM1 or p62) was performed on liver tissue from pediatric patients with and without NASH stratified by the KLB rs17618244 genotype. Immunofluorescence was performed on 2 μm-thick sections obtained from formalin-fixed tissue embedded in paraffin. Antigen retrieval was performed with EDTA (pH 9) (Dako, Glostrup, Denmark). The sections were incubated overnight at 4 °C with rabbit anti-KLB (dilution 1:300, Abcam, Cambridge, MA, USA) and with mouse anti-SQSTM1/p62 (dilution 1:300; Santa Cruz Dallas, Texas, USA) and revealed with Alexa Fluor 488 goat anti-rabbit (dilution 1:500, Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and with Alexa Fluor 488 goat anti-mouse (dilution 1:500, Applied Biosystems, Life Technologies, Carlsbad, 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. The confocal microscopy imaging was performed on an Olympus Fluoview FV1000 confocal microscope equipped with FV10-ASW version 4.1 software, using a 40x objective. Quantitative analysis of the imaging was performed as previously described.[24]

Statistical analysis

Results are expressed as means ± SD for normally distributed variables, median [IQR] for non-normally distributed variables which were log-transformed before analysis. Mean values were compared by ANOVA or by paired or unpaired 2-tailed Student's *t* test.

Association of the phenotypic trait with genetic variants was analyzed by fitting logistic (diagnosis of NAFLD *vs.* healthy control) and ordinal (histological features of liver damage in biopsied individuals) regression models, adjusted for relevant covariates (specified in the results section). Genetic traits were analyzed under additive models.

The association of KLB and FGF19 plasma levels with the *KLB* rs17618244 variant (R728Q protein variant) and histological features of liver damage was analyzed by generalized linear models, adjusted for sex, age, BMI and *PNPLA3* rs738409 genotype (I148M protein variant).

Statistical analyses were carried out using the JMP 14.0 Statistical analysis software (SAS Institute, Cary, NC, USA), and R statistical analysis software version 3.3.2. p values <0.05 were considered statistically significant. The study methods and results have been reported according to the STROBE/STREGA guidelines for genetic association studies.

In vitro methods

All *in vitro* methods are reported in the supplementary information.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

The *KLB* rs17618244 variant is associated with pediatric NAFLD

We first evaluated the impact of rs17618244 G>A *KLB* and rs1966265 G>A *FGFR4* variants, which may influence FGF19 signaling, on liver damage in 249 children with NAFLD and in 128 pediatric controls. The frequency distribution of the *KLB* rs17618244 and *FGFR4* rs1966265 variants was in Hardy-Weinberg equilibrium (Table S4). The *KLB* rs17618244 variant tended to be over-represented in patients than in healthy controls ($p = 0.038$; Fig. 1). By considering as further controls 502 healthy individuals included in the 1000 Genomes the association of the *KLB* rs17618244 variant with NAFLD remained significant ($p = 0.042$). Conversely, there was no difference in the frequency of the rs1966265 G>A *FGFR4* variant between patients with NAFLD and controls (Fig. 1).

The *KLB* rs17618244 variant increases the risk of ballooning and lobular inflammation in children with NAFLD

The impact of the *KLB* variant on NAFLD severity was then evaluated in pediatric patients with NAFLD. The clinical features of pediatric patients stratified by *KLB* rs17618244 variant are shown in Table S5. No differences in demographic and anthropometric features were found across rs17618244 genotypes.

The relationship between the *KLB* rs17618244 variant and the severity of liver damage is shown in Table 1. At multivariate ordinal regression analysis adjusted for age, sex, BMI, and *PNPLA3* rs738409 variant, carriers of the *KLB* rs17618244 variant had increased risk of both ballooning (Estimate: 0.45; 95% CI 0.035–0.88; $p = 0.032$) and lobular inflammation (Estimate: 0.45; 95% CI 0.034–0.87; $p = 0.036$). However, the *KLB* rs17618244 variant did not impact on steatosis, and was not significantly associated with fibrosis.

This data is consistent with the hypothesis that the *KLB* rs17618244 variant predisposes to progressive NAFLD by promoting hepatocellular damage.

Conversely, we did not find any association between the rs1966265 G>A *FGFR4* variant and the entire spectrum of liver damage (Table S6). Therefore, only the *KLB* rs17618244 variant was further considered.

Circulating KLB concentration is reduced and correlated with FGF19 in pediatric patients with NAFLD

To evaluate the mechanism underpinning the association between the *KLB* rs17618244 variant and progressive NAFLD, we next evaluated circulating KLB in 205 pediatric patients with NAFLD and in 36 healthy individuals.

Circulating KLB concentration was lower in pediatric patients with NAFLD compared to age-matched controls (p <0.0001; Fig. 2A). In children with NAFLD, KLB plasma levels were lower in carriers of the A allele of the *KLB* rs17618244 risk variant (p <0.0001; Fig. 2B). At multivariate generalized linear model,

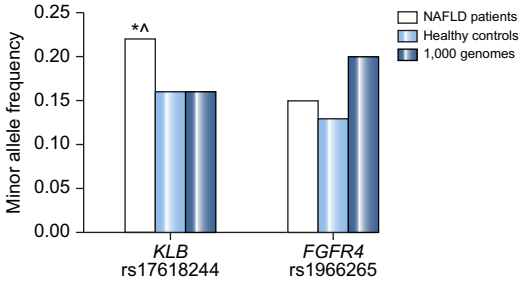

Fig. 1. Minor alleles frequency of *KLB* rs17618244 and *FGFR4* rs1966265. The histogram reports the frequency distribution of the *KLB* rs17618244 and *FGFR4* rs1966265 minor allele (A) in pediatric healthy controls (n = 128), children with NAFLD (n = 249) and healthy European individuals from the 1000 Genomes project (n = 502). Data were analyzed by ANOVA test, *$p = 0.038$ patients with NAFLD *vs.* healthy children ^$p = 0.042$ NAFLD patients *vs.* overall controls (healthy children and individuals included in the 1000 Genomes project). NAFLD, non-alcoholic fatty liver disease.

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Table 1. Impact of *KLB* rs17618244 variant on liver damage in 249 pediatric patients with NAFLD.

	Age (years)	Gender (M)	BMI (kg/m^2)	*PNPLA3* (I148M)	*KLB* (R728Q)
Steatosis					
Estimate	−0.08	0.04	−0.006	0.36	0.09
95% CI	−0.17–0.01	−0.20–0.29	−0.05–0.03	0.04–0.69	−0.34–0.69
p value*	0.09	0.74	0.75	0.03	0.52
Lobular Inflammation					
Estimate	−0.03	−0.08	−0.009	0.11	0.45
95% CI	−0.11–0.06	−0.32–0.16	−0.05–0.03	−0.20–0.43	0.03–0.88
p value*	0.52	0.53	0.64	0.49	**0.036**
Ballooning					
Estimate	−0.14	0.09	0.017	−0.07	0.45
95% CI	−0.22–0.00	−0.14–0.34	−0.02–0.05	−0.38–0.24	0.03–0.88
p value*	0.002	0.42	0.38	0.66	**0.032**
Fibrosis					
Estimate	−0.09	−0.06	0.004	0.29	0.25
95% CI	−0.17 to −0.01	−0.29 to −0.17	−0.03 to −0.04	−0.01–0.61	−0.14–0.65
p value*	0.03	0.62	0.85	0.06	0.22

*At multivariate generalized linear analysis; models were adjusted for age, sex, BMI, and *PNPLA3* 148M allele.
BMI, body mass index; NAFLD, non-alcoholic fatty liver disease.
Bold values represent the significant associations.

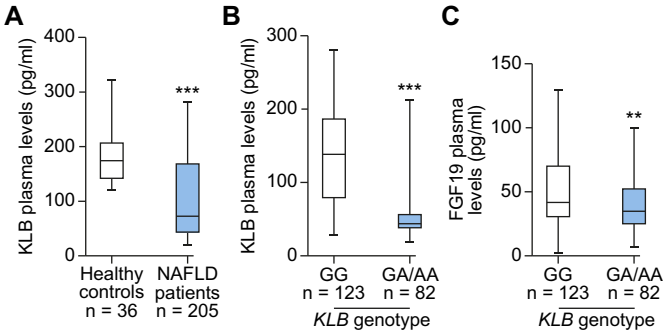

Fig. 2. Assessment of KLB and FGF19 circulating levels. The plots report KLB plasma levels according to the presence of (A) NAFLD and (B) *KLB* genotype. Data were analyzed by 2-tailed *t* tests, ***p <0.001 (Panel B refers to patients with NAFLD). (C) The plots report FGF19 plasma levels according to the *KLB* genotype. The panel refers to patients with NAFLD. Data were analyzed by 2-tailed *t* tests, **p = 0.01. NAFLD, non-alcoholic fatty liver disease.

adjusted for age, sex, BMI and *PNPLA3* genotype, KLB plasma levels were associated with *KLB* rs17618244 variant (Estimate −0.005; 95% CI -0.006 to −0.004; p <0.0001), lobular inflammation (Estimate −0.002; CI −0.003 to −0.0003; p = 0.02), ballooning (Estimate −0.003; CI −0.004 to −0.001; p = 0.001) and fibrosis (Estimate −0.003; CI −0.005 to -9.8×10^{-4}; p = 0.004) (Table S7). In a multivariate generalized linear model, adjusted for age, sex, BMI, *PNPLA3* genotype and the *KLB* rs17618244 variant, KLB plasma levels remained associated with lobular inflammation (Estimate −0.001; 95% CI −0.004 to −0.00002; p <0.047), ballooning (Estimate −0.003; CI −0.004 to −0.0008; p = 0.005) and fibrosis (Estimate −0.003; CI −0.006 to −0.001; p = 0.003).

The reduction of circulating KLB levels in the presence of the *KLB* rs17618244 variant could be the result of decreased hepatic protein levels or protein cleavage and release. Therefore, we assessed the hepatic expression of KLB in a subgroup of 69 children with NAFLD. As shown in the Fig. S1A, hepatic KLB was reduced in NAFLD patients who carry the rs17618244 A allele and this finding was confirmed even after stratification for the presence of NASH (Fig. S1B).

This data suggests that the *KLB* rs17618244 variant may predispose to more advanced liver damage by reducing KLB protein levels.

Circulating levels of FGF19, which requires KLB as co-receptor to facilitate its interaction with FGFR4, were also analyzed. FGF19 levels were lower in patients who carried the *KLB* rs17618244 risk variant compared to non-carriers (p = 0.01; Fig. 2C). In a multivariate generalized linear model adjusted for age, sex, BMI and *PNPLA3* genotype, FGF19 plasma levels were associated with the *KLB* rs17618244 variant (Estimate −0.003; 95% CI −0.006 to −0.0006; p = 0.02), ballooning (Estimate −0.005; 95% CI −0.01 to −0.001; p = 0.01) and fibrosis (Estimate −0.009; 95% CI −0.01 to −0.004; p = 0.0007).

This data suggests that liver damage is associated with both decreased FGF19 and KLB-dependent signaling.

Treatments inducing *in vitro* NAFLD reduce KLB expression and release, thus enhancing lipotoxicity

In order to evaluate the interplay between NAFLD and the KLB protein, an *in vitro* model of NAFLD was established. Firstly, HepG2 and Huh7 cells were genotyped for the *KLB* rs17618244 variant. Both cell lines expressed wild-type (GG) *KLB*. Next, cells were treated with 2 concentrations of palmitic acid and oleic acid in a 1:2 molar ratio for 24 h. As shown in Fig. 3A and 3B, after 24 h, free fatty acids (FFAs) induced statistically significant dose-dependent lipid accumulation, alongside a relevant reduction of cell viability, particularly after treatment with FFAs at high dose (Fig. 3C and 3D).

Exposure to FFAs caused a statistically significant reduction of KLB protein expression (Fig. 4A and Fig. S2A) that was not associated with changes in *KLB* mRNA levels (Fig. S2B). Next, the culture media of the cells treated with FFAs were collected to evaluate KLB release. As shown in Fig. 4B and Fig. S2C, exposure to FFAs was associated with reduced KLB secretion compared to untreated cells.

Exposure to FFAs mimics some aspects of lipotoxicity which occur in NAFLD. Indeed, FFAs stimulated p62 at mRNA and protein levels (Fig. 4C and 4D), which is also overexpressed in patients with NASH carrying the AA genotype (Fig. 4E).

KLB downregulation influences lipid accumulation, lipotoxicity and inflammation

In order to examine the hypothesis that *KLB* downregulation is associated with hepatic fat accumulation and lipotoxicity in hepatocytes, the *KLB* gene was silenced in HepG2 and Huh7 cells using commercial siRNAs. A pilot study was performed to iden-

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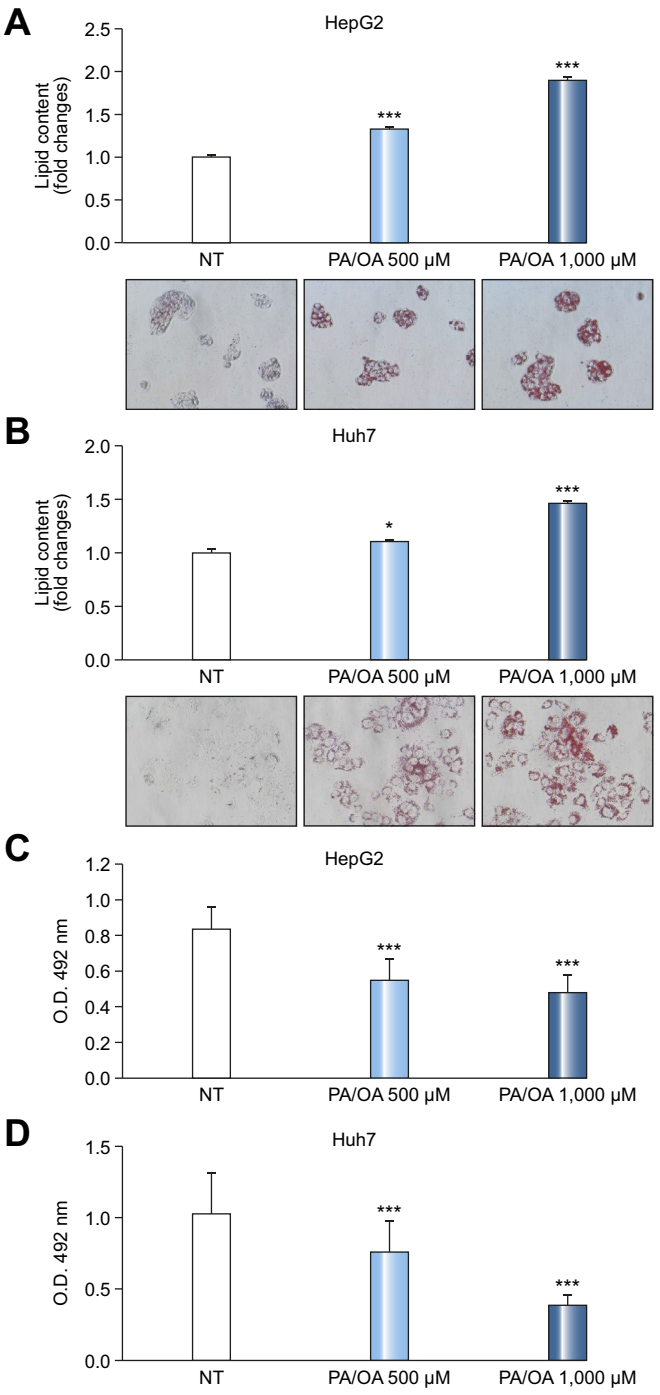

Fig. 3. Analysis of lipid content and cell viability in HepG2 and Huh7 cells after treatment with FFAs. The histograms (upper panels) report the fold change of the lipid content measured as O.D./mg protein by ORO in (A) HepG2 and (B) Huh7 cells treated with 500 or 1,000 μM PA/OA for 24 h compared to untreated (NT) cells. In the lower panels are reported representative images (40x) of the ORO staining. Data the mean ± SD of 2 independent experiments repeated at least in triplicate. Data were analyzed by 2-tailed *t* tests, $^{*}p$ <0.05, $^{***}p$ <0.001 *vs.* control cells. Effect of FFAs on (C) HepG2 and (D) Huh7 cell viability measured by XTT assay. Data refer to mean O.D. of 3 independent experiments repeated at least in triplicate. Data were analyzed by 2-tailed t tests, $^{***}p$ <0.001 *vs.* control cells. FFAs, free fatty acids; OA, oleic acid; ORO, Oil Red O; PA, palmitic acid.

tify the amount of KLB siRNAs required to obtain the best efficiency of silencing (Fig. S3A and B). The best efficiency of KLB silencing (40–50%) was obtained with 10 nM siRNA for HepG2 and 50 nM siRNA for Huh7, which did not affect cell viability (Fig. S3C and D).

As shown in Fig. 5A and Fig. S4A, *KLB* silencing was unable *per se* to cause changes in lipid accumulation in HepG2 and Huh7 cells, while it significantly increased the intracellular lipid accumulation in the presence of FFAs. However, the silencing-induced downregulation of *KLB* caused an upregulation of the expression of *p62, ACOX1* and *ACSL1* mRNA levels (Fig. 5B and Fig. S4B).

Next, we transfected HepG2 (GG) and Huh7 (GG) with the plasmid expressing the R728Q *KLB* mutant form (Fig. S5A). The overexpression of the R728Q *KLB* plasmid (Fig. S5B) had a dominant negative effect, downregulating the expression of KLB protein, while WT *KLB* plasmid transfection moderately increased protein levels (Fig. 6A and Fig. S5C). The downregulation of the KLB protein caused by the mutant plasmid was associated with an increase in lipid content (Fig. 6B).

As observed in patients with NASH who carry the rs17618244 AA genotype, the expression of the R728Q *KLB* mutant form in HepG2 and Huh7 induced an upregulation of lipotoxic and the pro-inflammatory genes, including *p62, ACOX1, ACSL1, IL-1β,* and *TNF-α* (Fig. 6C).

Discussion

In this study, we showed for the first time an association between the rs17618244 G>A genetic variant in the *KLB* gene and more severe liver damage in pediatric NAFLD. We found that the presence of the *KLB* variant was associated with an increased risk of ballooning and lobular inflammation. We also showed in the subgroup of children with NAFLD that the presence of the *KLB* variant was linked to a lower expression of circulating levels of FGF19 and hepatic and soluble KLB. The circulating levels of the latter were associated with lobular inflammation, ballooning and fibrosis. Supporting a causal role of reduced KLB expression in determining the susceptibility to liver damage, *in vitro* data confirmed that lipid accumulation after FFA treatment causes a reduction of cellular and soluble expression of the KLB protein in human hepatoma cell lines, which express wild-type *KLB*. Moreover, the reduction of KLB in the same cells increases lipid accumulation and induces upregulation of lipotoxic and pro-inflammatory genes.

KLB is a transmembrane protein mainly expressed in the liver. It acts as a co-receptor crucial for full activation of FGF19/FGFR4 complex that regulates BA synthesis by suppressing the expression of the CYP7A1. This process is tightly regulated since BA accumulation in the liver can lead to hepatotoxicity.[32,33] Indeed, intestinal FGF19 and the hepatic KLB/FGFR4 receptor system represents an endocrine network, essential for maintaining BA homeostasis. It is currently accepted that changes in the enterohepatic cycling and distribution of BAs may impair glucose and lipid metabolism and therefore BA levels are relevant for NAFLD development and progression.[34] Adults and children with NAFLD exhibit elevated hepatic and circulating concentrations of BAs that correlate with the severity of disease, mainly with fibrosis.[35,36]

In recent years, there has been a growing interest in BAs as signaling molecules and they are emerging as key players in the treatment of liver diseases.[19] Indeed, BAs, through activation of FXR, may regulate a wide range of target genes that modulate BA homeostasis, lipoprotein and glucose metabolism, and inflammatory responses.[37] In particular, it has been reported

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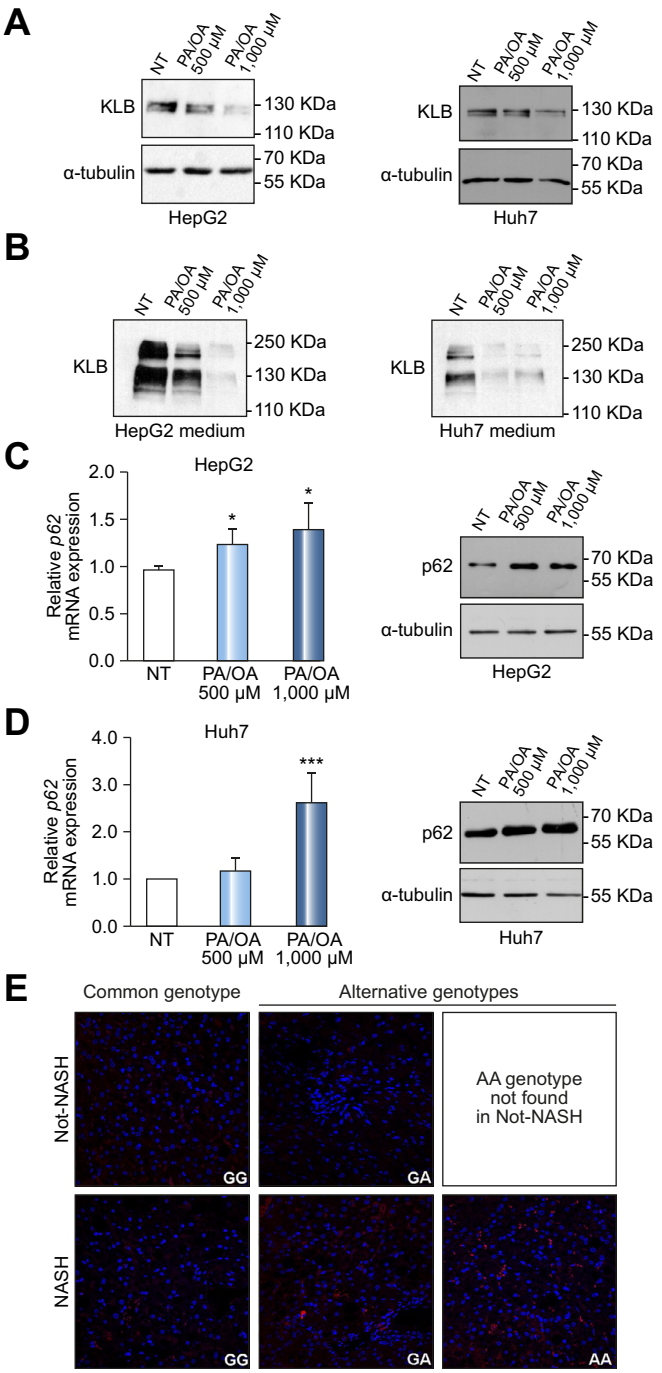
that FXR activation may repress *de novo* lipogenesis and lipoprotein export, as well as improve steatosis and reduce inflammation and fibrosis in mouse models of NASH.[38] Hence, FXR agonists or FGF19 analogues could be successful pharmacological strategies for NASH. Indeed, a recent multicenter, randomized, placebo-controlled clinical trial demonstrated that obeticholic acid (OCA), an FXR agonist, improved steatosis, inflammation and fibrosis.[39] Moreover, preliminary data from a multicenter, randomized, placebo-controlled phase II trial reported that an engineered FGF19 analogue (NGM282),



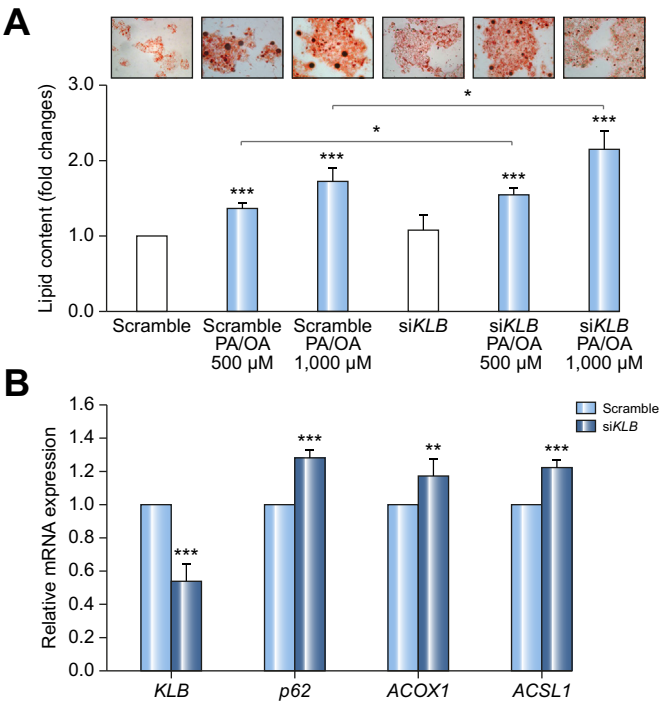


Fig. 5. Analysis of lipid content and lipotoxic genes in HepG2 cells silenced for KLB. (A) The histograms (lower panels) report the lipid content measured with ORO as O.D./mg protein in HepG2 cells silenced for KLB untreated (NT) or treated with 500 or 1,000 µM PA/OA for 24 h. In the upper panels are representative images (40x) of the ORO staining. Data are the mean ± SD of 2 independent experiments repeated at least in triplicate. Data were analyzed by ANOVA test, $^{*}p$ <0.05, $^{***}p$ <0.001. (B) Relative mRNA expression of *KLB, p62, ACOX1* and *ACSL1* genes measured by qRT-PCR in HepG2 cells transfected with 10 nM siRNA for KLB (siKLB) or control siRNA (scramble). Data are the mean ± SD of 3 independent experiments repeated at least in triplicate. Data were analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001 *vs.* scramble. FFAs, free fatty acids; OA, oleic acid; ORO, Oil Red O; PA, palmitic acid; qRT-PCR, quantitative reverse transcription PCR.

Fig. 4. Expression of KLB and p62 in HepG2 and Huh7 cells after treatment with FFAs and expression and intracellular distribution of p62 according to disease severity and *KLB* genotype. (A) Representative western blotting for KLB in HepG2 and Huh7 cells treated with 500 or 1,000 µM PA/OA for 24 h compared to untreated (NT) cells. α-Tubulin is reported as a loading control. (B) Representative Western blotting for circulating KLB in media from HepG2 and Huh7 cells treated or not with FFAs. All experiments were performed at least in duplicate. (C) p62 mRNA and protein levels were evaluated by qRT-PCR and western blotting in HepG2 cells incubated with 500 or 1,000 µM PA/OA for 24 h compared to untreated (NT) cells. α-Tubulin is reported as a loading control. Data are the mean ± SD of 2 independent experiments repeated at least in triplicate. Data were analyzed by 2-tailed *t* tests, $^{*}p$ <0.05 and $^{***}p$ <0.001 *vs.* control cells. (D) p62 mRNA and protein levels were evaluated by qRT-PCR and western blotting in Huh7 cells incubated with 500 or 1,000 µM PA/OA for 24 h compared to untreated (NT) cells. α-Tubulin is reported as a loading control. Data are the mean ± SD of 2 independent experiments repeated at least in triplicate. Data were analyzed by 2-tailed *t* tests, $^{***}p$ <0.001 *vs.* control cells. (E) The representative immunofluorescence was performed on 2 µm-thick sections obtained from formalin-fixed tissue embedded in paraffin. The staining of p62 is shown in red. The nuclei are revealed by specific DAPI staining, displayed in blue. 40x Magnification. FFAs, free fatty acids; OA, oleic acid; PA, palmitic acid; qRT-PCR, quantitative reverse transcription PCR.

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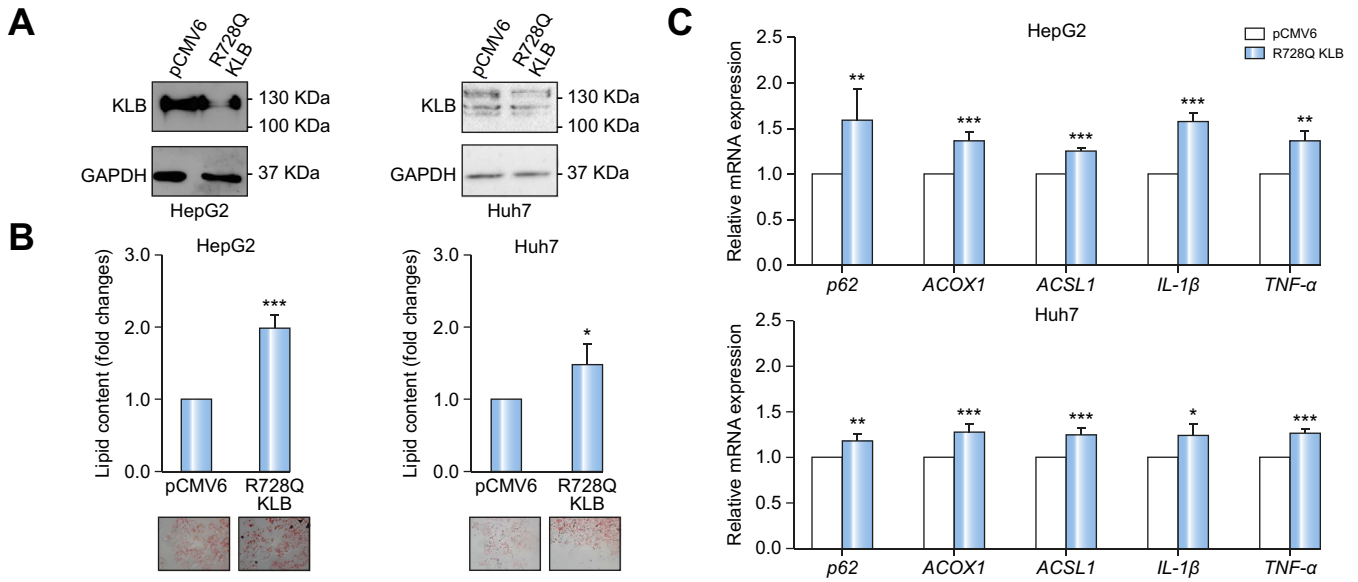

Fig. 6. Analysis of lipid content, lipotoxic and inflammatory genes in R728Q *KLB* mutant. (A) Representative western blotting of KLB protein expression in HepG2 and Huh7 cells transfected with plasmid for R728Q KLB (R728Q KLB). GAPDH is reported as a loading control. (B) The histograms (upper panel) report the lipid content measured with ORO as O.D./mg protein in HepG2 and Huh7 cells transfected with mutagenized plasmid for R728Q KLB (R728Q KLB). In the lower panel representative images (40x) of the ORO staining are displayed. Data are the mean ± SD of 3 independent experiments repeated at least in triplicate. Data were analyzed by 2-tailed *t* tests, ***p <0.001 *vs.* control cells (pCMV6). (C) Relative mRNA expression of *p62, ACOX1, ACSL1, IL-1β* and *TNF-α* genes measured by qRT-PCR in HepG2 and Huh7 cells transfected with mutagenized plasmid for R728Q KLB (R728Q KLB) or empty vector (pCMV6). Data are the mean ± SD of 3 independent experiments repeated at least in triplicate. Data were analyzed by 2-tailed *t* tests, *p <0.05, **p <0.01, ***p <0.001. ORO, Oil Red O; qRT-PCR, quantitative reverse transcription PCR.

reduced liver fat content and non-invasive biomarkers of fibrosis after 12 weeks of treatment.[40] The assessment of KLB levels could be relevant for evaluating the efficacy of these types of drugs. However, to date, no studies had investigated KLB co-receptor levels, either as a risk factor for NAFLD pathogenesis or as a predictive factor for treatment response.

In our study, we reported a relationship between the rs17618244 G>A *KLB* gene variant and the severity of liver damage. Carriers of this variant showed an increased risk of both ballooning and lobular inflammation and a decreased expression of hepatic KLB. We also observed a trend for more severe fibrosis (F2-F4; data not shown) in patients carrying the rs17618244 variant.

Furthermore, we analyzed the circulating KLB levels in our pediatric patients. We observed a significant reduction of KLB plasma levels in children with NAFLD. Our data represent the first evidence in the literature of a soluble form of KLB that could presumably act as soluble Klotho. Soluble forms of Klotho and KLB could be the result of a constitutive process named ectodomain shedding. Therefore, the levels of the soluble form of the protein should mirror the levels of the transmembrane and full-length form.[41] Noticeably, the reduction of circulating KLB was higher in patients who carried the rs17618244 *KLB* minor A allele. Indeed, at multivariate analysis, KLB plasma levels were associated with the rs17618244 *KLB* variant, lobular inflammation, ballooning and fibrosis. An association between KLB reduction and fibrosis was recently reported by Somm *et al.*[42] It was demonstrated that KLB-deficient mice were in a proinflammatory state with early evidence of fibrosis, defined by moderate deposition of collagen fibers and increased hepatic expression of fibrogenic genes.

The reduction of circulating KLB levels, associated with the presence of the rs17618244 gene variant, could be due to a decrease in hepatic protein levels, or protein cleavage and release. Our data seem to be in contrast to the study by Wong *et al.*,[29] wherein they showed that the rs17618244 *KLB* variant in HEK293 cells increased protein stability. These results were reported before the characterization of free and ligand-bound KLB extracellular regions,[43] so further investigations are needed to explore the role of this mutation in liver cells. Furthermore, since KLB also interacts with FGF21 and FGFR1c, the presence of the rs17618244 *KLB* variant could also influence adipose tissue homeostasis, which warrants further investigation.[41]

Notably, our data showed that hepatic expression of KLB was reduced in individuals with NASH compared to children without NASH, and more so in patients who carried the rs17618244 *KLB* variant. In addition, NASH carriers of the rs17618244 *KLB* variant displayed upregulated p62 protein expression. The downregulation of KLB and the upregulation of p62 were also confirmed in *in vitro* models of NAFLD. This data suggests that, independently of the presence of the variant, the accumulation of intra-hepatic lipids (steatosis) could be an indirect epigenetic regulator of KLB co-receptor expression, which in turn exacerbates lipotoxicity and promotes liver damage.

Furthermore, we found that the KLB downregulation, by the expression of the R728Q *KLB* mutant, increased the intracellular lipid accumulation and caused an upregulation of *p62, ACOX1, ACSL1, IL-1β* and *TNF-α* mRNA in HepG2 and in Huh7 cells.

The main limitation of this study is the lack of validation in other ethnic groups and in adults. Therefore, the replication of the study in independent cohorts from different ethnic groups is required to confirm the impact of rs17618244 *KLB* variant on the severity of liver damage in NAFLD. Further studies are also required to understand how this variant may impact on fibrosis.

In conclusion, we showed an association between rs17618244 *KLB* variant and the severity of NAFLD in Caucasian

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children. Moreover, we found that the KLB protein may protect against lipotoxicity and inflammation in hepatocytes. Further studies are needed to elucidate the mechanism linking altered hepatic KLB expression to NAFLD development and progression.

Abbreviations

BA, bile acid; BMI, body mass index; CRN, Clinical Research Network; CV, coefficient of variability; FFAs, free fatty acids; FGF, fibroblast growth factor; FXR, farnesoid X receptor; GWAS, genome-wide association studies; HCC, hepatocellular carcinoma; KLB, β-Klotho; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OA, oleic acid; ORO, Oil Red O; PA, palmitic acid; PNPLA3, patatin-like phospholipase domain–containing 3; qRT-PCR, quantitative reverse transcription PCR; TM6SF2, transmembrane 6 superfamily member 2.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Paola Dongiovanni, Annalisa Crudele, Luca Valenti, Anna Alisi: conception, design and drafting of the study. Annalisa Crudele, Nadia Panera, Ilaria Romito, Marica Meroni, Cristiano De Stefanis, Alessia Palma: execution and analysis of experiments.

Paola Dongiovanni, Donatella Comparcola, Anna Ludovica Fracanzani, Luca Miele, Luca Valenti, Valerio Nobili Anna Alisi: interpretation of data and critical revision of the manuscript.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.10.011.

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Author names in bold designate shared co-first authorship

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