Research article
MASLD and alcohol-related liver disease

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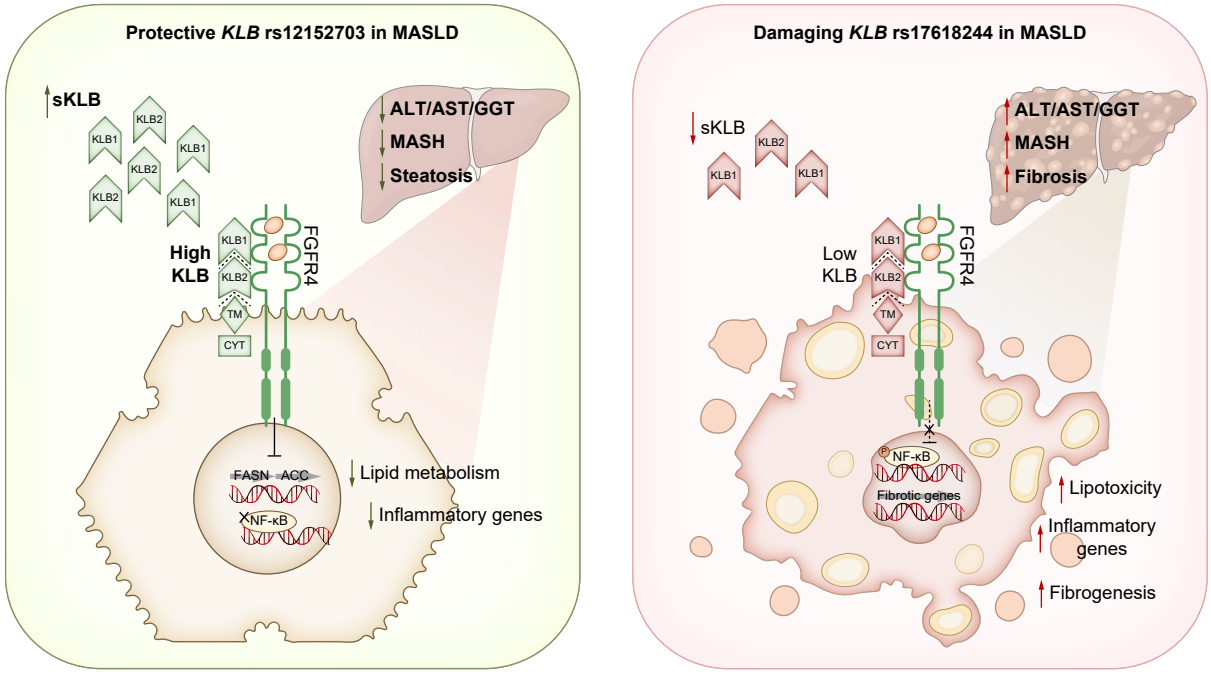
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Graphical abstract



Highlights:

- The *KLB* rs12152703 variant protected against hepatic inflammation in MASLD.
- The mutated T allele enhanced circulating and hepatic KLB protein levels.
- KLB overexpression in hepatocytes influenced lipid and inflammatory genes.
- KLB emerges as a novel druggable target for personalized treatment of MASLD.

Impact and implications:

This study highlights the protective effect of a genetic variant in the Klotho-beta (*KLB*) gene in attenuating hepatic inflammation and disease severity in both adults and children with metabolic dysfunction-associated steatotic liver disease. The favorable clinical associations appear to be mediated by increased circulating and hepatic KLB protein levels. Consistently, KLB overexpression alleviates lipid overload in hepatocytes by modulating the expression of genes involved in lipid metabolism. Together, these findings support the FGF19/KLB axis as a promising therapeutic target for the treatment of severe metabolic dysfunction-associated steatotic liver disease.

https://doi.org/10.1016/j.jhepr.2025.101717
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The *KLB* rs12152703 variant confers protection against hepatic inflammation in patients with MASLD by boosting Klotho-beta expression

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JHEP Reports **2026**. vol. 8 | 1–11

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Background & Aims: Metabolic dysfunction–associated steatotic liver disease (MASLD) is the most common cause of chronic liver disease worldwide, paralleling the rising prevalence of obesity. We previously reported that the rs17618244 variant in the Klotho-beta (*KLB*) gene, which encodes the hepatic obligate co-receptor of fibroblast growth factor receptor 4 (FGFR4), reduces hepatic and circulating KLB levels, leading to more severe MASLD in both children and adults. The present study aimed to evaluate the impact of another *KLB* variant, the intronic rs12152703 G>T polymorphism, on histological liver damage in patients with MASLD.

Methods: The rs12152703 variant was genotyped in 1,311 patients with biopsy-proven MASLD, including 261 children, and its association with the disease spectrum was assessed. We also investigated the relationship between this variant and hepatic and circulating KLB expression. Finally, we evaluated in an *in vitro* model whether KLB overexpression in HepG2 cells affects lipid homeostasis and inflammation.

Results: In multivariate analyses, the *KLB* rs12152703 variant was associated with lower serum aminotransferase levels and protection against steatosis, lobular inflammation, and steatohepatitis in the overall cohort (p <0.05). Hepatic and circulating KLB levels were increased in both adult and pediatric patients with MASLD carrying the variant (p <0.05). *In vitro*, KLB overexpression reduced intracellular lipid accumulation in free fatty acid-loaded HepG2 cells by modulating the expression of genes involved in lipid metabolism. Moreover, KLB induction counteracted lipopolysaccharide–induced activation of inflammatory genes and NF-κB (p65) phosphorylation.

Conclusions: The *KLB* rs12152703 variant confers protection against lobular inflammation and is associated with increased hepatic and circulating KLB levels, in contrast to the at-risk rs17618244 variant. Consistently, KLB overexpression ameliorated steatosis and the pro-inflammatory state in lipid-loaded hepatocytes. These findings suggest that KLB may represent a novel druggable target for the treatment of severe MASLD.

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Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) represents one of the most urgent global health burdens, affecting approximately 38% of the adult population and 10% of children/adolescents worldwide.[1,2] It entails a broad set of conditions, ranging from steatotic liver disease (SLD), characterized by excessive lipid build-up, to its progressive form, named metabolic dysfunction-associated steatohepatitis (MASH). The latter is a more challenging clinical issue, portrayed not only by hepatic fat-loading but also by lobular inflammation and ballooning, thus achieving a >10-fold increase in liver-related mortality. Indeed, this condition constitutes a fertile soil for the outbreak of fibrosis, cirrhosis, and eventually hepatocellular carcinoma.[3]

The ever-increasing prevalence of MASLD goes in tandem with the epidemic emergence of obesity and metabolic syndrome, already from childhood.[4] Despite extensive efforts to identify triggering factors and possible therapeutic strategies, an undisputed answer to this riddle has not yet been found.

In the past years, several genetic predictors have been pointed out as modifiers of the MASLD course. In particular, variants in genes involved in hepatic lipid handling, such as those in *PNPLA3*, *TM6SF2,* and *MBOAT7,* have been robustly linked to MASLD pathogenesis and progression in both adults and children.[5]

Among the plethora of inherited mutations that emerged, we identified the rs17618244 G>A variant in the Klotho-beta (*KLB*) gene as a novel candidate influencing lipid and

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https://doi.org/10.1016/j.jhepr.2025.101717

glucose homeostasis.[6,7] It encodes the obligate co-receptor of fibroblast growth factor receptor 4 (FGFR4) in the liver, thus mediating the binding of fibroblast growth factors 19 and 21 (FGF19/21) and the activation of the downstream signaling pathways.[8] These hormones are involved in a wide range of metabolic processes, especially in regulating glucose and lipid metabolism, as well as bile acid (BA) synthesis.[9] Moreover, the KLB/FGFR4/FGFs axis has also been suggested as a possible modulator of hepatic inflammation.[10]

In line with these observations, we reported that the *KLB* rs17618244 variant is associated with an increased risk of ballooning and lobular inflammation in pediatric patients, and it also boosted the odds of developing fibrosis in adults with MASLD, possibly due to the downregulation of circulating and hepatic KLB protein levels in carriers of the variant. In addition, we demonstrated that *KLB* silencing in HepG2 and Huh7 cells, using short-interfering RNA, promoted triglyceride accumulation, lipotoxicity, and pro-inflammatory cytokine secretion.[6,7]

Ji *et al.*[11] demonstrated that another *KLB* mutation, the intronic rs12152703 G>T, is related to obesity and negatively influences alanine aminotransferase (ALT) levels in a Chinese cohort of adults with MASLD, which displays a minor allele frequency (MAF) of this variant different from that observed in individuals of European ancestry. However, the effect size of this polymorphism was limited,[12] and its impact on histological liver damage has not yet been proven.

Therefore, we aimed to examine the impact of the less-studied *KLB* rs12152703 polymorphism on liver damage and serum/hepatic expression of the KLB protein in a large cohort of histologically characterized patients with MASLD (n = 1,311), of whom 261 were at pediatric age. We also sought to evaluate the *in vitro* effects of *KLB* overexpression on fat accumulation and inflammation.

In summary, our purpose was to recommend soluble KLB as a reliable, non-invasive biomarker for predicting liver damage and personalizing therapeutic options.

Material and methods

Patients

The overall cohort consisted of 1,311 unrelated patients with MASLD (Liver Biopsy cohort, [LBC]), including 1,050 adults and 261 children. Adult patients were consecutively enrolled from the metabolic liver diseases outpatient service at Fondazione IRCCS Ca' Granda of Milan between January 1999 and December 2023. Children were recruited at Bambino Gesù Children's Hospital between September 2011 and May 2016. Inclusion criteria were liver biopsy for suspected MASH (Liver Clinic, n = 603) or severe obesity (bariatric surgery, n = 447) and availability of blood and DNA samples and clinical data. Other causes of liver disease, including increased alcohol intake (>30/20 g/day in males/females), viral and autoimmune hepatitis, hereditary hemochromatosis, α1-antitrypsin deficiency, and history of infection with hepatitis B or hepatitis C were excluded. The study conformed to the Declaration of Helsinki and was approved by the Institutional Review Board of the Fondazione Ca' Granda of Milan and of Bambino Gesù Children's Hospital of Rome (CE approvals: 2366_2020 and 811_2021). All adults and parents of pediatric patients gave written informed consent. The demographic, anthropometric, genetic, and clinical features of the LBC, stratified according to enrolment criteria, are shown in Table S1. The frequency distribution of the *KLB* rs12152703 variant was stratified according to enrolment criteria, and the results were compared with those of non-Finnish European healthy individuals included in the 1000 Genomes Project (Table S2).

Gene expression in hepatic biopsy

Hepatic *KLB* gene expression was measured in a subset of 167 severely obese patients from the adult cohort (Bariatric surgery) (16), whose clinical features are shown in Table S3. The detailed protocol for the transcriptomic analysis is provided in the supplementary materials and methods.

Assessment of circulating KLB

Circulating KLB levels were evaluated in a subgroup of patients with MASLD belonging to the LBC, who were enrolled at the Fondazione IRCCS Cà Granda, Ospedale Policlinico, and at Bambino Gesù Children's Hospital, stratified according to the *KLB* rs12152703 genotype (GG: n = 137; GT: n = 78; TT: n = 21). Concentrations of KLB were measured in duplicate using ELISA (LS-F11894 LifeSpan BioSciences, Seattle, WA, USA), according to the manufacturer's instructions. 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.

Statistical analysis

Continuous variables were shown as mean and SD or median and interquartile range for highly skewed biological variables (*i.e.* aminotransferases). Variables with skewed distributions were log-transformed before analysis. Categorical variables were presented as numbers and proportions.

General linear, logistic and ordinal regression models were fit to examine continuous, binary, and categorical traits, respectively. The regression models were adjusted for sex, age, BMI kg/m^2, type 2 diabetes (T2D), PNPLA3 p.I148M, TM6SF2 p.E167K and *MBOAT7* rs641738 T alleles. Mean values were compared by one-way ANOVA or by two-tailed Student's *t* test (for continuous traits) and by Pearson's chi-square test for binary (impaired fasting glucose/T2D) or categorical traits (genetic data), where appropriate. For the *in vitro* study, results were expressed as means ± SD, and differences between samples were analyzed by two-tailed *t tests* or two-way ANOVA, where appropriate. *p* values were corrected for multiplicity by Tukey's honestly significant difference (HSD) multi-comparison *post hoc* test, and adjusted *p* values <0.05 were considered statistically significant. *p* <0.05 (two-tailed) was considered statistically significant. Additional information is included in the supplementary material and methods.

Results

The *KLB* rs12152703 variant protected against liver damage in patients with MASLD from the LBC

The allele frequencies of the *KLB* rs12152703 G>T variant in the LBC (N = 1,311, including 1,050 adults and 261 children) and the complete demographic, anthropometric, genetic, and clinical features of the LBC are represented in Tables S1 and

S2. In the LBC, the prevalence of the T allele was 27% compared to 21% in the 1000 Genomes Project (Fisher's exact test, $p = 0.0005$), mainly due to the enrichment of the T allele in obese patients belonging to the bariatric surgery cohort, as previously described.[11]

Demographic, anthropometric, and clinical features of the LBC (n = 1,311) stratified according to the *KLB* rs12152703 G>T variant are reported in Table 1. As expected, BMI paralleled the presence of the minor T allele ($p = 0.012$). Circulating aminotransferase levels (log-transformed ALT, aspartate aminotransferase [AST], and gamma-glutamyltransferase [GGT]) were reduced in patients who carry the variant (p <0.01 at one-way ANOVA for all comparisons).

In multivariate generalized linear models, the T minor allele was associated with lower ALT (β = −0.04; 95% CI −0.08 to −0.006; $p = 0.02$), AST (β = −0.03; 95% CI −0.06 to −0.006; $p = 0.017$), and GGT levels (β = −0.11; 95% CI −0.17 to −0.05; $p = 0.0002$), after adjustment for sex, age, BMI, type 2 diabetes, and the PNPLA3 p.I148M, TM6SF2 p.E167K, and *MBOAT7* rs641738 variants using a dominant genetic model (Table 1).

Next, we assessed the association between the *KLB* rs12152703 T allele and histological features of MASLD. In ordinal regression analyses adjusted as above, carriage of the *KLB* minor T allele was associated with lower grades of steatosis (β = −0.14; 95% CI −0.25 to −0.02; $p = 0.018$), lobular inflammation (β = −0.15; 95% CI −0.26 to −0.03; $p = 0.01$), and NAFLD activity score (NAS) (β = −0.15; 95% CI −0.26 to −0.04; $p = 0.005$) (Table 2). Consistently, the T allele was associated with reduced odds of MASH (odds ratio 0.70; 95% CI 0.54–0.90; $p = 0.006$), whereas no associations were observed with hepatocellular ballooning or fibrosis (Table 2). Notably, these findings remained robust after additional adjustment for the *KLB* rs17618244 at-risk variant (Table 2) and after stratification by disease severity (Table S4).

We previously showed that the *KLB* rs17618244 variant is associated not only with fibrosis but also with lobular inflammation and cirrhosis when patients are stratified by obesity status.[7] Therefore, we investigated whether obesity modifies the protective effect of the *KLB* rs12152703 T allele on liver damage. After stratifying patients by obesity status (non-obese, n = 553; obese, n = 758), the associations between the T allele and lobular inflammation (β = −0.21; 95% CI −0.35 to −0.06; $p = 0.005$), NAS (β = −0.15; 95% CI −0.29 to −0.015; $p = 0.03$), and MASH (odds ratio 0.65; 95% CI 0.46–0.91; $p = 0.01$) remained significant only in obese patients, whereas the association with steatosis was no longer observed (Table 3).

Overall, these findings suggest a possible protective effect of the *KLB* rs12152703 variant on non-invasive markers and MASH histological features, with a major effect in patients affected by obesity. The protection conferred by the *KLB* rs12152703 variant on liver damage was confirmed in both adult and pediatric patients with MASLD (Tables S5-S9 and supplementary results).

The *KLB* rs12152703 gene variant positively impacts hepatic and circulating KLB levels

We previously demonstrated that the *KLB* variant rs17618244 was related to an increased risk of developing severe liver damage in both adults and children by reducing circulating and hepatic KLB.[6,7] To discriminate whether the protective effect of the *KLB* rs12152703 mutation is mediated by alterations in KLB levels, we evaluated hepatic *KLB* mRNA levels in a subset of 167 patients with obesity belonging to the LBC. Transcriptomic data were available for these patients, who were stratified according to the *KLB* rs12152703 genotype (GG: n = 71; GT: n = 64; and TT: n = 32). The hepatic expression of KLB was enhanced in obese patients who carried the rs12152703 T allele (p <0.0001 at one-way ANOVA) (Fig. 1A). In addition, we confirmed the increased expression of KLB in the liver of patients carrying the variants after adjusting the analysis for age, sex, BMI, and T2D (beta: 99.91; 95% CI 24.8–175; $p = 0.009$). To further elucidate the impact of the variant on steatosis and lobular inflammation, we performed a differential gene expression analysis using the DESeq2 package, stratifying patients according to the presence of the *KLB* rs12152703 variant. Data were adjusted for potential confounding factors (age, sex, BMI, T2D, and batch effect). We then specifically tested, using gene set-enrichment analysis (GSEA), the enrichment of two biologically relevant pathways, namely lipid metabolism (including 123 genes) and inflammatory signaling

Table 1. Demographic, anthropometric, and clinical features of the liver biopsy cohort (N = 1,311) stratified by *KLB* rs12152703 G>T genotype.

	GG (n = 731)	GT (n = 452)	TT (n = 128)	*p* value
Sex, M	437 (59.8)	246 (54.5)	56 (43.7)	**0.009**
Age, years	40.8 ± 18.8	41.1 ± 18.1	44.8 ± 16.8	0.36
BMI, kg/m^2	32.3 ± 8.7	32.6 ± 8.8	36.6 ± 8.8	**0.012**
IFG/T2D, yes (%)	183 (25.1)	102 (22.5)	25 (19.8)	0.20
Glucose, mg/dl	102.1 ± 32.3	98.03 ± 24.2	97.7 ± 21.2	0.07*
HOMA-IR	4.7 ± 6.30	4.40 ± 3.20	4.50 ± 2.70	0.13*
Total cholesterol, mmol/L	4.9 ± 1.10	4.9 ± 1.10	4.8 ± 1.00	0.25*
LDL cholesterol, mmol/L	3.0 ± 0.99	3.00 ± 0.94	2.9 ± 0.89	0.60*
HDL cholesterol, mmol/L	1.26 ± 0.35	1.25 ± 0.35	1.28 ± 0.33	0.34*
Triglycerides, mmol/L	1.5 ± 0.86	1.5 ± 1.04	1.36 ± 0.65	0.55*
ALT, IU/L	33 {20-58}	31 {20-49}	26 {18-39}	**0.02***
AST, IU/L	26 {20-38}	25 {19-35}	21 {17-29}	**0.017***
GGT, IU/L	29 {17-59}	25 {15-55}	22 {16-39}	**0.0002***

Values are reported as mean ± SD, number (%) or median {IQR}, as appropriate. ALT, AST, and GGT values were log-transformed before the analyses. Characteristics of participants were compared across rs12152703 genotypes using generalized linear model (for continuous characteristics, *i.e.* aminotransferases) or nominal logistic regression model (for categorical characteristics).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; IFG, impaired fasting glucose; KLB, Klotho-beta; T2D, type 2 diabetes.

*Models were adjusted for sex, age, BMI, IFG (defined as fasting glucose >110 mg/dl)/T2D, PNPLA3 p.I148M, TM6SF2 p.E167K and *MBOAT7* rs641738 T alleles (dominant model). Bold values indicate significant associations.

Table 2. Associations between the *KLB* rs12152703 variant and the independent predictors of liver damage in patients from the liver biopsy cohort (n = 1,311).

	Steatosis			Lobular inflammation			Ballooning			Fibrosis			NAS			MASH		
	β	95% CI	*p* value	β	95% CI	*p* value	β	95% CI	*p* value	β	95% CI	*p* value	β	95% CI	*p* value	OR	95% CI	*p* value
Age, years	-0.01	-0.02- -0.007	**<0.0001**	-0.005	-0.01-0.001	**0.13**	-0.002	-0.003- -0.02	**<0.0001**	0.01	0.005-0.02	**0.0006**	-0.02	-0.02- 0.01	**<0.0001**	0.99	0.98-1.00	**0.03**
Sex, M	0.21	0.09-0.33	**0.0006**	0.18	0.06-0.31	**0.003**	0.20	0.06-0.35	**0.005**	0.25	0.13-0.37	**<0.0001**	0.24	0.13- -0.36	**<0.0001**	1.65	1.25-2.15	**0.0003**
BMI, kg/m^2	0.03	0.02-0.05	**<0.0001**	0.02	0.0008-0.03	**0.04**	-0.03	-0.05- -0.014	**0.0003**	-0.01	-0.02- -0.001	**0.03**	0.01	-0.003-0.02	**0.12**	1.02	1.0-1.03	**0.01**
IFG/T2D, yes	0.34	0.21-0.47	**<0.0001**	0.34	0.21-0.47	**<0.0001**	0.49	0.35-0.64	**<0.0001**	0.63	0.50-0.76	**<0.0001**	0.43	0.31-0.56	**<0.0001**	2.60	1.90-3.54	**<0.0001**
PNPLA3, p.I148M	0.43	0.27-0.59	**<0.0001**	0.33	0.17-0.47	**<0.0001**	0.17	-0.005-0.35	**0.05**	0.43	0.27-0.59	**<0.0001**	0.38	0.22-0.54	**<0.0001**	1.63	1.37-1.95	**<0.0001**
TM6SF2, p.E167K	0.73	0.42-1.03	**<0.0001**	0.39	0.10-0.68	**0.007**	0.06	-0.26-0.38	0.70	0.31	0.02-0.60	**0.003**	0.55	0.28-0.83	**<0.0001**	1.72	1.23-2.46	**0.002**
MBOAT7 rs641738 T	0.1	-0.06-0.25	0.20	0.07	-0.08- 0.22	0.38	-0.004	-0.18-0.17	0.96	0.08	-0.07-0.23	0.27	0.08	-0.06-0.22	0.26	1.14	0.96- 1.35	0.13
KLB, rs12152703 T yes	-0.14	-0.25- -0.02	**0.018**	-0.15	-0.26- -0.03	**0.01**	-0.06	-0.20-0.06	0.33	-0.07	-0.19-0.03	0.19	-0.15	-0.26- -0.04	**0.005**	0.70	0.54-0.90	**0.006**

Values were obtained at multivariate ordinal (for steatosis, lobular inflammation, ballooning, fibrosis and NAS) or nominal (for MASH) regression analysis adjusted for sex, age, BMI, IFG (defined as fasting glucose>110 mg/dl), T2D, and the PNPLA3 p.I148M, TM6SF2 p.E167K and *MBOAT7* rs641738 T alleles (dominant model). Additional adjustment for the *KLB* rs17618244 A allele yielded the following associations: steatosis (β = –0.13; 95% CI –0.25 to –0.02; $p = 0.015$), lobular inflammation (β = –0.16; 95% CI –0.27 to –0.04; $p = 0.008$), fibrosis (β = –0.07; 95% CI –0.18 to 0.03; $p = 0.19$), ballooning (β = –0.06; 95% CI –0.20 to 0.06; $p = 0.31$), NAS (β = –0.15; 95% CI –0.26 to –0.05; $p = 0.004$), and MASH (OR, 0.69; 95% CI 0.54–0.84; $p = 0.005$).

Bold values indicate significant associations.

IFG, impaired fasting glucose; KLB, Klotho-beta; MASH, metabolic dysfunction-associated steatohepatitis; NAS, NAFLD activity score; OR, odds ratio; T2D, type 2 diabetes.

Table 3. Associations between the *KLB* rs12152703 variant and the independent predictors of liver damage in 1,311 patients with biopsy-proven MASLD stratified according to the presence of obesity (n = 553 no obese patients and n = 758 obese patients).

	Steatosis			Lobular inflammation			Ballooning			Fibrosis			NAS			MASH		
	β	95% CI	*p* value	β	95% CI	*p* value	β	95% CI	*p* value	β	95% CI	*p* value	β	95% CI	*p* value	OR	95% CI	*p* value
Obesity no (n = 553)																		
KLB, rs12152703 T yes	-0.17	-0.36- 0.009	0.06	-0.03	-0.21-0.15	0.73	-0.006	-0.19-0.20	0.95	-0.13	-0.30-0.05	0.16	-0.12	-0.29- 0.05	0.15	0.80	0.54-1.20	0.28
Obesity yes (n = 758)																		
KLB, rs12152703 T yes	-0.08	-0.22-0.06	0.26	-0.21	-0.35- -0.06	**0.005**	-0.10	-0.28-0.07	0.25	-0.04	-0.19-0.11	0.60	-0.15	-0.29- -0.015	**0.03**	0.65	0.46-0.91	**0.01**

Values were obtained at multivariate ordinal (for steatosis, lobular inflammation, ballooning, fibrosis and NAS) or nominal (for MASH) regression analysis adjusted for sex, age, BMI, IFG (defined as fasting glucose >110 mg/dl), T2D, and the PNPLA3 p.I148M, TM6SF2 p.E167K and *MBOAT7* rs641738 T alleles (dominant model).

Bold values indicate significant associations.

IFG, impaired fasting glucose; KLB, Klotho-beta; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction–associated steatotic liver disease; NAS, NAFLD activity score; OR, odds ratio; T2D, type 2 diabetes.

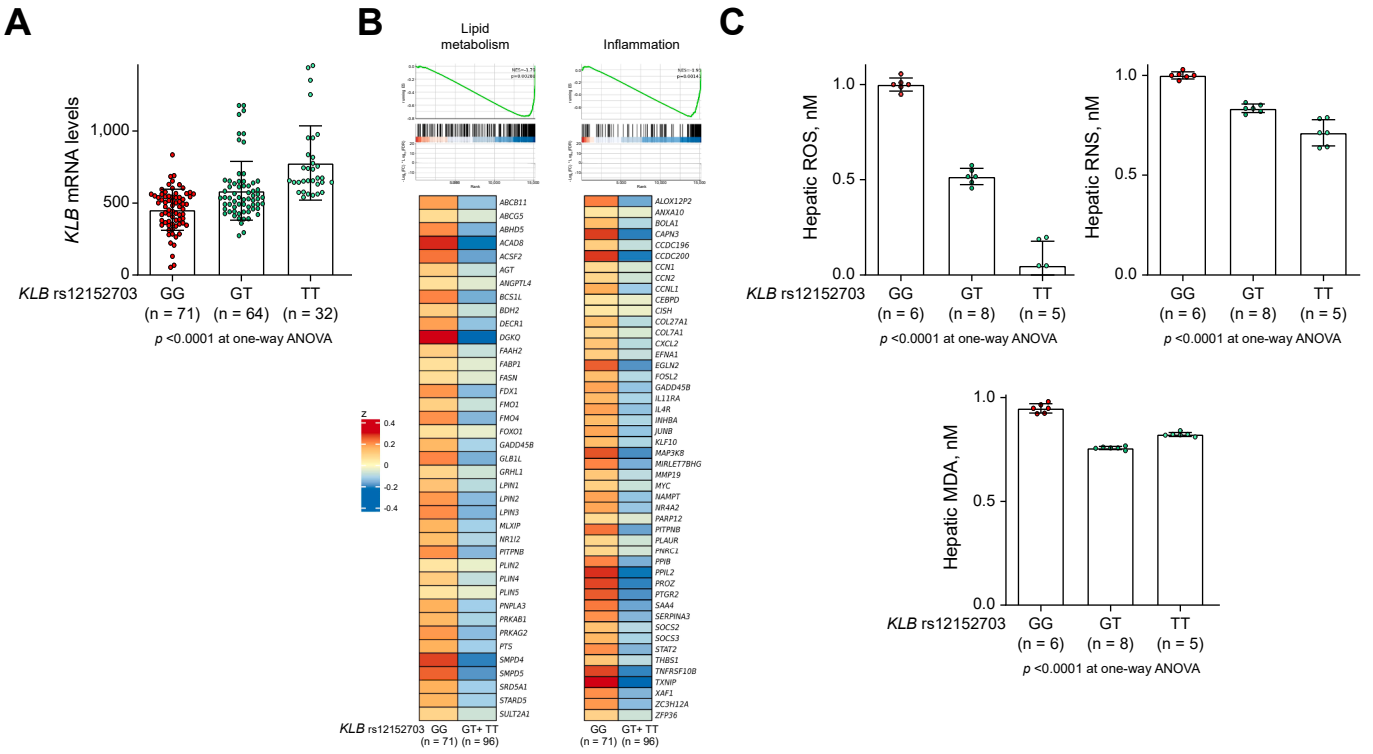


Fig. 1. Hepatic *KLB* mRNA levels were higher in patients with MASLD carrying the rs12152703 mutation. We evaluated *KLB* gene expression through transcriptome analysis of liver biopsies (n = 167) stratified according to the presence of the rs12152703 variant (GG, n = 71; GT, n = 64; TT, n = 32). Data are expressed as data points and SD. $p = 0.0001$ by one-way ANOVA (A). Running ES through the list of genes ranked by decreasing $-\log_{10}(p)*\log_2(FC)$, that is, from upregulated genes to downregulated genes (TT *vs.* GT+TT). Average relative expression (z) of the GSEA "leading edge" genes (those that contributed most to the gene set score) that belong to lipid metabolism (left) and inflammatory (right) pathways in 167 patients with MASLD stratified according to the presence of the rs12152703 variant (GG, n = 71; GT+TT, n = 96). The red shading represented induction, whereas the blue shading indicated repression. Z-scores were calculated from the values obtained by applying DESeq2 variance stabilizing transformation to gene counts (B). We evaluated oxidative stress by measuring dichlorodihydrofluorescein ROS/RNS levels, while lipid peroxidation was calculated by MDA production in homogenized liver samples (GG, n = 6; GT, n = 8; TT, n = 5). An equal amount of tissue homogenates were pooled, and all reactions were performed in triplicate. Three independent measurements were conducted. Data are expressed as data points and SD. p <0.0001 by one-way ANOVA for all comparisons (C). ES, enrichment score; FC, fold change; GSEA, gene set enrichment analysis; KLB, β-Klotho; MASLD, metabolic dysfunction–associated steatotic liver disease; MDA, malondialdehyde; ROS, reactive oxygen species; RNS, reactive nitrogen species.

(including 211 genes) in genes ranked by their differential expression between GG and GT/TT genotypes. Both pathways were significantly enriched in genes that are downregulated in carriers of the variant (GSEA; normalized enrichment score = -1.79, false discovery rate = 0.003 and normalized enrichment score = -1.95, false discovery rate = 0.001, respectively) (Fig. 1B).

In a subgroup of these patients (GG: n = 6; GT: n = 8; and TT: n = 10), negative for the rs17618244 at-risk variant and with a comparable NAS, we measured hepatic reactive oxygen species and reactive nitrogen species and the aldehyde derivatives (*i.e.* malondialdehyde), a product of lipid peroxidation of cell membranes, and we highlighted that they were all attenuated in the presence of the protective rs12152703 T allele (p <0.0001 at one-way ANOVA for all comparisons) (Fig. 1C). These results corroborate a reduced inflammatory milieu, independently of disease severity (see supplementary materials and methods).

To consolidate the above-described results, we also isolated primary hepatocytes from 25 patients with MASLD not belonging to the LBC who underwent hepatic resection or liver transplantation (GG: n = 13; GT/TT: n = 12; all patients were negative for the rs17618244 at-risk variant). *KLB* mRNA levels were significantly higher in carriers of the rs12152703 T allele compared to non-carriers ($p = 0.019$ at two-tailed Student's t test) (Fig. S1A).

To confirm these findings in the pediatric population, we assessed *KLB* expression in hepatic biopsies of children with MASLD, stratified by the *KLB* genotype (GG: n = 16; GT: n = 8; and TT: n = 4), and we found significantly increased *KLB* mRNA levels in individuals carrying the rs12152703 T allele (p <0.0001 by one-way ANOVA) (Fig. 2A). Next, we performed immunofluorescence on hepatic specimens obtained from the pediatric cohort (GG: n = 40; GT: n = 30; TT: n = 12), and we observed increased KLB protein expression in the presence of the T allele (p <0.0001 by one-way ANOVA) (Fig. 2B).

The analysis of linkage disequilibrium and studies regarding chromatin/TF binding annotations from ENCODE, RegulomeDB, and HaploReg publicly available datasets showed that the rs12152703 variant, along with the others included in the same haplotype, may alter the chromatin state, regulatory motifs, and chip-seq peaks, thereby possibly affecting KLB expression (Table S10 and supplementary results).

Finally, to investigate whether the enhanced hepatic KLB expression translated into higher circulating concentrations, we measured serum β-klotho levels in a subset of the LBC cohort (GG: n = 137; GT: n = 78; and TT: n = 21) and observed significantly elevated circulating KLB in patients carrying the

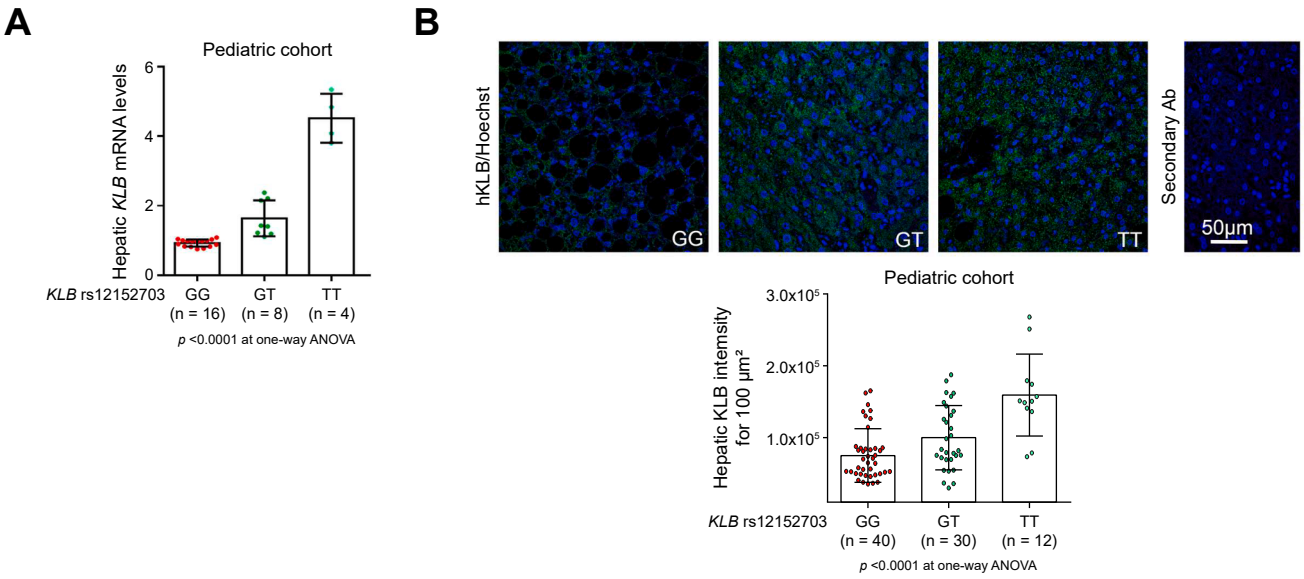


Fig. 2. Hepatic expression of Klotho-beta protein was enhanced in pediatric patients with MASLD carrying the rs12152703 mutation. *Klotho-beta* mRNA levels were assessed in 28 hepatic biopsies of pediatric patients with MASLD by qRT-PCR (GG, n = 16; GT, n = 8; TT, n = 4) and normalized to beta-actin as a housekeeping gene (A). KLB protein levels were measured by quantitative imaging of fluorescence (upper panel) on 2 µm-thick liver sections obtained from 82 children with MASLD (GG, n = 40; GT, n = 30; TT, n = 12). The staining of KLB is shown in green, whereas nuclei were colored by Hoechst staining in blue (lower panel). Magnification 40×, scale bar 50 µm (B). Data are expressed as data points and SD. *p* <0.0001 by one-way ANOVA for all comparisons. KLB, β-Klotho; MASLD, metabolic dysfunction–associated steatotic liver disease; qRT-PCR, quantitative reverse-transcription PCR.

variant (*p* <0.0001 at one-way ANOVA) (Fig. 3A). In the serum of the same patients with MASLD, we also quantified the pro-inflammatory cytokines IL1β, TNFα, and IL6 (GG: n = 145; GT: n = 86; and TT: n = 21), which were strongly reduced in individuals carrying the *KLB* T allele (*p* <0.0001 at one-way ANOVA for all comparisons) (Fig. 3B-D). These associations were also significant when adult and pediatric MASLD cohorts were considered separately (Figs S1,2).

KLB overexpression affects lipid accumulation in HepG2 cells

To gain insight into the protective role of the hepatic KLB protein in MASLD pathogenesis, we established an *in vitro* model in which the *KLB* gene was transiently overexpressed in HepG2 cells (Fig. S3A-C). As shown in Fig. 4A,B, 30 min of exposure to 40 ng/ml of FGF19 significantly increased FGFR4 activation/phosphorylation, as assessed by the pFGFR4/FGFR4 ratio, with an even greater effect in KLB-overexpressing cells. Similarly, the pERK/ERK ratio was significantly increased by FGF19 treatment and maintained by *KLB* overexpression (Fig. 4C,D). Of note, *KLB* upregulation can, *per se*, activate downstream pFGFR4/ERK signaling, suggesting a role for KLB in hepatic cells independent of its ligand FGF19.

Next, we further explored the anti-steatogenic effect of *KLB* overexpression by treating HepG2 cells with a mixture of free fatty acids (FFAs) for 24 h. Both *in vitro* fluorescence imaging and quantitative analysis showed that overexpressing *KLB* significantly attenuated FFA-induced lipid accumulation in HepG2 cells (Fig. 4E and Fig. S3D). The anti-steatogenic effect of *KLB* overexpression (Fig. S3E) may be explained by its

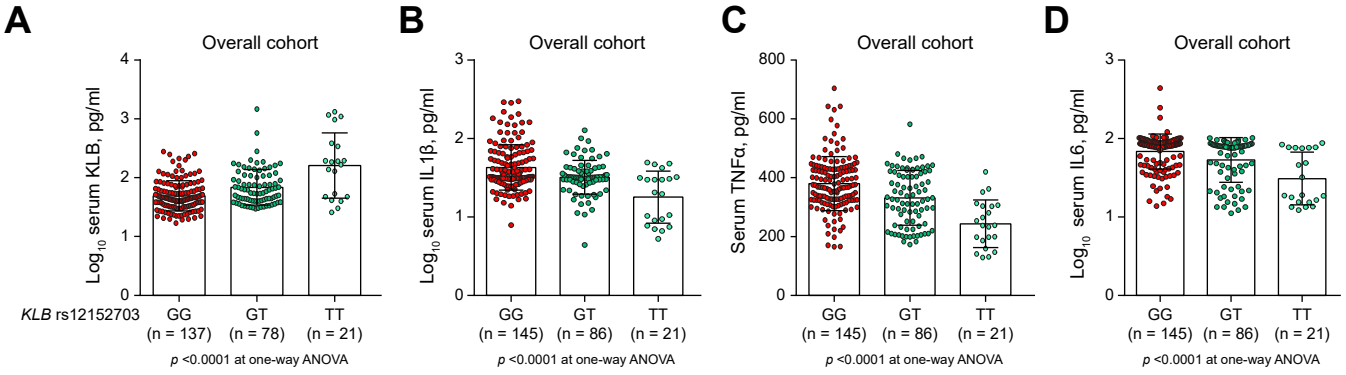


Fig. 3. The rs12152703 mutation improved circulating levels of Klotho-beta protein and ameliorated the pro-inflammatory microenvironment in patients with MASLD. Klotho-beta (A) serum levels were measured using an ELISA kit in 236 patients with MASLD belonging to the LBC (GG, n = 137; GT, n = 78; TT, n = 21). Circulating IL1b (B), TNFa (C), and IL6 (D) were evaluated by ELISA kits in 252 patients with MASLD belonging to the LBC (GG, n = 145; GT, n = 86; TT, n = 21). Data are expressed in pg/ml as data points and SD. All reactions were performed in duplicate. *p* <0.0001 by one-way ANOVA for all comparisons. Skewed values were log transformed before the analyses. LBC, liver biopsy cohort; MASLD, metabolic dysfunction–associated steatotic liver disease.

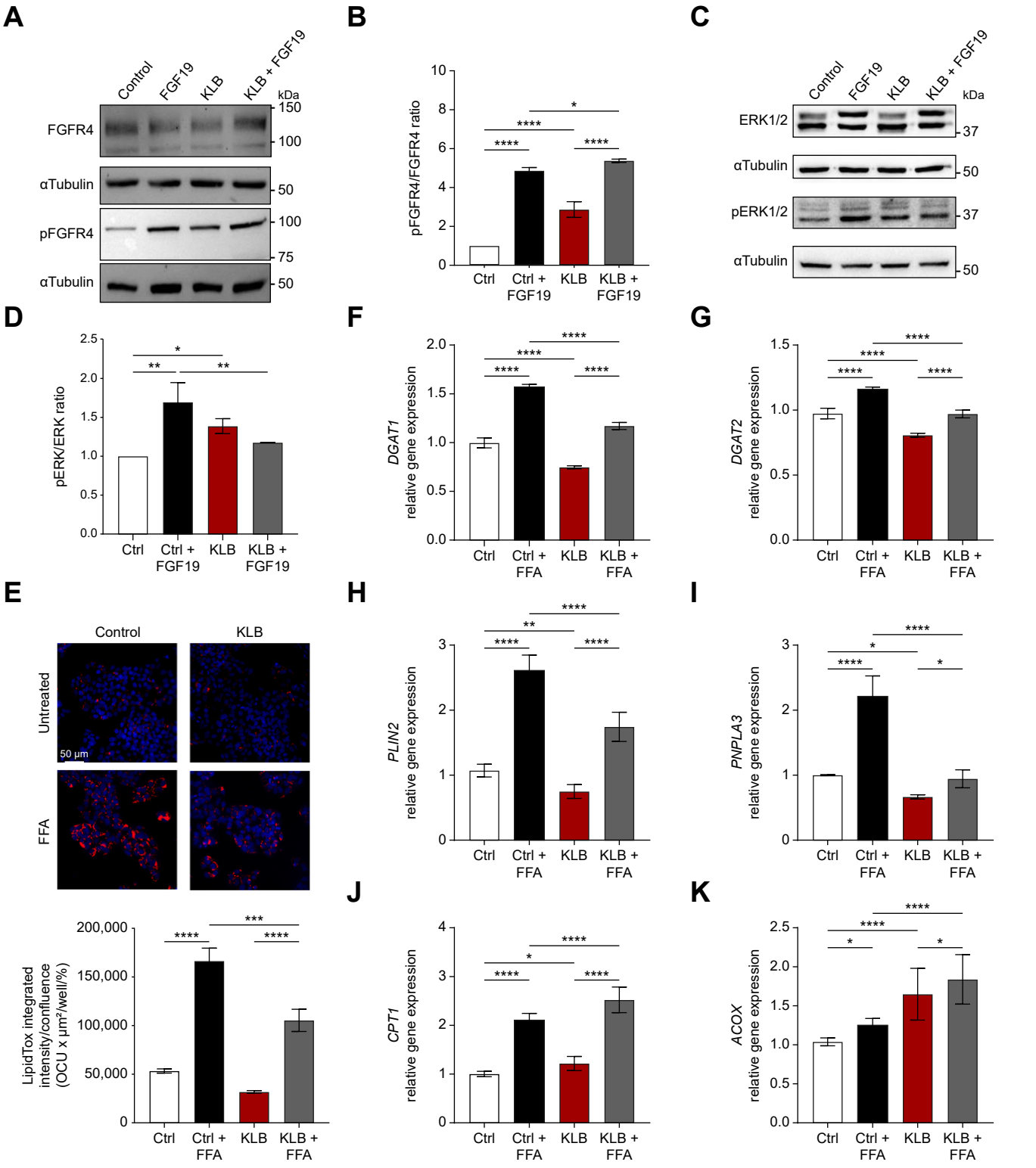


Fig. 4. The overexpression of KLB in HepG2 cells protects against fat accumulation. HepG2 control cells (Control) and KLB-overexpressing cells (KLB) were treated with 40 ng/ml FGF19 for 30 min. Protein levels of total FGFR4 and phosphorylated FGFR4 at Y642 (pFGFR4) were determined by WB analysis (A) and normalized to αTubulin (B). Protein levels of total ERK1/2 and phosphorylated ERK at Thr202/Tyr204 (pERK1/2) were determined by WB (C) and normalized to αTubulin (D). Control and KLB HepG2 cells were treated with 400 μM FFAs. We assessed lipid accumulation by LipidTOX and Hoechst staining, capturing lipid droplets in red and nuclei in blue (E, upper panel), and quantifying red fluorescence intensity as integrated intensity normalized to the cell phase confluency by Incucyte (E, lower panel). Magnification 40×, scale bar 50 μm. *DGTA1* (F), *DGTA2* (G), *PLIN2* (H), *PNPLA3* (I), *CPT1A* (J), and *ACOX* (K) mRNA levels were determined by qRT-PCR (normalized to GAPDH as a housekeeping gene). Statistical differences between groups were calculated using one-way ANOVA with Tukey's multiple comparisons test. Data are presented as the mean ± SD of at least three independent experiments and two or more biological replicates. **p* <0.05, ***p* <0.01, ****p* <0.001, and *****p* <0.0001. FFA, free fatty acid; KLB, Klotho-beta; qRT-PCR, quantitative reverse transcription PCR; WB, western blot.

ability to modulate genes involved in lipid metabolism, thereby corroborating the transcriptomic findings in patients with MASLD (Fig. 1B, Table S11). Indeed, we showed that *DGTA1*, *DGTA2*, *PLIN2,* and *PNPLA3* genes, involved in triglyceride synthesis and lipid droplet remodeling, were upregulated by FFAs and downregulated by *KLB* overexpression (Fig. 4F-I). On the contrary, *CPT1A* and *ACOX1* genes were upregulated by FFA treatment and *KLB* overexpression (Fig. 4J,K). Finally, *KLB* overexpression in HepG2 cells was ineffective in counteracting the FFA-dependent upregulation of *ACSL1*, *SREBP1*, *FASN*, *CD36*, and *MTTP* (Fig. S3F-J), as well as *ACC* and *SCD1* genes (Fig. S3K,L).

KLB overexpression in HepG2 cells affects the expression of inflammatory genes

Since the *KLB* rs12152703 variant was associated with reduced hepatic inflammation in patients with MASLD, we investigated whether *KLB* overexpression in HepG2 cells exerted anti-inflammatory effects. We first measured the expression of key proinflammatory genes in lipid-loaded HepG2 cells. As shown in Fig. 5A,B, we found that *KLB* overexpression reduced the FFA-mediated upregulation of *IL1β* and *TNFα* transcripts.

Next, we investigated the anti-inflammatory response in HepG2 cells overexpressing *KLB* under lipopolysaccharide (LPS) treatment (1 μg/ml). The LPS-dependent expression of *IL1β* and *TNFα* was attenuated by KLB upregulation (Fig. 5C,D), with the consequent reduction of pNF-κB(p65) nuclear translocation (Fig. S4).

Finally, we examined a panel of 607 inflammatory genes to identify additional targets potentially influenced by *KLB* overexpression. We outlined 182 significantly dysregulated genes across the four groups: Control, Control+LPS, KLB, and KLB+LPS (Table S12). Reactome pathway analysis revealed that these genes are mainly involved in cytokine and interleukin signaling, chemokine–receptor interactions, and TNF activation. Out of 182 genes, 78 were significantly upregulated in the Control+LPS group (Fig. 5E, Table S13), and the effect was attenuated in 64 genes by *KLB* overexpression (Fig. 5F, Table S14). In particular, KLB counteracted the LPS-induced upregulation of *IL1β* and *TNFα*, consistent with the reduced expression of these genes observed in both adult and pediatric patients carrying the T allele. In contrast, only 26 genes were significantly downregulated in the Control+LPS group (Fig. 5G, Table S15), and KLB reversed the transcriptional changes of 15 of these genes (Fig. 5H, Table S16).

Discussion

MASLD affects over 38% of the global population, including adults and children, since it is strongly intertwined with obesity, T2D, and metabolic syndrome.[1,2] Nonetheless, few novel therapeutic approaches have been approved for the treatment of MASH in clinical trials, and a pharmacological consensus for its management has not been reached yet. The present study demonstrated, for the first time, that the rs12152703 variant in the *KLB* gene mitigated liver inflammation in both adult and pediatric patients with MASLD, thereby enhancing the expression of KLB, which complexes with hepatic FGFR4 and serves as its obligate co-receptor for the FGF19 ligand. We corroborated the beneficial effects of *KLB* overexpression in *in vitro* models that mimic MASLD. Our findings were in line with our previous studies[6,7] in which we determined the involvement of KLB in modulating lipid metabolism and inflammation, two key processes that drive the progression toward advanced MASLD.

We demonstrated in patients with MASLD that the carriage of the minor T allele confers protection against elevated aminotransferase levels and histological liver damage. Indeed, in the LBC cohort, comprising 1,311 patients with MASLD, including 261 children, the rs12152703 variant was associated with a reduction in liver enzymes (*i.e*. AST, ALT, and GGT), along with decreased steatosis, lobular inflammation, and a lower risk of developing MASH. These associations remained significant even after adjusting for the main confounding factors, including obesity, T2D, and well-established genetic risk factors (*i.e*. p.I148M PNPLA3, p.E167K TM6SF2 and rs641738 *MBOAT7*). The positive impact of the variant on inflammation has been confirmed, even after separate analyses of subgroups of adults and children/adolescents with MASLD. We observed that the protective association of the *KLB* rs12152703 variant was more evident in younger individuals, including children and patients <30 years, whereas it appeared attenuated in adults ≥30 years (data not shown).

Moreover, the protective role of the rs12152703 mutation was unmasked when the LBC cohort was stratified by disease severity. Indeed, we found a significant association between the polymorphism and steatosis, MASH, NAS, and fibrosis in patients with NAS lower than 4, whereas the correlation with lobular inflammation was confirmed in both groups (NAS <4 and ≥4). These findings suggest that age and disease severity, which are strongly correlated in MASLD, may influence the phenotypic expression of the variant. A plausible explanation is that in long-standing disease, the beneficial effect of increased KLB expression may be partially overridden by cumulative metabolic injury and fibrogenic remodeling, thereby diminishing the impact of the genotype. We therefore clarify that the attenuation of associations observed in the pediatric cohort (n = 261) was largely attributable to limited sample size and differences in comorbidity distribution, whereas the attenuation seen in adults reflects an age-related modulation of the variant's effect. Taken together, these results underscore that the protective influence of rs12152703 is strongest earlier in the disease course and becomes progressively less pronounced with increasing age and disease severity.

These results conflict with those obtained by Ji *et al.*,[11] who observed a damaging effect of the rs12152703 variant. However, several differences in population characteristics, methodology, and outcome definitions distinguish the two studies. Firstly, our cohort comprises pediatric and adult patients with MASLD of European ancestry (*i.e*. European Non-Finnish from 1000 Genomes) with a minor allele frequency of 22%, whereas it is 8.8% in the East Asian population described by Ji *et al.*.[11] Secondly, in our cohorts, MASLD was diagnosed via liver biopsy, allowing precise evaluation of the variant's impact on histological features. Finally, lifestyle habits and gene-environment interactions may also contribute to the discrepancies between the two studies.

Notably, the protective effect of the rs12152703 variant was more pronounced in obese individuals, suggesting a potential interaction between obesity and KLB expression. In this subgroup, the minor T allele was enriched and significantly

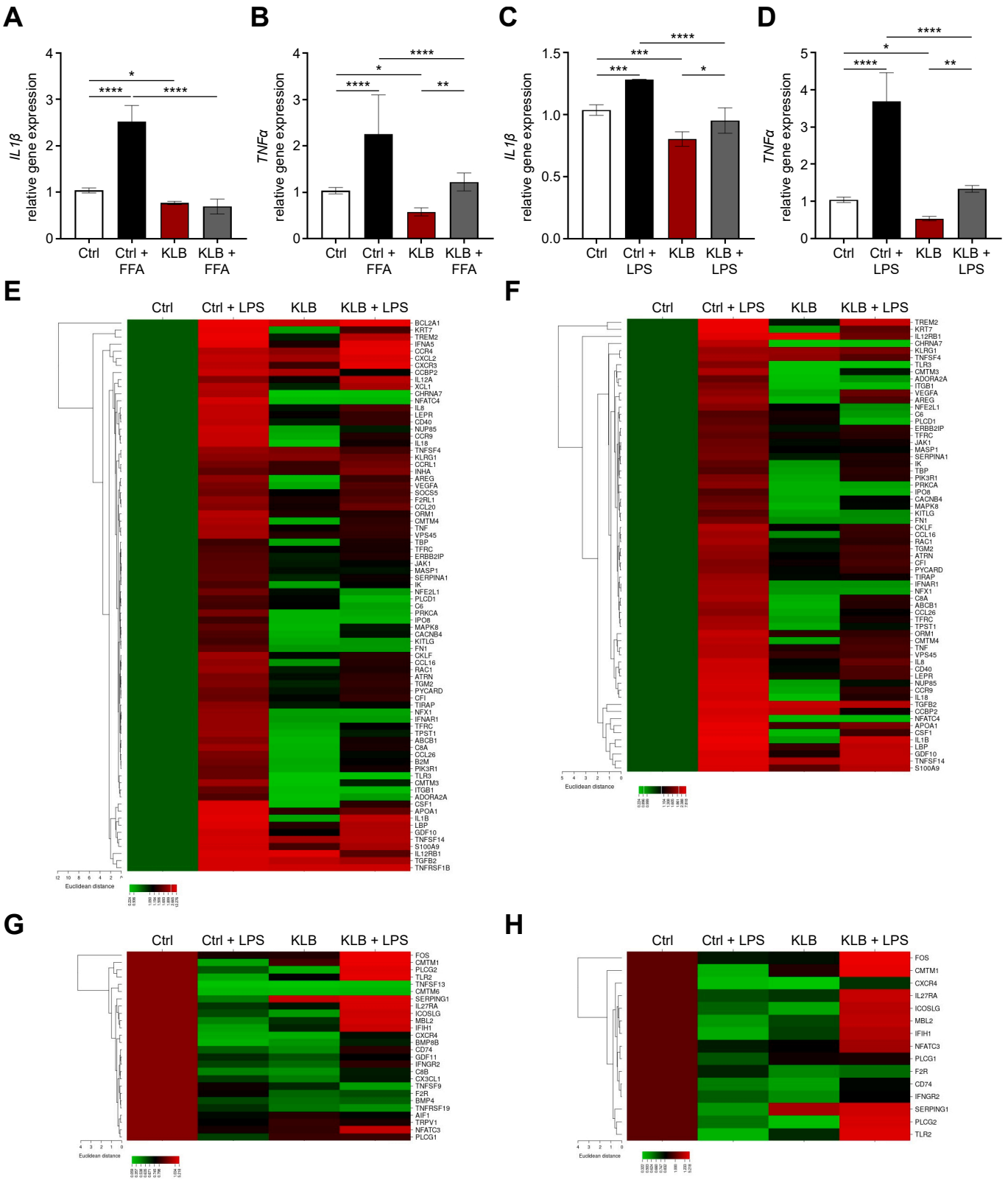


Fig. 5. The overexpression of KLB in HepG2 cells decreased FFA- and LPS-dependent pro-inflammatory responses. Control and KLB cells were treated with 400 μM of FFAs for 24 h. *IL1β* (A) and *TNFα* (B) mRNA levels were determined by qRT-PCR (normalized to GAPDH). (C–H) Control and KLB cells were treated with 1 μg/ml of LPS for 24 h. *IL1β* (C) and *TNFα* (D) mRNA levels were determined by qRT-PCR (normalized to GAPDH). Statistical differences between groups were calculated using one-way ANOVA with Tukey's multiple comparisons test. Data are presented as the mean±SD of at least three independent experiments and two or more biological replicates. **p* <0.05, ***p* <0.01, ****p* <0.001, and *****p* <0.0001. Heatmaps showing the 78 significantly upregulated genes by LPS (E), the 64 significantly upregulated genes by LPS and reversed by overexpression of KLB (F), the 26 significantly downregulated genes by LPS (G), and the 15 significantly downregulated genes by LPS reversed by overexpression of KLB (H). Upregulation and downregulation are indicated by red and green colors, respectively. FFA, free fatty acid; KLB, Klotho-beta; LPS, lipopolysaccharide; qRT-PCR, quantitative reverse transcription PCR.

associated with reduced lobular inflammation and a lower risk of developing MASH. In contrast, its protective effect against steatosis was lost, likely due to the dominant influence of obesity on hepatic fat accumulation. Consistently, we observed a significant interaction between obesity and the variant in modulating lobular inflammation, the histological feature most affected by rs12152703; however, this association was no longer significant after further adjustment for confounding factors (data not shown).

Furthermore, transcriptomic analyses revealed that the *KLB* rs12152703 variant influenced lipid metabolism by downregulating genes involved in *de novo* lipogenesis and upregulating, in turn, those implicated in fatty acid beta-oxidation. These genes were similarly modulated by *KLB* overexpression in an *in vitro* model of MASLD, thereby confirming KLB's ability to reduce lipid loading.

The nexus between obesity and KLB expression was also supported by our previously reported studies,[6,7] in which even the effect of the at-risk *KLB* rs17618244 variant was more evident in obese individuals. In addition, experimental evidence has demonstrated the protective role of KLB overexpression in adipose tissue in diet-induced obesity[13] and the inverse association between KLB depletion in adipose tissue and adiposity in mice.[14] Hence, it may be postulated that the effect size of the *KLB* variants was amplified by increased adiposity, and these polymorphisms may serve as genetic biomarkers for MASLD risk stratification, especially in patients with overweight/obesity who are at high risk of progressive MASLD.

Interestingly, unlike the at-risk *KLB* rs17618244 variant, which downregulated KLB expression, patients carrying the protective T allele of rs12152703 showed increased hepatic and circulating KLB levels and a reduction of hepatic oxidative stress. The increase in hepatic and serum KLB levels, due to the presence of the minor T allele, was also coupled with reduced circulating pro-inflammatory cytokines, further supporting the favorable influence of this mutation on low-grade systemic inflammation, in line with other previous evidence.[10] Accordingly, our *in vitro* data support the role of KLB overexpression in regulating several pro-inflammatory genes, underscoring the need to further investigate its contribution as an inflammatory regulator.

Overall, our results highlight the impact of the intronic rs12152703 variant on *KLB* gene and protein expression, conferring protection against MASH. This is consistent with a recent meta-analysis reporting a protective effect of the intronic *KLB* rs28712821 variant against cirrhosis in patients with alcohol-related liver disease.[15] Although observed in a different disease context, these findings support the concept that intronic variants – potential binding sites for transcription factors or targets of epigenetic regulation – can modulate KLB expression and, consequently, liver damage. This hypothesis is further supported by evidence that KLB expression is regulated by epigenetic mechanisms in obesity-related MASLD.[16]

Our study has some limitations. We did not identify a significant eQTL (expression quantitative trait locus) for the rs12152703 variant in the GTEx portal for adult livers. However, GTEx includes gene expression and genetic data from approximately 1,000 deceased individuals of diverse ancestries, with unreported liver histology, BMI ranging from 18.5–35, and ages 21–70 years. On the contrary, our transcriptomic data were derived from European individuals who underwent liver biopsy during bariatric surgery (BMI >35). Thus, we cannot rule out that liver damage and obesity may affect KLB expression by interacting with the rs12152703 variant. Another limitation is the lack of association between the rs12152703 variant and fibrosis, which is the primary determinant of long-term outcomes in MASLD. By stratifying the entire cohort according to disease severity, the mutation was also protective against fibrosis in patients with a NAS value <4.

In summary, the rs12152703 variant attenuated liver damage, particularly lobular inflammation, in both adult and pediatric patients with MASLD, effects that were associated with increased hepatic and serum KLB levels. Unlike previous studies, our work provides mechanistic insight into KLB's role through MASLD-related histological features. Moreover, our findings support the therapeutic potential of targeting the KLB/FGFs/FGFRs axis to improve MASLD and its metabolic comorbidities. They also suggest that strategies aimed at enhancing KLB expression or developing KLB-mimetic compounds may offer novel avenues for preventing or treating MASH, a critical turning point in MASLD progression.

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Health with "Current Research funds" to Anna Alisi; Ricerca Corrente 2025 to IRCCS Cà Granda Ospedale Maggiore Policlinico.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CV, coefficient of variability; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GGT, gamma-glutamyltransferase; KLB, Klotho-beta; LBC, liver biopsy cohort; LPS, lipopolysaccharide; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; NAS, NAFLD activity score; T2D, type 2 diabetes.

Financial support

This study was supported by the Italian Ministry of Health (GR-2019–12370172) to Bambino Gesù Children's Hospital, IRCCS, and IRCCS Cà Granda Ospedale Maggiore Policlinico. This work was supported also by the Italian Ministry of

Conflict of interest

The authors declare that they have no conflicts of interest.
Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study design: MM; NP; PD; AA; Statistical analysis: MM; EM; Patient recruitment: AM; ALF; MM; SB; Data collection: AM; ALF; MM; SB. Data analysis: MM; Data interpretation: MM; NP; PD; AA; Manuscript drafting: MM; NP; Manuscript revision: PD; AA. Funding acquisition: MM; NP; Supervision, and primary responsibility for final content: MM; NP; PD; AA; Read and approve the final manuscript: all authors.

Data availability

Further information and requests for resources, reagents and data should be directed to, and will be fulfilled by, the lead contact, PD (paola.dongiovanni@policlinico.mi.it).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2025.101717.

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Author names in bold designate shared co-first authorship

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Keywords: Liver Disease; genetics; MASH; gain-of-function.
Received 14 July 2025; received in revised form 10 December 2025; accepted 16 December 2025; Available online 23 December 2025