

# Low Lipoprotein(a) Levels Predict Hepatic Fibrosis in Patients With Nonalcoholic Fatty Liver Disease

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Dyslipidemia and cardiovascular complications are comorbidities of nonalcoholic fatty liver disease (NAFLD), which ranges from simple steatosis to nonalcoholic steatohepatitis, fibrosis, and cirrhosis up to hepatocellular carcinoma. Lipoprotein(a) (Lp(a)) has been associated with cardiovascular risk and metabolic abnormalities, but its impact on the severity of liver damage in patients with NAFLD remains to be clarified. Circulating Lp(a) levels were assessed in 600 patients with biopsy-proven NAFLD. The association of Lp(a) with liver damage was explored by categorizing serum Lp(a) into quartiles. The receiver operating characteristic curve was used to analyze the accuracy of serum Lp(a) in hepatic fibrosis prediction. Hepatic expression of lipoprotein A (*LPA*) and of genes involved in lipid metabolism and fibrogenic processes were evaluated by RNA sequencing in a subset of patients with NAFLD for whom Lp(a) dosage was available (n = 183). In patients with NAFLD, elevated Lp(a) levels were modestly associated with circulating lipids, carotid plaques, and hypertension ( $P < 0.05$ ). Conversely, patients with low serum Lp(a) displayed insulin resistance ( $P < 0.05$ ), transaminase elevation ( $P < 0.05$ ), and increased risk of developing severe fibrosis ( $P = 0.007$ ) and cirrhosis ( $P = 0.002$ ). In addition, the diagnostic accuracy of Lp(a) in predicting fibrosis increased by combining it with transaminases (area under the curve fibrosis stage 4, 0.87;  $P < 0.0001$ ). Hepatic *LPA* expression reflected serum Lp(a) levels ( $P = 0.018$ ), and both were reduced with the progression of NAFLD ( $P < 0.05$ ). Hepatic *LPA* messenger RNA levels correlated with those of genes involved in lipoprotein release, lipid synthesis, and fibrogenesis ( $P < 0.05$ ). Finally, transmembrane 6 superfamily member 2 (*TM6SF2*) rs58542926, apolipoprotein E (*ApoE*) rs445925, and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) rs7552841, known variants that modulate circulating lipids, may influence serum Lp(a) levels ( $P < 0.05$ ). **Conclusion:** Circulating Lp(a) combined with transaminases may represent a novel noninvasive biomarker to predict advanced fibrosis in patients with NAFLD. (*Hepatology Communications* 2021;0:1-15).

**N**onalcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disease in Western adult populations, and its prevalence has reached epidemic proportions.<sup>(1)</sup> It is closely related to a cluster of conditions, including insulin resistance (IR), obesity, type 2 diabetes mellitus (T2D), and atherogenic dyslipidemia,<sup>(2)</sup> that represent established risk factors for cardiovascular diseases (CVDs).<sup>(3,4)</sup> NAFLD encompasses liver disorders ranging from simple steatosis to the inflammatory

*Abbreviations:* ALT, alanine aminotransferase; ANOVA, analysis of variance; APO, apolipoprotein; apo(a)/(b), apolipoprotein(a)/(b); AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; CI, confidence interval; COL1A1, collagen type I alpha 1 chain; CVD, cardiovascular disease; F, fibrosis stage; FASN, fatty acid synthase; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IMT, intima-media thickness; IFG, impaired fasting glucose; IQR, interquartile range; IR, insulin resistance; IRCCS, Istituto di Ricovero e Cura a Carattere Scientifico; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); LPA, lipoprotein A; mRNA, messenger RNA; MTTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OR, odds ratio; PCSK9, proprotein convertase subtilisin/kexin type 9; PNPLA3, patatin-like phospholipase domain-containing 3; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; RNA-seq, RNA sequencing; ROC, receiver operating characteristic; SREBP1, sterol regulatory element-binding protein 1; T2D, type 2 diabetes mellitus; TGF $\beta$ , transforming growth factor beta; TM6SF2, transmembrane 6 superfamily member 2.

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form nonalcoholic steatohepatitis (NASH), which is characterized by hepatocellular degeneration and inflammation. NASH may then progress to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) and is likely to become the main indication for liver transplants in the near future.<sup>(5)</sup>

Environmental and genetic factors contribute to NAFLD pathogenesis. In the last decades, single nucleotide polymorphisms (SNPs) in genes regulating lipid handling and secretion, such as patatin-like phospholipase domain-containing 3 (PNPLA3) I148M, transmembrane 6 superfamily member 2 (TM6SF2) E167K, and rs641738 in the transmembrane channel-like 4/membrane bound  $\alpha$ -acyltransferase domain-containing 7 (*TMC4/MBOAT7*) locus, have been associated with fatty liver and its progressive forms.<sup>(6-10)</sup> Atherogenic dyslipidemia, defined by increased blood concentrations of small dense low-density lipoprotein (LDL) particles, decreased high-density lipoprotein (HDL), and increased triglycerides, has emerged as a major CVD risk factor in patients with NAFLD, particularly in those with advanced fibrosis.<sup>(4,11)</sup>

Although lipoprotein abnormalities have been related to cardiovascular risk in patients with NAFLD,

the impact of lipoprotein(a) (Lp(a)) alterations is still poorly explored. Lp(a) is a cholesteryl ester-rich lipoprotein synthesized by the liver and composed of one molecule of LDL particle-containing apolipoprotein(b) (apo(b))-100 and one of apo(a) linked by a disulfide bridge in a 1:1 molar ratio.<sup>(12,13)</sup>

Lp(a) levels are genetically determined depending on the number of identical Kringle repeats, which directly define apo(a) isoform size and inversely affect circulating Lp(a).<sup>(14)</sup> Thus, the lipoprotein A (*LPA*) gene represents the major determinant of serum Lp(a), accounting for 70%–90% of total variance.<sup>(15)</sup> Since Lp(a) levels remain stable throughout life, guidelines recommend that Lp(a) measurement should be considered at least once in the adult lifetime.<sup>(16-19)</sup> Although Lp(a) has no clear physiological function, genome-wide, epidemiological, and clinical studies have supported a causal association between elevated levels and CVD risk.<sup>(20,21)</sup> Conversely, few controversial reports indicate a relationship between Lp(a) concentration and other metabolic abnormalities.<sup>(22)</sup> Indeed, it has been described that Lp(a) levels are inversely associated with T2D risk,<sup>(23)</sup> although a Mendelian randomization study suggested that

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elevated Lp(a) levels are not causally associated with a lower risk of diabetes. In a large cohort of 2,242 Korean individuals with noninvasively assessed NAFLD, an inverse association between the presence of fatty liver and Lp(a) was reported.<sup>(24)</sup> These findings were confirmed in 22,534 participants who underwent a routine health screening program at Kangbuk Samsung Hospital in Korea, with a higher risk of developing NAFLD in patients with both low Lp(a) and high IR.<sup>(25)</sup> Finally, a recent study that included 176 patients with biopsy-proven NAFLD showed that serum Lp(a) was lower in subjects with advanced fibrosis compared to those with nonadvanced hepatic disease.<sup>(26)</sup>

In view of the inconclusive findings regarding the impact of Lp(a) alterations on NAFLD, the main goal of the present study was to evaluate the association between Lp(a) levels, advanced liver damage, and CVD risk (e.g., subclinical atherosclerosis) in patients with biopsy-proven NAFLD. Circulating Lp(a) was then correlated with the expression of *LPA* and other genes involved in lipid metabolism and liver damage in a subset of patients with severe obesity who had available transcriptomic data.

## Participants and Methods

### STUDY PARTICIPANTS

The study included 600 adult individuals with NAFLD who were consecutively enrolled at the Metabolic Liver Disease Laboratory outpatient service at Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca' Granda Ospedale Policlinico Milan, Italy. Inclusion criteria were availability of liver biopsies performed for suspected NASH or severe obesity, availability of DNA samples and clinical data. Individuals with excessive alcohol intake (men, >30 g/day; women, >20 g/day), viral and autoimmune hepatitis, hereditary hemochromatosis, and alpha-1 antitrypsin deficiency or other causes of liver disease were excluded. The clinical characteristics of patients are listed in Table 1.

Informed written consent was obtained from each patient, and the study protocol was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda, Milan (protocol code CE 401; 28/02/2019).

**TABLE 1. DEMOGRAPHIC, ANTHROPOMETRIC, AND CLINICAL FEATURES OF 600 PATIENTS WITH NAFLD WHOSE SERUM Lp(a) LEVELS WERE EVALUATED**

Parameter	Patients With NAFLD (n = 600)*
Sex, male (%)	404 (67)
Age, years	54 ± 13.2
BMI, kg/m <sup>2</sup>	30.2 ± 6.6
IFG/T2D, yes†	222 (37)
Total cholesterol, mmol/L	4.9 ± 1.1
Triglycerides, mmol/L	1.6 ± 0.9
LDL cholesterol, mmol/L	2.9 ± 1.1
HDL cholesterol, mmol/L	1.3 ± 0.3
ALT, IU/L	37 [21-63]
AST, IU/L	27 [20-41]
GGT, IU/L	39 [21-82]
Glucose, mg/dL	107.8 ± 31.2
Insulin, mIU/L‡	18.4 ± 13.5
HOMA-IR‡	4.5 ± 3.2
Carotid IMT, mm	0.86 ± 0.2
Carotid plaques, yes (%)	288 (48)
Statins, yes	138 (23)
Hypertension, yes (%)	277 (46)
Lp(a), nmol/L	19.9 [14-37]

\*Values are reported as mean ± SD, number (%), or median [IQR], as appropriate.

†IFG defined as fasting glucose >110 mg/dL.

‡HOMA-IR >2.5 and insulin levels >24 were present in 75% and 25% of patients with NAFLD (n = 600), respectively.

### BIOCHEMICAL EVALUATIONS

Body mass index (BMI) was measured using standard procedures. T2D was diagnosed when impaired fasting glucose (IFG) tolerance was present and fasting glucose was >110 mg/dL.<sup>(27)</sup> Alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, total cholesterol, HDL, and LDL were measured by standard laboratory methods.<sup>(27)</sup>

Systolic and diastolic blood pressures were measured twice on the same day, and the mean values were used for analysis. The presence of hypertension was defined when systolic blood pressure was over 140 mm Hg or diastolic blood pressure was over 90 mm Hg more than twice or in subjects treated with antihypertensive medication.

Mean carotid artery intima-media thickness (IMT), an index of the early atherosclerotic process, was determined by high-resolution B-mode ultrasonography with a 7.5-MHz transducer. Values of IMT

represent the mean IMT on the left and right sides. The presence of plaques was defined as a focal carotid thickening  $>1.2$  mm.

Serum samples were stored at  $-80^{\circ}\text{C}$  before analysis. Lp(a) was measured by a turbidimetric *in vitro* test (Tina-quant Lipoprotein (a) Gen. 2; Roche) by using a Cobas C501 from Roche.

For this assay, the measuring range was 7–240 nmol/L; a concentration up to 75 nmol/L was considered within the normal range, with an interassay and intra-assay coefficient of variation of 3% at 88.7 nmol/L. This assay is not influenced by apo(a) isoform size.<sup>(28)</sup>

Subjects were stratified according to Lp(a) distribution as follows: low Lp(a) levels were those below the twenty-fifth percentile ( $<13.9$  nmol/L [quartile I]; median value, 12.3 nmol/L; interquartile range [IQR], 11.3–13 nmol/L;  $n = 139$ ) and high levels were those above the seventy-fifth percentile ( $>36.3$  nmol/L [quartile IV]; median value, 63.7 nmol/L; IQR, 47.3–101.1 nmol/L;  $n = 146$ ). Intermediate values included in quartiles II and III ranged from the twenty-fifth to seventy-fifth percentiles (from 13.9 to 36.3 nmol/L; median value, 19.7 nmol/L; IQR, 16–27 nmol/L;  $n = 315$ ).

## HISTOLOGICAL EVALUATION

Steatosis was graded according to the percentage of affected hepatocytes as 0 (0%–4%), 1 (5%–32%), 2 (33%–65%), and 3 (66%–100%). Disease activity was assessed according to the NAFLD activity score with systematic evaluation of hepatocellular ballooning and necroinflammation; fibrosis was also staged according to recommendations of the NAFLD Clinical Research Network.<sup>(29)</sup> Scoring of liver biopsies was performed by independent pathologists unaware of patient status and genotype.<sup>(7,30)</sup> NASH was diagnosed when steatosis, lobular inflammation, and ballooning were concomitantly present.

## GENOTYPING

Patients were genotyped for the rs738409 C>G (PNPLA3 I148M), rs58542926 C>T (TM6SF2 E167K), apolipoprotein E (*ApoE*) rs445925, and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) rs7552841 variants, using TaqMan 5'-nuclease assays in duplicate (QuantStudio 3; Thermo Fisher, Waltham, MA), as described.<sup>(7,8)</sup> Genotyping success rate was  $>99\%$ .

## TRANSCRIPTOMIC ANALYSIS

RNA sequencing (RNA-seq) was performed in a subset of 183 patients with severe obesity (31 without and 152 with NAFLD) belonging to the cohort in which Lp(a) was measured ( $n = 600$ ) and in whom a percutaneous liver biopsy was performed during bariatric surgery at Fondazione IRCCS Cà Granda Ospedale Policlinico, Milan.<sup>(31)</sup> The study conformed to the Declaration of Helsinki and was approved by the institutional review boards and their ethics committees. All participants gave written informed consent. Clinical characteristics of patients for whom RNA-seq data were available are presented in Supporting Table S1. RNA-seq mapping descriptive statistics, detailed protocol, and the data analysis approach are described in the Supporting Materials and Methods.

## STATISTICAL ANALYSIS

Statistical analyses were performed using JMP 16.0 (SAS, Cary, NC), R statistical analysis, version 3.3.2 (<http://www.R-project.org/>), and Prism (version 9, GraphPad Software) by one-way analysis of variance (ANOVA) or chi-squared test, where appropriate.

For descriptive statistics, continuous variables are shown as mean and SD or median and IQR for highly skewed biological variables (i.e., AST, ALT, gamma-glutamyltransferase [GGT], triglycerides, Lp(a)). Variables with skewed distributions were logarithmically transformed before analyses. Categorical variables were presented as number and proportion. All genetic analyses were performed under a dominant (for *TM6SF2* E167K and *ApoE* rs445925 variants) or recessive (for *PCSK9* rs7552841 variant) model, according to the frequency distribution of the minor at-risk alleles among European non-Finnish healthy individuals included in the 1000 Genomes project. Analyses were performed by fitting data to multivariable models. In particular, generalized linear models were fit to examine continuous traits (i.e., Lp(a)). Multinomial logistic regression models were fit to examine binary traits (hypertension, carotid plaques, fibrosis  $>2$ , cirrhosis), and ordinal regression models were fit for ordinal traits (stage of fibrosis). When specified, confounding factors were included in a model.<sup>(26)</sup> Correlations were assessed by bivariate analysis. Differences between groups were calculated by one-way ANOVA, followed by post hoc *t* tests adjusted for the number of comparisons when

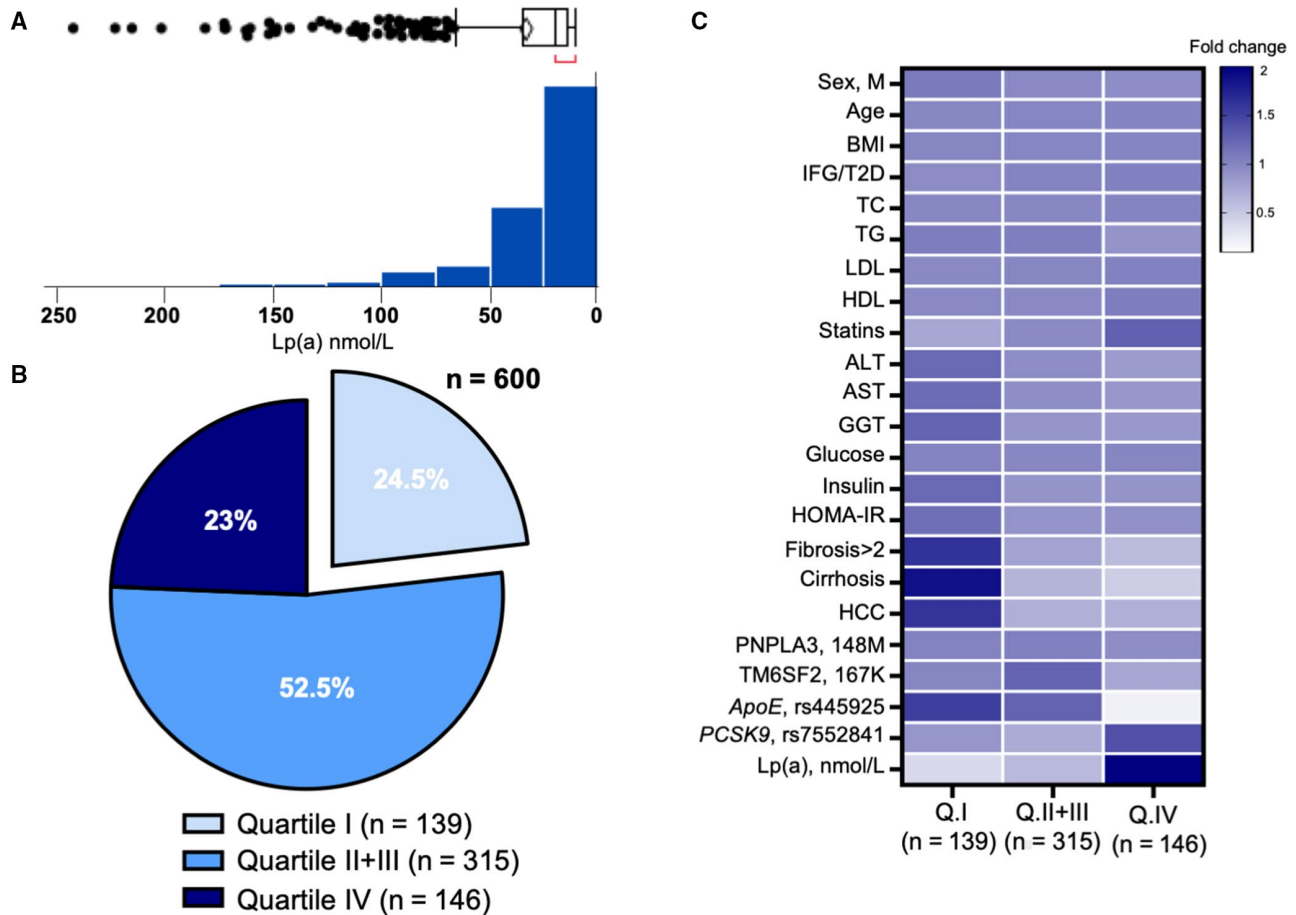


multiple groups were involved (Bonferroni correction or Benjamini-Hochberg false discovery rate correction, where indicated). To assess the utility of Lp(a) levels as a predictor of hepatic fibrosis, we constructed receiver operating characteristic (ROC) curves and calculated the area under the curves (AUCs). Bootstrap resampling (100 bootstrap resampling times) was conducted to calculate 95% confidence intervals (CIs). The frequency distribution of SNPs across quartiles was calculated by using the Fisher exact test (quartile IV vs. quartile I). Two-tailed  $P < 0.05$  was considered statistically significant.

## Results

### ASSOCIATION BETWEEN SERUM Lp(a) LEVELS AND BIOCHEMICAL FEATURES IN PATIENTS WITH NAFLD

The clinical features of patients enrolled in the study ( $n = 600$ ) are listed in Table 1 and Fig. 1. Lp(a) has a skewed distribution, and the median was 19.9 nmol/L (IQR, 14-37 nmol/L) (Fig. 1A). The mean age of participants was 54 years (SD,  $\pm 13.2$ ),



**FIG. 1.** Distribution of Lp(a) levels in patients with NAFLD. (A) Lp(a) serum concentration (nmol/L) was evaluated in 600 patients with NAFLD and showed a skewed distribution. Patients were stratified into the following three groups according to Lp(a) levels: quartile I  $<13.9$  nmol/L, quartile II+III from 13.9 to 36.3 nmol/L, and quartile IV  $>36.3$  nmol/L. (B) The pie chart displays the percentage distribution of quartiles across NAFLD cohorts (quartile I, 23% [ $n = 139$ ]; quartile II+III, 52.5% [ $n = 315$ ]; quartile IV, 24.5% [ $n = 146$ ]). (C) The heatmap represents clinical features of patients with NAFLD across Lp(a) quartiles. It was generated calculating the fold change of each clinical feature in the quartile compared to its mean value in the entire cohort. The intensity of blue indicates the enrichment of the trait in each quartile. Abbreviations: M, male; Q, quartile; TC, total cholesterol; TG, triglyceride.

and 404 participants (67%) were men. Thirty-seven percent of patients ( $n = 222$ ) had T2D, 46% ( $n = 277$ ) were hypertensive, 48% ( $n = 288$ ) had carotid plaques, and 23% ( $n = 138$ ) were treated with statins (Table 1).

We then stratified patients into quartiles according to Lp(a) levels (Fig. 1B); their clinical characteristics are summarized in Table 2 and Fig. 1C. Twenty-three percent of patients belonged to quartile I, 52.5% to quartile II+III, and 24.5% to quartile IV. No differences in demographic or anthropometric features were found across quartiles. The presence of elevated Lp(a) (quartile IV) was accompanied by higher HDL levels ( $P = 0.015$ , one-way ANOVA) and lower triglycerides ( $P = 0.06$ , one-way ANOVA). Patients with an elevated Lp(a) were more likely to be treated with statins ( $P = 0.05$ , one-way ANOVA). Insulin levels and homeostasis model assessment of insulin resistance (HOMA-IR) were raised in patients with NAFLD in quartile I, possibly suggesting an

inverse association between lower serum Lp(a) and IR ( $P = 0.008$  and  $P = 0.017$ , one-way ANOVA, respectively; adjusted  $P < 0.05$  for quartile IV vs. quartile I) (Table 2; Figs. 1C and 2A,B). Patients with lower Lp(a) levels showed increased ALT, AST, and GGT levels ( $P = 0.01$ ,  $P = 0.007$ , and  $P = 0.021$ , one-way ANOVA, respectively; adjusted  $P < 0.05$  for quartile IV vs. quartile I) (Table 2; Figs. 1C and 2C-E).

## LOW Lp(a) LEVELS ARE ASSOCIATED WITH HIGHER RISK OF FIBROSIS AND ADVANCED LIVER DISEASE IN PATIENTS WITH NAFLD

We next investigated whether Lp(a) levels may affect liver damage in patients with NAFLD. While there was no association with steatosis, necroinflammation,

**TABLE 2. DEMOGRAPHIC, ANTHROPOMETRIC, AND CLINICAL FEATURES OF 600 PATIENTS WITH NAFLD STRATIFIED BY Lp(a) QUARTILES**

Patients With NAFLD ( $n = 600$ )*	Quartile I ( $n = 139$ )	Quartile II+III ( $n = 315$ )	Quartile IV ( $n = 146$ )	$P^\dagger$
Sex, male (%)	102 (73)	208 (66)	94 (64)	0.21
Age, years	52.6 $\pm$ 12.9	53.4 $\pm$ 13.0	54.5 $\pm$ 13.7	0.47
BMI, kg/m <sup>2</sup>	30.2 $\pm$ 6.9	30.1 $\pm$ 6.5	30.4 $\pm$ 6.4	0.91
IFG/T2D, yes <sup>‡</sup>	49 (35)	117 (37)	56 (38)	0.88
Total cholesterol, mmol/L	4.9 $\pm$ 1.1	4.9 $\pm$ 1.2	5.0 $\pm$ 1.0	0.65
Triglycerides, mmol/L	1.64 $\pm$ 0.9	1.63 $\pm$ 1.13	1.39 $\pm$ 0.65	0.06
LDL cholesterol, mmol/L	2.85 $\pm$ 1.03	2.93 $\pm$ 1.06	3.0 $\pm$ 1.1	0.56
HDL cholesterol, mmol/L	1.27 $\pm$ 0.37	1.27 $\pm$ 0.34	1.38 $\pm$ 0.40	0.015
Statins, yes	23 (17)	69 (22)	42 (29)	0.05
ALT, IU/L	45 [28-74]	35 [21-59]	31 [20-52]	0.01
AST, IU/L	33 [22-49]	26 [20-39]	24 [20-40]	0.007
GGT, IU/L	51 [21-104]	36 [21-74]	35 [20-77]	0.021
Glucose, mg/dL	108.7 $\pm$ 37.6	107.2 $\pm$ 29.2	108.3 $\pm$ 28.9	0.88
Insulin mIU/L	22.8 $\pm$ 17.2	17.1 $\pm$ 12.1	17.4 $\pm$ 12.5	0.008
HOMA-IR	5.44 $\pm$ 3.8	4.2 $\pm$ 2.8	4.3 $\pm$ 3.4	0.017
Fibrosis >2, yes (%)	37 (27)	41 (13)	15 (10)	0.0002
Cirrhosis, yes (%)	28 (20)	22 (7)	7 (5)	<0.0001
HCC, yes (%)	10 (7)	11 (3)	4 (3)	0.11
PNPLA3, 148M allele yes (%)	85 (61)	195 (62)	82 (56)	0.48
TM6SF2, 167K allele yes (%)	17 (12)	47 (15)	13 (9)	0.23
ApoE rs445925 A allele, yes (%)	31 (22)	57 (18)	4 (3)	0.03
PCSK9 rs7552841 TT homozygous, yes (%)	24 (17)	44 (14)	39 (27)	0.04
Lp(a), nmol/L	12.3 [11.3-13]	19.7 [16-27]	63.7 [47.3-101.1]	<0.0001

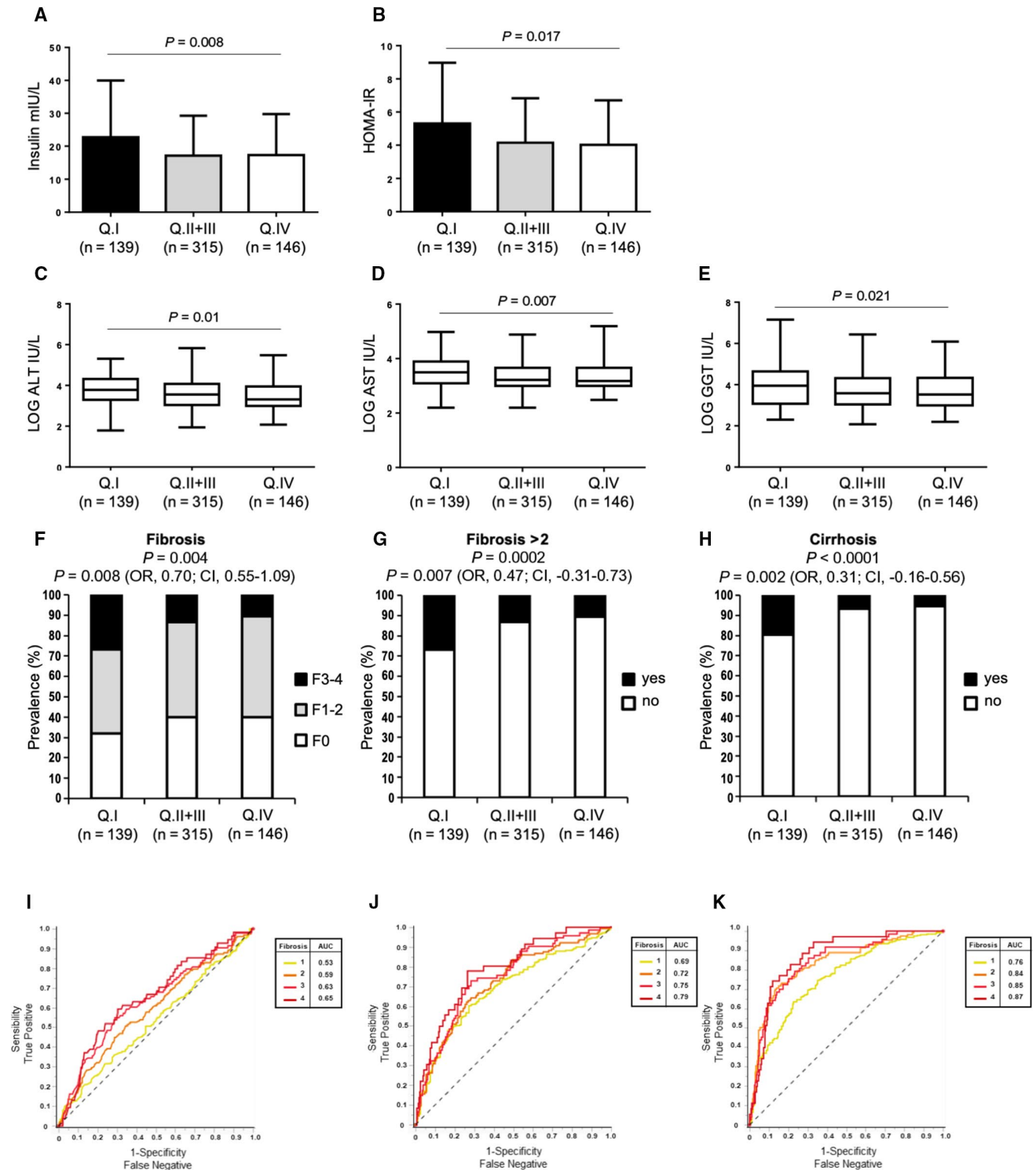
\*Values are reported as mean  $\pm$  SD, number (%), or median [IQR], as appropriate.

<sup>†</sup>Significant at  $P < 0.05$ .

<sup>‡</sup>IFG defined as fasting glucose >110 mg/dL).

and ballooning, the lowest Lp(a) levels were associated with fibrosis, fibrosis >2, and cirrhosis ( $P = 0.02$ ,  $P = 0.002$ , and  $P = 0.001$ , univariate analysis, respectively) (Supporting Fig. S1A-C). At multivariate

analysis, we observed an inverse correlation between Lp(a) levels and fibrosis (odds ratio [OR], 0.76; 95% CI, 0.59-0.96;  $P = 0.02$ ), fibrosis >2 (OR, 0.48; 95% CI, 0.30-0.76;  $P = 0.002$ ), and cirrhosis (OR, 0.31;



**FIG. 2.** Lp(a) levels are inversely associated with IR and with severity of liver damage in patients with NAFLD. (A) Total insulin, (B) HOMA-IR, (C) ALT, (D) AST, and (E) GGT were stratified according to quartiles. Boxes span from the twenty-fifth to seventy-fifth percentile, while whiskers indicate the tenth and ninetieth percentile.  $P < 0.05$  is considered significant, one-way ANOVA. Association of the Lp(a) quartiles with (F) fibrosis stage, (G) advanced fibrosis (fibrosis  $>2$ ), and (H) cirrhosis in patients with NAFLD. Univariable (not adjusted) and multivariable ordinal regression (for fibrosis) and nominal logistic regression analysis (for fibrosis  $>2$  and cirrhosis), adjusted for sex, age, BMI, T2D, ALT, total cholesterol, triglycerides, and statin use. (I) ROC curves of Lp(a) concentration among fibrosis grades (F0-F4). AUCs for Lp(a) were 0.53 (95% CI, 0.52-0.53;  $P = 0.09$ ) in F1, 0.59 (95% CI, 0.58-0.60;  $P < 0.0001$ ) in F2, 0.63 (95% CI, 0.63-0.64;  $P < 0.0001$ ) in F3, 0.65 (95% CI, 0.64-0.66;  $P < 0.0001$ ) in F4. (J) ROC curves of Lp(a) concentration adjusted for sex, age, BMI, T2D, total cholesterol, triglycerides, and statin use, among fibrosis grade (F0-F4). AUCs for Lp(a) were 0.69 (95% CI, 0.69-0.71;  $P < 0.0001$ ) in F1, 0.72 (95% CI, 0.72-0.73;  $P < 0.0001$ ) in F2, 0.75 (95% CI, 0.74-0.76;  $P < 0.0001$ ) in F3, 0.79 (95% CI, 0.78-0.80;  $P < 0.0001$ ) in F4. (K) ROC curves of Lp(a) concentration adjusted for sex, age, BMI, T2D, total cholesterol, triglycerides, ALT, AST, and statin use, among fibrosis grade (F0-F4). AUCs for Lp(a) were 0.76 (95% CI, 0.76-0.77;  $P < 0.0001$ ) in F1, 0.84 (95% CI, 0.83-0.84;  $P < 0.0001$ ) in F2, 0.85 (95% CI, 0.84-0.86;  $P < 0.0001$ ) in F3, 0.87 (95% CI, 0.86-0.88;  $P < 0.0001$ ) in F4. Abbreviation: Q, quartile.

**TABLE 3. ASSOCIATION BETWEEN CIRCULATING Lp(a) LEVELS AND ADVANCED LIVER DAMAGE IN PATIENTS WITH NAFLD (N = 600)**

	Fibrosis			Fibrosis $>2$			Cirrhosis		
	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>
Sex, male	1.23	1.01-1.52	0.04	1.25	0.65-2.40	0.50	1.14	0.48-2.71	0.77
Age, years	1.06	1.04-1.08	$<0.0001$	1.07	1.04-1.10	$<0.0001$	1.08	1.04-1.14	0.0002
BMI, kg/m <sup>2</sup>	1.08	1.05-1.12	$<0.0001$	1.06	1.00-1.12	0.04	1.06	0.99-1.14	0.08
IFG/T2D, yes	1.43	1.16-1.79	0.001	2.44	1.28-4.65	0.006	2.73	1.16-6.40	0.02
ALT, IU/L	3.63	2.69-4.95	$<0.0001$	3.49	2.17-5.59	$<0.0001$	3.11	1.63-5.92	0.0006
Total cholesterol, mmol/L	0.95	0.80-1.14	0.60	0.86	0.63-1.16	0.31	0.82	0.55-1.24	0.35
Triglycerides, mmol/L	1.00	0.81-1.23	0.99	0.98	0.71-1.36	0.91	1.12	0.73-1.71	0.59
Statin, yes	0.82	0.64-1.03	0.09	0.50	0.24-1.04	0.06	0.46	1.17-1.22	0.12
Lp(a), nmol/L	0.76	0.59-0.96	0.02	0.48	0.30-0.76	0.002	0.31	0.14-0.65	0.002

Values were obtained by multivariate ordinal regression analysis (for fibrosis) and nominal regression analysis (for fibrosis  $>2$  and cirrhosis) adjusted for sex, age, BMI, IFG (defined as fasting glucose  $>110$  mg/dL), T2D, ALT, total cholesterol, triglycerides, and statin drugs. The independent variable was circulating Lp(a) (continuous trait). Values of  $P < 0.05$  are significant.

95% CI, 0.14-0.65;  $P = 0.002$ ) after adjustment for sex, age, BMI, T2D, ALT, total cholesterol, triglycerides, and statin use (Table 3).

Furthermore, at multivariate analysis the inverse association between Lp(a) and fibrosis (OR, 0.70; 95% CI, 0.55-1.09;  $P = 0.008$ ), fibrosis  $>2$  (OR, 0.47; 95% CI, -0.31 to 0.73;  $P = 0.007$ ), and cirrhosis (OR, 0.31; 95% CI, -0.16 to 0.56;  $P = 0.002$ ) remained significant even after subdividing patients into quartiles according to Lp(a) levels after adjustment for the same confounders (Fig. 2F-H).

At ROC analysis, diagnostic accuracy of circulating Lp(a) in predicting fibrosis increased after adjustment for sex, age, BMI, T2D, total cholesterol, triglycerides, and statin use (AUC fibrosis stage [F]4, 0.65 vs. 0.79;  $P < 0.0001$ ) (Fig. 2I,J). At multivariate analysis, the AUC of the ROC curve of serum Lp(a) for fibrosis prediction was even higher by adding ALT and AST to

the confounders (AUC F4, 0.87 vs. 0.79;  $P < 0.0001$ ) (Fig. 2K), suggesting that circulating Lp(a) may be used together with transaminases as a biomarker to predict advanced fibrosis in patients with NAFLD.

### Lp(a) LEVELS ARE MODERATELY ASSOCIATED WITH CARDIOVASCULAR COMORBIDITIES IN PATIENTS WITH NAFLD

Since high Lp(a) levels contribute to CVD risk, which is a common feature of NAFLD, we analyzed the association between hypertension, carotid plaques, and Lp(a) concentration. At univariate analysis, Lp(a) levels raised the risk of hypertension (OR, 1.26; 95% CI, 0.99-1.60;  $P = 0.05$ ) and carotid plaque formation (OR, 1.37; 95% CI, 1.02-1.83;  $P = 0.03$ ) (Supporting



Fig. S1D,E). At multivariate analysis adjusted for sex, age, BMI, T2D, ALT, total cholesterol, triglycerides, and statin use, the effect of Lp(a) on these cardiovascular risk factors remained slightly significant for carotid plaques (OR, 1.41; 95% CI, 0.98-2.03;  $P = 0.06$ ) whereas it was lost for hypertension (OR, 1.26; 95% CI, 0.64-1.69;  $P = 0.13$ ) (Supporting Table S2).

By stratifying patients according to the presence of fibrosis and using univariate analysis, a stronger association was found between Lp(a) concentration and hypertension (OR, 1.46; 95% CI, 1.10-1.94;  $P = 0.008$ ) and Lp(a) concentration and carotid plaques (OR, 1.63; 95% CI, 1.13-2.36;  $P = 0.009$ ) only in those with mild fibrosis ( $n = 424$ , F0-F1). At multivariate analysis adjusted as above, the association between Lp(a) and carotid plaques remained significant (OR, 1.70; 95% CI, 1.05-2.74;  $P = 0.03$ ) (Supporting Table S3). Therefore, it could be speculated that in patients with NAFLD with advanced liver disease and in whom low Lp(a) levels reflect the impairment of hepatic function, Lp(a) measurement may not be a reliable predictor of cardiovascular risk.

### **Lp(a) LEVELS REFLECT LPA HEPATIC EXPRESSION AND CORRELATE WITH GENES INVOLVED IN LIPOPROTEIN SECRETION AND FIBROGENESIS**

We next evaluated the expression of the hepatic *LPA* gene in a subset of patients ( $n = 183$ ) who belonged to the entire cohort of patients with NAFLD ( $n = 600$ ). Serum Lp(a) concentration was positively correlated with its hepatic expression, supporting the concept that circulating Lp(a) reflects hepatic synthesis ( $y = 0.04x + 2.06$ ;  $P = 0.018$ ; Fig. 3A). In addition, both serum Lp(a) ( $P = 0.033$ , one-way ANOVA; adjusted  $P < 0.05$  for severe NAFLD vs. normal liver) and hepatic messenger RNA (mRNA) levels ( $P = 0.017$ , one-way ANOVA; adjusted  $P < 0.001$  for severe NAFLD vs. normal liver) were reduced in patients with mild and severe NAFLD versus those with normal liver (Fig. 3B,C).

LPA hepatic expression positively correlated with that of genes involved in lipoprotein export as APOB ( $y = 0.30x + 2.03$ ;  $P < 0.0001$ ), APOA1 ( $y = 0.12x + 2.53$ ;  $P = 0.04$ ), and microsomal triglyceride transfer protein (MTTP) ( $y = 0.18x + 1.98$ ;  $P < 0.0001$ ) (Fig. 3D-F).

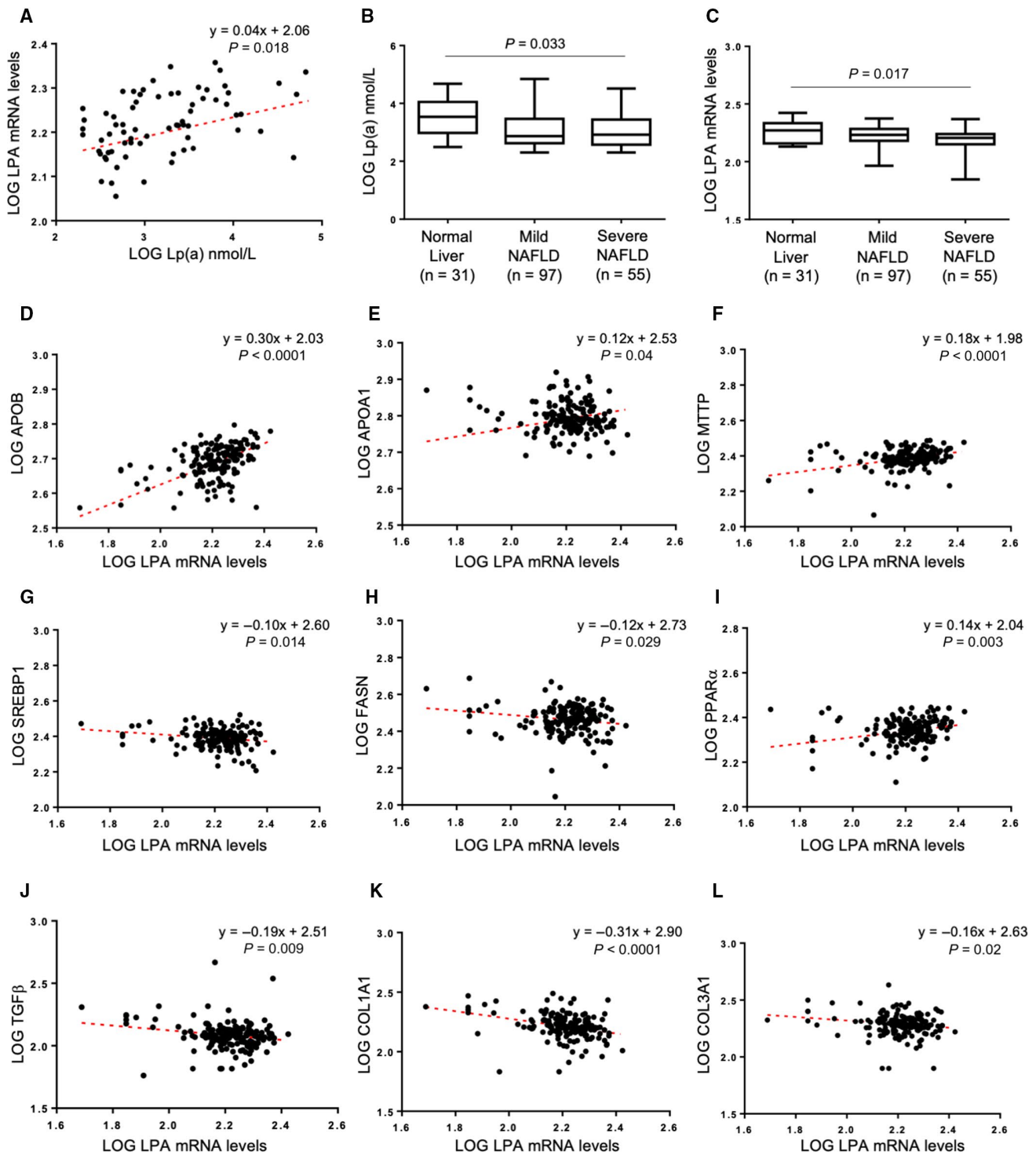
A negative correlation was also present between LPA mRNA levels and the expression of genes that regulate *de novo* lipogenesis (sterol regulatory element-binding protein 1 [SREBP1]  $y = -0.10x + 2.60$ ;  $P = 0.014$ ; fatty acid synthase [FASN]  $y = -0.12x + 2.73$ ;  $P = 0.029$ ) and a positive one with the expression of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) ( $y = 0.14x + 2.04$ ;  $P = 0.003$ ) (Fig. 3G-I).

Finally, LPA mRNA levels were inversely correlated with those of transforming growth factor beta (TGF $\beta$ ) ( $y = -0.19x + 2.51$ ;  $P = 0.009$ ), collagen type I alpha 1 chain (COL1A1) ( $y = -0.31x + 2.90$ ;  $P < 0.0001$ ), and COL3A1 ( $y = -0.16x + 2.63$ ;  $P = 0.02$ ), thus supporting a role of Lp(a) in the fibrogenic processes (Fig. 3J-L).

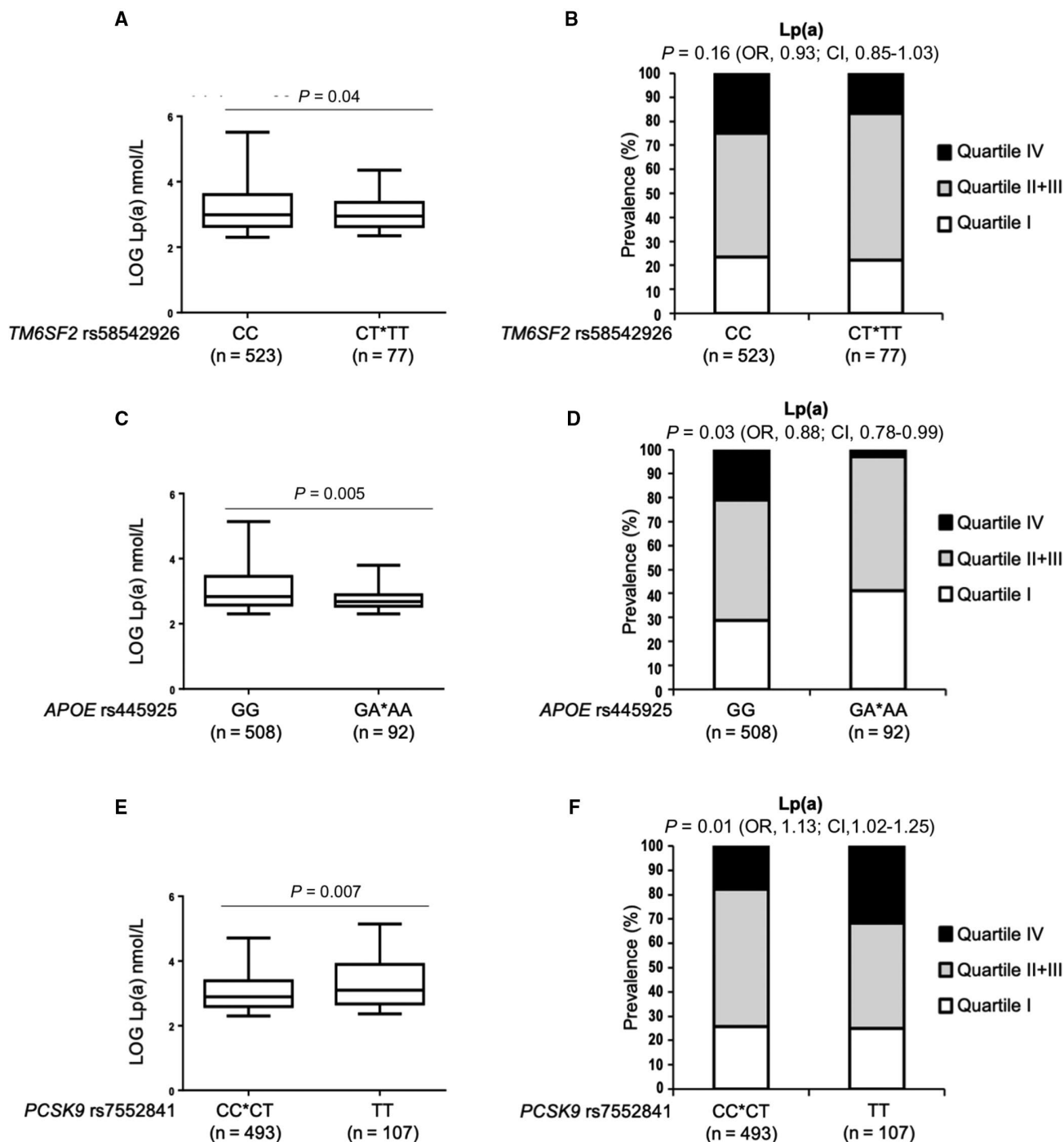
### **TM6SF2 rs58542926, ApoE rs445925, AND PCSK9 rs7552841 GENETIC VARIANTS AFFECT SERUM LP(A) LEVELS IN PATIENTS WITH NAFLD**

We next evaluated whether genetic variations that have been associated with lipoprotein metabolism could modulate circulating Lp(a) in patients with NAFLD. The frequency of PNPLA3 I148M and TM6SF2 E167K did not differ between Lp(a) quartiles, whereas the *ApoE* rs445925 A allele was less frequent in quartile IV (3% vs. 22% compared to quartile I;  $P = 0.03$ , Fisher exact test) (Table 2). Conversely, the frequency of *PCSK9* rs7552841 TT was higher in quartile IV (27% vs. 17% compared to quartile I;  $P = 0.04$ , Fisher exact test) (Table 2). At univariate analysis, carriers of the low-frequency minor *TM6SF2* T and *ApoE* A alleles had lower Lp(a) levels compared to noncarriers ( $P = 0.04$  and  $P = 0.005$ , two-tailed  $t$  test, respectively) (Fig. 4A,C). At multivariate analysis adjusted for sex, age, BMI, T2D, ALT, total cholesterol, triglycerides, and statin use, patients who carried the *ApoE* A allele had lower levels of Lp(a) compared to noncarriers (OR, 0.88; 95% CI, 0.78-0.99;  $P = 0.03$ ) whereas the association lost significance for the rs58542926 variant (OR, 0.93; 95% CI, 0.85-1.03;  $P = 0.16$ ) (Fig. 4B,D).

Conversely, homozygous patients (TT) for *PCSK9* rs7552841 had higher circulating Lp(a) levels compared to wild type or heterozygous patients (CC\*CT) at both univariate ( $P = 0.007$ , two-tailed  $t$  test) and



**FIG. 3.** Lp(a) hepatic mRNA levels correlate with serum Lp(a) concentration and with hepatic expression of genes involved in lipoprotein release, lipid synthesis, and fibrogenic processes. (A) Correlation analysis between hepatic LPA gene expression evaluated by transcriptome sequencing in liver biopsies (n = 183) and circulating Lp(a) (nmol/L). (B) Serum Lp(a) levels and (C) hepatic LPA expression were stratified according to severity of NAFLD. Boxes span from the twenty-fifth to seventy-fifth percentile, while whiskers indicate the tenth and ninetieth percentile.  $P < 0.05$  is significant, one-way ANOVA. Correlation analysis between hepatic Lp(a) expression and (D) APOB, (E) APOA1, (F) MTPP, (G) SREBP1, (H) FASN, (I) PPAR $\alpha$ , (J) TGF $\beta$ , (K) COL1A1, and (L) COL3A1 mRNA levels evaluated by transcriptome sequencing in liver biopsies (n = 183).



**FIG. 4.** Lp(a) serum levels are influenced by genetic variants. (A) Circulating Lp(a) was stratified according to the presence of *TM6SF2* rs58542926. (B) Distribution of Lp(a) quartiles in *TM6SF2* T allele carriers (CT\*TT) compared to noncarriers (CC). Association of serum Lp(a) with *TM6SF2* T allele by multivariable analysis adjusted for sex, age, BMI, T2D, ALT, total cholesterol, triglycerides and statin use. (C) Circulating Lp(a) was stratified according to the presence of *ApoE* rs445925. (D) Distribution of Lp(a) quartiles in *ApoE* A allele carriers (GA\*AA) compared to noncarriers (GG). Association of serum Lp(a) with *ApoE* A allele by multivariable analysis adjusted for sex, age, BMI, T2D, ALT, total cholesterol, triglycerides, and statin use. (E) Circulating Lp(a) was stratified according to the presence of *PCSK9* rs7552841. (F) Distribution of Lp(a) quartiles in *PCSK9* TT carriers compared to CC\*CT. Association of serum Lp(a) with *PCSK9* TT homozygosity by multivariable analysis adjusted for sex, age, BMI, T2D, ALT, total cholesterol, triglycerides, and statin use.

**TABLE 4. GENETIC VARIANTS INVOLVED IN LIPOPROTEIN METABOLISM IMPACT ON Lp(a) CIRCULATING LEVELS IN PATIENTS WITH NAFLD (N = 600)**

	Fibrosis			Fibrosis >2			Cirrhosis		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
Sex, male	1.55	1.17-2.29	0.02	1.36	0.25-7.50	0.72	0.89	0.12-6.41	0.91
Age, years	1.04	1.02-1.07	0.0005	1.16	1.07-1.25	0.0001	1.13	1.03-1.22	0.005
BMI, kg/m <sup>2</sup>	1.04	0.99-1.09	0.15	1.04	0.91-1.18	0.54	1.02	0.87-1.18	0.84
IFG/T2D, yes	2.27	1.55-3.35	<0.0001	4.42	1.27-15.4	0.01	4.99	1.12-21.4	0.03
Statin, yes	0.78	0.47-1.28	0.35	0.90	0.13-5.96	0.92	0.60	0.05-6.46	0.66
<i>TM6SF2</i> , 167K allele, yes	1.51	0.99-2.29	0.05	10.5	1.85-59.2	0.007	7.63	1.32-43.9	0.02
<i>ApoE</i> rs445925 A allele, yes	1.20	0.80-1.77	0.34	8.3	1.98-35.3	0.004	4.87	0.89-26.6	0.06
<i>PCSK9</i> rs7552841 TT homozygous, yes	1.38	0.98-1.92	0.07	1.46	0.36-5.87	0.59	4.29	0.98-18.8	0.06
Lp(a), nmol/L	0.93	0.59-1.48	0.77	0.72	0.24-2.15	0.55	0.88	0.27-2.87	0.84

Values were obtained by multivariate ordinal regression analysis (for fibrosis) and nominal regression analysis (for fibrosis >2 and cirrhosis), adjusted for sex, age, BMI, IFG (defined as fasting glucose >110 mg/dL), T2D, statin drugs, and *TM6SF2* rs58542926, *ApoE* rs445925, and *PCSK9* rs7552841 genetic variants. The independent variable was circulating Lp(a) (continuous trait). Values of  $P < 0.05$  are significant.

multivariate analysis (OR, 1.13; 95% CI, 1.02-1.25;  $P = 0.01$ ) (Fig. 4E,F).

At multivariate analysis adjusted for sex, age, BMI, T2D, and statin use, the association between Lp(a) levels and fibrosis was lost after the addition of *TM6SF2* T, *ApoE* A alleles, and *PCSK9* TT homozygosity as confounders (OR, 0.93; 95% CI, 0.59-1.48;  $P = 0.77$ ), suggesting that Lp(a) reduction in the presence of advanced liver damage may be dependent on genetics. Similar findings were observed for fibrosis >2 (OR, 0.72; 95% CI, 0.24-2.15;  $P = 0.55$ ) and cirrhosis (OR, 0.88; 95% CI, 0.27-2.87;  $P = 0.84$ ) (Table 4).

## Discussion

In this study, we evaluated Lp(a) serum levels in a large histologically and genetically characterized cohort of patients with NAFLD. We found that circulating Lp(a) was reduced in patients with fibrosis and cirrhosis, possibly representing a novel biomarker to predict advanced liver damage with an accuracy that further increases when combined with transaminases. Consistently, patients with low Lp(a) levels had hyperinsulinemia and IR, which are strong predictors of hepatic fibrosis.<sup>(32)</sup> Moreover, the reduced hepatic expression of the *LPA* gene accounted for lower circulating Lp(a) in patients with severe NAFLD. Finally, genetic variants already known to modulate circulating lipids affected serum Lp(a) concentration.

Lp(a) is a cholesteryl ester-rich lipoprotein synthesized by the liver and composed of one molecule of LDL particle-containing apo(b)-100 and one of apo(a) linked by a disulfide bridge. In the present cohort, the distribution of serum Lp(a) is highly skewed in line with the knowledge that roughly 80% of the Caucasian population has Lp(a) levels <50 mg/dL.<sup>(33)</sup> Several studies have evaluated the relationship between Lp(a) levels and chronic liver diseases, such as cirrhosis and HCC, independently of etiology, suggesting that circulating Lp(a) decreases when residual liver function declines.<sup>(34-36)</sup> Here, in a large cohort of 600 patients with biopsy-proven NAFLD, we found that Lp(a) levels decreased concomitantly with increasing fibrosis and cirrhosis. In keeping with our results, a recent study that included a smaller number of patients with biopsy-proven NAFLD showed that serum Lp(a) levels were reduced in subjects with advanced fibrosis compared to those with nonadvanced hepatic disease.<sup>(26)</sup>

To reinforce these findings, we evaluated the diagnostic accuracy of circulating Lp(a) to predict histologic fibrosis. The AUC for advanced fibrosis (F4) was 0.79 after adjustment for sex, age, BMI, T2D, total cholesterol, triglycerides, and statin use. Since transaminases are commonly used in clinical practice to noninvasively assess hepatic injury, we managed to combine ALT and AST to serum Lp(a) to improve their diagnostic accuracy. As a result, the AUC significantly increased to 0.87 when Lp(a) was combined



with transaminases (ALT and AST), suggesting that serum Lp(a) may be a novel biomarker to ameliorate the severe fibrosis estimation in patients with NAFLD.

Several epidemiological and observational studies indicate that patients with severe NAFLD are more susceptible to develop CVD.<sup>(37)</sup> In our cohort, Lp(a) concentration was mildly associated with hypertension and with carotid plaque formation by univariate analysis, and this effect was blunted at multivariate analysis adjusted for confounders. However, by stratifying patients according to the presence of fibrosis, Lp(a) concentration was more strongly associated with hypertension and carotid plaques only in those with mild fibrosis. Therefore, it could be speculated that in patients with advanced liver disease, in whom low Lp(a) levels reflect the impairment of hepatic function, its measurement may not be a reliable predictor of cardiovascular risk.<sup>(26)</sup>

Furthermore, it has also been demonstrated that IR, an independent predictor of severe fibrosis in patients with NAFLD,<sup>(32)</sup> may affect serum Lp(a). In a retrospective study, Lp(a) levels were inversely associated with the presence of IR in patients with NAFLD.<sup>(25)</sup> In a study performed in 2,242 Korean participants without diabetes in a health screening program, serum Lp(a) was inversely related to NAFLD, suggesting a higher risk of fatty liver in subjects with IR with low serum Lp(a).<sup>(24)</sup> Moreover, it has been demonstrated that individuals with glucose intolerance display a lower Lp(a) concentration and a significant increase in apo(a) size.<sup>(38)</sup> Consistently, we confirmed the previously described associations, demonstrating that patients with NAFLD with lower Lp(a) levels had hyperinsulinemia and a raised HOMA-IR index. It is not clear how IR may act as a mediator of reduced Lp(a) in patients with NAFLD, and a possible explanation relies on the ability of insulin to suppress apo(a) at the transcriptional levels, at least, in hepatocytes.<sup>(39)</sup>

Circulating Lp(a) is primarily synthesized and released by the liver and is determined by the *LPA* gene. Therefore, we next investigated the expression of hepatic *LPA* in a subset of 183 patients who belonged to the overall cohort. We found that Lp(a) dosage positively correlated with *LPA* mRNA levels and both were progressively down-regulated with the worsening of NAFLD. As we expected, hepatic *LPA* expression positively correlated with genes involved in the

secretion of very low-density lipoproteins (e.g., *APOB* or *MTTP*). In addition, according to the increase in HDL cholesterol observed in patients with higher Lp(a) levels, *LPA* hepatic expression was positively correlated with *APOA1*, which is the major lipoprotein constituent of HDL. Conversely, we found a negative correlation between Lp(a) and genes involved in *de novo* lipogenesis (e.g., *SREBP1* or *FASN*), triglyceride catabolism (*PPAR $\alpha$* ), or fibrogenesis (*TGF $\beta$* , *COL1A1*, and *COL3A1*), supporting the notion that patients with advanced liver damage secrete a reduced amount of Lp(a) due to the impairment of liver function. Another possible explanation of the negative correlation between the expression of Lp(a) and fibrogenic genes relies on the blunted inhibition of TGF $\beta$  by Lp(a), which contains plasminogen-like Kringle IV repeats, thus competing with plasminogen binding.<sup>(40)</sup> The loss of the inhibitory effect on TGF $\beta$  due to low Lp(a) levels results in enhanced smooth muscle cell activation and migration.<sup>(41)</sup> In addition, it has been reported that TGF $\beta$  downregulated the transcription of the *LPA* gene in primary hepatocytes.<sup>(42)</sup>

We then assessed the impact of *TM6SF2* rs58542926, *ApoE* rs445925, and *PCSK9* rs7552841 genetic variations, previously associated with cardiovascular comorbidities in patients with NAFLD, on circulating Lp(a). Patients carrying the *TM6SF2* T and *ApoE* A minor alleles showed reduced Lp(a) levels consistently with their opposite role in the protection against cardiovascular complications and in the induction of hepatic fibrosis.<sup>(7,43,44)</sup> In line with these findings, in 500,000 individuals from the publicly available United Kingdom Biobank Cohort (UKBBC), which is representative of the general population, the *TM6SF2* T allele and *ApoE* A allele carriage induced a protection against atherogenic dyslipidemia and against disorders of lipoprotein metabolism (data not shown). In contrast, we demonstrated that the homozygosity for the *PCSK9* gain-of-function variant was associated with increased serum Lp(a) levels in line with the hypothesis of the proatherogenic role of PCSK9 in addition to LDL regulation.<sup>(45)</sup> Supporting these observations, PCSK9 inhibitors reduce circulating Lp(a).<sup>(46)</sup> However, the effect of the *PCSK9* rs7552841 mutation on Lp(a) did not translate to significant protection against fibrosis onset in our cohort and in the UKBBC (data not shown).

This study has some limitations. Firstly, it is a single-center study with a retrospective nature. However, to

the best of our knowledge, this is the largest study on patients with biopsy-proven NAFLD (n = 600) in which Lp(a) levels were assessed in accordance with genetic polymorphisms that modulate cardiovascular risk and/or progressive NAFLD.<sup>(47)</sup> Secondly, we did not investigate inherited alterations in the *LPA* gene or the number of copies of Kringle repeats because it has been broadly demonstrated that 70%-90% of Lp(a) levels is genetically determined. Thirdly, the present findings refer only to patients with NAFLD of Caucasian origin.<sup>(48)</sup>

This study highlights for the first time the association between serum Lp(a) levels and advanced fibrosis in a large cohort of patients with biopsy-proven NAFLD. Furthermore, we demonstrated that circulating Lp(a) reflects its hepatic synthesis and that genetic variants previously associated with hepatic and cardiovascular damage may modulate serum Lp(a).

In conclusion, our results support the hypothesis that Lp(a) assessment in combination with transaminases may be considered as an early biomarker to predict hepatic fibrosis in patients with NAFLD.

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