Reduced Lysosomal Acid Lipase Activity in Blood and Platelets Is Associated With Nonalcoholic Fatty Liver Disease

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OBJECTIVES: To investigate whether blood total lysosomal acid lipase activity (BT-LAL) levels are uniquely associated with the noncirrhotic and cirrhotic stages of nonalcoholic fatty liver disease (NAFLD) and with protection from NAFLD in metabolically/genetically predisposed subjects and a normal liver. To clarify which enzyme-carrying circulating cells are involved in reduced BT-LAL of NAFLD.

METHODS: In a cross-sectional study, BT-LAL was measured by a fluorogenic method in patients with NAFLD (n = 118), alcoholic (n = 116), and hepatitis C virus-related disease (n = 49), in 103 controls with normal liver and in 58 liver transplant recipients. Intracellular platelet and leukocyte LAL was measured in 14 controls and 28 patients with NAFLD.

RESULTS: Compared with controls, (i) BT-LAL and LAL in platelets, but not in leukocytes, were progressively reduced in noncirrhotic NAFLD and in nonalcoholic steatohepatitis-related cirrhosis; (ii) platelet and leukocyte counts did not differ in patients with noncirrhotic NAFLD; and (iii) BT-LAL did not differ in alcoholic and hepatitis C virus noncirrhotic patients. BT-LAL progressively increased in controls with metabolic syndrome features according to their PNPLA3 rs738409 steatosis-associated variant status (II vs IM vs MM), and their BT-LAL was higher than that of noncirrhotic NAFLD, only when carriers of the PNPLA3 unfavorable alleles were considered. Liver transplant recipients with de novo NAFLD compared with those without de novo NAFLD had lower BT-LAL.

DISCUSSION: LAL in blood and platelets is progressively and uniquely reduced in NAFLD according to disease severity. High BT-LAL is associated with protection from NAFLD occurrence in subjects with metabolic and genetic predisposition. Low LAL in platelets and blood could play a pathogenetic role in NAFLD.

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A150, http://links.lww.com/CTG/A151, http://links.lww.com/CTG/A152, http://links.lww.com/CTG/A153, http://links.lww.com/CTG/A154, http://links.lww.com/CTG/A155, http://links.lww.com/CTG/A156, http://links.lww.com/CTG/A157

INTRODUCTION
Lysosomal acid lipase is an enzyme that hydrolyzes triglycerides and cholesteryl esters in several cells including hepatocytes, Kupffer cells, and bone marrow-derived monocyte-macrophages (1). The expression of lysosomal acid lipase is constitutive but can be further regulated by different stimuli. Lysosomal acid lipase activity (LAL) drives the hydrolysis of lipid droplets, and when LAL is too low, triglycerides and cholesteryl esters accumulate inside the lysosomes. In the liver, this accumulation occurs in both hepatocytes and Kupffer cells and favors hepatic steatosis. In addition, LAL inactivation in hepatocytes and immune cells is proinflammatory. In fact, the absence of LAL in hepatocytes causes an increased hepatic infiltration by macrophages (2), and the knockdown of this enzyme in some immunomodulator cell...
(myeloid derived suppressor cells, macrophages, and Treg and memory CD8+ T cells) induces a lipid metabolic switch, which favors inflammation in several organs, including the liver (1). In LAL deficiency, an autosomal recessive disease caused by mutations in the LIPA gene, which includes severe chronic liver disease that mimics nonalcoholic fatty liver disease (NAFLD), blood total LAL (BT-LAL), and intracellular leukocyte LAL is strongly reduced (3–5). Furthermore, it is known that the liver disease of LAL deficiency recurs after liver transplantation (LT), suggesting that the enzymatic deficiency in blood is sufficient to induce hepatic damage (6).

In addition to leukocytes, platelets have also been recently shown to contain LAL in healthy controls and BT-LAL is correlated more with LAL in platelets than with LAL in leukocytes (7). However, although platelets are known to play a role as modulators of liver diseases by nonthrombotic mechanisms that are incompletely understood (8,9), intracellular LAL in platelets has never been measured in patients with liver disease.

In the past years, a nongenetically determined reduction of BT-LAL with a residual enzymatic activity higher than that found in patients with LAL deficiency has been reported, in separate studies, regarding patients with noncirrhotic NAFLD (10) and with cryptogenic cirrhosis (11) compared with controls and in patients with cryptogenic cirrhosis compared with patients with cirrhosis of other etiologies (12).

Despite the evidence mentioned above, the association between reduced activity of BT-LAL and NAFLD throughout all disease stages is not considered very strong for several reasons. First of all, it is not known whether reduced BT-LAL is peculiar to NAFLD or it is also present in alcoholic liver disease (ALD), another condition characterized by hepatic fat accumulation. Second, it is unclear whether reduced BT-LAL in NAFLD is simply due to thrombocytopenia, which can be present even at the precirrhotic stage (10,13) or there is an intrinsic scarce enzymatic activity at the blood cell level. In fact, BT-LAL in platelets has not been measured in patients with NAFLD. Third, regarding the cirrhotic stage of NAFLD, comparative studies with healthy controls and with cirrhosis of other etiologies have taken into consideration patients with cryptogenic cirrhosis and not those with NASH-related cirrhosis (11,12). In this regard, it should be emphasized that recent data, although there is no agreement on the subject, suggest caution in equating the diagnosis of cryptogenic cirrhosis with that of NASH-related cirrhosis (14–17). Fourth, although the development of de novo NAFLD after LT in patients who were not affected by LAL deficiency represents a further model to test the association of BT-LAL levels with NAFLD, no data are available on this topic.

Finally, no data are available on BT-LAL in subjects with normal liver but metabolically and/or genetically predisposed to NAFLD.

Thus, we compared the following for the first time: (i) BT-LAL in controls with normal liver and in cirrhotic and noncirrhotic patients with only one isolated etiology of their chronic liver disease, namely, alcoholic, hepatitis C virus (HCV), and NAFLD, selecting patients with NASH-related cirrhosis and excluding those with cryptogenic cirrhosis; (ii) intracellular LAL in isolated platelets and leukocytes of subjects with normal liver vs patients with noncirrhotic NAFLD and with NASH-related cirrhosis; (iii) BT-LAL values after LT in patients with or without de novo NAFLD; and (iv) BT-LAL in subjects with noncirrhotic NAFLD and in those with normal liver but metabolically predisposed to NAFLD, according to their PNPLA3 rs738409 steatosis-associated variant status (18).

**METHODS**

**Study population**

Consecutive nontransplanted white patients older than 18 years were enrolled from October 2012 to September 2018 at the Department of Translational and Precision Medicine and the Day Service of Internal Medicine and Metabolic Diseases of the Department of Internal Medicine of the Sapienza University of Rome. Patients were enrolled to obtain groups strictly selected for one single etiology, namely, NAFLD (n = 118), alcoholic (n = 116), or HCV (n = 49), and for disease stage, i.e., cirrhotic vs noncirrhotic (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157). In particular, cirrhosis diagnosis, in general, independently from etiology, was based on liver biopsy or the presence of at least 2 of the following features: current or past cirrhosis complications (e.g., ascites, variceal gastrointestinal bleeding, and hepatic encephalopathy), the presence of hyperbilirubinemia, hypoalbuminemia, prolonged International Normalized Ratio, low platelet count, irregular liver surface at ultrasound/computed tomography, reduced portal vein flow at ultrasound, liver elastometry, and gastroesophageal varices at endoscopy. In the absence of pathological diagnosis or overt cirrhosis, individuals with the positivity of only one of the above-mentioned parameters were excluded from the study.

Inclusion criteria of noncirrhotic NAFLD and NASH-related cirrhotic patients were previous or current alcohol consumption of <20 g/d and the absence of any other etiology of chronic liver disease. For the diagnosis of noncirrhotic NAFLD, the ultrasonographic evidence of liver steatosis at ultrasounds was additionally requested, defined according to the Hamaguchi criteria (19), and/or NAFLD diagnosis at histology without cirrhosis and the absence of cirrhosis diagnosis as mentioned above. Noncirrhotic alcoholic or HCV patients with diabetes and/or obesity were also excluded. Regarding the cirrhotic stage of NAFLD, patients with cryptogenic cirrhosis were excluded and patients with NASH-related cirrhosis were only enrolled (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157).

To investigate whether post-transplant BT-LAL was associated with de novo NAFLD after LT, 58 stable liver-transplanted patients without cirrhosis recurrence and with a follow-up of at least 1 year after operation were consecutively enrolled and submitted to abdominal ultrasounds and blood sampling for BT-LAL measurement (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157). HCV-positive transplanted patients were excluded because HCV virus can directly cause steatosis even in the post-transplant setting.

Consecutive adult subjects with normal liver at ultrasounds and normal blood liver tests (n = 103) were enrolled as controls. These controls were not matched with any patient group and were either completely healthy subjects or patients with some features of metabolic syndrome.

All patients and controls signed the informed consent. The study was approved by the ethics committee of Policlinico Umberto I—Sapienza University of Rome (ref. no. 2277/2011 and 4591/2017) and was conducted according to the Declaration of Helsinki.
From all patients and controls was recorded a full clinical, drug, and alcohol consumption history (20), and blood was sampled for analyses (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157). Noncirrhotic NAFLD subjects and controls with at least one feature of metabolic syndrome were genotyped for the PNPLA3 rs738409 steatosis-associated variant (18).

LAL assays and PNPLA3 genotyping

All enzymatic assays were performed using a fluorimetric method at “Bambino Gesù Hospital” in Rome (Italy), within 1 week from the day of blood sampling, by a biologist (G.T.) unaware of clinical and biochemical characteristics of any enrolled subject.

BT-LAL was measured using the fluorimetric method as reported by Hamilton et al. (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157) (21).

To investigate intracellular LAL, platelets and leukocytes were isolated as proposed by Dagur and McCoy (22) with small modifications (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157). The morphology of isolated platelets and leukocytes was analyzed by using a FACS-Calibur cytometer (Becton Dickinson, Mountain View, CA), and purity was evaluated by immunophenotyping (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157).

Intracellular LAL in isolated leukocytes and platelets was measured as reported by Civallero et al. (23) with small modifications. Enzyme activities were expressed as nmol/mg protein/h (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157).

Inter- and intra-assay variations of BT-LAL and intracellular LAL determinations were lower than 5%. The intrapatient variability was lower than 10%.

PNPLA3 rs738409 was detected using dedicated TaqMan genotyping (Supplementary Methods, Supplementary Digital Content 8, http://links.lww.com/CTG/A157).

Statistical analyses

Continuous variables are presented as median (interquartile range). After the assessment of normality using the Kolmogorov–Smirnov test, the differences between groups were evaluated using Mann–Whitney U test or t test according to the variable normality. Categorical variables were expressed as count and percentages and compared with the χ2 test or Fisher exact test, as appropriate. Different multivariable binary logistic regression analyses were performed to investigate the association of BT-LAL with the noncirrhotic and cirrhotic stage of chronic liver diseases compared with the control group, with the stage of chronic liver disease of each etiology, and with etiology for each disease stage. Each multivariable model was adjusted for any demographic and clinical variable, including the features of metabolic syndrome and statin use, with a P value < 0.05 at univariate analyses. To avoid collinearity bias, blood platelet, leukocyte counts, and serum alanine aminotransferase were not introduced in the multivariate models even if they differed among groups because they showed correlation with BT-LAL. Computations were carried out with SPSS software 25.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Demographic, clinical, and biochemical parameters of the study groups are reported in Table 1. Daily alcohol consumption did not differ between the noncirrhotic ALD and the alcoholic cirrhotic groups, whereas the duration of at-risk alcohol consumption was longer in the alcoholic cirrhotic group. The time span of HCV infection did not differ between the noncirrhotic and cirrhotic HCV groups. Diagnosis in patients with noncirrhotic NAFLD was obtained in 53 (79%) cases by histology. BT-LAL did not differ between patients with histological diagnosis compared with those who had only ultrasounds and, among the biopsied subjects, between those with simple steatosis (n = 21) compared with those with NASH (n = 32) (data not shown).

BT-LAL in patients with NAFLD and ALD compared with subjects with normal liver

Because BT-LAL did not differ in patients with noncirrhotic NAFLD with nonhistological diagnosis and in those with histologically obtained diagnosis of NASH or of simple steatosis (data not shown), they were all considered as one group. As shown in Figure 1a, BT-LAL, compared with that of controls (0.94 [0.73–1.76] nmol/spot/hr), did not differ in patients with noncirrhotic ALD (1.04 [0.72–1.42] nmol/spot/hr), whereas it progressively significantly decreased in patients with noncirrhotic NAFLD (0.64 [0.50–0.91] nmol/spot/hr; P < 0.001), alcoholic cirrhosis (0.63 [0.46–0.89] nmol/spot/hr; P < 0.001), and NASH-related cirrhosis (0.48 [0.39–0.69] nmol/spot/hr); P < 0.001). Platelet count (Figure 1b), compared with that of controls, did not differ in the noncirrhotic ALD and NAFLD groups, whereas it was significantly reduced in the alcoholic (P < 0.001) and NASH-related (P < 0.001) cirrhotic groups. Leukocyte count (Figure 1b), compared with the controls, did not differ in patients with noncirrhotic NAFLD, whereas it was significantly higher in patients with noncirrhotic ALD (P < 0.001) and significantly lower in patients with alcoholic (P < 0.001) and NASH-related (P < 0.001) cirrhosis. After correction for the significant intergroup differences shown in Table 1, at multivariable logistic regression analyses comparing separately each group with the control group (Supplementary Table 1, Supplementary Digital Content 3, http://links.lww.com/CTG/A152), low BT-LAL was independently associated with noncirrhotic NAFLD (odds ratio [OR] 0.48 [0.22–0.96]; 95% confidence interval [CI]: 0.22–0.96; P = 0.028), NASH-related cirrhosis (OR 0.03 [95% CI: 0.001–0.03]; P < 0.001), and alcoholic cirrhosis (OR 0.227; 95% CI: 0.10–0.50; P = 0.005) (Supplementary Table 2, Supplementary Digital Content 3).
Table 1. Demographic, clinical, and biochemical parameters of the study groups

|                      | Men, n (%) | Age (yr) | BMI (kg/m²) | Diabetes, n (%) | Hypertension, n (%) | Dyslipidemia, n (%) | Statins, n (%) | ALT (UI/L) | MELD score | Daily alcohol intake (units) | Years of at-risk alcohol intake | Years since HCV infection |
|----------------------|------------|----------|-------------|-----------------|---------------------|---------------------|----------------|------------|------------|----------------------------|-----------------------------|--------------------------|
| CTRL (n = 103)       | 47 (46)    | 53 (30–67) | 24.4 (22.1–26.6) | 6 (6)           | 30 (29)             | 56 (54)             | 26 (25)       | 16 (12–20) | —          | —                          | —                           | —                        |
| Noncirrhotic NAFLD (n = 67) | 36 (54) | 53 (41–59) | 28.1 (26.0–31.0) | 18 (27)         | 24 (36)             | 47 (70)             | 14 (21)       | 51 (38–83) | —          | —                          | —                           | —                        |
| CIR-NASH (n = 51)    | 34 (67)    | 66 (58–72) | 27.0 (24.7–30.8) | 30 (59)         | 25 (49)             | 25 (49)             | 2 (4)          | 30 (20–43) | 13 (10–15) | —                          | —                           | —                        |
| Noncirrhotic ALD (n = 53) | 52 (98) | 50 (42–58) | 24.6 (22.3–26.5) | 0 (0)           | 7 (13)              | 41 (77)             | 2 (4)          | 21 (16–31) | —          | 13 (10–18)                 | 22 (14–29)                 | —                        |
| CIR-ALD (n = 63)     | 60 (95)    | 59 (54–64) | 26.4 (24.7–28.1) | 17 (27)         | 16 (25)             | 16 (25)             | 3 (5)          | 24 (17–38) | 14 (10–17) | 12a (9–16)                 | 31a (20–40)                | —                        |
| Noncirrhotic HCV (n = 19) | 14 (74) | 62 (54–72) | 24.6 (22.2–27.0) | 0 (0)           | 9 (47)              | 9 (47)              | 0 (0)          | 33 (25–46) | —          | —                          | 23 (15–31)                 | —                        |
| CIR-HCV (n = 30)     | 19 (63)    | 63 (55–75) | 24.8 (22.9–27.6) | 4 (13)          | 12 (40)             | 9 (30)              | 0 (0)          | 24 (16–64) | 10 (8–13) | —                          | 20 (9–25)                  | —                        |
| Noncirrhotic NAFLD vs CTRL, P value | 0.302 | 0.652 | <0.001 | <0.001 | 0.360 | <0.001 | <0.001 | <0.001 | <0.001 | NA | NA | NA | NA |
| CIR-NASH vs CTRL, P value | 0.014 | <0.001 | <0.001 | <0.001 | <0.001 | 0.015 | <0.001 | <0.001 | <0.001 | NA | NA | NA | NA |
| Noncirrhotic ALD vs CTRL, P value | <0.001 | 0.007 | 0.004 | <0.001 | 0.602 | <0.001 | 0.001 | <0.001 | NA | NA | NA | NA | NA |
| CIR-HCV vs CTRL, P value | 0.157 | <0.001 | 0.689 | 0.220 | 0.304 | 0.015 | 0.001 | <0.001 | NA | NA | NA | NA |
| CIR-NASH vs noncirrhotic NAFLD, P value | 0.624 | <0.001 | 0.173 | <0.001 | 0.149 | 0.020 | 0.013 | <0.001 | NA | NA | NA | NA |
| CIR-ALD vs noncirrhotic ALD, P value | 0.624 | <0.001 | 0.001 | <0.001 | 0.101 | <0.001 | 1.000 | 0.241 | NA | 0.404 | 0.001 | NA |
| CIR-HCV vs noncirrhotic HCV, P value | 0.532 | 0.604 | 0.737 | 0.137 | 0.582 | 0.188 | 1.000 | 0.330 | NA | NA | NA | 0.425 |
| Noncirrhotic NAFLD vs noncirrhotic ALD, P value | <0.001 | 0.847 | <0.001 | <0.001 | 0.005 | 0.375 | 0.006 | <0.001 | NA | NA | NA | NA |
| Noncirrhotic NAFLD vs noncirrhotic HCV, P value | 0.187 | <0.001 | <0.001 | 0.009 | 0.361 | 0.066 | 0.033 | 0.004 | NA | NA | NA | NA | NA |
We thus repeated the analyses comparing BT-LAL of patients with at least one feature of metabolic syndrome in the controls and in the NAFLD groups separately, according to the PNPLA3 genotype. As shown in Table 3, BT-LAL was significantly higher in the control group compared with the NAFLD group when the IM (P = 0.017) and, separately, according to the PNPLA3 genotype. As shown in Table 3, BT-LAL was significantly higher in the control group compared with the NAFLD group when the IM (P = 0.017) and,
even more, MM (P = 0.006) subjects were considered, whereas no difference was present in the II subjects. No difference in the platelet and leukocyte count was present, with the only exception of lower leukocyte counts in the control subjects as compared to the patients with noncirrhotic NAFLD when the PNPLA3 II subjects were considered.

### Table 2. Demographic, clinical, and biochemical characteristics of subjects with at least one feature of metabolic syndrome in the control and noncirrhotic NAFLD groups

|                        | CTRL (n = 62) | Noncirrhotic NAFLD (n = 57) | P value |
|------------------------|---------------|-----------------------------|---------|
| BT-LAL nmol/spot/hr    | 0.88 (0.73–1.19) | 0.76 (0.58–0.94)   | **0.005** |
| Men, n (%)             | 29 (47)       | 31 (54)                    | 0.407   |
| Age (yr)               | 58 (51–72)    | 53 (44–60)                 | **0.003** |
| BMI (kg/m²)            | 25.6 (22.2–27.6) | 28.9 (26.8–31.8)   | <**0.001** |
| Diabetes, n (%)        | 6 (10)        | 18 (32)                    | **0.003** |
| Hypertension, n (%)    | 30 (48)       | 24 (42)                    | 0.492   |
| Dyslipidemia, n (%)    | 56 (90)       | 47 (83)                    | 0.209   |
| Statins, n (%)         | 26 (42)       | 14 (25)                    | 0.45    |
| ALT (UI/L)             | 17 (13–20)    | 48 (38–83)                 | <**0.001** |
| Platelet count (n × 10⁹/mL) | 222 (184–263) | 231 (186–231)     | 0.411   |
| Leukocyte count (n × 10³/mm³) | 5.6 (4.9–6.5) | 6.0 (5.5–7.2) | **0.033** |
| PNPLA3, n (%)          |                |                            | **<0.001** |
| II                     | 22 (39)       | 26 (46)                    |         |
| IM                     | 12 (21)       | 21 (37)                    |         |
| MM                     | 22 (39)       | 10 (18)                    |         |

Continuous variables are expressed as median (25th–75th percentile), and the differences between groups were evaluated by Mann-Whitney U test or t test according to the variable normality. Categorical variables were expressed as count (percentages) and compared by the x² tests.

ALT, alanine aminotransferase; BMI, body mass index; BT-LAL, blood total lysosomal acid lipase activity; CTRL, controls with normal liver; NAFLD, nonalcoholic fatty liver disease; and noncirrhotic NAFLD, noncirrhotics with NAFLD.

Significant P values are reported in bold.

### Table 3. Demographic, clinical, and biochemical characteristics of subjects with at least one feature of metabolic syndrome in the control and noncirrhotic NAFLD groups, according to the PNPLA3 variant status

|                        | PNPLA3 II | PNPLA3 IM | PNPLA3 MM | P value |
|------------------------|-----------|-----------|-----------|---------|
|                        | CTRL      | NC-NAFLD  | P value   | CTRL    | NC-NAFLD  | P value   | CTRL    | NC-NAFLD  | P value   |
| BT-LAL (nanomoles/ spot/hr) | 0.76 (0.64–1.07) | 0.82 (0.65–1.10) | 0.885 | 0.88 (0.73–1.40) | 0.66 (0.51–0.89) | **0.017** | 0.96 (0.78–1.13) | 0.68 (0.61–0.80) | **0.006** |
| Platelet count (n × 10⁹/mL) | 215 (188–286) | 237 (200–282) | 0.598 | 231 (210–278) | 220 (179–257) | 0.427 | 207 (171–236) | 238 (183–270) | 0.184 |
| Leukocyte count (n × 10³/mm³) | 5.8 (4.3–6.3) | 6.9 (5.7–7.7) | **0.012** | 5.7 (5.3–7.4) | 5.9 (5.0–6.7) | 0.764 | 5.5 (4.8–6.4) | 5.8 (5.3–7.0) | 0.234 |
| Male sex               | 13 (59)   | 13 (50)   | 0.529   | 4 (33)   | 12 (57)   | 0.282   | 11 (50)  | 6 (60)    | 0.712 |
| Age (yr)               | 56 (47–71) | 53 (45–64) | 0.277   | 52 (37–71) | 52 (33–60) | 0.278   | 66 (55–75) | 50 (45–54) | **0.002** |
| BMI                    | 25.9 (23.1–27.5) | 28.4 (26.7–30.7) | **0.003** | 23.0 (20.0–26.5) | 29.4 (27.4–32.9) | <**0.001** | 26.1 (23.9–28.1) | 28.5 (26.2–32.5) | 0.082 |
| ALT (UI/L)             | 17 (13–25) | 47 (38–69) | <**0.001** | 14 (12–21) | 48 (33–96) | <**0.001** | 16 (13–19) | 81 (41–143) | **0.006** |

Continuous variables are expressed as median (25th–75th percentile), and the differences between groups were evaluated by Mann-Whitney U test or t test according to the variable normality. Categorical variables were expressed as count (percentages) and compared by the x² tests.

ALT, alanine aminotransferase; BMI, body mass index; BT-LAL, blood total lysosomal acid lipase activity; CTRL, controls with normal liver; NAFLD, nonalcoholic fatty liver disease; and NC-NAFLD, noncirrhotics with NAFLD.

Significant P values are reported in bold.
BT-LAL in patients with NAFLD, alcoholic, and HCV liver disease according to disease etiology and stage

We then separately investigated BT-LAL according to the stage of chronic liver disease within the NAFLD and ALD groups. As reported in Figure 1a, BT-LAL was significantly lower in alcoholic cirrhosis as compared to patients with noncirrhotic ALD (P < 0.001) and in NASH-related cirrhosis compared with noncirrhotic NAFLD (P < 0.001). Each cirrhotic group had significantly lower platelet and leukocyte counts compared with the respective noncirrhotic group (Figure 1b, c). After correction for the significant intergroup differences shown in Table 1, at multivariable logistic regression analyses (Supplementary Table 3, Supplementary Digital Content 5, http://links.lww.com/CTG/A154), low BT-LAL was independently associated with the presence of cirrhosis compared with the respective noncirrhotic group of either nonalcoholic (OR 0.059; 95% CI: 0.008–0.404; P = 0.004) or alcoholic (OR 0.080; 95% CI: 0.010–0.666; P = 0.020) etiology.

Finally, we compared BT-LAL according to the 2 different etiologies, NAFLD and ALD, considering separately the noncirrhotic and the cirrhotic stage. Regarding the noncirrhotic patients, as reported in Figure 1a, BT-LAL was significantly lower in the NAFLD group compared with the ALD group (P < 0.001). Platelet count did not differ between the noncirrhotic ALD and NAFLD groups. Leukocyte count was significantly (P < 0.001) lower in the noncirrhotic NAFLD group compared with the noncirrhotic ALD group. After correction for the significant intergroup differences shown in Table 1, at multivariable logistic regression analyses (Supplementary Table 3, Supplementary Digital Content 5, http://links.lww.com/CTG/A154), low total blood LAL was independently associated with noncirrhotic NAFLD compared with noncirrhotic ALD (OR 0.089; 95% CI: 0.017–0.452; P = 0.004).

Regarding the cirrhotic patients, as reported in Figure 1a, BT-LAL was significantly lower in the NASH-related group compared with the alcoholic group (P < 0.05). No intergroup difference was found regarding platelet and leukocyte counts. After correction for the significant intergroup differences shown in Table 1, at multivariable logistic regression analyses (Supplementary Table 3, Supplementary Digital Content 5, http://links.lww.com/CTG/A154), low BT-LAL was independently associated with NASH-related compared with alcoholic cirrhosis (OR 0.174; 95% CI: 0.033–0.923; P = 0.013).

Similar to ALD, BT-LAL was significantly lower in patients with HCV-related cirrhosis compared with both the controls and the noncirrhotic HCV group, whereas it did not differ between the noncirrhotic HCV group and controls (Supplemental results and Figure 1, Supplementary Digital Content 1, http://links.lww.com/CTG/A150).

Intracellular LAL in platelets and leukocytes according to the NAFLD stage

Because we found that BT-LAL compared with that in controls with normal liver was progressively reduced in NAFLD according to the disease stage, we then investigated LAL at the intracellular level of platelets and leukocytes isolated from control subjects (n = 14), noncirrhotic NAFLD (n = 14), and NASH-related cirrhosis (n = 14) (Supplementary Table 4, Supplementary Digital Content 6, http://links.lww.com/CTG/A155). Both leukocyte and platelet isolation procedures yielded high purity preparations (Supplementary Figure 2, Supplementary Digital Content 2, http://links.lww.com/CTG/A151).

Intracellular platelet activity normalized by cellular total protein content was significantly reduced in patients with noncirrhotic NAFLD (175 [108–270] nmol/mg of protein/hr; P = 0.022) and even more in patients with NASH-related cirrhosis (84 [51–177] nmol/mg of protein/hr; P < 0.001) compared with that in control subjects (314 [214–416] nmol/mg of protein/hr) (Figure 2a). Interestingly, intracellular platelet LAL was also significantly reduced in NASH-related cirrhosis compared with patients with noncirrhotic NAFLD (P = 0.022). At difference, intracellular leukocyte LAL did not differ among the 3 groups (Figure 2b), being 211 (123–290) nmol/mg of protein/hr in
preparations (Supplementary Figure 2, Supplementary Digital Content 2, http://links.lww.com/CTG/A151).

Intracellular platelet activity normalized by cellular total protein content was significantly reduced in patients with non-cirrhotic NAFLD (175 [108–270] nmol/mg of protein/hr; P = 0.022) and even more in patients with NASH-related cirrhosis (84 [51–177] nmol/mg of protein/hr; P < 0.001) compared with that in control subjects (314 [214–416] nmol/mg of protein/hr) (Figure 2a). Interestingly, intracellular platelet LAL was also significantly reduced in NASH-related cirrhosis compared with patients with noncirrhotic NAFLD (P = 0.022). At difference, intracellular leukocyte LAL did not differ among the 3 groups (Figure 2b), being 211 (123–290) nmol/mg of protein/hr in controls, 126 (95–291) nmol/mg of protein/hr in noncirrhotic NAFLD, and 169 (109–215) nmol/mg of protein/hr in patients with NASH-related cirrhosis.

BT-LAL after LT and de novo graft NAFLD
We then investigated BT-LAL according to the evidence at ultrasounds of post-transplant de novo graft NAFLD in stable patients who had undergone LT. As shown in Figure 3a, BT-LAL was significantly lower (P = 0.034) in recipients with post-transplant NAFLD (0.51 [0.43–0.72] nmol/spot/hr) than in those without NAFLD (0.69 [0.51–0.96] nmol/spot/hr), whereas no intergroup difference was found regarding platelet (Figure 3b) and leukocyte (Figure 3c) counts, any metabolic syndrome feature, statin treatment, and time since LT (Supplementary Table 5, Supplementary Digital Content 7, http://links.lww.com/CTG/A156).

We finally investigated whether the reduced BT-LAL levels that we found in nontransplanted cirrhotic patients was 4.6 years. As shown in Figure 4a, BT-LAL did not differ before and after LT for NASH-related cirrhosis (0.48 [0.39–0.69] nmol/spot/hr vs 0.52 [0.44–0.75] nmol/spot/hr, respectively) and for alcoholic cirrhosis (0.63 [0.46–0.89] nmol/spot/hr vs 0.73 [0.49–0.94] nmol/spot/hr, respectively). However, transplanted compared with nontransplanted cirrhotic patients had significantly higher platelet (Figure 4b) and leukocyte (Figure 4c) counts. Eight of these patients were studied both before and after LT. BT-LAL (0.50 ± 0.15 nmol/spot/hr vs 0.56 ± 0.33 nmol/spot/hr, respectively) and leukocyte count (4.89 ± 1.31 vs 5.65 ± 1.21 [n × 10^9/mL], respectively) did not differ before and after LT. However, platelet count was significantly (P = 0.012) higher after than before LT (134.0 ± 36.5 vs 48.9 ± 17.7 [n × 10^9/mL], respectively).

DISCUSSION
The main findings of our present study are as follows: (i) a progressive reduction of BT-LAL according to chronic liver disease stage, peculiar to nonalcoholic fatty liver disease (NAFLD) and independent from confounders; (ii) a progressive reduction of intracellular LAL in circulating platelets, but not in leukocytes, of patients with nonalcoholic fatty liver which was dependent on the disease stage and paralleled enzymatic activity reduction in whole blood; (iii) normal BT-LAL levels in heavy drinkers and in HCV infected subjects without evidence of cirrhosis; (iv) progressively high BT-LAL in PNPLA3 allele M carriers (IM and MM) with some of the metabolic syndrome features but without steatosis; and (v) significantly lower BT-LAL in LT recipients with post-
transplant de novo NAFLD compared with those without NAFLD.

We first investigated BT-LAL in patients at both the non-cirrhotic and cirrhotic stages of 3 chronic liver diseases selected to obtain groups with a single pure etiology, namely, NAFLD, alcoholic fatty liver disease, and HCV. We found that low BT-LAL was independently associated with the cirrhotic stage of each of the 3 different etiologies of chronic liver disease compared with controls. However, we found that only in NAFLD, there is a peculiar progressive reduction of BT-LAL, according to disease severity that also involves the noncirrhotic stage. Reduced BT-LAL in NAFLD could, in theory, be simply explained by the low number of platelets present in cirrhosis and reported also to occur in the precirrhotic stage of the disease (3,13). However, we found that low BT-LAL was independently associated with NASH-related cirrhosis compared with alcoholic cirrhosis, with non-cirrhotic NAFLD compared with controls and noncirrhotic ALD and, in the post-transplant setting, with de novo NAFLD compared with the absence of steatosis, even if platelets did not differ for each comparison.

In addition, we measured for the first time intracellular LAL in circulating cells of patients with different stages of NAFLD and controls with normal liver. Thus, we provided direct evidence for a progressively reduced LAL, expressed per mg of intracellular protein, carried by platelets, but not of that carried by leukocytes, in noncirrhotic NAFLD and, in a more severe fashion, in NASH-related cirrhosis. In particular, LAL carried by platelets in cirrhotic and noncirrhotic patients was 56% and 27% of that present in controls, respectively.

Our present demonstration of a progressively reduced BT-LAL according to the disease severity only in NAFLD, paralleled by a reduction of LAL in platelets, and the association of reduced BT-LAL with post-transplant de novo NAFLD strongly suggest that low LAL is peculiar to NAFLD, probably because of transcriptional or post-transcriptional reductions of LAL in platelets (7,10,24).

On the other hand, our data show that having normal BT-LAL values is associated with protection from NAFLD development in subjects with features of metabolic syndrome. In fact, normal BT-LAL was associated with the absence of steatosis even in subjects with metabolic syndrome features that should have been prone to the development of steatosis as carriers of the PNPLA3 variant that was found to be strongly associated with fat accumulation in hepatocytes (18). It should be noted that, in our study, BT-LAL was progressively increased in PNPLA3 variant II, IM, and MM subjects with features of metabolic syndrome but without steatosis, suggesting a protective role of high LAL in the sense that the more the genetic factor is unfavorable, the higher the BT-LAL values must be to protect against the development of NAFLD.

The concept that circulating cells with reduced LAL can induce NAFLD is in keeping with the well-described recurrence of liver steatosis and cirrhosis after LT in patients with LAL deficiency (6). To explain post-transplant liver disease recurrence in patients with LAL deficiency, it has been hypothesized that insufficient lipophagy in Kupffer cells and in bone marrow-derived myeloid cells would lead to their immunometabolic activation and migration into the liver with inflammation (1,6,25–27). We hypothesized that the reduced platelet LAL that we found in NAFLD causes subnormal lipophagy, cholesterol accumulation, and their metabolic activation (28–30). This metabolic activation
of platelets could increase their migration into the space of Disse and their demonstrated inflammatory capacity, as recently shown by Malehmir et al. (9). In addition, the involvement of platelet activation in NAFLD pathogenesis is reinforced by the beneficial effects of antiplatelet therapy that have been reported in animal models and in patients with NAFLD (9,31–33). Further studies will be needed to correlate LAL and lipodrome in platelets and their migration and activation into the liver. These studies will unravel the mechanisms underlying the reduction of LAL in platelets of patients with nonalcoholic fatty liver and its physiopathological role. A further unresolved question raised by our results is why BT-LAL levels of cirrhotic patients are similar before and after LT, although platelet and leukocyte counts increase after operation. A possible explanation could be that because LAL is inducible by fasting/starving (34), the opposite occurs after LT when calorie intake increases compared with that before operation with the reduction of LAL. In keeping with this hypothesis, it is known that LAL is induced by a low calorie intake (35).

A limitation of our study is that data on intracellular LAL have been obtained from a small number of patients. In addition, regarding the intergroup differences of BT-LAL, our study is limited by its monocentric and cross-sectional design and needs confirmation in larger studies because groups are relatively small. On the other hand, our study groups, including those with NAFLD, were carefully characterized for disease stage and unique etiology, avoiding the overlapping of liver disease cofactors, and we selected patients with a diagnosis of NASH-related cirrhosis and not those with cryptogenic cirrhosis (14–17).

Finally, our study was not designed to relate BT-LAL to histological NAFLD features and future prospective studies involving repeated liver biopsies of patients with NAFLD are needed to definitely unravel the strength of the association between reduced BT-LAL and disease severity. Studies involving a larger number of patients could also ascertain whether measurement of BT-LAL in patients with nonalcoholic fatty acid liver disease could be useful as a new noninvasive marker of liver disease severity across its different fibrosis stages.

Our results could also have possible implications for nonalcoholic fatty acid liver disease treatment, even if improving the LAL levels could result in a reduction of hepatic fat and/or inflammation in these patients is still unclear (35,36).

In conclusion, our data show that reduced LAL is associated with NAFLD because LAL, both in blood and in platelets, is progressively reduced according to disease stage. LAL reduction in blood at the noncirrhotic stage is unique for NAFLD and not for other etiologies of liver damage and is also associated with de novo post liver transplant NAFLD. In addition, normal LAL in blood is associated with the absence of steatosis even in subjects with metabolic syndrome features and LAL in blood progressively increases in PNPLA3 steatosis associated variant II, IM, and MM subjects without liver disease, suggesting that high LAL can be protective and low LAL detrimental for NAFLD development.

CONFLICTS OF INTEREST
Guarantor of the article: Stefano Ginanni Corradini, MD, PhD.
Specific author contributions: F.F., M.M., and S.G.C. contributed to the study concept and design. F.F. and S.G.C. performed the statistical analysis and wrote the manuscript. F.V. and F.A. supervised and oversaw the study. E.M., I.M., S.M., M.P., S.P., F.B., M.D.B., R.P., M.L.A., M.C., and A.D.S. collected sample and clinical information. M.M., R.M., and M.V. performed platelet and leukocyte isolation and flow cytometry of isolated platelets and leukocytes. G.T. performed LAL assay.

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