**Materials and methods**

**Study Population**

Consecutive non-transplanted Caucasian patients older than 18 years were enrolled from October 2012 to September 2018 at the Department of Translational and Precision Medicine and at the Day Service of Internal Medicine and Metabolic Diseases of the Department of Internal Medicine of 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 non-cirrhotic.

Liver steatosis at ultrasounds was defined according to the Hamaguchi criteria.19

Cirrhosis diagnosis was based on liver biopsy or on the presence of at least two of the following features: current or past cirrhosis complications (e.g. ascites, variceal gastrointestinal bleeding, hepatic encephalopathy), the presence of hyperbilirubinaemia, hypoalbuminaemia, prolonged INR, low platelet count, irregular liver surface at ultrasound/CT, reduced portal vein flow at ultrasound, liver elastometry, gastroesophageal varices at endoscopy. In the absence of pathological diagnosis or overt cirrhosis, individuals with positivity of only one of the above mentioned parameters were excluded from the study.

Inclusion criteria of non-transplanted non-cirrhotic NAFLD and NASH-related cirrhotic patients were prior or current alcohol consumption of <20 g/day, and the absence of any other aetiology of chronic liver disease. For the diagnosis of non-cirrhotic NAFLD, was additionally requested the ultrasonographic evidence of steatosis or NAFLD diagnosis at histology without cirrhosis and the absence of cirrhosis diagnosis as mentioned above. For the diagnosis of NASH-related cirrhosis, in addition to the general diagnosis of cirrhosis, it was requested at least one of the following features, reported to be present before the diagnosis of cirrhosis: 1) at least two features of the metabolic syndrome; 2) at least one feature of the metabolic syndrome plus evidence of liver steatosis at ultrasound or 3) histological evidence of steatosis or steatohepatitis.

Inclusion criteria of non-transplanted patients with non-cirrhotic alcoholic liver disease (ALD) were the ultrasonographic evidence of steatosis, a daily at-risk alcohol consumption ≥3 and ≥2 alcohol units for men and women respectively for at least 5 years (one unit of alcohol was defined as 12 g of ethanol), absence of cirrhosis, of any other etiology of chronic liver disease, of obesity and diabetes. Inclusion criteria of non-transplanted alcoholic cirrhosis were the same of non-cirrhotic ALD with respect to daily at-risk alcohol consumption, absence of any other etiology and the above mentioned general criteria for cirrhosis diagnosis.

Inclusion criteria of non-transplanted non-cirrhotic HCV patients were the presence of chronic hepatits C with HCV antibodies and serum HCV RNA positivity, increased serum aminotransferases, absence of cirrhosis, of any other aetiology of chronic liver disease, of obesity and diabetes. Inclusion criteria of non-transplanted HCV-related cirrhosis were the presence of HCV antibodies and serum HCV RNA positivity absence of any other aetiology and the above mentioned general criteria for cirrhosis diagnosis. Years from HCV infection were calculated from the presumed HCV transmission event.

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 one year after operation were consecutively enrolled and submitted to abdominal ultrasounds and blood sampling for BT-LAL measurement (supplementary methods). Inclusion criteria were NASH or alcoholic or hepatitis B virus (HBV) single aetiology of their pre-transplant cirrhosis and absence of post-transplant cirrhosis. HBsAg transplanted patients had undetectable HBV DNA in serum. HCV positive transplanted patients were excluded because HCV virus can directly cause steatosis even in the post-transplant setting.

Consecutive caucasian adult subjects with a normal liver at ultrasounds and normal blood liver tests (n= 103) were enrolled as controls. These controls were not matched to any patient group and were either completely healthy subjects or patients with some features of the 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. n° 2277/2011 and 4591/2017) and was conductedaccording to theHelsinki Declaration.

From all patients and controls was recorded a full clinical, drug and alcohol consumption history. In the case of a declared daily at-risk alcohol consumption ≥3 and ≥2 alcohol units for men and women respectively, a detailed alcohol consumption history was taken by Lifetime Drinking History (LDH) to calculate quantitative indices of alcohol consumption patterns, throughout the life span.20 Height and weight were recorded in order to calculate BMI. In the case of cirrhotic patients the dry weight was taken into account. Blood sampling was performed after a 12-hour fast to obtain BT-LAL, the lipid profile (total cholesterol, HDL-cholesterol, triglycerides), fasting blood glucose and blood cell counts. In addition, in cirrhotic patients, total bilirubin, creatinine and INR were measured to calculate the MELD score and in controls and in non-cirrhotic NAFLD subjects was performed genotyping of the PNPLA3 rs738409 steatosis-associated variant18.

**LAL activity assay in whole blood**

To measure BT-LAL, EDTA blood (BD Becton Dickinson S.p.A, Italia) was withdrawn by venipuncture and spotted on filter paper (Whatman grade 903, Schleicher & Schuell) according to the National Committee for Clinical Laboratory Standard (NCCLS) protocol. A total of 75 µl of blood was spotted in duplicate and dried, overnight, at room temperature. Hence, samples were stored double-bagged with desiccant at -20°C up to enzymatic assay. LAL activity present in whole blood was then measured using the fluorimetric method as reported by Hamilton et al.22 Briefly, activities were measured after being uninhibited or inhibited with Lalistat 2, and LAL activity was determined by subtracting the activity in the inhibited reaction from the uninhibited reaction (total lipase) and expressed as nmol/spot/h of 4-methylumbelliferyl palmitate used as substrate.

**Platelet and leukocyte isolation**

Platelets and leukocytes were isolated to investigate intracellular LAL activity under 12 hour fasting conditions. Platelets were isolated according to Dagur and McCoy,23 with small modifications. Eight ml of blood was drawn into BD Vacutainer® Citrate Tubes with 3.2% buffered sodium citrate solution (BD Becton Dickinson S.p.A, Italia). Then, it was centrifuged at 150 *x g* for 15 minutes at room temperature. The upper phase, representing platelet rich plasma, was carefully transferred into a 15 ml polypropylene lab ware and centrifuged at 100 *x g* for 15 minutes at room temperature. Platelet rich plasma was transferred into a new 15 ml polypropylene tube and combined with a citrate-dextrose solution (Santa Cruz Biotechnology, Inc.) as anticoagulant at 1:10 ratio. Finally, platelets were pelleted by centrifugation at 900 *x g* for 5 min at room temperature and then stored at -20°C until the enzymatic LAL assay was performed.

Leukocyte isolation was obtained according to Dagur and McCoy, 23 with small modifications. Specifically, 6 ml of EDTA blood (BD Becton Dickinson S.p.A, Italia) was collected by venipuncture and centrifuged at 300 *x g* for 5 minutes. After removing plasma upper phase, BD Pharm Lyse™ solution (BD Becton Dickinson S.p.A, Italia) was added to induce red blood cell lysis (1:10, volume to volume) incubating for 10 minutes under light oscillation. Hence, red blood cell lysed blood was centrifuged at 300 *x g*, for 5 minutes and the resulting surnatant was discarded. The remaining pellet was washed with 1X cold phosphate-buffered saline (pH 7.4, 10% fetal bovine serum) by centrifugation at 200 *x g* for 5 minutes. All steps were performed at room temperature. Next, surnatant was discarded and the leukocyte pellet was frozen for further use.

**Flow cytometry of isolated platelets and leukocytes**

Morphology and purity of isolated platelets and leukocytes were analysed using a FACSCalibur cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW, 488 nm, air-cooled argon ion laser for excitation of FITC (FL1), PE (FL2) and PerCP (FL3), and with a 10 mW, 635 nm, red diode laser for excitation of APC (FL4). The cytometer stability and sensitivity were checked before each acquisition session, by measuring the intensity and the variation coefficient of scatters and fluorescence signals of microbeads Nile Red (Becton Dickinson). FL4 detection was optimized by time delay calibration using APC microbeads (Becton Dickinson). Samples were acquired and analysed using CELLQuest 3.3 (Becton Dickinson, Mountain View, CA) and FlowJo (TreeStar, Ashland, Ore) software.

After isolation, 1 × 106 of platelets were labeled with 5μl of anti‐CD41‐PE–conjugated monoclonal antibody (platelet glycoprotein IIb, PC-309-T100 EXBIO) and 10μl of anti-CD45-FITC (leukocyte common antigen, 347463 BD Biosciences) for 20 min at room temperature in 100μl of Tyrode’s solution (TS). Then other 200μl of TS were added before analysis. To avoid activation, platelets were immediately stained and analysed after isolation.

Leukocytes (2x105) were stained with 10μl of anti-CD45-FITC and 5μl of anti-CD41-PerCP for 20 min on ice in 100μl of 1XPBS-10%FBS (PF10), then 1ml of PF10 was added and the sample was centrifugated at 200g for 4 min at 4°C and resuspended in 300μl of PF10 before analysis. Platelets were distinguished from debris and other cells (red blood cells and white blood cells) based on forward light scatter (FSC-H) *vs*. side light scatter (SSC-H) plot characteristics on a log/log scale with a FSC threshold set to 150 to exclude debris. Leukocytes were analysed with FCS-H and SSC-H in linear scale setting and the FSC threshold was set to 0 to verify if contaminating platelets were present.

**Intracellular LAL activity assay in isolated platelets and leukocytes**

The isolated leukocyte and platelet pellets were resuspended in bidistilled water, sonicated three times for 2 seconds  (Sonics Vibra Cell, Sonics & Material Inc., Newtown, CT), and the total protein concentration was measured using the BCA protein assay (Pierce™) in duplicate. Then, a sample corresponding to 40 µg of proteins was used to measure LAL activity in duplicate, by 4-methylumbelliferyl palmitate fluorimetry, as described by Civallero et al.24

**DNA extraction and PNPLA3 genotyping**

Whole blood containing EDTA (BD Becton Dickinson S.p.A, Italia) was centrifuged at 1,376 g for 10 minutes, the buffy coat fraction was recovered and DNA extraction was performed using QIAmp® DNA Blood Kit (QIAGEN S.p.A, Milan, Italy) according to the manufacturer’s instructions. DNA yield of all samples was determined by measuring its concentration in the eluate by absorbance at 260 nm and then 1 to 10 ng of purified genomic DNA sample per template well was directly used for the genotyping assays. PNPLA3 rs738409 was detected using a dedicated TaqMan® genotyping assay that allows to distinguish both wildtype and variant alleles at the SNP site in the DNA target sequence (TaqMan Applied Biosystems, Foster City, CA, USA). All samples were tested in triplicate with a success rate of 99%.

**Supplementary results:**

**BT-LAL in patients with HCV-related chronic liver disease compared to subjects with normal liver**

Supplementary figure 1 depicts BT-LAL (panel A), platelet (panel B) and leukocyte (panel C) counts in the non-cirrhotic and cirrhotic HCV groups and in controls with normal liver. BT-LAL did not differ between the controls (0.94 [0.73-1.76] nmol/spot/h) and the non-cirrhotic HCV (0.96 [0.83-1.06] nmol/spot/h) group, while it was significantly lower in patients with HCV-related cirrhosis (0.64 [0.50-0.91] nmol/spot/h) compared to both the controls (p<0.001) and the non-cirrhotic HCV (p<0.01) group. Platelet count, compared to that of controls, was progressively and significantly reduced in the non-cirrhotic and in the cirrhotic HCV group. Leukocyte count was significantly lower in the cirrhotic HCV compared either to the non-cirrhotic HCV and to the control. After correction for the significant intergroup differences shown in Table 1, at multivariable logistic regression analysis (supplementary table 1), low BT-LAL was independently associated with HCV-related cirrhosis (OR 0.139; 95% CI: 0.027–0.725; P = 0.019) compared to the control group.

**Supplementary figure legends**

**Supplementary figure 1:** blood total lysosomal acid lipase activity (panel A), platelet count (panel B) and leukocyte count (panel C) in controls with normal liver (CTRL), in non-cirrhotic HCV patients (non-cirrhotic HCV) and patients with HCV-related cirrhosis (CIR-HCV)

\*P<0.05; \*\*P<0.01; \*\*\*P<0.001

**Supplementary figure 2:** Isolated platelet and leukocyte preparation purity analysed by flow cytometry. (A) Representative morphology of platelets after isolation; FSC and SSC logarithmic scale identifies platelets (R1 gate) from debris (left panel). Surface marker staining with anti‐CD41‐PerCp and anti-CD45-FITC antibodies confirmed that platelet purity was greater than 98% (analysis performed on R1‐gated events; right panel). (B) Representative morphology of leukocytes. FSC and SSC linear scale showing lymphocytes, monocytes, granulocytes and other cells or debris (left panel). Staining with anti-CD45-FITC and anti-CD41-PerCP on total acquired events shows 86,9% of CD45+ leukocytes, 0,4% of CD41+ platelets and 12% of debris (right panel).