Pennisi Grazia (Orcid ID: 0000-0001-9518-4751)
Romero-G mez Manuel (Orcid ID: 0000-0001-8494-8947)
Vigan Mauro (Orcid ID: 0000-0003-0769-1767)
Wong Vincent Wai-Sun (Orcid ID: 0000-0003-2215-9410)
Fracanzani Anna Ludovica (Orcid ID: 0000-0001-5918-0171)
Sebastiani Giada (Orcid ID: 0000-0003-2655-8283)
Berzigotti Annalisa (Orcid ID: 0000-0003-4562-9016)
Vernuccio Federica (Orcid ID: 0000-0003-0350-1794)
de Ledinghen Victor (Orcid ID: 0000-0001-6414-1951)
Petta Salvatore (Orcid ID: 0000-0002-0822-9673)

TITLE: A CHOLESTATIC PATTERN PREDICTS MAJOR LIVER-RELATED OUTCOMES IN PATIENTS WITH NONALCOHOLIC FATTY LIVER DISEASE

AUTHORS: Grazia Pennisi[1*], Rosaria Maria Pipitone[1*], Daniela Cabibi[2*], Marco Enea[2], Manuel Romero-Gomez[3], Mauro Viganò[4], Elisabetta Bugianesi[5], Vincent Wai-Sun Wong[6], Anna Ludovica Fracanzani[7], Giada Sebastiani[8], Annalisa Berzigotti[9], Francesca Di Salvo[11], Antonino Giulio Giannone[2], Claudia La Mantia[1], Giulia Lupo[1], Rossana Porcasi[2], Federica Vernuccio[12], Rossella Zito[2], Vito Di Marco[1], Calogero Cammà[1], Antonio Craxì[1], Victor de Ledinghen[10], Stefania Grimaudo[1°], Salvatore Petta[1°].

*These authors equally contributed to the paper.

°These authors equally contributed to the paper.

AFFILITATION: [1]Section of Gastroenterology and Hepatology, Dipartimento Di Promozione Della Salute, Materno Infantile, Medicina Interna e Specialistica Di Eccellenza (PROMISE), University of Palermo, Italy

[2]Dipartimento Di Promozione Della Salute, Materno Infantile, Medicina Interna e Specialistica Di Eccellenza (PROMISE), University of Palermo, Italy

[3]Digestive Diseases Unit, Hospital Universitario Virgen del Rocío, Biomedical Research Networking Center in Hepatic and Digestive Diseases, Instituto de Biomedicina de Sevilla, University of Seville, Seville, Spain.

[4]Hepatology Unit, Ospedale San Giuseppe, University of Milan, Milan, Italy.

[5]Division of Gastroenterology, Department of Medical Sciences, University of Torino, Turin, Italy

[6]Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/liv.15232

[7]Department of Pathophysiology and Transplantation, Ca' Granda IRCCS Foundation, Policlinico Hospital, University of Milan, Milan, Italy.

[8]Division of Gastroenterology and Hepatology, McGill University Health Centre, Montreal, Quebec, Canada.

[9]Hepatology Group, University Clinic for Visceral Surgery and Medicine, Inselspital, Department for Biomedical Research, University of Bern, Bern, Switzerland.

[10]Centre d'Investigation de la Fibrose Hépatique, Hôpital Haut-Lévêque, Bordeaux University Hospital, Pessac, & INSERM U1053, Université de Bordeaux, France.

[11]Department of Agricultural, Food and Forest Sciences, University of Palermo, 90128, Palermo, Italy

[12]Dipartimento di Biomedicina, Neuroscienze e Diagnostica avanzata (BIND), , University of Palermo, Italy

CORRESPONDING AUTHOR: Salvatore Petta. Section of Gastroenterology and Hepatology, PROMISE, Policlinico Universitario Paolo Giaccone, Piazza delle Cliniche, 2, 90127 Palermo, Italy. Phone: +39 091 23890673. Fax +39 091 6552156. E-mail: salvatore.petta@unipa.it.

NUMBER OF FIGURES/SUPPLEMENTAL FIGURES: 5/7

NUMBER OF TABLES/SUPPLEMENTAL TABLES: 2/4

LIST OF ABBREVIATIONS: NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; MALO: liver-related events; HCC: hepatocellular carcinoma; LD: liver decompensation; US: ultrasound; LSM: liver stiffness mesurement; T2D: type 2 diabetes; AST: aspartate aminotrasferase; ALT: alanine aminotransferase; GGT, gamma glutamiltrasferase; ALP, alkaline phosphatase; BMI: body mass index; HDL: high density lipoprotein; PLT: Platelets; IHC: immunohistochemical; FXR: Farnesoid X Receptor; RXRα: retinoid X receptors α; LXRα: liver X receptor α; VCAM-1: vascular cell adhesion molecule-1.

CONFLICT OF INTEREST: None. GS has acted as speaker for Pfizer, Merck, Novonordisk, Novartis, Gilead, and AbbVie, served as an advisory board member for Merck, Gilead, Pfizer,

Allergan, Novonordisk, Intercept and Novartis and has received research funding from Merck and Theratec.

FUNDING: None. GS is supported by a Senior Salary Award from Fonds de la Recherche en Santé du Quebéc (FRQS) (#296306).

CONTRIBUTORS: G. Pennisi1, R.M. Pipitone, D. Cabibi, M. Enea, M. Romero-Gomez, M. Viganò, E. Bugianesi, V.W. Wong, A.L. Fracanzani, G. Sebastiani, A. Berzigotti, F. Di Salvo1, A.G. Giannone, C. La Mantia, G. Lupo, R. Porcasi, F. Vernuccio, R. Zito, V. Di Marco, C. Cammà, A. Craxì, V. de Ledinghen, S. Grimaudo, S. Petta had full control of the study design, data analysis and interpretation, and preparation of article. All authors were involved in planning the analysis and drafting the article. The final draft article was approved by all the authors.

DATA AVAILABILITY STATEMENT: Data are available on reasonable request to the authors.

ABSTRACT

BACKGROUND&AIMS: NAFLD patients usually have increase in AST/ALT levels but cholestasis can also be observed. We aimed to assess in subjects with NAFLD the impact of the (cholestatic)C pattern on the likelihood of developing of major liver-related outcomes(MALO).

METHODS:582 consecutive patients with biopsy-proven NAFLD or a clinical diagnosis of NAFLD-related compensated cirrhosis were classified as hepatocellular(H), C and mixed(M) patterns, by using the formula (ALT/ALT Upper Limit of Normal-ULN)/(ALP/ALP ULN). MALO were recorded during follow-up. An external cohort of 1281 biopsy-proven NAFLD patients was enrolled as validation set.

RESULTS:H, M and C patterns were found in 153(26.3%), 272(46.7%) and 157(27%) patients, respectively. During a median follow-up of 78 months, only 1(0.6%) patient with H pattern experienced MALO, while 15(5.5%) and 38(24.2%) patients in M and C group had MALO. At multivariate Cox regression analysis, age>55 years(HR2.55,95%C.I.1.17-5.54;p=0.01), platelets<150,000/mmc(HR0.14,95%C.I.0.06-0.32;p<0.001), albumin<4g/L(HR0.62,95%C.I.0.35-1.08;p=0.09), C vs M pattern(HR7.86,95%C.I.1.03-60.1;p=0.04), C vs H pattern(HR12.1,95% C.I.1.61-90.9;p=0.01) and fibrosis F3-F4(HR35.8,95%C.I.4.65-275.2;p<0.001) were independent risk factors for MALO occurrence. C vs M pattern(HR14.3,95%C.I.1.90-105.6;p=0.008) and C vs H pattern(HR15.6,95%C.I. 2.10-115.1;p=0.0068) were confirmed independently associated with MALO occurrence in the validation set. Immunohistochemical analysis found a significant higher prevalence of moderate-high grade ductular metaplasia combined with low grade ductular

proliferation in C pattern when compared with biochemical H pattern. Gene expression analysis showed a lower expression of *NR1H3, RXRα, VCAM1* in patients with the C pattern.

CONCLUSIONS: The presence of a cholestatic pattern in patients with NAFLD predicts a higher risk of MALO independently from other features of liver disease.

Key Words: NAFLD, NASH, CIRRHOSIS, CHOLESTASIS

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most emergent chronic liver disease of the last decade, affecting more than a quarter of population worldwide, representing a growing cause of cirrhosis, hepatic complications, mortality, and liver transplantation [1]. Due to the growing global incidence of diabetes and obesity, the prevalence, and the severity of NAFLD is expected to increase quickly in the nearest future, leading NAFLD to become a burden for global public health in the next years [2].

The development and the progression of NAFLD towards cirrhosis and its complications (e.g. liver decompensation and hepatocellular carcinoma – HCC) are influenced by genetic background and by individual metabolic risk factors [3-7]. Genetic susceptibility and metabolic comorbidities act synergically to induce and to perpetuate liver damage through lipotoxicity, which prompts the activation of hepatic inflammation cascade, resulting in activation of fibrogenesis pathways [8].

NAFLD is usually an asymptomatic condition where cytolysis with raised alanine aminotransferase (ALT) - expression of liver inflammation - can be present in a relevant proportion of patients [9]. Conversely, a cholestasis with increased alkaline phosphatase (ALP) is less frequently observed than in other biliary tract liver diseases. However, the prevalence and the clinical significance of cholestatic pattern in NAFLD is still uncertain.

Some small studies reported a link between cholestasis and liver damage in NAFLD [10-13]. Along this line, recent data in a small cohort of biopsy-proven NAFLD patients found that cholestasis,

evaluated by a simple formula considering ALT and ALP values, was associated with advanced stage of histological fibrosis, more severe liver disease and metabolic setting [14]. Thus, the data about cholestatic pattern in NAFLD is poor and fragmentary, its clinical role is not completely clear, and its biological plausibility is still unexplained.

The aim of the present study is to investigate the prevalence of a biochemical pattern of cholestasis in a cohort of patients with biopsy-proven NAFLD or with a clinical diagnosis of compensated cirrhosis related to NAFLD, and to evaluate its impact on the development of major liver related outcomes (MALO). Results were validated in an external independent cohort, and immunohistochemical and transcriptomic analyses were performed to identify specific histological changes and gene expression pathways associated with biochemical cholestasis.

METHODS

Patient selection

We retrospectively analyzed data from 582 patients prospectively recruited at the Gastrointestinal & Liver Unit of the Palermo University Hospital (training set) and with histological diagnosis of NAFLD or clinical diagnosis of Child Pugh A5 cirrhosis related to NAFLD and without previous history of liver decompensation (LD), portal thrombosis, esophageal varices band ligation and HCC. Specifically, in patients without histology, cirrhosis was diagnosed by liver stiffness measurement (LSM) >11.5 kPa for M probe [15] or >11 kPa for XL probe [16], and the diagnosis of NAFLD required the presence of ultrasonography-assessed steatosis plus at least one criterion of the metabolic syndrome (obesity, diabetes, arterial hypertension, dyslipidemia).

A multicenter cohort of patients with histological diagnosis of NAFLD enrolled at Centre d'Investigation de la Fibrose Hépatique of the Bordeaux University Hospital, at Division of Gastroenterology and Hepatology of McGill University Health Centre of Montreal QC, at Hepatology Unit of Ospedale San Giuseppe University of Milan, at Hospital Universitario Virgen del Rocío de Sevilla, at Department of Medicine and Therapeutics of the Chinese University of

Hong Kong, at the Department of hepatology, UVCM, University Hospital of Bern, Switzerland, at Division of Gastroenterology, Department of Medical Sciences of University of Torino, and at Department of Pathophysiology and Transplantation, Ca' Granda IRCCS Foundation of Policlinico Hospital of University of Milan, was retrospectively evaluated as validation set.

Only patients with a follow-up of at least 6 months were included. Other causes of liver disease were ruled out, including alcohol intake (>30 g/day in men, >20 g/day in women) as evaluated by a questionnaire, viral (hepatitis B surface antigen, anti–hepatitis C virus, and anti–human immunodeficiency virus negativity), autoimmune hepatitis including primary biliary cholangitis of primary sclerosing cholangitis, hereditary hemochromatosis, and alpha-1 antitrypsin deficiency. Patients with an episode of extra-hepatic cholestasis at the diagnosis, and patients who took part to randomized controlled trials with new NASH drugs during the follow-up were also excluded.

The Kleiner scoring system [17] was used for histological assessment of NAFLD and specifically to grade steatosis, lobular inflammation, and hepatocellular ballooning and to stage fibrosis from 0 to 4.

The study was carried out in accordance with the principles of the Helsinki Declaration and with local and national laws. Approval was obtained by the Ethical Committee of the University Hospital “Paolo Giaccone” in Palermo.

Patient evaluation

Clinical and metabolic data were collected at the time of enrollment. Body mass index (BMI) was calculated in kilograms for weight and in meters for height. Obesity was defined as BMI $\geq$ 30 Kg/m^2. The diagnosis of type 2 diabetes (T2D) was made according to the American Diabetes Association [18], using a value of fasting blood glucose $\geq$ 126 mg/dl. In patients with a previous diagnosis of T2D, current medications and their changes were documented. Arterial hypertension

was defined by systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or use of blood-pressure-lowering agents [19].

A 8-hour overnight fasting blood sample was drawn to determine serum levels of AST, ALT, GGT, ALP, PLT, albumin, total bilirubin, total cholesterol, HDL cholesterol, triglycerides and plasma glucose concentration.

Patients were categorized into three groups based on the pattern of elevated liver enzymes as follows: predominantly cholestatic pattern (C pattern), predominantly hepatocellular pattern (H pattern) and mixed (M) pattern. The pattern of elevated liver enzymes was calculated by using the following formula: R = (ALT/ALT Upper Limit of Normal)/(ALP/ALP Upper Limit of Normal) [20,21]. As previous published [20,21], C pattern group included patients with a ratio of less than 2, the H pattern group included patients with a ratio of more than 5, and the M pattern group included patients with a ratio between 2 and 5. The upper limit of normal for ALT is 19 and 31 IU\L for women and men [21], respectively, while the ALP upper normal level is 115 IU/L based on the laboratory reference of the Palermo University Hospital. Fib-4 score was also calculated according to the published formula [22].

Major Liver-Related Outcomes

MALO were recorded during entire follow-up, and they were defined as development of LD (occurrence of ascites and/or bleeding varices and/or encephalopathy and/or jaundice) or of HCC. Ultrasound examination for HCC surveillance was carried out yearly in patients with F0-F2 fibrosis, and every 6 months in patients with F3 fibrosis or cirrhosis, according to international guidelines [23].

In patient with cirrhosis, upper gastrointestinal endoscopy was performed at baseline and repeated as recommended by clinical guidelines. Patients with progression to medium or large (F2 or F3) esophageal varices were treated with β -blockers or underwent elastic banding, whereas no prophylaxis was scheduled for patients with small (F1) varices.

Patients developing hepatic events during follow-up were evaluated for available therapies and/or for liver transplantation, as appropriate [23,24].

Immunohistochemical analysis

Only samples measuring more than 1,5 cm and containing more than 10 portal tracts were assessed. The slides were stained with hematoxylin-eosin, and with Shikata's orcein, Masson Trichrome and Sirius Red special histochemical stains. Immunohistochemical (IHC) stains were carried out with the Ventana BenchMark Ultra automated slide staining system (Ventana/Roche Diagnostics) according to the manufacturer's instructions, using the following prediluted primary antibodies: anti-Cytokeratin 7 (CK7, clone SP52; rabbit monoclonal; Ventana/Roche), anti-Cytokeratin 19 (CK19, clone A53-B/A2.26; mouse monoclonal; Cell Marque) and anti-Ep-CAM (clone Ber-EP4; mouse monoclonal; Cell Marque). The slides were observed on Leica DM2000 microscope; microphotographs were obtained using a Leica DFC320 Camera.

The expression of CK7, CK19 and EpCAM was assessed semiquantitatively [25]. We evaluated the presence and the degree of ductular reaction, defined as the presence of newformed small ductules, situated outside the portal tracts, with CK7/CK19 positive immunostaining. Analogously, we assessed the presence of biliary metaplasia, defined as single cells or small groups of cells without clear central lumen, with an intermediate hepatobiliary phenotype, with CK7/EpCAM positive expression. We used a four-tiered semiquantitative scoring method for all the above mentioned antibodies: score 0: Absence of immunohistochemical expression in the liver parenchyma outside the portal tracts; score 1: focal presence of immunohistochemical expression next to the portal tracts, in the range of 1 HPF, in less than 50% of the portal tracts; score 2: moderate presence of immunohistochemical expression next to the portal tracts in less than 50% of the portal tracts, in the range of more than 1 HPF and/or in more than 50% of the portal tracts , in the range of 1 HPF; score 3: diffuse presence of immunohistochemical expression adjacent to the portal tracts in more than 50% of the portal tracts, in the range of more than 1 HPF.

Gene expression

Tissues stored at –80°C were homogenized, and total RNA was extracted using the miRNeasy micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Total RNA (1 μg) was retro-transcribed, using the iScript™ gDNA Clear cDNA Synthesis Kit, according to manufacturer's recommendations (Bio-Rad, CA, USA).

Quantitative Real Time PCR was performed using the 384 well-plate pre-designed Prime PCR Cholestasis panel (24 target genes) and Prime PCR custom panel specific for genes involved in liver cirrhosis (184 target genes) (Bio-Rad, CA, USA). All the plates contained primers for genomic DNA detection (gDNA), positive PCR control (PCR), RNA Quality Assay (RQ1 and RQ2), Reverse Transcription Control (RT) and 3 housekeeping genes: TATA-box binding protein (TBP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phospho -ribosyl-transferase 1 (HPRT1). Data were expressed as fold change using $2^{-\Delta\Delta Ct}$ method referred to LX2 cell line as control sample. Differences among experimental groups were analyzed by Student t test and used for comparison with PRIME PCR analysis software (Bio-Rad).

On the basis of the relative quantification method, the amount of target, normalized to the endogenous reference GAPDH and relative with respect to the control sample (LX2 cells line), was computed; a list of gene expression for which RQ was statistically significant, were selected and analysed among experimental groups.

Statistical Analysis

The statistical analysis followed three steps. In the first step, a descriptive analysis of the patients' characteristics by biochemical pattern was performed, with p-values of the differences adjusted by using the Benjamini-Hochberg correction for multiple tests [26]. Survival outcomes (time to HCC, time to decompensation, and time to hepatic event) were analyzed by Kaplan-Meier survival curves. In the second step, univariate, and multivariate proportional hazards Cox regression models [27,28] were fitted to estimate the effect of the covariates on the outcomes. Firth's penalized maximum likelihood bias reduction [29,30] was used to avoid divergent parameter estimates, with infinite standard errors, due to monotone likelihood.

The selection of covariates for the multivariate final models was performed by following statistical and clinical criteria. For each of the three outcomes, the starting point was a multivariate

PH Cox model including all the significant risk factors ($p<0.05$) from the univariate models. Then the final models were chosen by a backward selection based on the p-value. The third step was to firstly assess the accuracy of the predictions on the training set, for each of three models, by time-dependent ROC analysis. Then, the ROC analysis was repeated on the test set to validate the final models.

RESULTS

TRAINING SET

Patient features and outcomes

Baseline characteristics of the 582 patients with NAFLD stratified for liver enzyme biochemical pattern are shown in **Table 1**. One hundred fifty-three patients had H, while 157 and 272 had C and M patterns, respectively. Eighty-five (14.6%) patients had presence of ULN ALP values. **Supplemental Figure 1** shows the distribution of each pattern by fibrosis stage.

The diagnosis of NAFLD was supported by histology in 435 cases (74.7%). Cirrhosis was diagnosed in 147 patients: by histology in 54 cases, and on clinical criteria in 93 cases (63.3%) (Supplemental table 1). Specifically, 80 of the advanced fibrosis/cirrhosis patients had a liver stiffness measurement > 15 kPa [31] and 13 were between 11 kPa and 15 kPa (eleven of these cases also had esophageal varices and the other two had signs of portal hypertension: splenomegaly and portal vein ectasia).

During a median follow-up of 78 months, only 1 patient with H pattern experienced MALO (HCC) with a likelihood of 0.8% at 5 years. Fifteen patients with M pattern experienced MALO (14 LD and 3 HCC) during a median follow-up of 76.9 months, with an actuarial rate of MALO at 1, 3 and 5 years of 1.5, 3.1 and 4.7%, respectively. Finally, 38 patients with C pattern developed MALO (36 LD and 12 HCC) during a median follow-up of 66.3 months, with an actuarial rate of MALO occurrence at 1, 3 and 5 years of 3.3, 16 and 22.9%, respectively.

Association of biochemical pattern with MALO

At univariate Cox Regression analysis, C vs M pattern (HR 9.86, 95% C.I. 1.31-74.15; p=0.02) and C vs H pattern (HR 47.36, 95% C.I. 6.5-345.21; p<0.001) were associated with MALO occurrence. At multivariate Cox Regression analysis, age > 55 (HR 2.55, 95% C.I. 1.17-5.54; p=0.01), platelets <150,000/mmc (HR 0.14, 95% C.I. 0.06-0.32; p<0.001), albumin <4 g/L (HR 0.62, 95% C.I. 0.35-1.08; p=0.09), C vs M pattern (HR 7.86, 95% C.I. 1.03-60.1; p=0.04), C vs H pattern (HR 12.1, 95% C.I. 1.61-90.9; p=0.01) and fibrosis F3-F4 (HR 35.8, 95% C.I. 4.65-275.2; p<0.001) were independent risk factors for MALO occurrence (**Table 2**). When including into the model log GGT serum levels this last was (HR 1.44, 95% C.I. 1.07-1.94; p=0.01) independently associated with a higher risk of developing MALO, and both C vs M pattern (HR 8.02, 95% C.I. 1.05-61.1; p=0.04) and C vs H pattern (HR 12.6, 95% C.I. 1.69-95.2; p=0.01) were confirmed as independent risk factors. When replacing into the model the liver enzyme score with presence of abnormal ALP values this last remained significantly associated with a higher risk of developing MALO (HR 1.79, 95% C.I. 1.02-3.13; p=0.03).

The Kaplan-Meier curves in **Figure 1A** show the probability of MALO occurrence over time, according to the liver enzyme biochemical pattern (C vs M vs H). **Supplemental Figure 2A and 2D** show the AUC at 1, 3, 5 and 10 years of the model (AUC=0.92, 0.93, 0.94 and 0.93, respectively) and of the liver enzyme biochemical score (AUC= 0.82 at 1, 3, 5 and 10 years) for the prediction of MALO. Notably, the AUC of the liver enzyme biochemical score performed better than that of FIB-4 (AUC at 1, 3, 5 and 10 years 0.75, 0.74, 0.74 and 0.59, respectively; p<0.05 for all) and of AST/ALT ratio (AUC at 1 year 0.78, p=0.13; 3 years 0.76, p=0.05; 5 years 0.73, p=0.03 and 10 years 0.63 p=0.002).

Because of all MALO developed in patients with baseline F3-F4 fibrosis, we repeated analyses in this subgroup where age > 55 (HR 2.42, 95% C.I. 1.12-5.24; p=0.02), albumin <4 g/L (HR 0.15, 95% C.I. 0.07-0.34; p<0.001), C vs M pattern (HR 7.80, 95% C.I. 1.01-59.8; p=0.04), and C vs H pattern (HR 12.1, 95% C.I. 1.61-91.3; p=0.01) were confirmed as independently associated with MALO occurrence (**Table 2**). **Supplemental Figure 2B and 2D** show the AUC of

the model and of the liver enzyme biochemical score in this group of patients. Further, sub-group analysis by excluding from the entire cohort patients with clinical diagnosis of cirrhosis confirmed C vs M pattern (HR 7.47, 95% C.I. 1.01-66.1; p=0.04), and C vs H pattern (HR 9.03, 95% C.I. 1.03-78.6; p=0.03) as independent risk factors for MALO occurrence.

Considering LD and HCC separately, at multivariate Cox Regression analysis, C vs M pattern (HR 10.5, 95% 1.4-1345, p=0.01), and C vs H pattern (HR 20.1, 95% C.I. 2.7-2558; p<0.001) were independently associated with LD occurrence (**Table 2**), these associations being also confirmed in the subgroup of patients with F3-F4 fibrosis (**Table 2**). **Figure 2A** shows Kaplan-Meier curves and AUC of LD occurrence, according to the liver enzyme biochemical pattern (C vs M vs H). **Supplemental Figure 3** reports the AUCs at 1, 3, 5 and 10 years of the model and of the score in the entire population and in F3-F4 patients. Looking at HCC, univariate Cox Regression analysis showed that C vs H pattern was linked with HCC occurrence in the entire population (HR 14.10, 95% C.I. 1.83-108.65; p=0.01) and in subjects with fibrosis F3-F4 (HR 8.40, 95% C.I. 1.09-64.88; p=0.04), even if these associations were not confirmed at multivariate analyses (**Table 2**). **Figure 3A** shows the probability of HCC, according to the liver enzyme biochemical pattern (C vs M vs H) while **Supplemental Figure 4** shows the AUC of the model and of the score for the prediction of HCC in the entire cohort and in patients with F3-F4 fibrosis.

VALIDATION SET

Validation cohort included 1281 subjects: 269 of these had H, 603 M and 409 C pattern (**Supplemental Table 2)**. **Supplemental Table 3** compares training versus validation cohort.

During a median follow-up of 62.9 months, 6 patients with H pattern experienced MALO (4 LD and 2 HCC) with a likelihood of 0.3% and 0.6% at 3 and 5 years. Fifty-four patients with M pattern experienced MALO (29 LD and 27 HCC) during a median follow-up of 76.9 months, with an actuarial rate of MALO at 1, 3 and 5 years of 0.7%, 1.5% and 2.5%, respectively. Finally, 98 patients

with a C pattern developed MALO (73 LD and 39 HCC) during a median follow-up of 66.3 months, the actuarial rate of MALO occurrence at 1, 3 and 5 years being 2.5%, 6.6% and 8.2%, respectively.

At multivariate Cox Regression analysis C vs M pattern (HR 14.3, 95% C.I. 1.90-105.6; p=0.008) and C vs H pattern (HR 15.6, 95% C.I. 2.10-115.1; p=0.0068) were independently associated with MALO occurrence, and these associations were maintained in the subgroup of subjects with F3-F4 fibrosis (**Supplemental Table 4)**. **Supplemental Figure 5** shows the AUC at 1, 3, 5 and 10 years of the model and of the score for the prediction of MALO in the entire cohort and in the subgroup of subjects with F3-F4 fibrosis. Analyses on LD and HCC considered separately were reported in **Supplemental Table 4, Supplemental Figure 6** and **Supplemental Figure 7**.

When looking at patients with baseline F0-F2 fibrosis only ten patients developed MALO during follow-up: none of them had baseline H pattern, while M and C pattern were observed in six and four patients, respectively.

IMMUNOHISTOCHEMICAL ANALYSIS

In a subgroup of 38 patients with F3-F4 fibrosis (57.9% males, mean age 57.4 years, mean BMI 32.1 Kg/m2, 52.6% with diabetes) and stratified according to H (N=26) or C (N=12) pattern, we searched for liver morphological changes associated with the biochemical profile. Patients with C pattern had a nonsignificant statistical trend (66.6% vs 38.4%, p=0.09) for CK7 expression when considered alone, compared to patients with a H biochemical pattern. Notably, patients with a biochemical C pattern were characterized by a significant higher prevalence of moderate-high ductular metaplasia combined with low ductular proliferation respect to what observed in their counterpart with a biochemical H pattern (58.3% vs 11.5%, p=0.002), this association being also confirmed after adjusting for age, gender, BMI and obesity (OR 13.5, 95% C.I. 2.00-91.8, p=008).

Figure 4 depicts hepatic morphological changes by immunohistochemistry related to H or C biochemical patterns.

GENE EXPRESSION

Gene expression analyses were performed in 14 patients with F3-F4 fibrosis and available frozen liver biopsy (6 with cholestatic and 8 with cytolytic pattern; 50% males, mean age 61 years, mean BMI 32.9 Kg/m2, 57.1% with diabetes). Using an RT Profiler PCR microarray approach, we found that three genes, among the 208 analyzed showed significant different of expression: *NR1H3* (alias *LXRα* - liver X receptor α), *RXRα* (retinoid X receptors α), *VCAM-1* (vascular cell adhesion molecule-1).

In order to test for conditional independence between Up-Down regulated results and cholestatic or noncholestatic pattern in each gene, the Mantel-Haenszel X-squared test (MH) was implemented on the 2x2 frequencies tables up-down regulated versus patterns, after stratifying by genes and significant differences were identified among patterns, MH= 16.219 with P value=0.0003.

The Student t-test detected statistically significant differences in fold expression of genes *NR1H3* (p=0.01), *RXRα* (p=0.03) and *VCAM-1* (p=0.04) **(Figure 5).**

NR1H3 and *RXRα*, normalized respect to LX2, were regulated in differential manner in the two experimental groups: for *NR1H3* the percentage of up-regulation increases from 83% (17% no changes) in cholestatic pattern to 100% in noncholestatic pattern; the fold expression mean increases from 10.88 in cholestatic pattern to 19.23 in noncholestatic pattern. Percentage of up regulation of *RXRα* is 67% in cholestatic pattern and increases up to 88% in non-cholestatic (33% and 12% are no change respectively in the two groups); the mean value increases from 2.59 to 6.36.

Expression of *VCAM-1*, normalized respect to LX2, has a percentage of 50% of down-regulation (mean=-6.16) in cholestatic pattern and 25% (mean=-0.75) in noncholestatic pattern.

DISCUSSION

The present study in a large cohort of individuals with histological diagnosis of NAFLD or clinical diagnosis of compensated cirrhosis related to NAFLD, shows that a biochemical cholestatic pattern -associated with specifical liver morphological and gene expression changes- independently

predicts a higher risk of developing MALO. These results were replicated in a large external cohort of biopsy-proven NAFLD patients.

NAFLD is the most growing asymptomatic liver disease of last decades, suspected often because of abnormal liver function tests, with the increase in AST and ALT levels being the most common alteration. However, in some patients a cholestatic pattern with uncertain prevalence and clinical significance can also be present. In our study, conducted in a large cohort of NAFLD patients, we found that 27% of the population had a C pattern, the prevalence further increasing according to the severity of liver fibrosis. This rate is lower compared to the 43.6% reported by Shirin et al. [14] in a small cohort of 106 patients with histological diagnosis of NAFLD, and in range to what reported in other small studies [10-13]. Differences in baseline characteristics of the populations and in sample size can explain discordant results.

NAFLD is an increasing cause of MALO, such as LD and HCC, up to liver transplantation and death [32-35]. The identification of predictive features, possibly based on non-invasive tests, able to stratify patients according to prognosis is an unmet need. To the best of our knowledge our study is the first demonstrating that the presence of a biochemical cholestatic pattern identifies a subgroup of NAFLD patients at higher risk of developing MALO. In particular, the 5-year risk probability of MALO occurrence progressively reduced from 22.9% in NAFLD subjects with the C pattern, to 4.7% and 0.8% in M and H pattern, respectively. Notably, the association of the C pattern with MALO occurrence was confirmed at multivariate Cox regression analysis after adjusting for confounders, and in the at higher risk subgroup of patients with F3 fibrosis or cirrhosis. Notably the C pattern was also confirmed to predict the development of LD and HCC considered separately, even if this last association was not maintained at multivariate analysis for HCC probably due to the small number of observed events. Our results are consistent with a recent study reporting an association between the C pattern and portal hypertension in both cirrhotic and noncirrhotic NAFLD patients [36].

The present study is not designed to clarify the pathogenic link between presence of C pattern and risk of MALO development; however, we did some analyses to propose a biological plausibility.

Specifically, first we searched by IHC for morphological changes in liver histology in cholestatic and hepatocellular settings, and we found that patients with the C pattern had a significant higher prevalence of moderate-high ductular metaplasia combined with low ductular proliferation than those with the H pattern. Bile duct proliferation is the pathological expression of ductular reaction (DR), typically observed in chronic liver diseases, especially in biliary disorders such as primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC) and biliary atresia [37]. Liver injury is the main trigger of DR, that encompasses not only bile duct proliferation, but also a wide spectrum of liver tissue reactions, including the activation of several inflammatory pathways [38,39]. Chronic liver injury activates hepatic progenitor cells (HPCs), quiescent in normal liver, with a consequent DR that entails to different ways. First, HPCs can differentiate in intermediate cell and towards hepatocytes or cholangiocytes lineages, according to the pathogenesis of liver damage; conversely, HPCs can dedifferentiate, leading to metaplasia [40]. This phenomenon was widely studied by Carpino et al. [37] in biliary disorders, in which DR resulted more pronounced in patients with higher severity of fibrosis, than in those with lower stage of fibrosis and in controls. In addition, authors have found a direct association of DR with biochemical cholestasis in patients with both PBC and PSC. In this setting, the activation of HPCs compartment seems to indicate a secondary response of the hepatic parenchyma to cholestasis more than a primary reaction to the biliary damage as a tentative of hepatocyte regeneration [41]. Furthermore, relative bile acid overload may be an early trigger in ductular metaplasia of hepatocytes, exerting a primary effect on parenchymal cells themselves, which in turn stimulate HSCs [42-44]. The biological significance of DR and of HPCs activation was also explored in NAFLD. Prominent DR emerges in patients with definite NASH, not in those with simple steatosis and it was associated with more severe fibrosis by activation of hepatic stellate cells (HSCs). [38,45,46]. Consistent with the above quoted literature data and with our evidence about the association between biochemical cholestasis, a IHC profile characterized by higher ductal metaplasia and lower ductular proliferation, and a higher risk of MALO occurrence, we can speculate that the intrinsic biliary disfunction associated with the presence metaplasia -as response to liver injury or

induced by biliary acid overload-, and not fully compensated by differentiate effective ductular proliferation, could explain the biochemical manifestation of cholestasis and could identify a pattern of liver disease at higher risk of progression. Further analyses are needed to better clarify our results.

To further explore the association between the C pattern and adverse outcomes of NAFLD we searched for a differential gene expression according to the biochemical pattern. In a small subgroup of patients stratified for C and H patterns we found a significant down-expression of *NR1H3, RXRα* and *VCAM-1* genes in patients with the C compared to those with the H pattern. Retinoid X receptors α (*RXRα*) belongs to nuclear receptors that mediate the biological effects of retinoids by their involvement in retinoic acid-mediated gene activation. This receptor functions as transcription factor by binding as homodimers or heterodimers to specific sequences in the promoters of target genes. *RXRα* creates a functional heterodimer with liver X receptor α (*LXRα* alias *NR1H3*), representing the active ligand-binding subunit [47]. The heterodimer represents a key regulator of macrophage function, controlling transcriptional programs involved in lipid and cholesterol homeostasis and inflammation [48]. *LXRα* activated pathways have the dual functions: on one hand, they are involved in maintaining cholesterol and bile acid homeostasis by increasing cholesterol catabolism and, on the other hand, they can prevent toxicity from bile acid accumulation [49]. On this scenario the low expression of *RXRα* and *LXRα* in NAFLD patients with the C pattern, and the consequent lack of their beneficial effects, is consistent with the observed link between the C pattern and poor clinical outcomes. On the other side, Vascular cell adhesion molecule 1 (*VCAM-1*) is a surface protein that, inducing vascular endothelial dysfunction, adherence and extravasation of monocytes to blood vessels leading to a proinflammatory status in different setting [50], acts as trigger and worsen liver inflammation in NAFLD with as a consequence a possible commitment versus a cytolytic hepatocellular pattern [51,52]. However, a study showed that bile acids can exert a direct downregulation on *VCAM-1* expression, and that inhibits the cell growth and proliferation and enhances the cell apoptosis [50]. According to this data, we suspected that cholestasis *per se* could be a possible trigger of reduction of *VCAM-1* expression. However, the biological and clinical

significance of the different expression of *RXRα, NR1H3* and *VCAM-1* in our cohort of NAFLD patients with C and H patterns needs further clarifications.

From a clinical standpoint, our data suggest that the presence of a cholestatic biochemical pattern identifies a sub-group of patients at higher risk of MALO. The evaluation of biochemical pattern is widely affordable and available because of the use of a simple formula including ALT and ALP. For this reason, the biochemical pattern, together with other easy-to-calculate scores like FIB-4 [53] and together with LSM [54] -when available- could be assessed during routine visits to predict and differentiate the liver outcomes in order to personalize the follow-up. Notably, our results were largely validated in an external independent multicenter cohort of patients with histological diagnosis of NAFLD, even if lack of widely accepted cut-off for defining ULN ALP and, therefore, differences in used cut-offs among centers could affect the interpretation of our results.

The main limitation of our study lies in the enrollment of patients with NAFLD from tertiary care centers that can be different in terms of clinical features, metabolic comorbidities and genetic background from NAFLD individuals from general population. Another potential limitation is that the prognostic significance of the C pattern could be driven not only by increased ALP levels but also by low ALT levels as expression of burnout NASH in patients with advanced liver disease. However, when replacing into the model the liver enzyme score with presence of abnormal ALP values this last -expression of cholestasis- remained independently associated with a higher risk of developing MALO confirming the negative impact of cholestasis on liver-related prognosis. Anyway, we cannot exclude that the presence of C pattern as well as the increase in ALP levels, instead of being a "a priory" condition increasing the risk of MALO, were only expression of a more advanced liver disease. The small number of patients with baseline F0-F2 fibrosis who developed MALO makes not possible sub-group analyses, and lack of data on changes over time in the pattern of liver enzymes and on baseline and follow-up biliary acid serum levels do not allow us to draw definitive conclusions about this topic. Finally, the lack of discrimination between acute and chronic LD [55], as well as the

use of LSM in a proportion of cases for diagnosing cirrhosis and potentially overestimating the severity of baseline liver disease, could affect the interpretation of results.

In conclusion, in patients with NAFLD and the presence of a cholestatic biochemical pattern, associated with specific liver morphological changes, predicts a higher risk of developing MALO. Gene expression and immunohistochemical analysis, if externally validated, could be underlie new pathogenic mechanism and potential target therapeutic strategies.

LEGENDS

Figure 1. MALO probability, in training (A) and validation cohort (B), according to the biochemical pattern (C vs M vs H).

Figure 2. LD probability, in training (A) and validation cohort (B), according to the biochemical pattern (C vs M vs H).

Figure 3. HCC probability, in training (A) and validation cohort (B), according to the biochemical pattern (C vs M vs H).

Figure 4. A,B,C: Patients with biochemical C pattern showed low ductular proliferation (B) and diffuse biliary metaplasia (C) respect to patients with biochemical H pattern (D,E,F). CK7 expression didn't show significant difference.

A,D: CK7; B,E: CK19; C,F: EpCAM.

Original magnification A-F: 40x

Figure 5. Relative changes in fold expression of genes involved in cholestatic pathway in subjects with (red bars) and without (blue bars) cholestatic pattern respect to LX2 cell line. $P^{\S}$ =0.01 (NR1H3); P^{ς}=0.03 (RXRA); $P^{\ddagger}$=0.04 (VCAM1).

Supplemental Figure 1. Distribution of biochemical patterns according to the fibrosis stage.

Supplemental Figure 2. AUC of the entire model in training cohort (A) and in subgroup of subjects with fibrosis F3-F4 (B), and of the biochemical score in training cohort (C) and in subgroup of subjects with fibrosis F3-F4 (D) for the prediction of MALO.

Supplemental Figure 3. AUC of the entire model in training cohort (A) and in subgroup of subjects with fibrosis F3-F4 (B), and of the biochemical score in training cohort (C) and in subgroup of subjects with fibrosis F3-F4 (D) for the prediction of LD.

Supplemental Figure 4. AUC of the entire model in training cohort (A) and in subgroup of subjects with fibrosis F3-F4 (B), and of the biochemical score in training cohort (C) and in subgroup of subjects with fibrosis F3-F4 (D) for the prediction of HCC.

Supplemental Figure 5. AUC of the entire model in validation cohort (A) and in subgroup of subjects with fibrosis F3-F4 (B), and of the biochemical score in validation cohort (C) and in subgroup of subjects with fibrosis F3-F4 (D) for the prediction of MALO.

Supplemental Figure 6. AUC of the entire model in validation cohort (A) and in subgroup of subjects with fibrosis F3-F4 (B), and of the biochemical score in validation cohort (C) and in subgroup of subjects with fibrosis F3-F4 (D) for the prediction of LD.

Supplemental Figure 7. AUC of the entire model in validation cohort (A) and in subgroup of subjects with fibrosis F3-F4 (B), and of the biochemical score in validation cohort (C) and in subgroup of subjects with fibrosis F3-F4 (D) for the prediction of HCC.

Table 1. Baseline features of training cohort stratified for hepatocellular (H), mixed (M) and cholestatic (C) pattern.

Table 2. Cox multivariate analysis of variables associated with MALO, LD and HCC occurrence in overall training cohort and subgroup with fibrosis F3-F4.

Supplemental Table 1. Baseline features of cirrhotic patients from the training cohort stratified for histological or clinical diagnosis.

Supplemental Table 2. Baseline features of the validation cohort stratified for hepatocellular (H), mixed (M) and cholestatic (C) pattern.

Supplemental Table 3. Comparison of baseline features of the training and validation cohorts.

Supplemental Table 4. Cox multivariate analysis of variables associated with MALO, LD and HCC occurrence in overall validation cohort and subgroup with fibrosis F3-F4.

REFERENCES

1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global Epidemiology of Non-Alcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence and Outcomes. Hepatology. 2016 Jul;64(1):73-84.
2. Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. Hepatology. 2018 Jan;67(1):123-133. doi: 10.1002/hep.29466. Epub 2017 Dec 1. PMID: 28802062; PMCID: PMC5767767.
3. Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. Hepatology 1999;30:1356-1362.
4. Neuschwander-Tetri BA, Clark JM, Bass NM, Van Natta ML, Unalp-Arida A, Tonascia J, et al. Clinical, laboratory and histological associations in adults with nonalcoholic fatty liver disease. Hepatology 2010;52:913-924.
5. Angulo P, Bugianesi E, Bjornsson ES, Charatcharoenwitthaya P, Mill PR, Barrera F, et al. Simple noninvasive systems predict longterm outcomes of patients with nonalcoholic fatty liver disease. Gastroenterology 2013;145:782-789.
6. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-1465.

7. Kozlitina J, Smagris E, Stender S, Nordestgaard BG, Zhou HH, Tybjærg-Hansen A, et al. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fattyliver disease. Nat Genet 2014;46:352-356.
8. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism. 2016 Aug;65(8):1038-48. doi: 10.1016/j.metabol.2015.12.012. Epub 2016 Jan 4. PMID: 26823198.
9. Rinella ME. Nonalcoholic fatty liver disease: a systematic review. JAMA. 2015 Jun 9;313(22):2263-73. doi: 10.1001/jama.2015.5370. Erratum in: JAMA. 2015 Oct 13;314(14):1521. PMID: 26057287.
10. Shipovskaya AA, Dudanova OP. Intrahepatic cholestasis in nonalcoholic fatty liver disease. Ter Arkh. 2018 Feb 15;90(2):69-74.
11. Sorrentino P, Tarantino G, Perrella A, Micheli P, Perrella O, Conca P. A clinical-morphological study on cholestatic presentation of nonalcoholic fatty liver disease. Dig Dis Sci. 2005 Jun;50(6):1130-5.
12. Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, Irvine K, Clouston AD. The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. Hepatology. 2014 Apr;59(4):1393-405.
13. Jüngst C, Berg T, Cheng J, Green RM, Jia J, Mason AL, Lammert F. Intrahepatic cholestasis in common chronic liver diseases. Eur J Clin Invest. 2013Oct;43(10):1069-83.
14. Shirin D, Peleg N, Sneh-Arbib O, Cohen-Naftaly M, Braun M, Shochat T, Issachar A, Shlomai A. The Pattern of Elevated Liver Function Tests in Nonalcoholic Fatty Liver Disease Predicts Fibrosis Stage and Metabolic-Associated Comorbidities. Dig Dis. 2019;37(1):69-76. doi: 10.1159/000491428. Epub 2018 Jul 17. PMID: 30016799.
15. Wong VW, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, et al. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. Hepatology. 2010 Feb;51(2):454-62.

16. Wong VW, Vergniol J, Wong GL, Foucher J, Chan AW, Chermak F, et al. Liver stiffness measurement using XL probe in patients with nonalcoholic fatty liver disease. Am J Gastroenterol. 2012 Dec;107(12):1862-71.
17. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology2005; 411:313–21
18. Introduction: Standards of Medical Care in Diabetes-2020. Diabetes Care. 2020 Jan;43(Suppl 1):S1-S2. doi: 10.2337/dc20-Sint. PMID: 31862741
19. Williams B, Mancia G, Spiering W, et al; ESC Scientific Document Group. 2018 ESC/ESH Guidelines for the management of arterial hypertension. Eur Heart J. 2018 Sep 1;39(33):3021-3104. doi: 10.1093/eurheartj/ehy339. Erratum in: Eur Heart J. 2019 Feb 1;40(5):475. PMID: 30165516.
20. DeLeve LD, Kaplowitz N: Mechanisms of drug-induced liver disease. Gastroenterol Clin North Am 1995;24:787–810.
21. Kwo PY, Cohen SM, Lim JK: ACG Clinical Guideline: Evaluation of Abnormal Liver Chemistries. Am J Gastroenterol 2017; 112: 18–35
22. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al; APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology. 2006 Jun;43(6):1317-25.
23. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J Hepatol. 2018 Jul;69(1):182-236
24. Cillo U, Burra P, Mazzaferro V, Belli L, Pinna AD, Spada M, et al; I-BELT (Italian Board of Experts in the Field of Liver Transplantation). A Multistep, Consensus-Based Approach to Organ Allocation in Liver Transplantation: Toward a "Blended Principle Model". Am J Transplant. 2015 Oct;15(10):2552-61
25. Bateman AC and Hübscher SG. Cytokeratin expression as an aid to diagnosis in medical liver biopsies. (2010) Histopathology 56, 415–425

26. Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological), 57(1), 289-300.

27. Cox David R. Regression models and life-tables. Journal of the Royal Statistical Society: Series B (Methodological), 1972, 34.2: 187-202

28. Grambsch Patricia M.; Therneau, Terry M. Proportional hazards tests and diagnostics based on weighted residuals. Biometrika, 1994, 81.3: 515-526

29. Firth D (1993). Bias reduction of maximum likelihood estimates. Biometrika 80:27–38.

30. Heinze G and Schemper M (2001). A Solution to the Problem of Monotone Likelihood in Cox Regression. Biometrics 57(1):114–119.

31. Wong VW, Irles M, Wong GL, Shili S, Chan AW, Merrouche W, Shu SS, Foucher J, Le Bail B, Chan WK, Chan HL, de Ledinghen V. Unified interpretation of liver stiffness measurement by M and XL probes in non-alcoholic fatty liver disease. Gut. 2019 Nov;68(11):2057-2064.

32. Adams LA,LympJF,St Sauver J,et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study.Gastroenterology. 2005;129(1):113–121.

33. Ekstedt M,Franzén LE, Mathiesen UL, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology. 2006; 44(4):865–873.

34. Angulo P, Kleiner DE, Dam-Larsen S, et al. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. Gastroenterology. 2015; 149(2):389–97.e10.

35. Bhala N, Angulo P, van der Poorten D, et al. The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study. Hepatology. 2011; 54(4):1208–1216

36. Dor Shirin, Ana Tobar, Ahinoam Glusman Bendersky, Rabab Naamneh, Amir Shlomai. Liver test-derived R factor is associated with portal hypertension in patients with non-alcoholic fatty liver disease. PO-2791 EASL 2021

37. Sato K, Marzioni M, Meng F, Francis H, Glaser S, Alpini G. Ductular Reaction in Liver Diseases: Pathological Mechanisms and Translational Significances. Hepatology. 2019 Jan;69(1):420-430. doi: 10.1002/hep.30150. Epub 2018 Dec 27. Erratum in: Hepatology. 2019 Sep;70(3):1089. PMID: 30070383; PMCID: PMC6324973.

38. Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, Irvine K, et al. The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. *Hepatology.* 2014;59:1393–1405

39. Lorenzini S, Bird TG, Boulter L, Bellamy C, Samuel K, Aucott R, Clayton E, et al. Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver. *Gut.* 2010;59:645–654.

40. Manco R, Clerbaux LA, Verhulst S, Bou Nader M, Sempoux C, Ambroise J, Bearzatto B, Gala JL, Horsmans Y, van Grunsven L, Desdouets C, Leclercq I. Reactive cholangiocytes differentiate into proliferative hepatocytes with efficient DNA repair in mice with chronic liver injury. J Hepatol. 2019 Jun;70(6):1180-1191.

41. Carpino G, Cardinale V, Folseraas T, Overi D, Floreani A, Franchitto A, Onori P, Cazzagon N, Berloco PB, Karlsen TH, Alvaro D, Gaudio E. Hepatic Stem/Progenitor Cell Activation Differs between Primary Sclerosing and Primary Biliary Cholangitis. Am J Pathol. 2018 Mar;188(3):627-639. doi: 10.1016/j.ajpath.2017.11.010. Epub 2017 Dec 15. PMID: 29248458.

42. Desmet VJ. Ductal plates in hepatic ductular reactions. Hypothesis and implications. I. Types of ductular reaction reconsidered. Virchows Arch. 2011; 458:251–259.

43. Desmet VJ. Ductal plates in hepatic ductular reactions. Hypothesis and implications. II. Ontogenic liver growth in childhood. Virchows Arch. 2011; 458:261–270.

44. Desmet VJ. Ductal plates in hepatic ductular reactions. Hypothesis and implications. III. Implications for liver pathology. Virchows Arch. 2011; 458:271–279.

45. Richardson MM, Jonsson JR, Powell EE, Brunt EM, Neuschwander-Tetri BA, Bhathal PS, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. Gastroenterology 2007;133:80-90.

46. Overi D, Carpino G, Franchitto A, Onori P, Gaudio E. Hepatocyte Injury and Hepatic Stem Cell Niche in the Progression of Non-Alcoholic Steatohepatitis. Cells. 2020 Mar 2;9(3):590. doi: 10.3390/cells9030590. PMID: 32131439; PMCID: PMC7140508.

47. Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* 1995;**9**:1033–1045. doi: 10.1101/gad.9.9.1033.

48. Kalaany NY, Mangelsdorf DJ. LXRS AND FXR: The yin and Yang of cholesterol and fat metabolism. *Annu Rev Physiol.* 2006;68:159–191. doi:10.1146/ annurev.physiol. 68.033104.152158.

49. Uppal, H., Saini, S.P., Moschetta, A., Mu, Y., Zhou, J., Gong, H., Zhai, Y., Ren, S., Michalopoulos, G.K., Mangelsdorf, D.J. and Xie, W. (2007), Activation of LXRs prevents bile acid toxicity and cholestasis in female mice. Hepatology, 45: 422-432.

50. Qin X, Ni X, Mao X, Ying H, Du Q. Cholestatic pregnancy is associated with reduced VCAM1 expression in vascular endothelial cell of placenta. Reprod Toxicol. 2017 Dec;74:23-31. doi: 10.1016/j.reprotox.2017.08.002. Epub 2017 Aug 26. PMID: 28851649.

51. Carr RM. VCAM-1: closing the gap between lipotoxicity and endothelial dysfunction in nonalcoholic steatohepatitis. J Clin Invest. 2021 Mar 15;131(6):e147556. doi: 10.1172/JCI147556. PMID: 33720049; PMCID: PMC7954588.

52. Hammoutene A, Biquard L, Lasselin J, et al. A defect in endothelial autophagy occurs in patients with non-alcoholic steatohepatitis and promotes inflammation and fibrosis. J Hepatol. 2020 Mar;72(3):528-538. doi: 10.1016/j.jhep.2019.10.028. Epub 2019 Nov 11.

53. Younes R, Caviglia GP, Govaere O, Rosso C, Armandi A, Sanavia T, et al. Long-term outcomes and predictive ability of non-invasive scoring systems in patients with non-alcoholic fatty liver disease. J Hepatol. 2021 Jun 4:S0168-8278(21)00343-3.

54. Petta S, Sebastiani G, Viganò M, Ampuero J, Wai-Sun Wong V, Boursier J, et al. Monitoring Occurrence of Liver-Related Events and Survival by Transient Elastography in Patients With Nonalcoholic Fatty Liver Disease and Compensated Advanced Chronic Liver Disease. Clin Gastroenterol Hepatol. 2021 Apr;19(4):806-815.e5.

55. D'Amico G, Bernardi M, Angeli P. Towards a new definition of decompensated cirrhosis. J Hepatol. 2022 Jan;76(1):202-207.

Table 1. Baseline features of training cohort stratified for hepatocellular (H), mixed (M) and cholestatic (C) pattern.

	Hepatocellular (N=153)	**Mixed (N=272)**	**Cholestatic (N=157)**	**P value H vs M**	**P value H vs C**	**P value M vs C**
Age (*)	45.6±13.6	49.4±13.7	57.5±12.8	0.009	<0.001	<0.001
Male (#)	55%	66%	70%	0.04	0.03	0.5
BMI > 30 kg/m^2 (#)	44%	45%	59%	0.8	0.01	0.01
Diabetes (#)	30%	34%	47%	0.4	0.008	0.01
Arterial Hypertension (#)	36%	37.5%	49%	0.8	0.04	0.04
Glucose – mg/dl (§)	92 (83-105)	95 (86-114)	100 (89-118.5)	0.05	<0.001	0.1
Total cholesterol – mg/dl (*)	204.8±49.7	191.2±43.4	182 ±43.6	0.008	<0.001	0.04
HDL cholesterol - mg/dl (§)	47 (40-55)	48 (38-57)	47 (39-56)	0.8	0.6	0.5
Tryglicerides – mg/dl (§)	132.5 (94-175)	121 (84-167)	124 (88.5-168)	0.1	0.2	0.7
PLT – mmc (*)	248.9±84.1	225.4±72.4	184±88.1	0.007	<0.001	<0.001
AST – U/L (§)	54 (41-71)	35 (28-46)	28 (23-39)	<0.001	<0.001	<0.001
ALT – U/L (§)	105 (78-145)	55 (43-74)	34 (24-41)	<0.001	<0.001	<0.001
GGT – U/L (§)	63 (40-103)	65.5 (31-119)	77 (33-134)	0.8	0.5	0.4
ALP – U/L (§)	64 (53-77)	75 (62-90)	100 (77-143)	<0.001	<0.001	<0.001
Total bilirubin – mg/dl (§)	0.60 (0.47-0.9)	0.64 (0.46-0.9)	0.70 (0.5-1.02)	0.8	0.1	0.1
Albumin – g/dl (*)	4.54±0.35	4.47±0.40	4.21±0.46	0.07	<0.001	<0.001
NASH (#) (°)	80%	75%	66%	0.3	0.07	0.1
Steatosis grade 2-3 (#) (°)	80%	62%	41%	<0.001	<0.001	<0.001
Fibrosis F3-F4 (#)	26%	37.5%	55%	0.02	<0.001	<0.001
Cirrhosis (#)	7%	22%	48%	<0.001	<0.001	<0.001
Time of Follow-up - months (*)	78±38.7	76.9±43.7	66.3±42	0.79	0.01	0.01

Abbreviations: BMI, body mass index; HDL, high density lipoprotein; PLT, platelets; AST, aspartate aminotrasferase; ALT, alanino aminotrasferase; GGT, gamma glutamiltrasferase; ALP, alkaline phosphatase; NASH, nonalcoholic steatohepatitis.
Data are given as: (*) mean ± standard deviations, (§) median and interquartile range, or (#) percentage of cases (%). P-values of the differences were adjusted by using the Benjamini-Hochberg correction for multiple tests.

° Data on steatosis grade and NASH are referred to the 435 patients with histological diagnosis of NAFLD.

Table 2. Cox multivariate analysis of variables associated with MALO, LD and HCC occurrence in overall training cohort and subgroup with fibrosis F3-F4.

Abbreviations: LRE, liver-related event; LD, liver decompensation; HCC, hepatocellular carcinoma; H, hepatocellular; M, mixed; C, cholestatic; PLT, platelets.

		Overall			*Fibrosis F3-F4*		
Events	**Variable**	**HR**	**95% C.I.**	**P**	**HR**	**95% C.I.**	**P**
MALO	**Age≥55 yrs**	2.55	[1.17-5.54]	0.01	2.42	[1.12-5.24]	0.02
	PLT<150000/mmc	6.63	[3.00-14.6]	<0.001	6.63	[3.00-14.6]	<0.001
	Albumin<4g/dl	1.35	[0.76-2.39]	0.29	1.35	[0.76-2.39]	0.29
	M vs C pattern	7.86	[1.03-60.1]	0.04	7.80	[1.01-59.8]	0.04
	H vs C pattern	12.1	[1.61-90.9]	0.01	12.1	[1.61-91.3]	0.01
	Fibrosis F3-F4	35.8	[4.65-275.2]	<0.001			
LD	**Albumin<4g/dl**	1.36	[0.76-2.44]	0.29	1.39	[1.16-2.49]	0.01
	PLT<150000/mmc	9.70	[3.76-25.0]	<0.001	9.04	[3.47-23.5]	<0.001
	M vs C pattern	10.5	[1.4-1345]	0.01	10.4	[1.4-1330]	0.01
	H vs C pattern	20.1	[2.7-2558]	<0.001	19.8	[2.7-2529]	<0.001
	Fibrosis F3-F4	24.1	[5.9-221.8]	<0.001			
HCC	**Age≥55 yrs**	5.32	[1.26-50.4]	0.01	7.61	[0.97-59.4]	0.05
	PLT<150000/mmc	4.62	[1.29-16.5]	0.02	4.62	[1.29-16.5]	0.03
	Albumin<4g/dl	2.00	[1.16-5.38]	0.02	2.00	[1.26-5.35]	0.02
	Fibrosis F3-F4	15.4	[1.6-2075]	0.01			

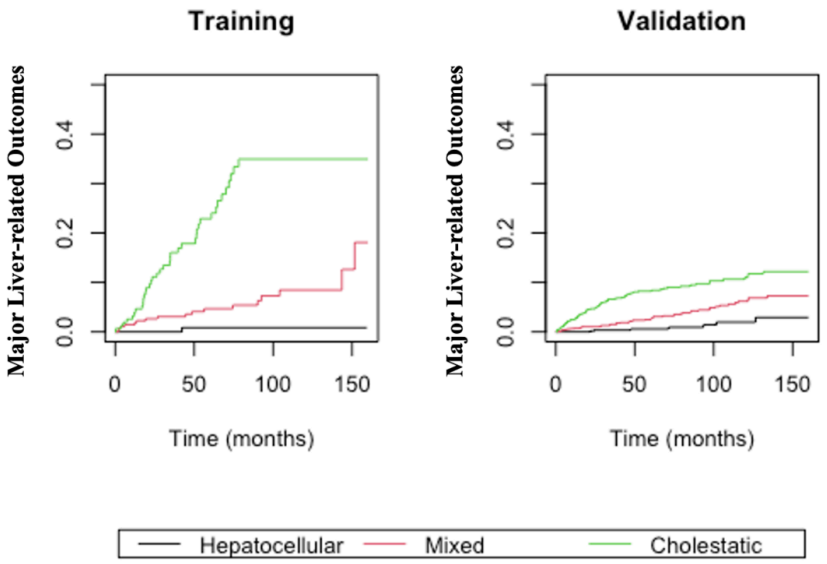


Figure 1. MALO probability, in training (A) and validation cohort (B), according to the biochemical pattern (C vs M vs H).

LIV_15232_Figure 1 08022022.png

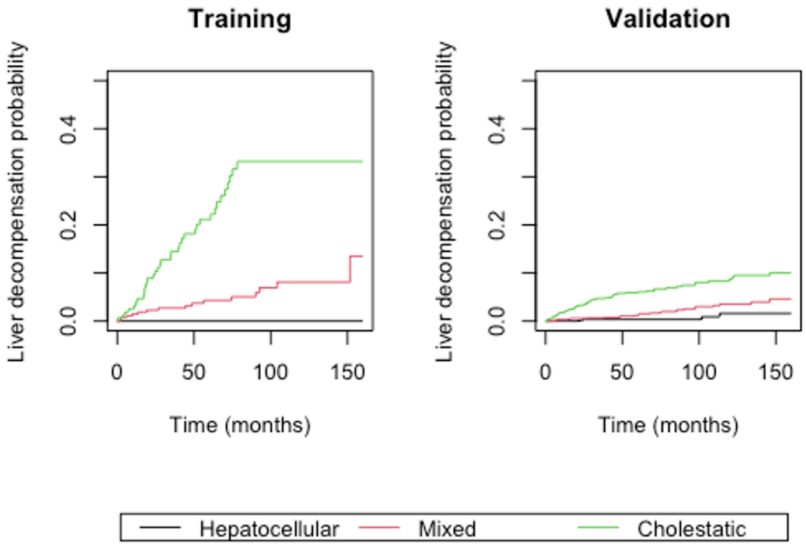


Figure 2. LD probability, in training (A) and validation cohort (B), according to the biochemical pattern (C vs M vs H).

LIV_15232_Figure 2.png

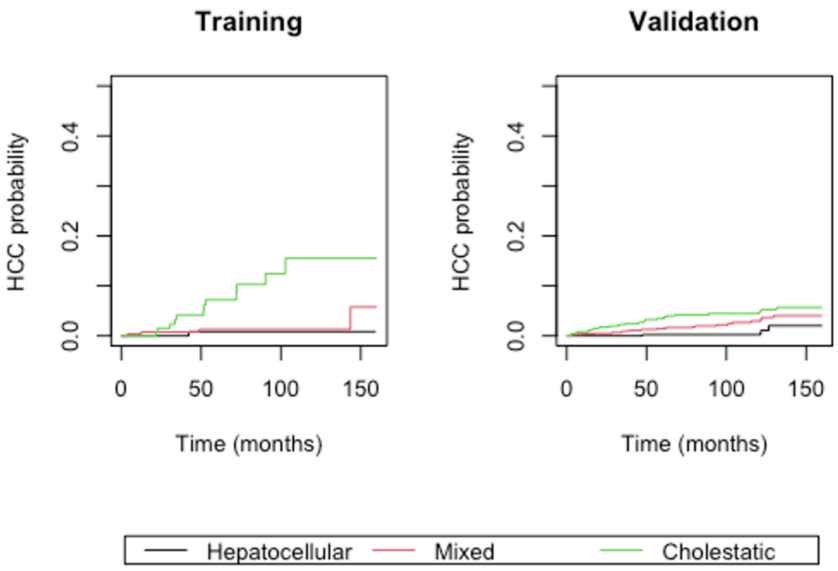
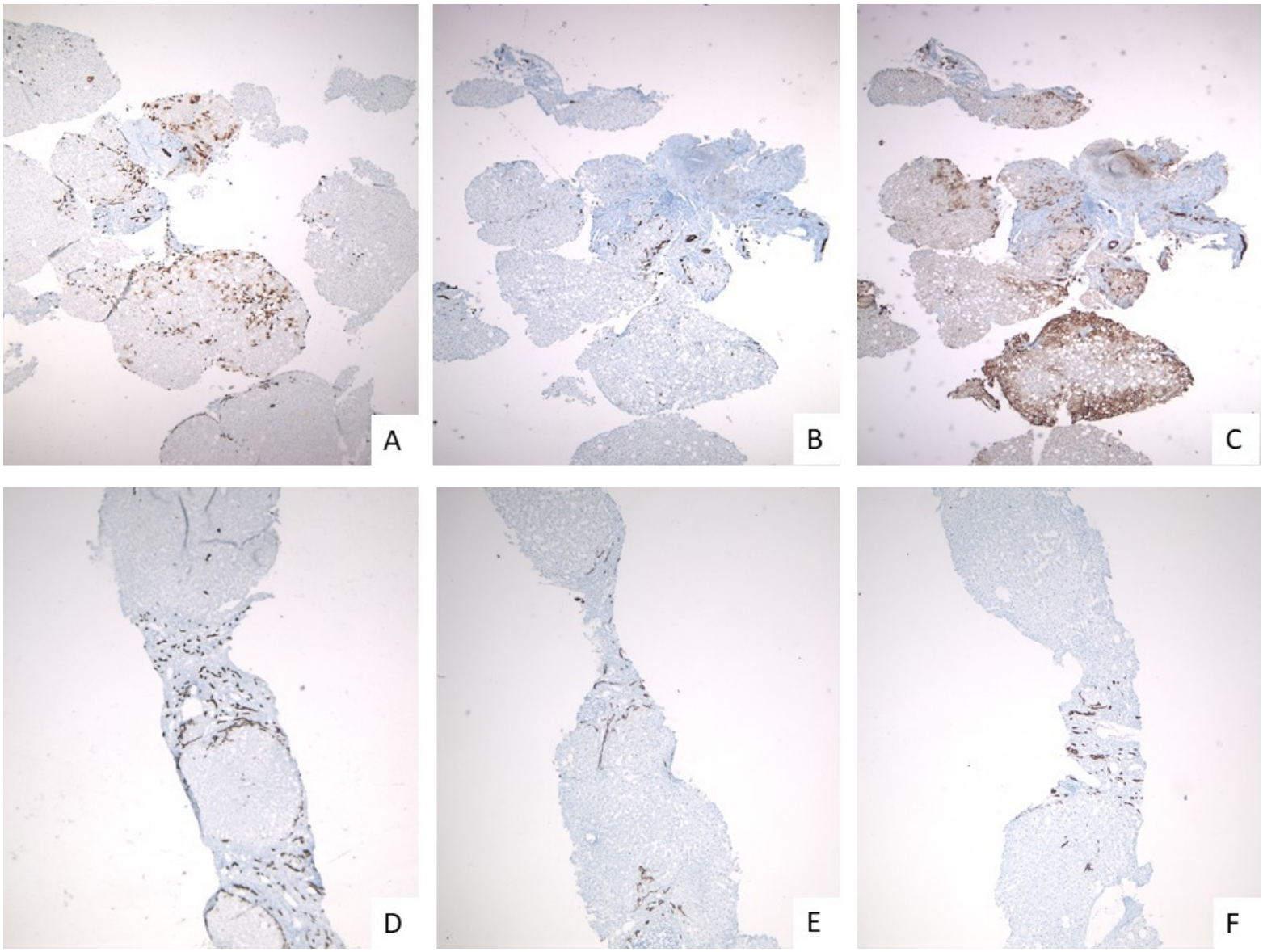


Figure 3. HCC probability, in training (A) and validation cohort (B), according to the biochemical pattern (C vs M vs H).

LIV_15232_Figure 3.png



LIV_15232_FIGURE 4.jpeg

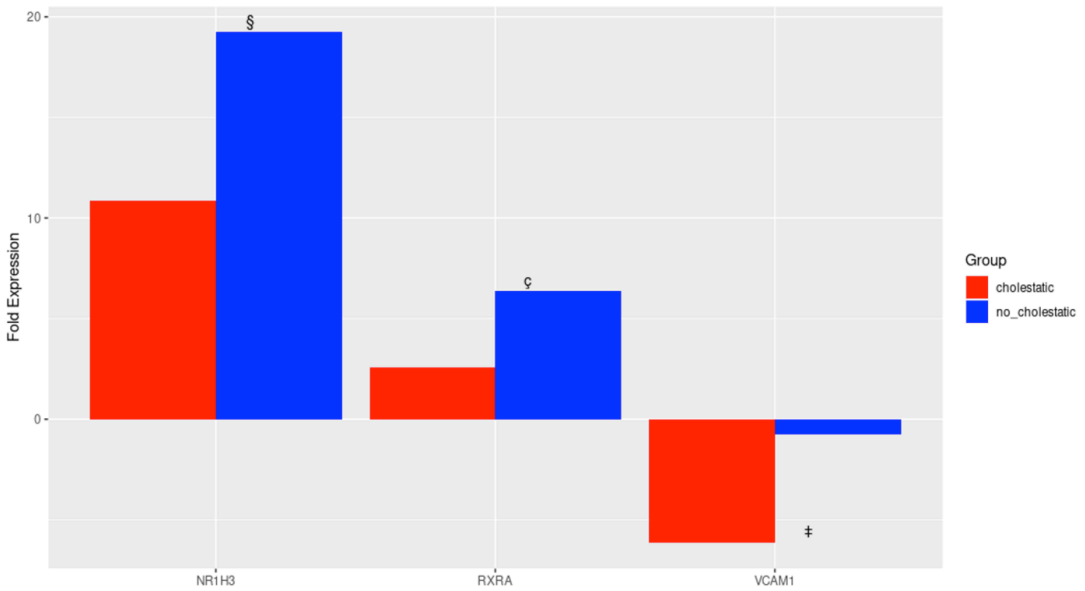


Figure 5. Relative changes in fold expression of genes involved in cholestatic pathway in subjects with (red bars) and without (blue bars) cholestatic pattern respect to LX2 cell line. $P^{§}$ =0.01 (NR1H3); $P^{ç}$=0.03 (RXRA); $P^{‡}$=0.04 (VCAM1).

Abbreviations: NR1H3(Nuclear Receptor subfamily 1 group H member 3), RXRA (Retinoid X Receptor Alpha), VCAM1 (Vascular Cell Adhesion Molecule 1).

LIV_15232_Figure 5.png