A cholestatic pattern predicts major liver-related outcomes in patients with non-alcoholic fatty liver disease

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A cholestatic pattern predicts major liver-related outcomes in patients with non-alcoholic fatty liver disease

Grazia Pennisi1| Rosaria Maria Pipitone1| Daniela Cabibi2| Marco Enea2|Manuel Romero- Gomez3| Mauro Viganò4| Elisabetta Bugianesi5|Vincent Wai-Sun Wong6| Anna Ludovica Fracanzani7| Giada Sebastiani8|Annalisa Berzigotti9| Francesca Di Salvo10| Antonino Giulio Giannone2|Claudia La Mantia1| Giulia Lupo1| Rossana Porcasi2| Federica Vernuccio11|Rossella Zito2| Vito Di Marco1| Calogero Cammà1| Antonio Craxì|Victor de Ledinghen12| Stefania Grimaudo1| Salvatore Petta1

AbstractBackground & Aims: NAFLD patients usually have an increase in AST/ALT levels, but cholestasis can also be observed. We aimed to assess in subjects with NAFLD the im-pact of the (cholestatic) C pattern on the likelihood of developing major liver- related outcomes (MALO).Methods: Five hundred and eighty-two 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, whilst 15 (5.5%) and 38 (24.2%) patients in M and C groups had MALO. At multivariate Cox regression analysis, age >55 years (HR 2.55, 95% CI 1.17–5.54; p= .01), platelets <150 000/mmc (HR 0.14, 95% CI 0.06–0.32; p< .001), albumin <4 g/L(HR 0.62, 95% CI 0.35–1.08; p= .09), C versus M pattern (HR 7.86, 95% CI 1.03–60.1; p= .04), C versus H pattern(HR 12.1, 95% CI 1.61–90.9; p= .01) and fibrosis F3–F4(HR 35.8, 95% CI 4.65–275.2; p< .001) were independent risk factors for MALO occurrence. C versus M pattern(HR 14.3, 95% CI 1.90–105.6; p= .008) and C versus H pattern (HR 15.6, 95% CI 2.10–115.1; p= .0068) were con-firmed independently associated with MALO occurrence in the validation set. The immunohistochemical analysis found a significantly higher prevalence of moderate-high- grade ductular metaplasia combined with low- grade ductular proliferation in C pattern when compared with the biochemical H pattern. Gene expression analysis showed a lower expression of NR1H3, RXRα and 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.

INTRODUCTION

Non- alcoholic fatty liver disease (NAFLD) is the most emergent chronic liver disease of the last decade, affecting more than a quar-ter of population worldwide, representing a growing cause of cir-rhosis, hepatic complications, mortality and liver transplantation.[1]Because of 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 cirrho-sis and its complications (e.g. liver decompensation and hepatocellular carcinoma—HCC) are influenced by genetic background and by indi-vidual metabolic risk factors.[3 – 7] Genetic susceptibility and metabolic comorbidities act synergically to induce and perpetuate liver damage through lipotoxicity, which prompts the activation of hepatic inflam-mation cascade, resulting in the activation of fibrogenesis pathways.[8]NAFLD is usually an asymptomatic condition where cytoly-sis with raised alanine aminotransferase (ALT)—expression of liver inflammation—can be present in a relevant proportion of patients.[9]Conversely, 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 dis-ease and metabolic setting.14 Thus, the data about the cholestatic pattern in NAFLD are 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 devel-opment of major liver-related outcomes (MALO). Results were vali-dated 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.

2 | METHODS

2.1 | Patient selection

We retrospectively analysed data from 582 patients prospec-tively 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, oesophageal varices band ligation and HCC. Specifically, in patients without histology, cirrhosis was diagnosed by liver stiffness measurement (LSM) >11.5 kPa for M probe15 or >11 kPa for XL probe,16 and the diagnosis of NAFLD required the presence of ultrasonography-assessed steatosis plus at least one cri-terion of the metabolic syndrome (obesity, diabetes, arterial hyper-tension and dyslipidaemia).A multicentre 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 in-cluded. Other causes of liver disease were ruled out, including al-cohol 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), auto-immune hepatitis including primary biliary cholangitis of primary sclerosing cholangitis, hereditary haemochromatosis and alpha-1 antitrypsin deficiency. Patients with an episode of extra-hepatic cholestasis at the diagnosis and patients who took part in random-ized controlled trials with new NASH drugs during the follow-up were also excluded. The Kleiner scoring system17 was used for histological assess-ment of NAFLD and specifically to grade steatosis, lobular inflamma-tion 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.

2.2 | Patient evaluation

Clinical and metabolic data were collected at the time of enrolment. Body mass index (BMI) was calculated in kilograms for weight and in meters for height. Obesity was defined as BMI ≥30 kg/m2. The diagnosis of type 2 diabetes (T2D) was made according to the American Diabetes Association,18 using a value of fasting blood glucose ≥126 mg/dl. In patients with a previous diagnosis of T2D, current medications and their changes were documented. Arterial hypertension was de-fined by systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or use of blood pressure-lowering agents.19An 8- h 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 pat-tern 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 previously pub-lished,20,21 the 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, whilst 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.

2.3 | Major liver-related outcomes

MALO were recorded during the entire follow-up, and they were de-fined 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.23In 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) oesophageal 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 eval-uated for available therapies and/or for liver transplantation, as appropriate.23,24

2.4 | Immunohistochemical analysis

Only samples measuring more than 1.5 cm and containing more than 10 portal tracts were assessed. The slides were stained with haematoxylin–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) ac-cording 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 semi-quantitatively.25 We evaluated the presence and the degree of ductular reaction, defined as the presence of newly formed 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 semi-quantitative scoring method for all the above-mentioned antibodies: score 0: Absence of immunohistochemical expression in the liver parenchyma outside the portal tracts; score 1: the 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: the 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: the diffuse presence of immunohistochem-ical expression adjacent to the portal tracts in more than 50% of the portal tracts, in the range of more than 1 HPF.

2.5 | Gene expression

Tissues stored at −80°C were homogenized, and total RNA was ex-tracted using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) ac-cording 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).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 cir-rhosis (184 target genes) (Bio-Rad). All the plates contained prim-ers for genomic DNA detection (gDNA), positive PCR control (PCR), RNA Quality Assay (RQ1 and RQ2), Reverse Transcription Control (RT) and three housekeeping genes: TATA-box binding protein (TBP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxan-thine phosphoribosyltransferase 1 (HPRT1). Data were expressed as fold change using the $2 - \Delta\Delta Ct$ method referred to LX2 cell line as control sample. Differences among experimental groups were ana-lysed 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 rela-tive with respect to the control sample (LX2 cell line), was computed; a list of gene expression for which RQ was statistically significant, were selected and analysed among experimental groups.2.6 | Statistical analysisThe statistical analysis followed three steps. In the first step, a de-scriptive 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.26Survival outcomes (time to HCC, time to decompensation and time to hepatic event) were analysed by Kaplan–Meier survival curves. In the second step, univariate and multivariate proportional hazards Cox regression models27,28 were fitted to estimate the effect of the covariates on the outcomes. Firth's penalized maximum likelihood bias reduction29,30 was used to avoid divergent parameter estimates, with infinite standard errors, owing 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< .05) from the uni-variate 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.

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3 | RESULTS

3.1 | Training set

3.1.1 | Patient features and outcomes

Baseline characteristics of the 582 patients with NAFLD stratified for liver enzyme biochemical pattern are shown in Table 1. One hun-dred and fifty-three patients had H, whilst 157 and 272 had C and M patterns respectively. Eighty-five (14.6%) patients had the presence of ULN ALP values. Figure S1shows 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 histol-ogy in 54 cases and on clinical criteria in 93 cases (63.3%) (Table S1). Specifically, 80 of the advanced fibrosis/cirrhosis patients had a liver stiffness measurement >15 kPa31 and 13 were between 11 and 15 kPa (11 of these cases also had oesophageal varices and the other two had signs of portal hypertension: splenomegaly and portal vein ectasia).During a median follow-up of 78 months, only one 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% re-spectively. 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.

3.1.2 | Association of biochemical pattern with MALO

At univariate Cox regression analysis, C versus M pattern (HR 9.86, 95% CI 1.31–74.15; p= .02) and C versus H pattern (HR 47.36, 95% CI 6.5–345.21; p< .001) were associated with MALO occurrence. At multivariate Cox regression analysis, age >55 (HR 2.55, 95% CI 1.17–5.54; p= .01), platelets <150 000/mmc (HR 0.14, 95% CI 0.06– 0.32; p< .001), albumin <4 g/L (HR 0.62, 95% CI 0.35–1.08; p= .09), C versus M pattern (HR 7.86, 95% CI 1.03–60.1; p= .04), C versus H pattern (HR 12.1, 95% CI 1.61–90.9; p= .01) and fibro-sis F3–F4 (HR 35.8, 95% CI 4.65–275.2; p< .001) were independ-ent risk factors for MALO occurrence (Table 2). When including into the model log GGT serum levels, this last was (HR 1.44, 95% CI 1.07–1.94; p= .01) independently associated with a higher risk of developing MALO, and both C versus M pattern (HR 8.02, 95% CI 1.05–61.1; p= .04) and C versus H pattern (HR 12.6, 95% CI 1 . 6 9 – 9 5 . 2 ; p= .01) were confirmed as independent risk factors. When replacing into the model the abnormal ALP values with the presence of abnormal ALP values this last remained significantly associated with a higher risk of developing MALO (HR 1.79, 95% CI 1.02–3.13; p= .03).The Kaplan–Meier curves in Figure 1A show the probability of MALO occurrence over time, according to the liver enzyme bio-chemical pattern (C vs. M vs. H). Figure S2A and D 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< .05 for all) and of AST/ALT ratio (AUC at 1 year 0.78, p= .13; 3 years 0.76, p= .05; 5 years 0.73, p= .03 and 10 years 0.63 p= .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% CI 1.12–5.24; p= .02), albumin <4 g/L (HR 0.15, 95% CI 0.07– 0.34; p< .001), C versus M pattern (HR 7.80, 95% CI 1.01–59.8; p= .04) and C versus H pattern (HR 12.1, 95% CI 1.61–91.3; p= .01) were confirmed as independently associated with MALO occurrence (Table 2). Figures S2B and D 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 the clinical diagnosis of cirrhosis confirmed C versus M pattern (HR 7.47, 95% CI 1.01–66.1; p= .04) and C versus H pattern (HR 9.03, 95% CI 1 . 0 3 – 7 8 . 6 ; p= .03) as independent risk factors for MALO occurrence.Considering LD and HCC separately, at multivariate Cox re-gression analysis, C versus M pattern (HR 10.5, 95% 1.4–1345, p= .01) and C versus H pattern (HR 20.1, 95% CI 2.7–2558; p< .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). Figure S3 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, univar-iate Cox regression analysis showed that C versus H pattern was linked with HCC occurrence in the entire population (HR 14.10, 95% CI 1.83–108.65; p= .01) and in subjects with fibrosis F3–F4 (HR 8.40, 95% CI 1.09–64.88; p= .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 bio-chemical pattern (C vs M vs H), whilst Figure S4 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.

3.2 | Validation set

During a median follow-up of 62.9 months, six patients with H pattern experienced MALO (four LD and two HCC) with a like-lihood 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 me-dian 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 versus M pattern (HR 14.3, 95% CI 1.90–105.6; p= .008) and C versus H pattern (HR 15.6, 95% CI 2.10–115.1; p= .0068) were independently asso-ciated with MALO occurrence, and these associations were main-tained in the subgroup of subjects with F3–F4 fibrosis (Table S4). Figure S5 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 Table S4, Figures S6 and S7.When looking at patients with baseline F0–F2 fibrosis, only 10 patients developed MALO during follow-up: none of them had base-line H pattern, whilst M and C patterns were observed in six and four patients respectively.3.3 | Immunohistochemical analysisIn 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 pro-file. Patients with C pattern had a non-significant statistical trend (66.6% vs. 38.4%, p= .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 sig-nificantly higher prevalence of moderate-high ductular metaplasia combined with low ductular proliferation with respect to what was observed in their counterpart with a biochemical H pattern (58.3% vs. 11.5%, p= .002); this association being also confirmed after ad-justing for age, gender, BMI and obesity (OR 13.5, 95% CI 2.00–91.8, p= 08).Figure 4 depicts hepatic morphological changes by immunohis-tochemistry related to H or C biochemical patterns.

3.4 | Gene expression

Gene expression analyses were performed in 14 patients with F3– F4 fibrosis and available frozen liver biopsy (six with cholestatic and eight with the 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 analysed, showed a significant difference in expression: NR1H3(alias LXRα - liver X receptor α), RXRα (retinoid X receptors α) and V C A M - 1 (vascular cell adhesion molecule-1).In order to test for conditional independence between up- down regulated results and cholestatic or non-cholestatic pattern in each gene, the Mantel–Haenszel X-squared test (MH) was implemented on the 2 × 2 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= .01), RXRα (p= .03) and V C A M - 1(p= .04) (Figure 5).NR1H3 and RXRα, normalized with respect to LX2, were regu-lated in a 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 non-cholestatic pattern; the fold expression mean increases from 10.88 in cholestatic pattern to 19.23 in non-cholestatic pattern. The percentage of upregulation 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 V C A M - 1, normalized respect to LX2, has a percent-age of 50% of down-regulation (mean = −6.16) in cholestatic pattern and 25% (mean = −0.75) in non-cholestatic pattern.

4 | 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 predic-tive 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 to demonstrate 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 patterns respectively. Notably, the association of the C pat-tern with MALO occurrence was confirmed at multivariate Cox re-gression 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 because of 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 non-cirrhotic NAFLD patients.36The present study is not designed to clarify the pathogenic link between the 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 significantly 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 bili-ary disorders such as primary biliary cholangitis (PBC), primary scle-rosing cholangitis (PSC) and biliary atresia.[37] Liver injury is the main trigger of DR, which encompasses not only bile duct proliferation, but also a wide spectrum of liver tissue reactions, including the ac-tivation of several inflammatory pathways.[12,38] Chronic liver injury activates hepatic progenitor cells (HPCs), quiescent in normal liver, with a consequent DR that entails different ways. First, HPCs can differentiate in intermediate cell and towards hepatocytes or chol-angiocytes lineages, according to the pathogenesis of liver damage; conversely, HPCs can dedifferentiate, leading to metaplasia.[39] This phenomenon was widely studied by Carpino et al.[37] in biliary disor-ders, in which DR resulted more pronounced in patients with higher severity of fibrosis than in those with a 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 indi-cate a secondary response of the hepatic parenchyma to cholestasis more than a primary reaction to the biliary damage as a tentative of hepatocyte regeneration.[40] Furthermore, relative bile acid overload may be an early trigger in ductular metaplasia of hepatocytes, exert-ing a primary effect on parenchymal cells themselves, which, in turn, stimulate HSCs.[41–43] 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)..[12,44,45] Consistent with the above-quoted lit-erature data and with our evidence about the association between biochemical cholestasis, a IHC profile characterized by higher duc-tal metaplasia and lower ductular proliferation, and a higher risk of MALO occurrence, we can speculate that the intrinsic biliary dys-function associated with the presence metaplasia—as a response to liver injury or induced by biliary acid overload—and not fully com-pensated by differentiate effective ductular proliferation, could ex-plain the biochemical manifestation of cholestasis and could identify a pattern of liver disease at higher risk of progression. Further anal-yses 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 ex-pression 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 V C A M - 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.[46] The heterodimer represents a key regu-lator of macrophage function, controlling transcriptional programs involved in lipid and cholesterol homoeostasis and inflammation.[47]LXRα activated pathways have dual functions: on one hand, they are involved in maintaining cholesterol and bile acid homoeostasis by increasing cholesterol catabolism and, on the other hand, they can prevent toxicity from bile acid accumulation.[48] In 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 con-sistent with the observed link between the C pattern and poor clin-ical outcomes. On the other side, vascular cell adhesion molecule 1 (V C A M - 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,[49]acts as a trigger and worsen liver inflammation in NAFLD with as a consequence a possible commitment versus a cytolytic hepatocel-lular pattern.[50,51] However, a study

showed that bile acids can exert a direct downregulation on V C A M - 1 expression and that inhibits the cell growth and proliferation and enhances the cell apoptosis.[49] According to these data, we suspected that cholestasis per se could be a possible trigger of reduction of V C A M - 1 expression. However, the biological and clinical significance of the different expression of RXRα, NR1H3 and V C A M - 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 subgroup 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 in-cluding ALT and ALP. For this reason, the biochemical pattern, to-gether with other easy-to-calculate scores like FIB-4[52] and together with LSM[53]— when available—could be assessed during routine visits to predict and differentiate the liver outcomes in order to personal-ize the follow-up. Notably, our results were largely validated in an external independent multicentre cohort of patients with histolog-ical diagnosis of NAFLD, even if lack of widely accepted cut-off for defining ULN ALP and, therefore, differences in used cut-offs among centres could affect the interpretation of our results. The main limitation of our study lies in the enrolment of pa-tients with NAFLD from tertiary care centres that can be different in terms of clinical features, metabolic comorbidities and genetic background from NAFLD individuals from the 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 the presence of abnormal ALP values this last— expression of cholestasis—remained independently associated with a higher risk of developing MALO confirming the negative im-pact 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 | 1047 PENNISI et al. developed MALO makes not possible subgroup 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,[54] 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 mor-phological changes, predicts a higher risk of developing MALO. Gene expression and immunohistochemical analysis, if externally validated, could underlie new pathogenic mechanism and potential target therapeutic strategies.

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Tables

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

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

Figures

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–C) Patients with biochemical C pattern showed low ductular proliferation (B) and diffuse biliary metaplasia (C) with respect to patients with biochemical H pattern (D–F). CK7 expression did not show a 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 the cholestatic pathway in subjects with (red bars) and without (blue bars) cholestatic pattern with respect to LX2 cell line. p§= .01 (NR1H3); pÇ= .03 (RXRA); p‡= .04 (VCAM1)