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Collagen formation assessed by PRO-C3 is an heritable trait and is associated with liver fibrosis assessed by MRE

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Abstract

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Rohit Loomba: study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript, obtained funding, study supervision, approved final submission.

Conflict of interests: Ida F. Villesen, Dr Natasja S. Gudmann, Dr Diana J. Leeming and Dr Morten A. Karsdal are full-time employees of Nordic Biosciences. Dr Morten A. Karsdal and Dr Diana J. Leeming hold stocks in Nordic Biosciences. Dr Diana J. Leeming and Dr Morten A. Karsdal hold patents for PRO-C3, PRO-C5, PRO-C6, C3M, C4M2 and P4NP7S. All other authors report no other conflict of interests.

Background—PRO-C3 (N-terminal pro-peptide of type III collagen) is a biomarker of liver fibrosis in nonalcoholic-fatty-liver-disease (NAFLD). This study examines the association between PRO-C3 concentration and liver fibrosis assessed by magnetic resonance (MR) elastography (MRE)-measured stiffness (MRE-stiffness) and the heritability of PRO-C3 concentration in a cohort of twins and families with and without NAFLD. We performed a cross-sectional analysis of a well-characterized prospective cohort of 306 participants including 44 probands with NAFLD-cirrhosis and their 72 first-degree-relatives, 24 probands with NAFLD without advanced fibrosis and their 24 first-degree-relatives and 72 non-NAFLD controls and their 72 first-degree-relatives. Liver steatosis was assessed by MR imaging proton density fat fraction (MRI-PDFF) and liver fibrosis by MRE-stiffness. Serum PRO-C3 was assessed by competitive ELISA. We assessed the familial correlation of PRO-C3 concentration, shared gene effects between PRO-C3 concentration and liver steatosis and fibrosis, and association between PRO-C3 concentration and genetic variants in *PNPLA3*, *TM6SF2*, *MBOAT* and *CGKR*.

In multivariable-adjusted models including age, sex, body mass index and ethnicity, serum PRO-C3 correlated strongly with liver fibrosis (r^2=0.50, p<0.001), and demonstrated robust heritability [$\boldsymbol{h^2}$:0.36, 95%confidence-interval (CI):0.07–0.59, p=0.016]. PRO-C3 concentration and steatosis had a strong genetic correlation [$\boldsymbol{r_G}$:0.62,95%CI:0.236–1.001, p=0.002] whereas PRO-C3 concentration and fibrosis had a strong environmental correlation [$\boldsymbol{r_E}$:0.55,95%CI:0.317–0.717, p<0.001]. PRO-C3 concentrations were higher in carriers of *TM6SF2*rs58542926-T-allele versus non-carriers: 15.7 (±10.5) versus 10.8 (±5.7) ng/L, (p=0.047).

Conclusion—Serum PRO-C3 correlates with MRE-assessed fibrosis, is heritable, shares genetic correlation with liver steatosis and shares environmental correlation with liver fibrosis. PRO-C3 concentration appears to be linked to both fibrosis and steatosis and increased in carriers of *TM6SF2*rs58542926 risk allele.

Keywords

Biomarker; non-invasive; cirrhosis; TM6SF2

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is now the most prevalent cause of chronic liver disease worldwide(1, 2). NAFLD encompasses a spectrum of histological liver phenotype ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), the more progressive form of NAFLD. NASH has a significantly increased risk of progression to advanced fibrosis and cirrhosis(3). Several studies have demonstrated that the presence of advanced fibrosis is the most important predictor of mortality in NAFLD(4, 5).

Previous studies have reported a familial aggregation of NAFLD and NAFLD-related cirrhosis (6–8), demonstrated that both liver steatosis and fibrosis are heritable, and shown that they share a gene-effect in twins with and without NAFLD(9, 10). We have recently demonstrated that first-degree relatives of probands with NAFLD-cirrhosis had a significant higher risk of having advanced fibrosis compared to first-degree relatives of non-NAFLD controls(11). Genome-wide association studies (GWAS) have identified genetic risk variants contributing to NAFLD incidence and progression located in *PNPLA3*(12), *TM6SF2*(13,

14), *GCKR*(15) and *MBOAT7*(16). While these risk alleles have advanced our understanding of the genetic susceptibility towards NAFLD, they do not account for all of the variance observed in NAFLD(15, 17, 18). Therefore, there is still an unmet need to better characterize the factors involved in the heritability of liver fibrosis in NAFLD. Identifying factors associated with fibrogenesis among families would help to determine therapeutic targets as well as potential biomarkers for the screening of individuals at high risk of advanced fibrosis in these families.

Liver fibrosis is characterized by the accumulation of excess extracellular matrix (ECM). Over the past decade, a panel of collagen-derived biomarkers specifically assessing ECM remodeling, which is a key component of fibrogenesis, has been developed(19). These biomarkers measure the end-products of tissue remodeling, known as neo-epitopes, resulting from specific ECM proteins undergoing posttranslational modification such as protease cleavage. These neo-epitopes are released into the circulation and thus may potentially reflect the dynamic activity of either the formation or degradation of the ECM involved in the fibrogenesis. Among them, PRO-C3 detects the N-terminal pro-peptide of type III collagen (PIIINP) released by A Disintegrin and Metalloproteinase with Thrombospondin motifs 2 (ADAMTS-2) during ECM formation. PRO-C3 has emerged as a key non-invasive biomarker of fibrogenesis(20). It has been shown that PRO-C3 is associated with the presence of liver fibrosis in chronic liver disease including NAFLD(20–24). In addition, PRO-C3 has recently been found to accurately detect the presence of advanced fibrosis in individuals with NAFLD when incorporated in a clinical prediction rule(25). However, there are no data regarding whether PRO-C3 concentration, or other biomarkers of ECM activity are heritable and if they share any gene effect with liver steatosis and fibrosis.

Using a unique twin and family study design including well-characterized and prospectively recruited probands encompassing the entire spectrum of NAFLD and their first degree-relatives, we aimed to examine the association between PRO-C3 concentration and liver fibrosis assessed by magnetic-resonance-elastography (MRE)-measured stiffness and to assess the heritability of PRO-C3 concentration in a cohort of twins and families with and without NAFLD.

MATERIAL AND METHODS

Study design

This is a cross-sectional analysis of a prospective Familial cohort study of participants from the Familial Cirrhosis Study and Twins and Family Study prospectively recruited at the University of California at San Diego (UCSD) NAFLD Research Center between December 2011 and October 2017. All participants underwent a standardized rigorous clinical research visit including detailed medical history, physical examination, and testing to rule out other causes of chronic liver diseases (see inclusion and exclusion criteria for further details), fasting laboratory tests at the University of California at San Diego (UCSD) NAFLD Research Center(9–11, 26, 27). Participants also underwent an advanced magnetic resonance examination, including confounder-corrected chemical-shift-encoded magnetic resonance imaging to measure hepatic proton density fat fraction (MRI-PDFF) and magnetic resonance elastography (MRE) to measure liver stiffness at the UCSD Liver Imaging Group for the

screening of NAFLD and advanced fibrosis(28–31). Written informed consent was obtained from all participants.

Study participants

Proband with NAFLD-Cirrhosis and first-degree relatives—This study included 44 probands with NAFLD-cirrhosis and 70 of their first-degree relatives from the Familial Cirrhosis cohort prospectively recruited at the UCSD NAFLD Research Center(11). Probands with NAFLD-cirrhosis had a documented evidence of NAFLD with either biopsy-proven or meeting imaging criteria for cirrhosis. Definition for NAFLD was based upon American Association for the Study of Liver Study (AASLD) Practice Guidelines(32). Recruitment of these participants was approved by the UCSD Institutional Review Board number 140084.

Inclusion/exclusion criteria for the Familial Cirrhosis cohort—Probands and first-degree relatives had to be at least 18 years old. Probands were required to have documented diagnosis of NAFLD-cirrhosis either by liver biopsy or by documented imaging evidence by a protocol specified criteria via ultrasound, computed tomography (CT), or MRI. First-degree relatives (sibling, child, or parent) with written informed consent who did not meet any exclusion criteria were included in the study.

Please see the Supplementary Material for detailed exclusion criteria.

Proband with NAFLD without advanced fibrosis and non-NAFLD control and first-degree relatives—The study included a total of 192 participants from the Twin and Family study corresponding to 144 twins (72 twin-pairs; 47 monozygotic twin-pairs, 25 dizygotic twin-pairs) and 24 siblings or parents-offspring pairs (9, 11, 26, 27). These twin, sib-sib, and parent-offspring pairs were prospectively recruited and they reside in southern California. Recruitment of these participants was approved by the UCSD Institutional Review Board number 111282.

Participants from the Twin and Family study were classified based upon their liver status as NAFLD controls (group A, defined by no evidence of NAFLD [MRI-PDFF<5%] or advanced fibrosis [MRE <3.63 kPa]) and participants with evidence of NAFLD without evidence of advanced fibrosis (group B, defined by MRI-PDFF≥5% and MRE-stiffness<3.63 kPa). Group A included 72 pairs (n=144) of community-dwelling controls (52 twin pairs, 10 sibling-sibling pairs and 10 parent-offspring pairs); randomly assigned as probands (group A1, n=72) or first-degree relatives (group A2, n=72). Group B included 24 pairs (n=48) 20 twin pairs, 1 sibling-sibling pair and 3 parent-offspring pairs, randomly assigned as probands (group B1, n=24) or first-degree relatives (group B2, n=24) Supplemental Table 1.

Inclusion and exclusion criteria for Twin and Family cohort—Please see Supplementary Material for detailed inclusion and exclusion criteria.

Clinical assessments and laboratory test: Please see Supplementary material.

MRI assessment

MRI was performed on a 3T research scanner (GE Signa EXCITE HDxt; GE Healthcare, Waukesha, WI) with all participants in the supine position. MRI-PDFF was used to measure hepatic steatosis and MRE was used to measure liver fibrosis. The details of the MRI protocol have been previously described in references(33, 34).

Justification for not using liver biopsy for assessment of liver steatosis and fibrosis in controls and first-degree relatives—Liver biopsy was not used for hepatic steatosis and fibrosis assessment of controls and first-degree relatives as they were asymptomatic with no suspected liver disease and therefore performing a liver biopsy would have been unethical. A non-invasive, accurate quantitative imaging method was used to estimate liver steatosis and fibrosis. We have previously shown that MRI-PDFF is a precise, accurate and reproducible non-invasive biomarker for the quantification of liver steatosis(35, 36). In addition, MRE is the most accurate, currently available, non-invasive quantitative biomarker of liver fibrosis(28, 29, 37) with an excellent diagnostic accuracy in differentiating between normal liver and mild fibrosis (stage 0–2) and between non-advanced fibrosis and advanced fibrosis (stage 3–4)(29).

Definition of NAFLD, cirrhosis and advanced fibrosis

Please see supplemental data

Biomarkers of ECM activity assessment

The serological biomarkers of interstitial matrix turnover was assessed by type III, V and VI collagen formation (PRO-C3(38), PRO-C5(39) and PRO-C6(40)) and type III collagen degradation by MMP-9 (C3M(41)). Basement membrane formation was evaluated by type IV collagen formation (P4NP7S(42)) and degradation mediated by MMPs (C4M2 (43)). All markers were assessed by ELISAs. The Nordic Bioscience ELISA assays were performed as follows: 96-well pre-coated streptavidin plates (Roche Diagnostics, Mannheim, Germany) were coated with the appropriate biotinylated synthetic peptides and incubated for 30 minutes at 20°C. Twenty μL of standard peptide or pre-diluted sample were added to appropriate wells, followed by peroxidase-conjugated specific monoclonal antibodies and incubated for 1 hour or overnight at 20°C or 4ºC. Finally, tetramethylbenzinidine (TMB) (cat.438OH, Kem-En-Tec Diagnostics, Taastrup, Denmark) was added, and the plates were incubated for 15 minutes at 20ºC in darkness. All the above incubation steps included shaking at 300 rpm. After each incubation step, the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The TMB reaction was stopped by adding 0.18 M H_2SO_4 as stopping solution and measured at 450 nm with 650 nm as reference. A calibration curve was plotted using a 4-parametric mathematical fit model. Detailed specification the biomarkers of ECM activity assessed in the study are summarized in the Supplemental Table 2.

Genotyping: whole-blood specimens collected during the research visit were used and DNA was extracted. *PNPLA3*, *TM6SF2*, *GCKR* and *MBOAT7* genotyping was conducted in a subgroup of the cohort (n=135) and their association in explaining the variance in PRO-C3

and other biomarkers of ECM activity was examined. The genotyping was performed by Human Longevity, Inc (San Diego, CA) and has been previously described (10, 11).

Primary outcome

The study assessed two primary outcomes. The first primary outcome was the association between PRO-C3 and liver fibrosis as assessed by MRE. The second primary outcome was the heritability of PRO-C3 and genetic or environmental correlation between PRO-C3 concentration and liver steatosis or liver fibrosis.

The secondary outcomes were the association between other ECM activity biomarker and liver fibrosis as assessed by MRE and the heritability of other biomarkers of ECM activity and genetic or environmental correlation between biomarkers of ECM activity and liver steatosis or liver fibrosis.

Statistical analysis

Data analysis—Patients' demographic, anthropometric, clinical, and biochemical characteristics were summarized. Categorical variables were shown as counts and percentages, and associations were tested using a chi-squared test or Fisher's exact test. Normally distributed continuous variables were shown as mean (± standard deviation), and differences between groups were analyzed using a two-independent samples t- test or Wilcoxon-Mann-Whitney test. Spearman correlation between biomarkers of ECM activity and liver fibrosis assessed by MRE were performed. Sensitivity analyses were performed using partial correlation between biomarkers of ECM activity liver fibrosis assessed by MRE adjusted for age, sex, BMI, and Hispanic ethnicity to account for potential cofounders. Familial correlation was assessed by comparing spearman correlation within related pairs and within random unrelated pairs. The association between PRO-C3 concentration and genetic variant was assessed using, generalized estimating equations (GEE) to account for intrapair correlations within twinships. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA) or SPSS 25.0 (IBM, Chicago, IL). A two-tailed P value <0.05 was considered statistically significant.

Heritability estimates and share gene-effect—AE models were used to estimate the shared genetic determination (rG) and shared environmental determination (rE) between twin pairs as described in previous studies(9, 26). In the classical twin study of sets of monozygotic and dizygotic twins, four latent factors can account for the variance of any phenotype: additive genetic effects (A); nonadditive genetic effects, including dominance (D); common or shared environmental effects (C); and nonshared or individual-specific environmental effects (E)(44). Because monozygotic twins are presumed to be genetically identical, they correlate perfectly ($r = 1.0$) with respect to both additive and nonadditive genetic effects. Dizygotic twins share, on average, 50% of their genes, resulting in correlations of 0.50 for additive genetic effects and 0.25 for nonadditive genetic effects. The C term is defined as environmental factors that make twins similar; hence, common environmental factors correlate 1.0 across twin pairs, regardless of zygosity. The E term represents environmental factors that lead to differences between twins. Because these are individual-specific factors, they are assumed to be uncorrelated across twins. Error is

assumed to be random across individuals, so measurement error forms part of the estimate of E in these analyses. These latent factors comprise what are referred to as the univariate ACE or ADE models; due to model underidentification, an ACDE model cannot be tested in the classical twin design (44). The analyses were performed using OpenMx, a structural equation modeling software package for genetically informative data (http://openmx.psyc.virginia.edu). Prior to the model fitting, the measures were adjusted for controlling age, gender, and ethnicity. Overall, AE models tended to provide the best fits to the data. Consequently, the genetic effects estimated in these AE models refer to broad-sense heritability, reflecting the proportion of phenotypic variance accounted for by the combined effect of all genetic influences (A+D).

Sample size estimation

We have previously reported that median heritability estimates of serum metabolites was 0.4 ranging from 0.3 to 0.9 in the Twin cohort(26), and median heritability estimates of serum metabolites was 0.5 ranging from 0.2 to 0.8 in a UK Twins cohort(45). We have also previously estimated the heritability of hepatic steatosis to be approximately 0.5(10). Therefore, we anticipated that the heritability of PRO-C3 concentration or other biomarkers of ECM activity and hepatic steatosis or liver fibrosis with one another would be approximately in the range from 0.4 to 0.6. It has been shown that, to detect an additive genetic component of 0.4–0.8 in an ACE model, approximately 36–74 twin pairs are needed to produce a power of 0.95 with an alpha value of 0.05(46). Therefore, the 72 twin pairs included in this study would be adequate to assess the heritability of PRO-C3 concentration and other biomarkers of ECM activity and their genetic correlation with liver steatosis and fibrosis in this cohort.

RESULTS

Baseline characteristics

This cross-sectional analysis included a total of 306 participants who were prospectively recruited including 44 probands with NAFLD-cirrhosis and 70 of their first-degree relatives, 24 probands with NAFLD (MRI-PDDF ≥ 5% and without advanced fibrosis (MRE < 3.63 kPa) and 24 of their first-degree relatives, and 72 non-NAFLD controls (MRI-PDFF <5%) and 72 of their first-degree relatives. The detailed derivation of study cohort is shown in Supplemental Figure 1. The participants underwent serum PRO-C3 and other ECM activity biomarker profiling, clinical evaluation and advanced MRI assessment. The detailed demographic, biochemical, imaging data and biomarkers of ECM activity of the probands stratified by their metabolic and liver phenotype are provided in Table 1a. The detailed demographic, biochemical, imaging data and biomarkers of ECM activity of the first-degree relatives stratified by the liver phenotype of the probands are provided in Table 1b.

Association between PRO-C3 and others Biomarkers of ECM activity and liver fibrosis—Serum PRO-C3 concentration showed a strong correlation with liver fibrosis as assessed by MRE (p= 9.0E-09). In addition, serum PRO-C6 (p=0.0001), C3M (p=0.0009) and C4M2 (p=0.005) were also significantly associated with liver fibrosis in the cohort (Supplemental Table 3, Figure 1). In multivariable-adjusted models including age, sex, body

mass index and ethnicity, the results remained statistically and clinically significant, and PRO-C3 concentration showed a significant correlation with liver fibrosis r=0.62, p=4.0E-11 (Supplemental Table 3, Figure 1). Only PRO-C3 concentration was significantly correlated with the presence of advanced fibrosis (MRE>3.63 kPa) in the multi-variable adjusted model: r=0.36, p=0.0004 (Supplemental Table 3). Finally, PRO-C3 concentrations were significantly higher in the subjects with NAFLD-cirrhosis median (±SD) 20.4 ng/mL (±29.2) vs non-NAFLD controls: 9.1 ng/mL (±4.8) p<0.001, and versus subjects with NAFLD without advanced fibrosis 9.1 (±6.0), p<0.001 Figure 2.

Familial correlation and heritability of PRO-C3 concentration—The heritability estimates were assessed in the subgroup of twin pairs and are provided in Table 2. Only PRO-C3 concentration was significantly heritable with a heritability estimate (h^2) of: 0.37 (95% confidence interval [CI], 0.097–0.592, p=0.009). The PRO-C3 concentration remained statistically significant even in a multivariable-adjusted model including age, sex, ethnicity and BMI with an h^2 of 0.36 (95%CI: 0.072–0.585). The twinship correlation by PRO-C3 concentration is shown in Figure 3A and 3B.

The familial correlation of PRO-C3 concentration and other biomarkers of ECM activity were assessed by comparing the spearman correlation within related pairs compared to correlation within random unrelated pairs. The concentration of P4NP7S and PRO-C3 had the most significant correlation within related pairs without overlap of the 95%CI between correlation coefficient within related pairs and within random unrelated pairs indicating a significant familial correlation of the concentration of PRO-C3 and P4NP7S concentration Figure 3C.

Shared genetic correlation between PRO-C3 and liver steatosis—The genetic correlation and environmental correlation between PRO-C3 concentration and liver steatosis assessed by MRI-PDFF and with liver fibrosis assessed by MRE was further investigated Table 3. PRO-C3 concentration had a significant shared gene effect with liver steatosis with a genetic correlation estimates r_G of 0.62, 95%CI: 0.236–1.001; p=0.002, whereas PRO-C3 concentration and liver fibrosis MRE demonstrated a strong environmental correlation: r_E : 0.55, 95%CI: 0.317–0.717; p<0.001 Table 3, Figure 4A.

TM6SF2 is associated with PRO-C3 concentration—As PRO-C3 concentration and liver steatosis share a significant gene effect, we further investigated whether genetic variant associated with NAFLD such as *PNPLA3* rs738409, *TM6SF2* rs58542926, *MBOAT7* rs641738, *GCKR* rs1260326, were associated with PRO-C3 concentration Supplemental Table 4. PRO-C3 concentrations were higher in carriers of *TM6SF2*rs58542926-T-allele versus non-carriers: 15.7 (±10.5) versus 10.8 ±5.7) ng/L, (p=0.047) Figure 4B.

DISCUSSION

Main findings

Using a uniquely well-phenotyped familial cohort, we have demonstrated that serum PRO-C3 concentration is strongly correlated with liver fibrosis as assessed by MRE. In addition, we have demonstrated that level of PRO-C3 is heritable, shares significant gene-effect with

liver steatosis whereas it shares environmental effect with liver fibrosis. Finally, we have shown that PRO-C3 concentration is associated with the rare genetic variant located in *TM6SF2* rs58542926. These results indicate a plausible common genetic basis between fibrogenesis and liver steatosis and provide new insights underlying the mechanism involved in the familial susceptibility towards NAFLD-related fibrosis. In addition, these findings show that that the quantity of liver steatosis is associated with fibrogenesis which involves at least partially the variant *TM6SF2* rs58542926. These data indicate that beyond the genetic factors involved in liver steatosis accumulation which is known as the initial step towards fibrogenesis, environmental factors have an additive effect triggering an increase in fibrogenesis and thus accelerating the development of severe liver injury such as advanced fibrosis or cirrhosis.

While there is currently no FDA approved therapy for the treatment of NASH, these data have important implication for developing therapeutic approaches of NASH-related fibrosis. Reducing liver steatosis e.g by targeting TM6SF2 pathway could reduce ECM accumulation or fibrogenesis and prevent the pejorative evolution towards advanced fibrosis and cirrhosis. Thus, identifying the pathways involved in the development of advanced fibrosis especially in individuals at high risk such as first-degree relatives of probands with NAFLD-related cirrhosis may have important clinical implications. In the future, this will potentially help to address future guidelines for the screening of this high-risk population.

In context of published literature

Previous studies have demonstrated that PRO-C3 concentration is associated with degree and progression in liver fibrosis in chronic liver disease such as chronic hepatitis C (20, 21, 23). Recently, Daniels and colleagues have shown that PRO-C3 can accurately detect the presence of advanced fibrosis when associated with other clinical prediction rules in NAFLD patients(25). In this study, we confirm previously reported association between PRO-C3 concentration and liver fibrosis in a well-characterized cohort of participant with and without NAFLD. In addition, we report significant correlation between liver fibrosis and additional biomarkers of ECM activity including C3M, C4M2, P4NP7S in the cohort. Interestingly, preliminary data from a therapeutic trial suggest that serum PRO-C3 could be useful to determine the therapeutic response to anti-fibrotic agent(22), especially in the setting of clinical trials in NASH (47).

We have previously demonstrated that both liver steatosis and fibrosis are heritable and that they share a gene-effect (9, 10). In addition, we have recently demonstrated that first-degree relatives of probands with NAFLD-cirrhosis had a significant higher risk of advanced fibrosis compared to first-degree relatives of non-NAFLD controls (11). This study is novel because we demonstrate the heritability of PRO-C3 concentration to be significant, and showed that PRO-C3 concentration has shared gene effect with liver steatosis and shared environmental effect with liver fibrosis. In addition, we have identified a significant association between PRO-C3 concentration and the non-synonymous genetic variant located in *TM6SF2* rs58542926. This loss of function variant has been associated with increased liver steatosis(13, 14) and NAFLD severity including liver fibrosis in GWAS studies(13, 18). *TM6SF2* rs58542926 leads to an increased accumulation of fat in the hepatocytes and a

defect of very-low density lipoprotein secretion(14). Studies also shown a reciprocal association between TM6SF2 rs58542926 and cardiovascular disease (48). Interestingly, the association between TM6SF2 rs58542926 and advanced fibrosis has been reported to be dependent on liver steatosis. Indeed, Dongiovanni et al. have shown that genetically determined liver steatosis is associated with the severity of NAFLD such as fibrosis in a cross-sectional study of a large European cohort with liver biopsy(18). Accordingly, we have recently demonstrated that higher hepatic fat content is associated with liver fibrosis progression in individuals with NAFLD and paired liver biopsy(30). Our study provides additional evidence of the role of liver steatosis genetically determined by TM6SF2 rs58542926 and now link it with PRO-C3, a marker of fibrogenesis. Further investigations are needed to determine the precise pathophysiological mechanism involved in the accelerated development of liver fibrosis when liver steatosis increases and its potential association with cardiovascular disease.

Strengths and limitations

There are several notable strengths of this study including the prospectively recruited study cohort including probands encompassing the entire spectrum of NAFLD and their first degree-relatives. In addition, all participants underwent a systematic and standardized liver disease assessment and other causes of liver disease were systematically excluded.

However, we acknowledge the following limitations to this study. Liver biopsy assessment could not be justified as previously noted in the methods section, and instead we utilized the most accurate non-invasive modalities for the assessment of hepatic steatosis and hepatic fibrosis (35, 36). As this study screened asymptomatic first-degree relatives of patients with NAFLD- cirrhosis, and controls with no suspected liver disease, exposing the study population to the risks associated with a liver biopsy such as pain, risk of bleeding, and in rare cases death, would not be justifiable and appropriate. Therefore, we are not able to determine whether a high PRO-C3 value is of greater predictive value in an individual if his/her family member also has NASH fibrosis, as compared to if his/her family member has NASH but no fibrosis and further study are needed to determine the clinical relevance of the use of PRO-C3. Finally, the association between PRO-C3 concentration and the four major genetic variant known to be associated with NAFLD have been assessed in this study while other genetic association with PRO-C3 concentration cannot be excluded.

Implication for future study

In this study, we confirm that PRO-C3 concentration is strongly correlated with liver fibrosis as assessed by MRE. Furthermore, PRO-C3 concentration is heritable and share significant gene-effect with liver steatosis that involves at least partially the variant located in *TM6SF2* rs58542926. Future identification of the pathway involved in this common genetic association may lead to individualized, targeted therapies that may prevent and/or reverse the development of liver fibrosis. Finally, longitudinal studies are needed to determine whether higher concentration of PRO-C3 can predict the development of advanced fibrosis in high-risk population such as first-degree relative of proband with NAFLD-cirrhosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DZ	dizygotic
ECM	extracellular matrix
GWAS	genome-wide association studies
GEE	generalized estimating equations
MMPs	matrix metalloproteinases
MRI-PDFF	magnetic resonance imaging proton-density-fat-fraction
MRE	magnetic resonance elastography
MZ	monozygotic
NAFLD	Nonalcoholic fatty liver disease
NAFL	nonalcoholic fatty liver
NASH	nonalcoholic steatohepatitis
PIINP	peptide of type III procollagen
PRO-C3	N-terminal pro-peptide of type III procollagen
UCSD	University of California at San Diego

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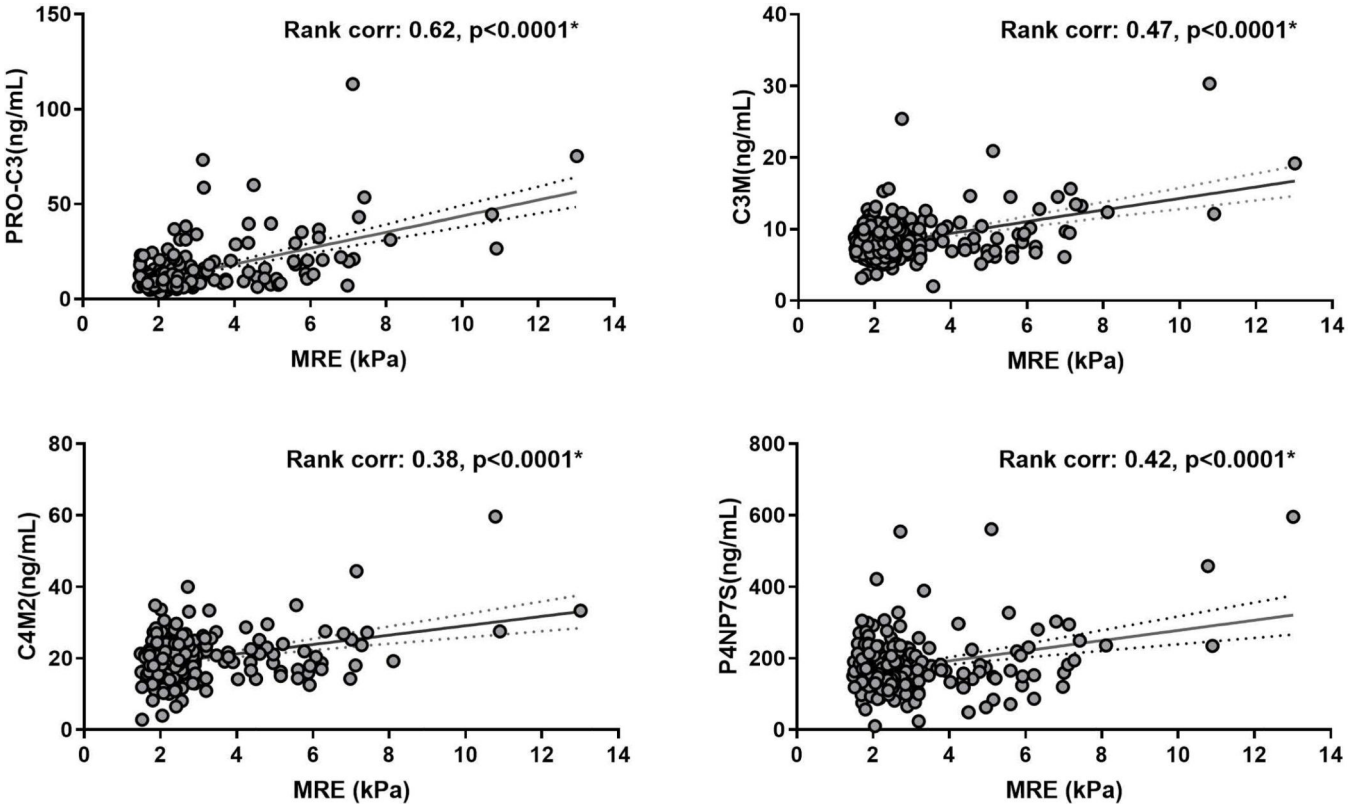

Figure 1. Correlation between PRO-C3 and other ECM remodeling biomarkers and liver fibrosis assessed by MRE

*rank coefficient an p-value determined using partial correlation adjusted for age, sex, BMI, and Hispanic ethnicity are shown for PRO-C3, C3M, C4M2 and P4NP7S.

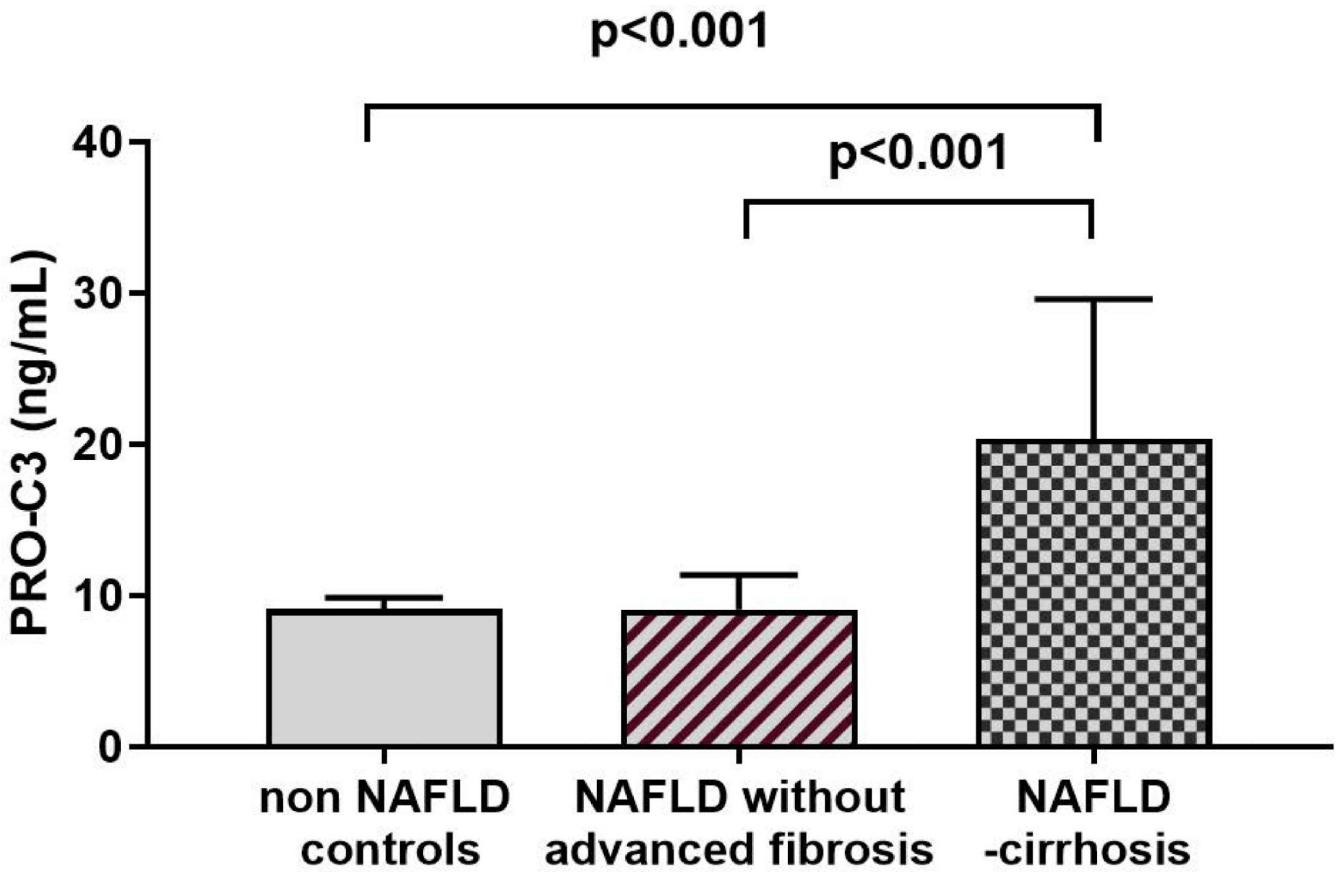

Figure 2. Serum PRO-C3 levels are significantly increased in NAFLD-cirrhosis

Median and 95% confidence interval of PRO-C3 levels across 3 independent group: non-NAFLD control (blue), proband with NAFLD without advanced fibrosis (green), and NAFLD-cirrhosis (pink) are shown. P-value were determined using nonparametric Mann-Whitney test

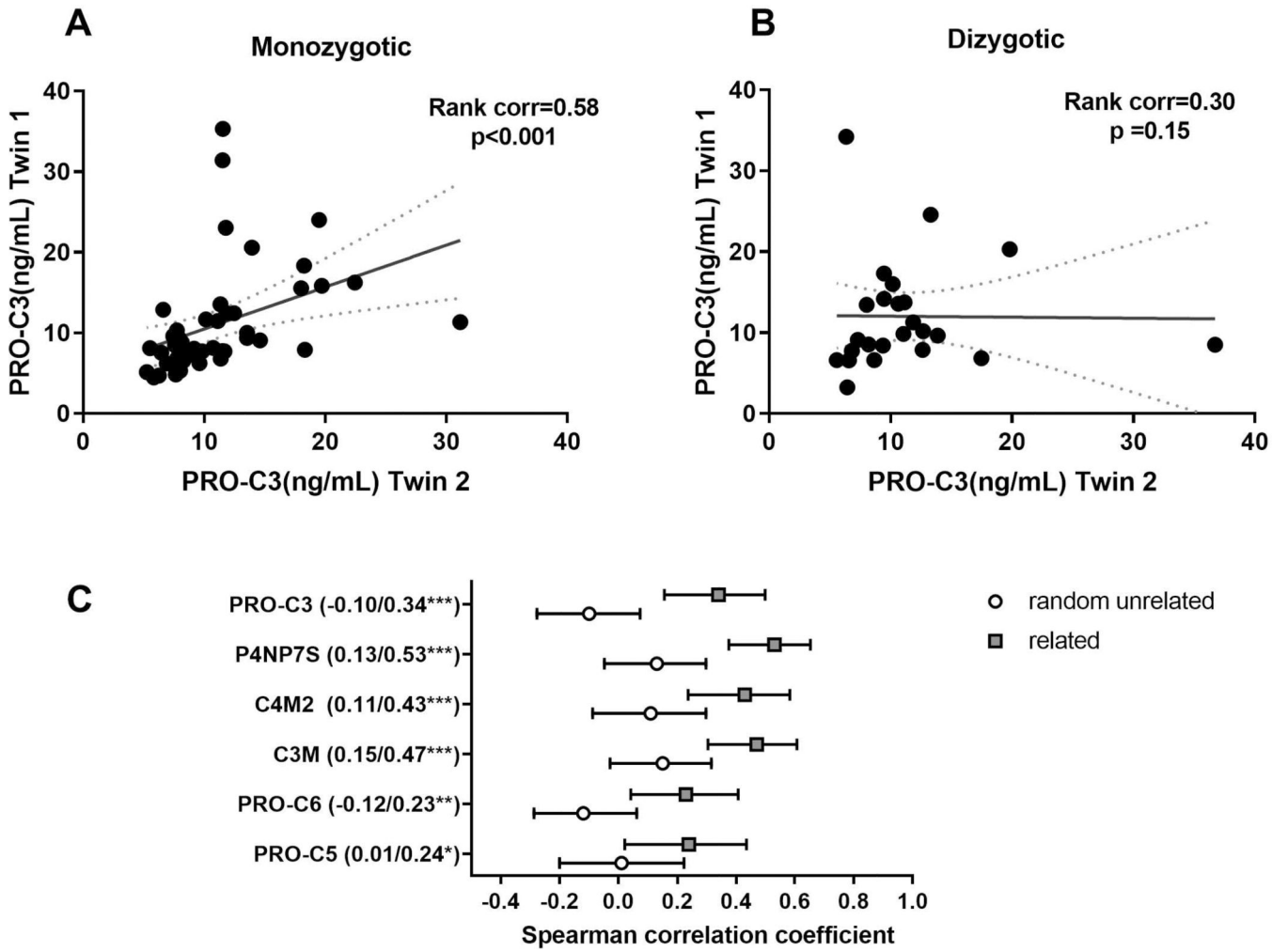

Figure 3. Familial and Twinship correlation of PRO-C3 levels

Twinship correlations; A. The monozygotic twin-pairs showed a robust correlation in PRO-C3 (r2=0.58; p <0.001) but not B. the dizygotic twin-pairs (r= 0.30; p=0.15), showing that PRO-C3 concentration is a heritable trait. C. Familial correlation shown as Spearman correlation coefficient and 95% confidence interval within random unrelated pairs (white dots, n=135 pairs) and within related pairs (black squares, n=115 pairs) as a significant association was found between PRO-C3 and NAFLD-cirrhosis, individuals with cirrhosis were excluded from the analysis. Spearman coefficient values are indicated in the in Y axis legend (unrelated/related pairs), *p<0.05, **p<0.01, ***p<0.001.

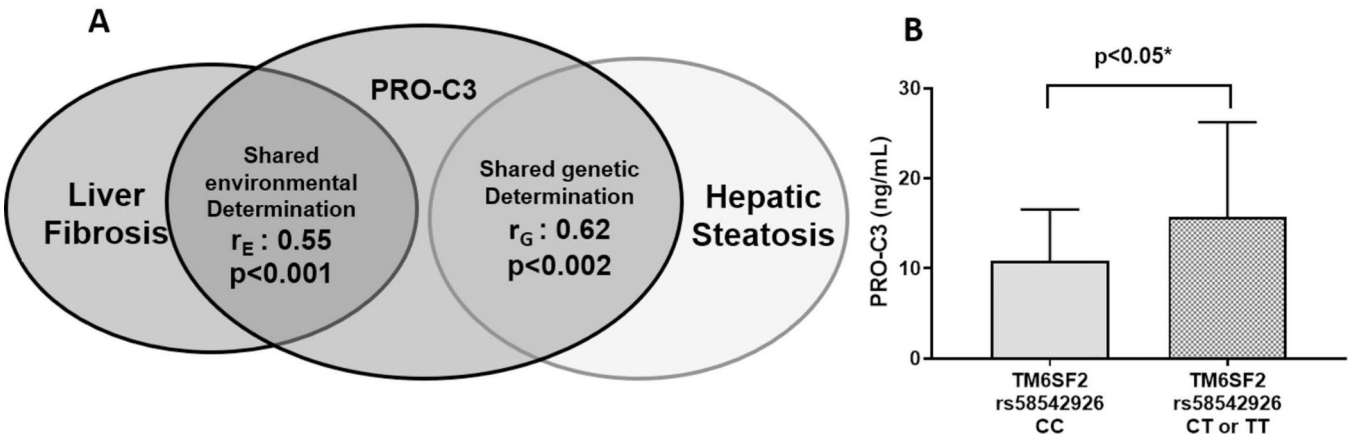

Figure 4. Shared genetic and environmental determination of PRO-C3 and higher PRO-C3 levels in TM6SF2 risk allele

A. AE Model for genetic correlation between PRO-C3 and hepatic steatosis assessed by MRI-PDFF and environmental correlation between PRO-C3 and liver fibrosis assessed by MRE. B. Mean and standard deviation of PRO-C3 concentration in TM6SF2 rs5854296 rare allele T carriers compared to non-carriers. *P-value derived from Generalized Estimating Equations to account for correlation within twinship adjusted for age and sex.

Table 1a:

Baseline Characteristics of probands with NAFLD-cirrhosis, non-NAFLD controls and proband with NAFLD without advanced fibrosis

Characteristics	Group A1 Non-NAFLD control (n=72)	Group B1 Proband with NAFLD without Advanced fibrosis (n=24)	Group C1 Probands with NAFLD-cirrhosis (n=44)	Overall p-value
Demographics				
Age (years)	44.9 (20.3)	52.8 (16.5)	62.2 (10.3)	**<0.0001** $^{\beta,\delta}$
Female, n (%)	54 (75.0%)	15 (62.5%)	35 (79.6%)	0.3009
White, n (%)	57 (80.3%)	19 (79.2%)	12 (27.3%)	**<0.0001**
Hispanic or Latino, n (%)	9 (12.5%)	3 (12.5%)	26 (59.1%)	**<0.0001**
BMI (kg/m^2)	25.2 (6.5)	30.9 (6.0)	31.4 (6.5)	**<0.0001** $^{\alpha,\beta}$
Clinical				
Type 2 Diabetes, n (%)	0	4 (16.7%)	35 (81.4%)	**<0.0001**
Biological data				
AST (U/L)	20.9 (5.4)	22.7 (6.6)	50.8 (28.2)	**<0.0001** $^{\beta,\delta}$
ALT (U/L)	17.7 (7.4)	25 (11.5)	47.8 (37.4)	**<0.0001** $^{\beta,\delta}$
Alk P (U/L)	67.2 (19.5)	69.2 (19.6)	107.6 (39.9)	**<0.0001** $^{\beta,\delta}$
GGT (Ui/L)	17.6 (8.2)	27.3 (21.3)	100.4 (61.7)	**<0.0001** $^{\beta,\delta}$
Total Bilirubin (mg/dL)	0.5 (0.2)	0.4 (0.2)	1.6 (5.3)	0.1282
Direct Bilirubin (mg/dL)	0.1 (0)	0.1 (0)	0.9 (4.1)	0.1669
Albumin (g/dL)	4.6 (0.3)	4.5 (0.2)	4.1 (0.4)	**<0.0001** $^{\beta,\delta}$
Glucose (mg/dl)	86.2 (8.7)	94.7 (30.7)	125.2 (54.8)	**<0.0001** $^{\beta,\delta}$
Hemoglobin A1c (%)	5.6 (0.3)	5.9 (0.7)	6.8 (1.6)	**<0.0001** $^{\beta,\delta}$
Insulin (U/ml)	8.1 (4.5)	15.2 (12.2)	48.8 (58.7)	**<0.0001** $^{\beta,\delta}$
Triglycerides (mg/dL)	75.6 (26.1)	132 (62.5)	155.4 (117.6)	**<0.0001** $^{\alpha,\beta}$
Total cholesterol (mg/dL)	188 (38.3)	196.4 (30.1)	144.6 (62.7)	**<0.0001** $^{\beta\delta}$
HDL-cholesterol (mg/dL)	69.3 (18.4)	52.8 (15.7)	55.8 (33.1)	**0.0017** $^{\alpha,\beta}$
LDL-cholesterol (mg/dL)	103.6 (30.3)	118.3 (27.6)	84.6 (27.8)	**<0.0001** $^{\alpha,\beta,\delta}$
Platelet count (10^3/μL)	255.7 (49.3)	268.3 (51.0)	169.3 (73.3)	**<0.0001** $^{\beta,\delta}$
Prothrombin time	10.7 (0.9)	11.4 (4.2)	12.2 (2.8)	**0.0039** $^{\beta}$
INR	1 (0.1)	1.1 (0.4)	1.1 (0.3)	**0.0306** $^{\beta}$
Ferritin (ng/mL)	89.4 (70.8)	120.8 (91.5)	165.9 (215.3)	**0.0176** $^{\beta}$
ECM biomarkers				
PRO-C3 (ng/mL)	10.3 (4.8)	10.8 (6.1)	30 (29.2)	**<0.0001** $^{\beta,\delta}$

Characteristics	Group A1 Non-NAFLD control (n=72)	Group B1 Proband with NAFLD without Advanced fibrosis (n=24)	Group C1 Probands with NAFLD-cirrhosis (n=44)	Overall p-value
PRO-C5 (ng/mL)	234.4 (152)	258.5 (141.9)	244.3 (191.3)	0.8482
PRO-C6 (ng/mL)	8.9 (6.1)	10.3 (4.9)	13.7 (13.0)	**0.0195** $^{\beta}$
P4NP7S (ng/mL)	176.2 (52.3)	161.1 (41.5)	193.1 (124)	0.2761
C3M (ng/mL)	8 (2.1)	7.3 (1.3)	10.5 (4.8)	**<0.0001** $^{\beta, \delta}$
C4M2 (ng/mL)	18 (5.3)	18.7 (5.0)	22.4 (9.0)	**0.0050** $^{\beta, \delta}$
Imaging data				
MRI-PDFF %	2.3 (0.8)	11.4 (6.9)	7.0 (5.4)	**<0.0001** $^{\alpha, \beta, \delta}$
MRE kPa	2.1 (0.4)	2.5 (0.4)	5.5 (2.3)	**<0.0001** $^{\beta, \delta}$

Mean values are provided with standard deviation in parentheses, unless otherwise noted as n (%) BMI: body mass index, HbA1c: glycated hemoglobin, ALT: alanine aminotransferase, AST: aspartate aminotransferase, INR: International Normalized Ratio, APRI: AST to platelet ratio, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, Alk P: Alkaline Phosphatase, MRI-PDFF: magnetic resonance imaging proton density fat fraction, MRE: magnetic resonance elastography.

*P-value determined by chi-square or F-test from ANOVA.

Bold indicates significant P values <0.05.

Superscripts indicate individual significant mean differences between

$^{\alpha}$non-NAFLD control versus patients with NAFLD without advanced fibrosis

$^{\beta}$non-NAFLD control versus proband with NAFLD-cirrhosis and

$^{\delta}$patients with NAFLD without advanced fibrosis versus proband with NAFLD-cirrhosis

Table 1b:

Baseline Characteristics of first-degree relatives of NAFLD-cirrhosis, first-degree relatives of controls, first degree relative of Proband with NAFLD without Advanced fibrosis

Characteristics	Group A2 First Degree relatives of controls (n=72)	Group B2 First Degree relatives of NAFLD without Advanced fibrosis (n=24)	Group C2 First Degree relatives of NAFLD-cirrhosis (G3R) (n=70)	Overall p-value
Demographics				
Age (years)	44.4 (20.4)	53.9 (15.8)	50.6 (17.8)	**0.0464** [ϒ]
Female, n (%)	52 (72.2%)	16 (66.7%)	52 (74.3%)	0.7717
White, n (%)	57 (80.3%)	19 (79.2%)	13 (18.8%)	**<0.0001**
Hispanic or Latino, n (%)	9 (12.5%)	3 (12.5%)	49 (70.0%)	**<0.0001**
BMI (kg/m^2)	25.5 (5.3)	29.5 (6.6)	34 (11.6)	**<0.0001** [Ω, Δ]
Clinical				
Type 2 Diabetes, n (%)	2 (2.9%)	4 (16.7%)	18 (25.7)	**0.0006**
Biological data				
AST (U/L)	23.4 (7.6)	34.5 (21.3)	27.1 (19)	**0.0112** [ϒ, Δ]
ALT (U/L)	21.8 (14.2)	40.2 (37)	30.7 (26.8)	**0.0038** [ϒ, Ω]
Alk P (U/L)	68.4 (20.8)	81.3 (21.4)	80.8 (28.2)	**0.0059** [ϒ, Ω]
GGT (Ui/L)	21.8 (17.8)	64.7 (85)	44.8 (38.8)	**<0.0001** [ϒ, Ω]
Total Bilirubin (mg/dL)	0.4 (0.2)	0.6 (0.4)	0.5 (0.3)	**0.0257** [ϒ, Ω]
Direct Bilirubin (mg/dL)	0.1 (0)	0.2 (0.1)	0.2 (0.1)	**0.0016** [ϒ, Ω]
Albumin (g/dL)	4.5 (0.3)	4.4 (0.4)	4.4 (0.2)	**0.1053** [Ω]
Glucose (mg/dl)	87.9 (11.4)	89.7 (14.5)	95.5 (26.1)	0.0649
Hemoglobin A1c (%)	5.7 (0.4)	5.8 (0.5)	5.9 (0.9)	0.2779
Insulin (U/ml)	8.3 (4.7)	15.0 (13.3)	22.7 (18.2)	**<0.0001** [ϒ, Ω, Δ]
Triglycerides (mg/dL)	89.6 (44.4)	156.8 (117.1)	152.6 (76.2)	**<0.0001** [ϒ, Ω]
Total cholesterol (mg/dL)	194.3 (44)	200.4 (39.3)	175.5 (56.8)	**0.0323** [Ω, Δ]
HDL-cholesterol (mg/dL)	65 (18.6)	56.7 (22.7)	54.1 (26)	**0.0168** [Ω]
LDL-cholesterol (mg/dL)	111.4 (40.6)	113.8 (30.2)	108.7 (35.6)	0.8293
Platelet count (10^3/μL)	247.1 (49.3)	237 (71.4)	266.4 (74.9)	0.0849
Prothrombin time	10.9 (2.1)	11.7 (4.3)	11.5 (3.5)	0.3542
INR	1.1 (0.3)	1.1 (0.4)	1.1 (0.3)	0.5815
Ferritin (ng/mL)	90.1 (68.7)	117 (86.5)	126.2 (128.5)	0.0973
ECM biomarkers				
PRO-C3 (ng/mL)	10.4 (4.1)	13.7 (9.4)	14.4 (13.3)	**0.0494** [Ω]
PRO-C5 (ng/mL)	261.6 (153.9)	349.3 (134.6)	254.9 (219.1)	0.1263

Characteristics	Group A2 First Degree relatives of controls (n=72)	Group B2 First Degree relatives of NAFLD without Advanced fibrosis (n=24)	Group C2 First Degree relatives of NAFLD-cirrhosis (G3R) (n=70)	Overall p-value
PRO-C6 (ng/mL)	7.7 (4.5)	10.2 (5.6)	9 (5.8)	0.1001
P4NP7S (ng/mL)	173.8 (57.2)	180.4 (52.8)	180.3 (86.1)	0.8413
C3M (ng/mL)	8.1 (2.0)	7.5 (2.4)	9.7 (3.1)	**0.0001** Ω, Δ
C4M2 (ng/mL)	19.8 (5.1)	19.6 (4)	21.7 (6.7)	0.1195
Imaging data				
MRI-PDFF %	2.5 (1.8)	6.1 (5.2)	10.1 (8.5)	**<0.0001** γ, γ„, Δ
NAFLD (MRI-PDFF≥5%), n (%)	1 (1.4)	7 (29.2)	47 (67.1)	**< 0.0001** γ, γ„, Δ
MRE kPa	2.1 (0.4)	3.0 (1.4)	2.6 (0.9)	**0.0001** γ, Ω

Mean values are provided with standard deviation in parentheses, unless otherwise noted as n (%) BMI: body mass index, HbA1c: glycated hemoglobin, ALT: alanine aminotransferase, AST: aspartate aminotransferase, INR: International Normalized Ratio, APRI: AST to platelet ratio, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, Alk P: Alkaline Phosphatase, MRI-PDFF: magnetic resonance imaging proton density fat fraction, MRE: magnetic resonance elastography.

*P-value determined by chi-square or F-test from anova.

Bold indicates significant P values <0.05.

Superscripts indicate individual significant mean differences between

γ first degree relatives of non-NAFLD control versus first degree relatives of patients with NAFLD without advanced fibrosis

Ω first degree relatives of non-NAFLD control versus first degree relatives of proband with NAFLD-cirrhosis and

Δ first degree relatives of patients with NAFLD without advanced fibrosis versus first degree relatives of proband with NAFLD-cirrhosis.

Table 2.

Heritability estimates of Pro-C3 and other ECM remodeling biomarkers in AE model

Trait	Heritability estimates h2	95% CI	P value
Primary outcome			
Pro-C3 (ng/mL)			
Unadjusted	0.373	(0.097–0.592)	**0.0089**
Age-sex-adjusted	0.336	(0.051–0.567)	**0.0215**
Age-sex-ethnicity-BMI-adjusted	0.362	(0.072–0.585)	**0.0157**
Secondary outcome			
P4NP7S (ng/mL)			
unadjusted	0.136	(0.000–0.337)	0.460
Age-sex- adjusted	0.119	(0.000–0.322)	0.279
Age-sex-ethnicity-BMI-adjusted	0.078	(0.000–0.289)	0.488
PRO-C6 (ng/mL)			
unadjusted	0.256	(0.005–0.476)	**0.0449**
Age-sex- adjusted	0.241	(0.000–0.463)	0.0605
Age-sex-ethnicity-BMI-adjusted	0.075	(0.000–0.327)	0.572
C3M (ng/mL)			
unadjusted	0.126	(0.000–0.324)	0.235
Age-sex- adjusted	0.117	(0.000–0.316)	0.272
Age-sex-ethnicity-BMI-adjusted	0.082	(0.000–0.287)	0.453
C4M2 (ng/mL)			
unadjusted	0.087	(0.000–0.313)	0.472
Age-sex- adjusted	0.043	(NA −0.278)	0.7297
Age-sex-ethnicity-BMI-adjusted	0	(NA −0.23)	1
Pro-C5 (ng/mL)			
unadjusted	0.048	(NA −0.282)	0.6996
Age-sex- adjusted	0.019	(NA −0.258)	0.8816
Age-sex-ethnicity-BMI-adjusted	0	(NA −0.212)	1

Table 3.

AE Model for genetic and environmental correlation between Pro-C3 concentration and hepatic steatosis and fibrosis

Pro-C3 (ng/mL)	**Genetic correlation**			
	Hepatic steatosis (MRI-PDFF)		**Liver fibrosis (MRE)**	
	r_G Estimate(95% CI)	P value	r_G Estimate(95% CI)	P value
unadjusted	0.593 (0.240, 1.000)	**0.00109**	0.245 (−0.332, 0.580)	0.303
Age - sex-adjusted	0.671 (0.302, 1.000)	**0.00041**	0.301 (NA, 0.680)	0.320
Age-sex-ethnicity-BMI-adjusted	0.619 (0.236, 1.000)	**0.00152**	0.227 (−1.000, 0.830)	0.596
Pro-C3 (ng/mL)	**Environmental correlation**			
	Hepatic steatosis (MRI-PDFF)		**Liver fibrosis (MRE)**	
	r_E Estimate(95% CI)	P value	r_E Estimate(95% CI)	P value
unadjusted	−0.210 (−0.451, 0.066)	0.132	0.528 (0.291, 0.702)	**<0.001**
Age – sex-adjusted	−0.217 (−0.457, 0.059)	0.121	0.519 (0.281, 0.695)	**<0.001**
Age-sex-ethnicity-BMI-adjusted	−0.231 (−0.470, 0.045)	0.0982	0.549 (0.317, 0.717)	**<0.001**