Binge drinking induces an acute burst of markers of hepatic fibrogenesis (PRO-C3)

Torp, Nikolaj; Israelson, Mads; Nielsen, Mette Juul; Åstrand, Claus Philip; Juhl, Pernille; Johansen, Stine; Hansen, Camilla Dalby; Madsen, Bjørn; Villesen, Ida Falk; Leeming, Diana Julie; Thiele, Maja; Hansen, Torben; Karsdal, Morten; Krag, Aleksander

Published in:
Liver International

DOI:
10.1111/liv.15120

Publication date:
2022

Document version:
Accepted manuscript

Citation for published version (APA):

Go to publication entry in University of Southern Denmark's Research Portal

Terms of use
This work is brought to you by the University of Southern Denmark. Unless otherwise specified it has been shared according to the terms for self-archiving. If no other license is stated, these terms apply:

• You may download this work for personal use only.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim. Please direct all enquiries to puresupport@bib.sdu.dk
TITLE PAGE

TITLE: Binge drinking induces an acute burst of markers of hepatic fibrogenesis (PRO-C3)

AUTHORS: Nikolaj Torp*1,2, Mads Israelsen*1,2, Mette Juul Nielsen3, Claus Philip Åstrand1,2, Pernille Juhl1,4, Stine Johansen1,2, Camilla Dalby Hansen1,2, Bjørn Madsen1,2, Ida Falk Villesen3, Diana Julie Leeming3, Maja Thiele1,2, Torben Hansen5, Morten Karsdal3,6, Aleksander Krag1,2

Affiliations: 1) Department of Gastroenterology and Hepatology, Odense University Hospital, Denmark 2) Institute of Clinical Research, University of Southern Denmark, Denmark 3) Nordic Bioscience A/S, Herlev Hovedgade, Herlev, Denmark 4) Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark 5) Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen, Denmark 6) Department of Molecular Medicine, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark

* shared first co-authorship

Corresponding Author:

Aleksander Krag

Odense Liver Research Centre, Department of Gastroenterology and Hepatology,

Odense University Hospital, Kloevervaenget 10, 5000 Odense C, Denmark

E-mail: aleksander.krag@rsyd.dk

Electronic word count text: 3.620

Number of figures and tables: 8
Electronic word count abstract: 240

Abbreviations: ALD, alcohol-related liver disease; ECM, extracellular matrix; NAFLD, non-alcoholic fatty liver disease; PRO-C3, N-terminal pro-peptide of type III collagen; PRO-C4, Internal epitope in the 7S domain of type IV collagen; PRO-C8, C-terminal of type VIII collagen; C3M, degraded type III collagen neo-epitope; C4M, degraded type IV collagen neo-epitope; HSC, hepatic stellate cell; BMI, body mass index; HC, healthy control; MMP, matrix metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinase-1; LSM, liver stiffness measurement; CAP, controlled attenuated parameter; ELF, enhanced liver fibrosis test; ALT, alanine aminotransferase; AST, asparagine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; HDL, high-density lipoprotein

Conflict of interest: MJN, PJ, DJL and MK are full time employees of Nordic Bioscience, Herlev, DK. DJL and MK own stock in Nordic Bioscience, Herlev, DK. Remaining authors have nothing to disclose.

Financial support: This study was supported by a Challenge Grant from the Novo Nordisk Foundation, “MicrobLiver”, NNF15OC0016692

Trial registration number: NCT03018990

Ethics approval statement: This study was approved by the Ethical Committee of Southern Denmark (S-20160083).

Patient consent statement: All participants signed informed consent forms prior to any investigations.

Data availability statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions

Statement of contribution: NT, MI, CDH and BM collected the data. NT, MI, CPÅ and SJ performed the statistical analyses. MJN, PJ IFV and DJL performed biomarker analyses. NT, MI and
CPÅ wrote the draft manuscript. MT, TH, MK and AK critically reviewed the draft manuscript. All authors approved the final version of the manuscript.

Permission to reproduce material from other sources: Graphical abstract created with BioRender.com reproduced with permission.

Graphical abstract
Abstract

**Background and aims:** Binge drinking is associated with increased risk of liver-disease. Morbidity and mortality of alcohol-related liver disease (ALD) is associated with collagen deposition in the hepatic extracellular matrix (ECM). However, acute effects of binge drinking on ECM turnover are unknown. We aimed to investigate the effects on hepatic ECM turnover following a binge drinking episode.

**Methods:** We performed a pathophysiological intervention study with 15 non-alcoholic fatty liver disease (NAFLD) patients, 15 ALD patients and 10 healthy controls. We used 40% ethanol in 9 mg/mL NaCl administered through a nasogastric tube to simulate binge drinking. Hepatic vein catheterisation allowed simultaneous hepatic- and systemic vein sampling. Markers of ECM formation and degradation were measured with competitive ELISA.

**Results:** The interstitial matrix formation marker PRO-C3 increased by 1.2 ng/mL (10%, P<0.001) 24 hours after binge drinking. In participants with existing liver fibrosis determined by elevated baseline PRO-C3, hepatic levels increased by 0.09 ng/mL (95% CI: 0.03; 0.15, P=0.005) while systemic PRO-C3 decreased 0.11 ng/mL (95% CI: -0.15; -0.06, P<0.001) in 3-hours. PRO-C8 increased by 30% (+0.9 ng/mL, P=0.014) in liver-diseased patients with F0-F1 but not in any other group. 24-hour changes in systemic C3M and PRO-C3 were not associated (P=0.911).

**Conclusions:** Binge drinking induced an acute burst of PRO-C3 in healthy individuals and patients with liver-disease. Markers of ECM degradation were not correlated to markers of ECM formation, suggestive that even a single episode of binge drinking promotes excessive hepatic fibrogenesis.

**Keywords:** acute intoxication, fibrosis, extracellular matrix, cirrhosis, biomarker

**Layman summary**

The harmful effects of long-term binge drinking on the liver is well-known due to the development of scar tissue, known as fibrosis. We investigated if markers of fibrosis formation and degradation was affected by a single binge drinking episode in healthy individuals and in patients with known liver
disease. We showed that binge drinking has immediate effects on liver fibrosis formation and that the effect was measurable by the biomarker PRO-C3 within 24 hours after the acute insult.
**Introduction**

In a global context, alcohol consumption is the 7th leading risk factor for both death and burden of disease.\(^1\) Indeed, nearly half of all chronic liver disease deaths in 2016 was caused by alcohol consumption.\(^2\) Furthermore, drinking in the form of “binges” has been associated with an increased risk of liver-disease independent of average alcohol intake.\(^3,4\) Although no universal definition of “binge drinking” exists, the National Institute of Alcohol Abuse and Alcoholism defines a binge drinking episode as the consumption of four or five drinks in about two hours for women and men, respectively.\(^5\) Liver-related morbidity and mortality in alcohol-related liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are largely driven by liver fibrosis, a process that evolves over decades and is characterized by the accumulation of fibrous tissue in the extracellular matrix (ECM).\(^6,7\) The hepatic ECM is rich in collagens, produced by hepatic stellate cells (HSC) and myofibroblasts that ensure tissue integrity and architecture.\(^8\) Structurally, the hepatic ECM is divided into a pericellular basement membrane dominated by network-forming collagens (e.g., type IV and VIII) produced partly by hepatocytes, and a interstitial matrix rich in dense fibrillar type III collagen, produced by myofibroblasts.\(^9\) In the liver, the basement membrane remodeling is believed to reflect hepatic injury in patients with mild fibrosis, whereas interstitial matrix remodeling mirror fibrosis progression. When alcohol induces liver fibrosis, it is due to a net hepatic collagen deposition, a process known as fibrogenesis.\(^10,11\) However, the pathophysiological role of binge drinking on ECM production by hepatocytes and myofibroblasts is unknown.\(^12,13\) We hypothesized that a single binge drinking episode activates hepatic fibrogenesis and fibrolysis, differentiated according to location of the components (i.e., basement membrane or interstitial matrix) and the severity of existing liver fibrosis. Therefore, in the setting of hepatic vein access, our primary aim was to identify the effect of binge drinking on interstitial matrix formation after 24 hours and during intoxication in both hepatic and systemic blood. Our secondary aims included I) if binge drinking had similar effects on the basement membrane formation after 24 hours and during intoxication and II) how binge drinking affected both interstitial matrix and basement membrane degradation.

**Methods**
Participants

This is a pathophysiological intervention study, approved by the local Ethical Committee of Southern Denmark (S-20160083) and registered at clinicaltrials.gov (NCT03018990). We conducted the study in compliance with the Declaration of Helsinki, and all participants signed informed consent forms prior to any investigations. Forty participants were recruited with three distinct phenotypes, namely healthy controls (HC), NAFLD patients and ALD patients. Prior to investigations, eligible liver-diseased patients (ALD and NAFLD) had biopsy-proven liver fibrosis according to NASH Clinical Research Network. During the study patients with ALD and NAFLD were biopsied once again, whereafter they were divided according to their stage of fibrosis with no-to-mild fibrosis (F0-F1) or significant fibrosis (>F2). Since the collagen turnover is associated with severity of disease in both ALD and NAFLD patients, liver-diseased participants were split according to their fibrosis stage instead of etiological background in the main analyses. Participants were individuals 18-75 years of age, with a bodyweight of >50kg and a willingness to be abstinent from alcohol for a period of 48 hours. For liver-diseased patients, phenotypes of liver disease were classified according to biochemistry, transient- and shear wave elastography, liver histology and a medical history of risk factors. All ALD patients were active drinkers and expressed no desire to become abstinent. NAFLD patients had histological features of NAFLD and no history of alcohol overuse. Healthy controls were required not to have any concomitant diseases, intake of any medication, signs (normal elastography and blood tests) of ALD, non-alcoholic steatohepatitis or a current/previous alcohol overuse (>21 units/weekly for men and >14 units/weekly for women).

Investigations

An overview of the study design and setup can be seen in Figure 1. Results of the primary study are reported elsewhere. The 48-hour alcohol abstinence was confirmed by measuring blood ethanol levels on the day of the investigation before the start of the study. The intervention was in the form of a weight adjusted ethanol-NaCl mixture that was administered via a nasogastric tube and infusion pump.

The ethanol-NaCl mixture consisted of 40% ethanol in 9 mg/ml NaCl solution produced by the hospital pharmacy. A dose of 2.5 mL per kg body weight was scheduled for all participants to be
administered by pump infusion via nasogastric tube, over a period of 30 minutes. However, all participants with a body mass index (BMI) above 25 kg/m\(^2\) were given a dose adjustment of 0.5 mL per kg bodyweight. This was done as a precaution against severe intoxication in obese participants.

Blood samples were drawn from two sites simultaneously during the intervention period. The collecting sites were the right external jugular vein and a hepatic vein, with the assistance of ultrasound- and X-ray guided hepatic vein catherization. In total, blood samples were collected at ten timepoints during the research period. Within one hour of sampling, the blood samples for quantification of ECM remodeling biomarkers were centrifuged and stored at -80°C.

The acute effects of binge drinking were analyzed by 1) changes in systemic circulation over 24 hours and 2) changes in both systemic and hepatic circulation over 3 hours.

Quantification of ECM remodeling biomarkers

To assess interstitial matrix and basement membrane turnover, hepatic and systemic blood samples were analyzed. Measurements of type III, IV and VIII collagen formation (PRO-C3, PRO-C4 and PRO-C8) and type III and IV collagen degradation (C3M and C4M) were performed according to previously defined protocols.\textsuperscript{18,19} Briefly, competitive ELISA assays utilizing monoclonal antibodies towards an array of collagen formation and degradation were analyzed. For collagen formation, the N-terminal pro-peptide of type III collagen (PRO-C3), internal epitope in the 7S domain of type IV collagen (PRO-C4) and the C-terminal of type VIII collagen (PRO-C8) were all measured. Matrix metalloproteinase (MMP) mediated fragments of type III and IV collagen degradation (C3M and C4M) were used to determine hepatic and systemic levels for each participant (Nordic Bioscience, Herlev, Denmark). For measurements below or above the lower- and upper limit of measurement range, values were recorded as the lowest or highest value within the detection range of the specific assay, respectively.

Statistics
We report categorical data as counts with proportions, continuous, normal distributed data as means with standard deviations, and continuous, non-normal distributed data as medians with 25th and 75th percentiles. Statistical tests of equality between the three study groups (healthy controls, F0-F1 and >F2) were performed with one-way ANOVAs for continuous, parametric data and by Kruskal-Wallis tests for continuous non-parametric data. Pairwise comparisons of continuous parametric data were done with Student T-tests and Wilcoxon rank-sum tests for continuous non-parametric data. For categorical data $\chi^2$-tests were performed as appropriate. We applied mixed effect models for time-series of repeated measurements with the individual participant as the random-effect parameter, and interactions between following explanatory parameters: sampling site, concentration over time, baseline concentrations, study groups and phenotypes. Spearman’s rank correlation coefficient was used to assess the association between continuous variables. All computations were performed in STATA version 17.0 (College Station, TX, USA). P-values < 0.050 were considered significant.
Results

Baseline characteristics

We performed investigations between November 2016 and November 2018. Due to non-compliance with the 48-hour alcohol abstinence requirement we excluded one ALD patient. Baseline characteristics for the remaining participants can be viewed in Table 1. The three groups for the primary outcome analysis consisted of 10 healthy control subjects, 13 patients with F0-F1, and 16 patients with >F2. The mean age was 54±11 years and 62% were male. Overall, the serum alcohol concentration increased from 0 ± 0 mmol/L at baseline, to peak at 34 ± 4 mmol/L after one hour and declined to 21 ± 3 mmol/L after three hours.

At baseline, PRO-C3 was 17.2 ng/mL in >F2 patients and hereby 79% and 48% higher compared to healthy controls (P=0.002) and F0-F1 patients (P=0.024), respectively. We found PRO-C8 elevated only in >F2 patients compared to healthy controls, while we observed no difference between groups for PRO-C4 (Table 1).

By site of sampling, baseline PRO-C3 was lower in the hepatic compared to the systemic vein (-0.74 ng/mL, 95% CI: -1.08; -0.40, P<0.001) (Figure 2A). For PRO-C8 and PRO-C4, hepatic vein levels at baseline were greater than the systemic vein (0.66 ng/mL, 95% CI: 0.47; 0.86, P<0.001 and 89.00 ng/mL, 95% CI: 68.71; 109.96, P<0.001, respectively) (Figure 3A & 4A).

Binge drinking induces release of interstitial matrix formation markers within 24 hours in healthy and liver diseased patients

We investigated the acute effects of binge drinking on interstitial matrix formation assessed by the type III collagen formation marker PRO-C3. Analyses were performed on changes in systemic circulation over 24 hours, as well as changes in both systemic and hepatic circulation over 3 hours. Finally, we analyzed the net deposition ratio defined as the ratio between in the PRO-C3 formation marker and its corresponding degradation marker (C3M).

PRO-C3 increased significantly 24 hours after a single binge drinking episode with 1.2 ng/mL, equivalent to a 10% increase from baseline, in all study groups without significant between-group...
differences (P<0.001) (Table 2). We did not detect a difference according to phenotype in 24-hour PRO-C3 increases, as ALD patients had an increase of 1.5 ng/mL (P=0.025) and NAFLD an increase of 1.8 ng/mL (P=0.018) (Supporting Information, Table 1).

In response to the binge drinking episode, systemic PRO-C3 among patients with >F2 decreased by 1.06 ng/mL (95% CI: -21.15; -0.02, P=0.045) in the 3-hour analysis (Table 3). No significant changes in systemic PRO-C3 during the 3-hour analysis were observed in healthy controls (0.41 ng/mL, 95% CI: -1.24; 0.41, P=0.328) nor in patients with F0-F1 (0.22 ng/mL, 95% CI: -1.31; 0.88, P=0.698). During the 3-hour analyses, PRO-C3 did not increase significantly in neither the systemic- nor the hepatic vein for ALD and NAFLD patients compared to healthy controls (Supporting Information, Table 2).

In participants with elevated PRO-C3 levels in systemic circulation at baseline, the binge drinking episode increased hepatic PRO-C3 by 0.09 ng/mL (95% CI: 0.03; 0.15, P=0.005), while systemic PRO-C3 decreased by 0.11 ng/mL (95% CI: -0.15; -0.06, P<0.001) during the 3-hour analysis, suggestive of hepatic output (Supporting Information, Table 3). We confirmed a net deposition profile in analyses of the hepatic PRO-C3/C3M ratio of participants at baseline (Figure 2C). Here patients with >F2 had a higher type III collagen formation versus degradation compared to patients with none-to-mild fibrosis (P=0.006) and healthy controls (P=0.003) with no difference between the two latter (P=0.494). In contrast, the systemic PRO-C3/C3M ratio was not different between any of the three groups (>F2 vs. F0-F1, P=0.350; >F2 vs. HC, P=0.192; F0-F1 vs. HC, P=0.410).

**Basement membrane formation increases for only liver-diseased with none-to-mild fibrosis within 24 hours**

Basement membrane formation dynamics were investigated in analyses of circulating markers of PRO-C8 and PRO-C4. For each of the two basement membrane formation markers, we performed analyses of changes in the systemic circulation over 24 hours, as well as changes in both systemic and hepatic circulation over 3 hours.

The 24-hour systemic PRO-C8 concentration increased by 0.9 ng/mL, or 30% from baseline, (P=0.014) in patients with F0-F1 after the single binge drinking episode (Table 2). Healthy controls
and patients with >F2 did not experience an increase (0.2 ng/mL, P=0.473, 0.3 ng/mL, P=0.776, respectively) nor when divided as ALD and NAFLD patients (Supporting Information, Table 1).

Systemic PRO-C8 did not change in 3-hour analyses due to binge drinking when grouped by HC, F0-F1 and >F2 (Figure 3B). Neither did the hepatic vein levels of PRO-C8 change significantly during 3-hour analyses (Table 3). However, divided by etiological background, ALD patients had higher levels of PRO-C8 in both the systemic vein (2.61 ng/mL, P<0.001) and in the hepatic vein (0.65 ng/mL, P=0.010) compared to healthy controls but no significant increase or decrease of PRO-C8 in either was observed during the 3-hour analysis (Supporting Information, Table 3).

The systemic PRO-C4 did not significantly increase or decrease in any of the three study groups, 24-hour after a single episode of binge drinking (Table 2). No response was neither observed when divided according to clinical phenotype for liver-diseased patients with ALD and NAFLD (Supporting Information, Table 1).

In isolated 3-hour analyses of sampling site, PRO-C4 decreased by 10% in the hepatic vein (43 ng/mL) (95% CI: -79.87; -6.59, P=0.021), while systemic PRO-C4 remained unchanged in the same period (95% CI: -2.70; 49.12, P=0.079) (Fig 4A). No differences were observed in systemic and hepatic PRO-C4 between healthy controls, NAFLD or ALD during 3-hour analysis (Supporting Information, Table 3). Analyses were robust when results were corrected for sex but with a tendency for higher levels of PRO-C4 in female participants (95.06 ng/mL, 95% CI: -16.71; 206.84, P=0.096).

**Interstitial matrix and basement membrane degradation response correlate following a binge drinking episode**

We finally investigated the 24-hour changes in C3M and C4M as markers of MMP-mediated interstitial matrix and basement membrane degradation. Hereafter, we analyzed the association between the degradation markers, their corresponding formation markers (PRO-C3 and PRO-C4, respectively) and tissue inhibitor of metalloproteinase-1 (TIMP1) as an inhibitor of MMP activity.
Systemic C3M and C4M remained unchanged 24-hours after a single binge drinking episode both when divided by fibrosis stage and by etiological background (Table 2 & Supporting Information, Table 1). Baseline systemic C3M was 24% (P=0.001) and 21% (P=0.021) higher in patients with >F2 compared to healthy controls and F0-F1 patients, respectively (Table 1). There were no differences in baseline C4M among the three study groups (P=0.133).

The C3M and C4M 24-hour systemic responses to a binge drinking episode, were strongly associated (r = 0.83, P<0.001) (Figure 5A). The 24-hour changes of C3M were not associated with PRO-C3 changes (r = -0.02, P=0.911) (Figure 5B), while C4M 24-hour changes were independent of PRO-C4 changes (r = 0.16, P=0.344) (Figure 5C).

Interstitial matrix and basement membrane degradation marker responses, as evaluated by C3M and C4M, were both independent of TIMP1 levels at baseline (r = 0.14, P=0.414 and r = 0.18, P=0.293, respectively).
Discussion

This first pathophysiological intervention study demonstrated that a single episode of binge drinking stimulates circulating markers of fibrogenesis within 24-hours. We observed an acute burst of the interstitial matrix marker PRO-C3, a well-known biomarker of liver fibrosis severity.\textsuperscript{20,21} The basement membrane marker PRO-C8 displayed a distinct characteristic since elevated levels after 24 hours were observed for liver-diseased patients with none-to-mild fibrosis, but not healthy controls nor patients with significant fibrosis. Finally, dynamics of interstitial and basement membrane degradation markers C3M and C4M did not correlate with changes of their corresponding formation markers but C3M was elevated in patients with liver disease compared to healthy controls.

PRO-C3 displays high diagnostic accuracy of advanced fibrosis and predicts fibrosis progression and outcomes in several liver disease etiologies.\textsuperscript{15,22-27} In our study, PRO-C3 increased in all three study groups within 24 hours, and when liver-diseased patients were divided according to etiological background. This indicates that binge drinking induces a fibrogenic response independent of disease state and etiology. The basal formation turnover rate of type III collagen is estimated to be between 0.2% and 0.6% per day.\textsuperscript{28} Since participants in our study had PRO-C3 increases in the range of 10% after 24 hours, we believe this suggests a “burst” due to increased hepatic interstitial matrix formation. This is most likely due to alcohol’s activating effects on hepatic stellate cells and myofibroblasts, known to induce fibrogenesis.\textsuperscript{10} The magnitude of observed PRO-C3 increase is remarkable, since liver fibrosis progression due to alcohol-induced liver injury normally develop in the order of years.\textsuperscript{29} In this context, another interesting finding is the apparent decrease in systemic PRO-C3 within three hours after binge drinking for patients with significant fibrosis, whereafter we observed a 24-hour increase. We attribute this observation to the fact that MMP-mediated degradation of existing type III collagen precede the deposition of the newly synthesized type III collagen during the ECM remodeling process.\textsuperscript{30} This could also explain why we only observed the dynamic in patients with significant fibrosis, since the healthy controls and patients with none-to-mild fibrosis had too low levels of existing type III collagen in their ECM for a measurable preceding degradation response.

We also observed increased 3-hour hepatic PRO-C3 levels, for participants with high systemic PRO-C3 at baseline, as an indication of liver fibrosis.\textsuperscript{9} Hence, our findings indicate that existing liver
fibrosis predispose to additional fibrosis formation in the liver. Studies show that large interindividual differences in liver fibrosis formation due to alcohol exist and an increased hepatic fibrosis formation rate with fibrosis stage. Binge drinking may therefore aggravate the fibrogenic effect in patients with existing fibrosis.

PRO-C8 was not as sensitive to acute alterations as PRO-C3. However, the biomarker of basement membrane remodeling displayed its own distinct pattern, possibly due to other cells being involved in basement membrane remodeling including hepatocytes and sinusoidal cells. First, after 24 hours there was a 30% systemic increase, a change was only observed in patients with fibrosis grades of F0-F1. Networking collagens such as type VIII collagen are known to dominate early-stage fibrosis where they affect cell behaviour. For type VIII collagen the non-collagen domain is known as vastatin and have been shown to hold hormonal anti-angiogenic effects. However, the role of PRO-C8 in liver fibrosis is still largely unknown.

We found PRO-C4 to be more abundant in the hepatic vein compared to the systemic circulation at baseline. Previous studies have demonstrated that the liver is a source of PRO-C4. We were not able to demonstrate that binge drinking exerts a notable effect on PRO-C4 levels. At the same time, we were unable to identify clear sex-specific differences in PRO-C4 levels in post-hoc analyses, even though other studies have shown PRO-C4 to be more abundant in females. This is most likely due the few number of females included in our study.

C3M and C4M are formed in part by MMP9-derived degradation of type III and IV collagen, but where C4M also is a product of MMP2- and MMP12-derived degradation. Animal models of MMP-9 activity after acute liver injury have provided conflicting results. We observed a strong correlation between the MMP9-derived biomarkers 24 hours after binge drinking. In a human setting, binge drinking therefore likely represents an indirect or direct regulator of MMP9 activity, irrespective of liver-disease status and fibrosis stage. However, in contrast to the formation markers, the collagen degradation markers did not increase significantly in response to binge drinking. This points towards a fibrogenic response with a net deposition of ECM fragments. Our analyses of the net deposition ratio of PRO-C3 and C3M in the hepatic blood showed that the ratio between the two also
increased with liver fibrosis stage at baseline. This confirms another recent study by our group where turnover in general was found to be increased in patients with ALD.27

Markers of hepatic collagen formation and degradation have been studied and measured for several years in different etiologies and stages of disease. When comparing our results to that of other cohorts we find similar levels according to fibrosis stage for the markers why we believe this enhances the external validity of the findings.16

In the interpretation of the current findings, there are some limitations to consider. First, due to the study design, hepatic measurements of ECM formation and degradation markers were not available at the 24-hour timepoint. Naturally, due to the invasive nature of hepatic vein measurements, through hepatic vein characterization, we were only able to infer systemic dynamics in the 24-hour analyses. Potentially, this could lead to a source of bias when hepatic measurements were not available at the 24-hour timepoint. However, since we only observed this dynamic nature for PRO-C3, and not any of the other biomarkers, we consider this risk negligible. Second, since we did not analyze markers of systemic inflammation, we were unable to infer how binge drinking affected this. Our initial analyses did not reveal any significant alterations in ALT or CRP as surrogates of inflammation, why it should be investigated further. Especially more sensitive markers of inflammation are of significant interest since they have been associated with a poor prognosis in the liver disease trajectory.39 Finally, due to the exploratory nature of our study, we performed data analyses where our results were unadjusted for multiplicity. However, post-hoc false discovery rate analysis revealed that the overall increase in PRO-C3 after 24 hours was robust, why we believe this reflects a true effect.

Important methodological strengths also apply to this study. We applied a study setup including hepatic vein measurements whereby we were able to distinguish the ECM turnover from the liver to that of other tissues in the intervention period. Furthermore, studying binge drinking and ECM turnover in a controlled setting allowed us to infer causal relations in comparison to observational associations.

This study unravels new potential perspectives of ECM turnover in relation to binge drinking. ECM formation seems to be more sensitive to alcohol intake than inflammation-mediated degradation, why binge drinking in the acute phase stimulate a myofibroblast response. The sensitiveness of PRO-C3 in
response to an acute alcohol insult and fibrosis formation provides intriguing perspectives. Potentially, PRO-C3 could be applied to monitor the efficacy of anti-fibrotic drugs. Hereby the liver fibrosis reducing effect could be evaluated in days instead of years, which could have implications for future liver fibrosis drug development.

Conclusions

The hepatic interstitial matrix and basement membrane are dynamic entities that seem to respond to a single binge drinking episode. We have displayed that binge drinking appears to induce an acute release of biomarkers from the ECM, particularly in patients with existing fibrosis. The most abundant and sensitive marker to reflect this dynamic state is PRO-C3. Markers for basement membrane remodeling are more elusive and future research should be focused on identifying the appropriate individual drivers of remodeling and differences in hepatic ECM turnover response.
References

Author names in bold designate shared co-first authorship


This article is protected by copyright. All rights reserved


### Tables & figures

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>F0-F1</th>
<th>≥F2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 13)</td>
<td>(n = 16)</td>
<td>(overall)</td>
</tr>
<tr>
<td>ALD (n)</td>
<td>-</td>
<td>7 (54%)</td>
<td>7 (44%)</td>
<td>0.588</td>
</tr>
<tr>
<td>NAFLD (n)</td>
<td>-</td>
<td>6 (46%)</td>
<td>9 (56%)</td>
<td>0.588</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.3 ± 10.1</td>
<td>48.1 ± 11.9†</td>
<td>58.2 ± 9.0</td>
<td>0.043</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>5 (50%)</td>
<td>9 (69%)</td>
<td>10 (63%)</td>
<td>0.640</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.3 [23.1-27.3]†</td>
<td>30.9 [24.8-41.5]</td>
<td>31.4 [28.3-33.4]§</td>
<td>0.008</td>
</tr>
<tr>
<td>LSM (kPa)</td>
<td>4.6 [3.9-5.0]†</td>
<td>8.9 [5.9-9.6]‡</td>
<td>11.4 [9.8-13.6]§</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CAP (dB/m)</td>
<td>230 ± 49†</td>
<td>316 ± 75</td>
<td>339 ± 45§</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ELF</td>
<td>8.5 [8.1-9.2]</td>
<td>9.2 [8.5-9.5]‡</td>
<td>9.8 [9.3-10.4]§</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>44.9 ± 3.9</td>
<td>44.2 ± 2.7</td>
<td>44.4 ± 3.9</td>
<td>0.698</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>26 [17-35]</td>
<td>34 [25-57]</td>
<td>50 [33-77]§</td>
<td>0.028</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>23 [22-28]</td>
<td>29 [26-49]</td>
<td>48 [29-80]§</td>
<td>0.005</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>58 [49-63]†</td>
<td>72 [70-105]</td>
<td>68 [59-86]§</td>
<td>0.010</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>10 [9-13]</td>
<td>10 [8-13]</td>
<td>12 [6-14]</td>
<td>0.900</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>22 [15-26]†</td>
<td>103 [46-255]</td>
<td>83 [61-145]§</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>35 [31-37]</td>
<td>38 [31-45]</td>
<td>43 [37-51]§</td>
<td>0.025</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.5 [1.1-2]</td>
<td>1.2 [1.1-1.6]</td>
<td>1.2 [1.1-1.4]</td>
<td>0.238</td>
</tr>
<tr>
<td><strong>ECM biomarkers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRO-C3 (ng/mL)</td>
<td>9.6 [8.6-10.8]</td>
<td>11.6 [11.0-13.8]‡</td>
<td>17.2 [12.0-24.0]§</td>
<td>0.004</td>
</tr>
<tr>
<td>PRO-C4 (ng/mL)</td>
<td>246.8 ± 119.0</td>
<td>330.4 ± 121.7</td>
<td>347.5 ± 188.3</td>
<td>0.355</td>
</tr>
<tr>
<td>PRO-C8 (ng/mL)</td>
<td>2.0 [1.0-3.3]</td>
<td>3.0 [2.4-3.9]</td>
<td>4.9 [3.2-6.6]§</td>
<td>0.014</td>
</tr>
</tbody>
</table>

This article is protected by copyright. All rights reserved.
<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>F0-F1</th>
<th>&gt;F2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3M (ng/mL)</td>
<td>8.2 [7.6-8.8]</td>
<td>8.4 [7.6-10.4]‡</td>
<td>10.2 [9.2-12.8]§</td>
<td>0.007</td>
</tr>
<tr>
<td>C4M (ng/mL)</td>
<td>23.5 [18.4-27.8]</td>
<td>24.1 [19.8-29.9]</td>
<td>27.8 [22.8-33.4]</td>
<td>0.133</td>
</tr>
</tbody>
</table>

Table 1. Baseline characteristics.

Data are presented as n (%). Parametric continuous data are presented as mean ± SD, and non-parametric continuous data are presented as medians (p25-p75). ALD, Alcohol-related liver disease; NAFLD, Non-alcohol-related liver disease, ELF, Enhanced liver fibrosis test, PRO-C, pro-collagen; BMI, Body mass index; LSM, liver stiffness measurement; CAP, controlled attenuation parameter; ELF, enhanced liver fibrosis test; ALT, alanine aminotransferase; AST, asparagine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; HC, healthy controls; F0-F1, patients with liver disease and no to mild fibrosis; >F2, patients with liver disease and significant fibrosis. P-value represent test between all three study groups. († p<0.05 between HC and F0-F1, ‡ p<0.05 between F0-F1 and >F2, § p<0.05 between >F2 and HC)
<table>
<thead>
<tr>
<th></th>
<th>PRO-C3 (ng/mL)</th>
<th>PRO-C4 (ng/mL)</th>
<th>PRO-C8 (ng/mL)</th>
<th>C3M (ng/mL)</th>
<th>C4M (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall (n = 39)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td><strong>12.6 [9.8-16.2]</strong></td>
<td>287.1 [220.6-402.7]</td>
<td>3.3 [2.3-5.3]</td>
<td>9.2 [8.0-10.4]</td>
<td>24.6 [20.3-30.4]</td>
</tr>
<tr>
<td>24 hours</td>
<td><strong>13.8 [10.8-17.8]</strong></td>
<td>339.2 [181.9-412.7]</td>
<td>3.6 [1.9-5.5]</td>
<td>9.2 [7.6-10.4]</td>
<td>26.3 [19.0-32.3]</td>
</tr>
<tr>
<td>P-value</td>
<td><em>&lt;0.001</em></td>
<td>0.199</td>
<td>0.068</td>
<td>0.961</td>
<td>0.635</td>
</tr>
<tr>
<td><strong>HC (n = 10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td><strong>9.6 [8.6-10.8]</strong></td>
<td>274.5 [182.6-317.5]</td>
<td>2.0 [1.0-3.3]</td>
<td>8.2 [7.6-8.8]</td>
<td>23.5 [18.4-28.0]</td>
</tr>
<tr>
<td>24 hours</td>
<td><strong>10.6 [9.6-12.4]</strong></td>
<td>299.0 [180.5-366.6]</td>
<td>2.2 [1.6-2.8]</td>
<td>8.0 [7.2-8.8]</td>
<td>25.0 [18.1-26.8]</td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.012</strong></td>
<td>0.647</td>
<td>0.473</td>
<td>0.442</td>
<td>0.879</td>
</tr>
<tr>
<td><strong>F0-F1 (n = 13)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This article is protected by copyright. All rights reserved.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>24 hours</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-value</td>
<td>0.030</td>
<td>0.345</td>
<td>0.014</td>
</tr>
</tbody>
</table>

>F2 (n = 16)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>24 hours</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-value</td>
<td>0.021</td>
<td>0.605</td>
<td>0.776</td>
</tr>
</tbody>
</table>

Table 2. Changes in collagen formation markers (PRO-C3, PRO-C4 and PRO-C8) and collagen degradation markers (C3M and C4M) 24 hours after a binge drinking episode. Data is presented as median (p25-p75). HC, healthy controls; F0-F1, patients with liver-disease and no to mild fibrosis; >F2, patients with liver-disease and significant fibrosis. P-value represent test between baseline and 24 hours. Significant results (P < 0.050) are marked in bold.
<table>
<thead>
<tr>
<th></th>
<th>PRO-C3</th>
<th>PRO-C4</th>
<th>PRO-C8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient [95% CI]</td>
<td>Coefficient [95% CI]</td>
<td>Coefficient [95% CI]</td>
</tr>
<tr>
<td>Time</td>
<td>-0.4140 [-1.2290; +0.4066]</td>
<td>+31.0335 [-18.0741; +81.7610]</td>
<td>-0.0161 [-0.4912; +0.4590]</td>
</tr>
<tr>
<td>Sample site (Hepatic)</td>
<td>-0.9905 [-1.6441; -0.3395]</td>
<td>+62.9141 [+22.5329; +103.2953]</td>
<td>+0.3962 [+0.0182; +0.7742]</td>
</tr>
<tr>
<td>Group</td>
<td>F0-F1 +1.527 [-4.2239; +7.2782]</td>
<td>+129.8085 [-12.3672; +271.9841]</td>
<td>+1.4283 [-0.1077; +2.9643]</td>
</tr>
<tr>
<td></td>
<td>&gt;F2 +10.04 [+4.5260; +15.5493]</td>
<td>+106.4000 [-20.8948; +242.6197]</td>
<td>+2.0343 [+0.5621; +3.5064]</td>
</tr>
<tr>
<td>Time#Hepatic</td>
<td>-0.4343 [-1.5079; +0.7222]</td>
<td>-7.1183 [-78.8580; +64.6213]</td>
<td>+0.2282 [-0.4411; +0.8974]</td>
</tr>
<tr>
<td>Time#Group</td>
<td>F0-F1 -0.2160 [-1.3046; +0.8709]</td>
<td>-39.6520 [-107.1259; +27.8220]</td>
<td>+0.1958 [-0.4340; +0.8256]</td>
</tr>
<tr>
<td></td>
<td>&gt;F2 -1.0653 [-2.1150; -0.2288]</td>
<td>-0.0730 [-51.5282; +77.8023]</td>
<td>-0.4603 [-1.0642; +1.4350]</td>
</tr>
<tr>
<td>Hepatic#Group</td>
<td>F0-F1 +0.0506 [-0.8153; +0.9164]</td>
<td>+19.1100 [-34.6054; +72.8186]</td>
<td>+0.3684 [-0.1328; +0.8696]</td>
</tr>
<tr>
<td></td>
<td>&gt;F2 +0.5752 [-0.2547; +1.4050]</td>
<td>+49.87 [-2.6056; 100.3466]</td>
<td>+0.3543 [-0.1265; +0.8351]</td>
</tr>
<tr>
<td>Time#Hepatic#Group</td>
<td>F0-F1 +0.3860 [-1.1523; +1.9243]</td>
<td>-34.0103 [-129.4329; +61.4123]</td>
<td>-0.3818 [-1.2710; +0.5070]</td>
</tr>
</tbody>
</table>
Table 3. Changes in collagen formation markers (PRO-C3, PRO-C4 and PRO-C8) during 3-hour analyses. The unit of time-dependent variables are ng/mL/180 minutes. F0-F1, patients with liver-disease and no to mild fibrosis; >F2, patients with liver-disease and significant fibrosis. Significant results (P < 0.050) are marked in **bold**.

| >F2   | +1.4435 [−0.0308; +2.9177] | 0.055 [−151.8162; +31.0846] | 0.196 [−0.7165; +0.9874] | +0.1355 [−0.7165; +0.9874] | 0.755 |
Figure 1. Study setup.

Vertical blue arrows indicate timepoints for blood sampling. The intervention period lasted from the start of alcohol infusion up until 4 hours post-infusion. The total research period lasted from the start of alcohol infusion until 24 hours post-infusion.
Figure 2. Dynamics of interstitial matrix formation PRO-C3. A) 3-hour changes of PRO-C3 in the hepatic- and systemic vein for all participants (n = 39). B) 3-hour changes in systemic PRO-C3 for healthy controls (n = 10), F0-F1 patients (n = 13) and >F2 patients (n = 16). C) Hepatic vein PRO-C3/C3M ratio at baseline. *P<0.05, **P<0.01. 1440 minutes denotes the 24-hour systemic measurement. Note the broken x-axis in Figure 2A and 2B. SEM, standard error of the mean.
Figure 3. Dynamics of basement membrane formation PRO-C8. A) 3-hour changes of PRO-C8 in the hepatic- and systemic vein for all participants (n = 39). B) 3-hour changes in systemic PRO-C4 for healthy controls (n = 10), F0-F1 patients (n = 13) and >F2 patients (n = 16). Note the broken x-axis in all graphs. SEM, standard error of the mean.
Figure 4. Dynamics of basement membrane formation PRO-C4. A) 3-hour changes of PRO-C4 in the hepatic- and systemic vein for all participants (n = 39). B) 3-hour changes in systemic PRO-C4 for healthy controls (n = 10), F0-F1 patients (n = 13) and >F2 patients (n = 16). 1440 minutes denotes the 24-hour systemic measurement. Note the broken x-axis in all graphs. SEM, standard error of the mean
Figure 5. 24-hour systemic changes in C3M, C4M, PRO-C3 and PRO-C4. Datapoints for each of the three study groups are displayed according to the best fitting line. A) Association between 24-hour systemic C3M and C4M changes. Spearman’s rank correlation coefficient was $r = 0.83 (P<0.001)$. B) No association existed between C3M and PRO-C3 in 24-hour changes ($r = -0.02, P=0.911$). C) There were no association between 24-hour C4M and PRO-C4 responses ($r = 0.16, P=0.344$).
Figures

Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.