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Extracellular matrix components indicate remodelling activity in different fibrosis stages of human non-alcoholic fatty liver disease

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Extracellular matrix components indicate remodelling activity in different fibrosis stages of human non-alcoholic fatty liver disease

Aims: The composition of several important extracellular matrix components (ECM) has not yet been elucidated in human non-alcoholic fatty liver disease (NAFLD). We aim to investigate the proportion of hepatic stellate cells (HSCs) and activity of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) in human NAFLD liver tissue with respect to severity of inflammation and fibrosis.

Methods and results: Histopathological features were quantified by NAFLD activity score and grading assignment. The collagen proportionate area (CPA) was measured. Slides were stained with alpha-smooth muscle actin (α-SMA), as a marker of activated HSCs, and α-SMA was quantified digitally. Zymography was performed to measure the proteolytic activity of MMP-2 and MMP-9. TIMP-1 and TIMP-2 protein concentration was measured with enzyme-linked immunosorbent assay (ELISA). α-SMA was higher in severe fibrosis (6.3%, interquartile range 2.9–13.1) than mild and no fibrosis (median 1.1 and 0.9%, $P < 0.001$) and correlated strongly with CPA ($Rs = 0.870$, $P < 0.001$). ProMMP-2 activity in severe (4.1%, IQR 2.6–16.2) and mild fibrosis (2.7%, IQR 1.9–3.9) was higher than in no fibrosis (1.5%, (IQR 0.95–2.1); $P = 0.001$ and $P = 0.046$) and showed a moderate positive correlation with CPA ($Rs = 0.495$, $P = 0.001$). TIMP-1 and TIMP-2 were significantly higher in severe fibrosis than mild or no fibrosis. Both showed moderate correlation with CPA (TIMP-1: $Rs = 0.471$, $P = 0.002$ and TIMP-2: $Rs = 0.325$, $P = 0.036$). MMP-9 correlated as the only ECM component to inflammation severity.

Conclusions: Advanced human NAFLD-fibrosis has a distinct ECM composition with increased HSCs and increased TIMP inhibition, but there is also ongoing remodelling activity of MMP-2.

Keywords: extracellular matrix, histopathology, human study, liver fibrosis, NAFLD

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease marked by fat accumulation. Prevalence is increasing, and this disease now affects approximately 25% of the global population.[1] NAFLD consists of a wide disease spectrum,

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extending from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH is a risk factor for fibrosis, and depending on host-related factors may progress to cirrhosis and even hepatocellular carcinoma.[2]

The role of the extracellular matrix (ECM) in fibrosis progression in NAFLD is complex and dynamic, and our understanding is incomplete.[3,4] The onset of inflammation in a steatotic liver triggers a cascade that results eventually in liver fibrosis. Inflammatory injury activates hepatic stellate cells (HSCs) to differentiate into a proliferative and profibrogenic phenotype, and to secrete several fibrotic components (collagens, proteoglycans and elastin), profibrogenic enzymes and cytokines.[5]

The histopathological pattern of fibrosis in NAFLD has a typical distribution, usually starting in the pericentral, zone 3, region.[6] It is believed that as fibrosis progresses, accumulated ECM components mature, cross-link and destroy normal liver architecture.[7] A recent study, however, shows that even in advanced NAFLD cases, there is active fibrosis matrix remodelling signalling the possibility of reversibility of fibrosis.[8]

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are involved in the turnover of fibrosis and play an important role in ECM homeostasis.[9] TIMP-1 and -2 are secreted by HSCs, and are abundantly present in the human liver. Blocking MMPs, such as MMP-2 and -9, prevents degradation of fibrosis. Investigation in experimental models of liver fibrosis suggests that an imbalance between TIMP and MMP drives fibrogenesis. Specifically, the increase of TIMPs and blocked MMP activity advances fibrosis.[10,11]

Although these model systems have allowed us to gain a deeper understanding of the complexity of liver inflammation and repair, the composition of the ECM in human NAFLD is less well studied.[12] A number of studies have attempted to shed light on this issue. One study found that MMP-9 mRNA expression, but not MMP-2, was up-regulated in liver tissue from NASH patients.[13] Another study saw that MMP-2 mRNA and serum levels were up-regulated in NASH, but a third study could not confirm this and found only increased TIMP-1 serum levels.[14,15] Human data on pro- and antifibrotic ECM components in relation to fibrosis stage or inflammation grade are lacking. Exploring this balance could lead to clearer insight into the activity of ECM remodelling. Therefore, we embarked upon a study to investigate the tissue activity of HSCs, MMP-2, MMP-9, TIMP-1 and TIMP-2 in relation to NAFLD fibrosis and disease severity.

Methods

STUDY DESIGN

We performed a cross-sectional study using biopsy material from patients with non-alcoholic fatty liver disease. Patients with evidence of alternative or coexistent liver disease (viral hepatitis, autoimmune hepatitis, Wilson's disease, haemochromatosis or alpha-1-antitrypsin deficiency) during standard work-up were excluded. Patients who consumed more than 210 g of alcohol per week for males and 140 g for females were also excluded.

Biopsies were collected between 2011 and 2016. Immediately after collection, liver tissue was divided in two parts: one formalin-fixed and paraffin-embedded (FFPE) for histopathological scoring and a second part snap-frozen in liquid nitrogen and stored at −80°C until activity assays [zymography, enzyme-linked immunosorbent assay (ELISA)] were performed [for the Biospecimen Reporting for Improved Study Quality (BRISQ) TIER-1 list], see Table S1. The study was approved by the Institutional Review Board of the Radboud University Medical Centre (no. 2016-2823) and patient material was used according to the Code of Conduct for Responsible Use of Human Tissue and Medical Research.[16] Clinical data and laboratory results up to 3 months prior to liver biopsy were obtained through chart review.

HISTOLOGY

Histopathological scoring was performed on FFPE slides. Slides were stained with a haematoxylin and eosin and picrosirius red stain and reviewed by experienced liver pathologists. Staging of fibrosis (0–4) was assessed using the ordinal scale associated with the NAFLD activity score, and patients were grouped into no fibrosis (stage 0); mild (stages 1–2) and severe fibrosis (stages 3–4).[17] Furthermore, the collagen proportionate area (CPA; the tissue percentage occupied by collagen, as stained with picrosirius red) was assessed digitally, as described previously.[18] Disease activity was graded with NAFLD activity score (NAS; 0–8) by summing the scores of steatosis (0–3), lobular inflammation (0–3) and hepatocyte ballooning (0–2). Histological NASH was determined with the fatty liver inhibition of progression (FLIP) algorithm, in which at least 1 point for ballooning and inflammation, in addition to steatosis, has to be present to

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define histological NASH.[19] Groups were also divided into patients with a total NAS of 0–2, NAS of 3–4 and NAS ≥5.[17] Tissue sections were cut consecutively and immunostained with antibody against α-SMA (1:3200, clone 1A4; Sigma-Aldrich, St Louis, MO, USA). Of the slides, 10 pictures were taken randomly at ×20 magnitude (Zeiss microscope, AxioVision version 2.0 software; Carl Zeiss Ltd, Cambridge, UK, attached to a personal computer), and images were quantified digitally (after exclusion of artefacts and blood vessels) with use of a colour deconvolution plugin in the ImageJ processing program (FIJI).[20]

A mean α-SMA proportionate area in % (area positive α-SMA staining/total area of the biopsy specimen) was calculated for each slide.

GELATIN ZYMOGRAPHY

Protein extraction from snap-frozen liver tissue, determination of protein concentrations and gelatin zymography were performed as described previously;[21] see Supporting information. In short, gelatinolytic activity of pro- and active forms of MMP-2 and MMP-9 were determined on a gelatin-impregnated sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Samples were loaded with a fixed amount of protein *in duplo*. An internal standard was included as a reference for quantification of proteolytic activity. After electrophoresis and incubation, gels were stained with Coomassie Blue. Quantification of proteinase activity, which is expressed as relative percentage to the reference standard, was performed with computerised densitometry. The mean of duplicates is presented. For the layout of the gels and positions of the pro- and active forms of MMP-2 and MMP-9 see Figure 1.

ELISA

TIMP-1 and -2 concentrations were measured in the supernatant of the snap-frozen liver tissue using sandwich ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Absorbance was read at 450 nm on an Infinite200-PRO plate reader (Tecan, Zürich, Switzerland). TIMP-1 and TIMP-2 concentrations (in pg/μg protein) were calculated using the Magellan program.

STATISTICAL ANALYSIS

Categorical variables (gender) are expressed in number and percentage and analysed by Fisher's exact test. Continuous data are presented as mean with standard deviation (SD) or median with interquartile range (IQR, 25th–75th percentile) and analysed with an independent-samples *t*-test or Mann–Whitney *U*-test, one-way analysis of variance (ANOVA) or Kruskal–Wallis test, Pearson's or Spearman's rank correlation coefficient, according to distribution. A two-sided level of $P < 0.05$ is considered statistically significant.

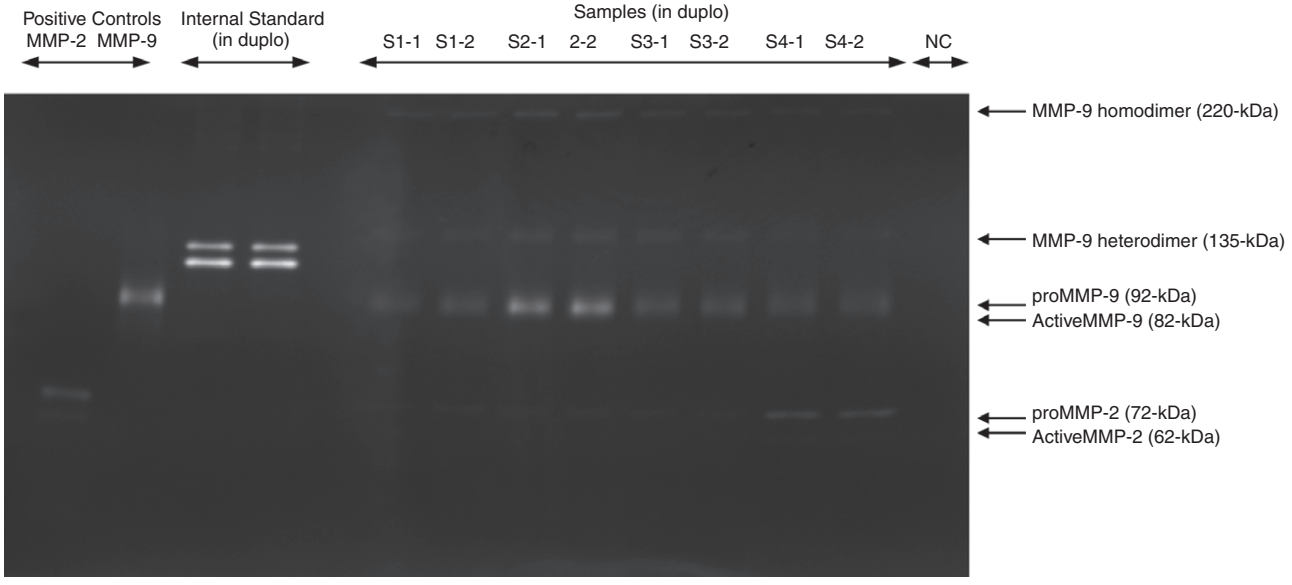

Figure 1. Zymography (layout). Proteolytic matrix metalloproteinase (MMP) activity in human liver tissue extracts detected by quantitative zymography. Proteolytic activity was measured with Total Lab Quant, in which the intensity of the bands was measured as a relative percentage of the internal standard. NC = sample buffer was loaded as a negative control on position 15. Position 5 remained blank. Active MMP-9, located at 82 kDa and active MMP-2 at 62 kDa are not identifiable by eye in these tissue extracts.[10]

Table 1. Characteristics of patients with and without histological NASH

	No NASH (n = 14)	Histological NASH† (n = 28)	P-value
Demographics			
Age (years)	50.6 ± 12.1	50.6 ± 10.8	0.985
Male gender	7 (50.0%)	16 (57.1%)	0.661
Diabetes mellitus type 2	3 (21.4%)	9 (32.1%)	0.719
BMI (kg/m^2)	28.3 ± 2.6	32.7 ± 5.7	0.020
Laboratory results			
Alanine transaminase (IU/l)	66 (47–103) n = 13*	77 (59–139) n = 24	0.422
Aspartase transaminase (IU/l)	36 (30–53) n = 12	54 (42–78) n = 21	0.030
Alkaline phosphatase (IU/l)	86 (65–125) n = 12	109 (82–131) n = 21	0.187
Gamma-glutamyltransferase (IU/l)	135 (53–335)	102 (59–260) n = 23	0.922
Bilirubin (μmol/l)	10 (6–11) n = 12	10 (7–16) n = 21	0.518
C-reactive protein (mg/l)	1 (1–8) n = 5	9 (5–45) n = 7	0.073
Ferritin (μg/l)	258 (205–553) n = 6	214 (96–804) n = 16	0.590
ECM components			
α-SMA (%)	1.2 (0.7–2.4)	1.8 (0.7–6.0)	0.272
ProMMP-2 (relative %)	2.0 (1.4–2.8)	2.8 (1.8–4.3)	0.147
ActMMP-2 (relative %)	0.4 (0.2–0.9)	0.4 (0.2–0.8)	0.927
ProMMP-9 (relative %)	40.5 (23.2–71.1)	34.0 (23.0–62.5)	0.626
ActMMP-9 (relative %)	0.2 (0.1–0.4)	0.2 (0.1–0.3)	0.607
TIMP-1 (pg/μg)	2.4 (2.1–2.8)	2.7 (2.0–3.4)	0.607
TIMP-2 (pg/μg)	3.2 (2.8–3.6)	3.4 (2.7–3.9)	0.589

Results are shown as count and percentage (n, %), mean with standard deviation or median and interquartile range (25th–75th percentiles). NASH, Non-alcoholic steatohepatitis; BMI, Body mass index; ECM, Extracellular matrix components; α-SMA, Alpha-smooth muscle actin; MMP, Matrix metalloproteinases; TIMP, Tissue inhibitors of MMP; FLIP, Fatty liver inhibition of progression.
*Number of patients with laboratory results within 3 months prior to liver biopsy are specified in the columns.
†Histological NASH, based on FLIP algorithm.[19]

Statistics are performed using SPSS statistical software package (version 22.0; SPSS Inc., Chicago, IL, USA). Figures are created using Graphpad Prism (version 5.03; Graphpad Software Inc., La Jolla, CA, USA).

Results

CLINICOPATHOLOGICAL CHARACTERISTICS

The total cohort consisted of 42 patients with a clinical and histological diagnosis of NAFLD or NASH. The mean age was 51 ± 11 years and 55% were male. Median NAS was 4 (IQR = 3–5), and 16 (38%) patients had histological evidence of NASH, as indicated by NAS ≥5. Based on the FLIP algorithm, 28 (67%) patients had histological NASH. Characteristics of patients with and without histological NASH are shown in Table 1. Patients with histological NASH had significantly higher aspartate transaminase (AST) levels compared to those without NASH (see Table 1). AST was 144 (IQR = 63–155) U/l in severe (NAS 2) inflammation and 75 (IQR = 50–134) U/l in mild (NAS 0–1) inflammation (P = 0.020). Alanine transaminase (ALT) was 67 (IQR = 48–101) U/l in severe compared to 42 (IQR = 33–54) U/l in patients with mild inflammation (P = 0.028).

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Table 2. Characteristics of patients within fibrosis groups

	No fibrosis F0 (n = 9)	Mild fibrosis F1–2 (n = 22)	Severe fibrosis F3–4 (n = 11)	P-value
Demographics				
Age (years)	48.9 ± 10.0	47.1 ± 10.3	58.9 ± 9.8	0.011
Male gender	6 (66.7%)	13 (59.1%)	4 (36.4%)	0.335
Diabetes mellitus type 2	1 (11.1%)	5 (22.7%)	6 (54.5%)	0.069
BMI (kg/m^2)	30.6 ± 4.1	32.1 ± 5.6	30.0 ± 7.1	0.570
Histology				
Collagen proportionate area	1.54 (0.91–2.59)	4.07 (2.56–5.86)	13.02 (10.46–17.84)	<0.001
NAFLD activity score 0–2	5 (55.6%)	4 (18.2%)	0	0.005
NAFLD activity score 3–4	4 (44.4%)	6 (27.3%)	7 (63.6%)	0.005
NAFLD activity score ≥5	0	12 (54.5%)	4 (36.4%)	0.005
Histological NASH*	2 (22.2%)	17 (77.3%)	9 (81.8%)	0.006
ECM components				
α-SMA (%)	0.9 (0.5–2.1)	1.1 (0.6–1.9)	6.3 (2.9–13.1)	<0.001
ProMMP-2 (relative %)	1.5 (1.0–2.1)	2.8 (1.9–3.9)	4.2 (2.6–16.2)	<0.001
ActMMP-2 (relative %)	0.4 (0.2–0.7)	0.3 (0.1–0.7)	0.7 (0.2.2.0)	0.278
ProMMP-9 (relative %)	36.9 (16.7–78–.1)	36.8 (24.5–69.6)	34.0 (28.0–46.1)	0.848
ActMMP-9 (relative %)	0.2 (0.1–0.5)	0.1 (0.1–0.2)	0.2 (0.1–0.5)	0.286
TIMP-1 (pg/μg)	2.4 (1.9–2.6)	2.3 (2.0–3.1)	3.4 (2.9–5.0)	0.007
TIMP-2 (pg/μg)	3.0 (2.8–3.4)	3.3 (2.3–3.6)	4.1 (3.5–4.6)	0.007

Results are shown as count and percentage (n, %), mean ± standard deviation or median and interquartile range (25th–75th percentiles). NASH, Non-alcoholic steatohepatitis; BMI, Body mass index; ECM, Extracellular matrix components; α-SMA, Alpha-smooth muscle actin; MMP, Matrix metalloproteinases; TIMP, Tissue inhibitors of MMP; FLIP, Fatty liver inhibition of progression; NAFLD, Non-alcoholic fatty liver disease.
*Histological NASH, based on FLIP algorithm.[19]

Fibrosis was absent from the liver biopsy in nine patients, while 22 patients had mild fibrosis (15 stage 1, seven stage 2), and 11 patients had severe fibrosis (six stage 3, five stage 4). Median CPA in the whole cohort was 4.2% (IQR = 2.3–10.6), and CPA correlated strongly with the histopathologically determined fibrosis stage (Rs = 0.749, P < 0.001). See Table 2 for characteristics of the fibrosis groups.

CORRELATION EXTRACELLULAR MATRIX COMPONENTS AND FIBROSIS STAGING

The area of α-SMA staining was significantly higher in samples with severe fibrosis compared to samples with mild or absent fibrosis, and there was a strong correlation of α-SMA with CPA (Rs = 0.870, P < 0.001, see Table 2 and Figure 2A, B). The gelatinolytic activity of proforms of both MMPs was increased compared to the active forms regardless of stage; median proMMP-2: 2.7% (IQR = 1.7–4.1), proMMP-9: 36.2% (IQR = 23.3–61.0), actMMP-2: 0.4% (IQR = 0.2–0.8) and actMMP-9: 0.2% (IQR = 0.1–0.3).

Median proMMP-2 activity was significantly higher in severe and mild fibrosis compared to samples without fibrosis, and showed a moderate positive correlation with CPA (Rs = 0.495, P = 0.001). The activity of actMMP-2 was not statistically significantly different between groups (see Table 2 and Figure 3A). Both forms of MMP-9 were distributed similarly among different fibrosis groups (see Table 2 and Figure S1), and were not correlated with CPA (see

Table 3 for all correlations on ECM components and fibrosis staging).

TIMP-1 and TIMP-2 (in pg/μg protein) levels were significantly higher in severe fibrosis than mild or absent fibrosis (see Table 2 and Figure 3B). Both TIMP-1 and TIMP-2 levels correlated with CPA (TIMP-1: $Rs = 0.471$, $P = 0.002$ and TIMP-2: $Rs = 0.325$, $P = 0.036$; see Table 3).

CORRELATION EXTRACELLULAR MATRIX COMPONENTS AND DISEASE ACTIVITY/ INFLAMMATION

Median α-SMA in patients with histological NASH was comparable to that of patients without histological NASH (see Table 1). α-SMA did not correlate with total NAS grade or individual components of NAS grading (steatosis, ballooning or inflammation; see Table 3). ProMMP-9 levels were higher in patients with severe inflammation (58.0% IQR = 29.2–85.5) compared to mild inflammation (30.1% IQR = 21.8–44.7, $P = 0.035$). ActMMP-9 was similar in mild and severe inflammation (see Figure S1). Both actMMP-9 and proMMP-9 were similar between patients with and without histological NASH (see Table 1). α-SMA and MMP-9 showed no correlation to ALT or AST levels (data not shown).

Discussion

This study documents that liver biopsy samples from patients with advanced NAFLD-associated fibrosis possess a distinct ECM composition, with increased levels of α-SMA, proMMP-2 and both TIMP-1 and -2. These results suggest that advancing fibrosis in

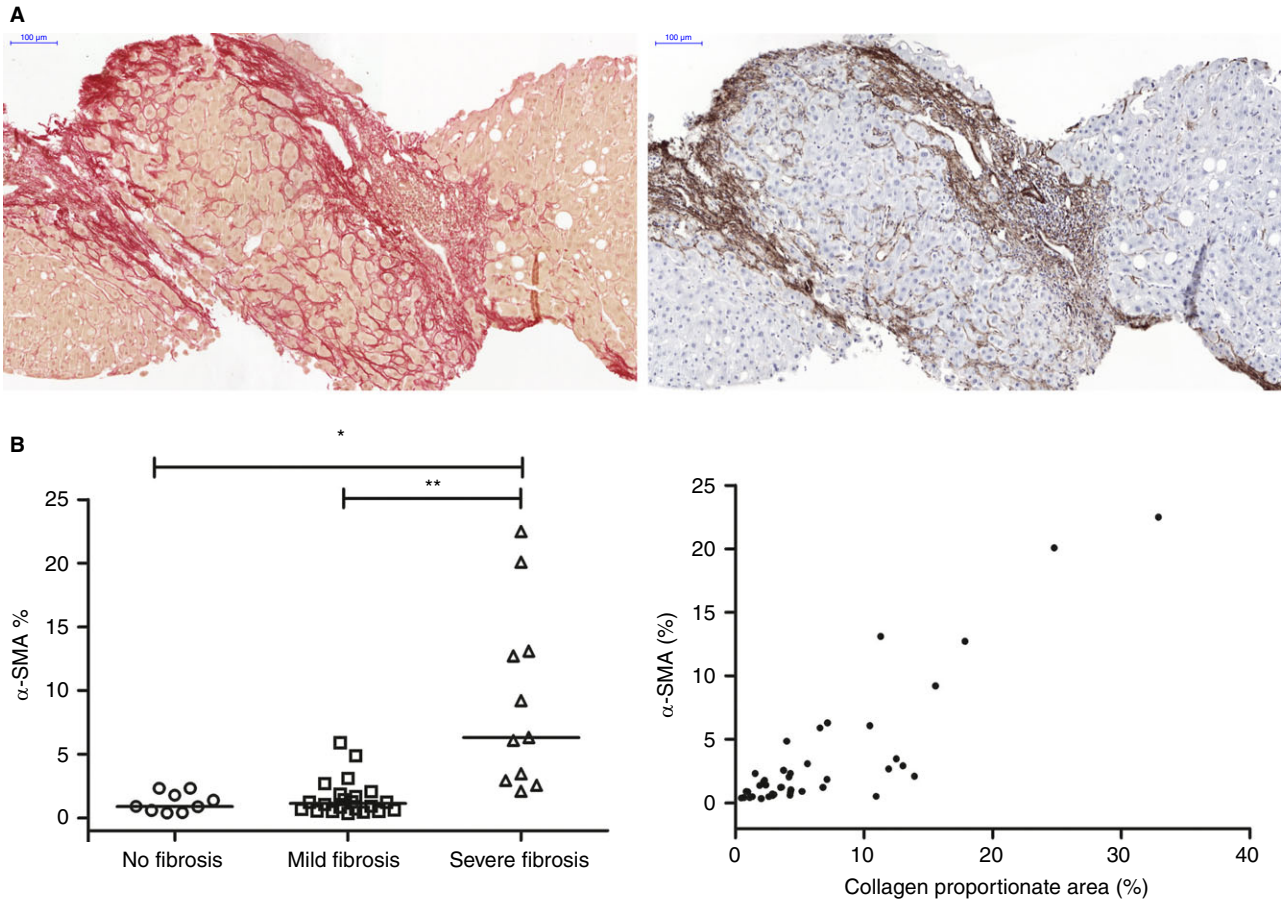

Figure 2. **A**, Example of alpha-smooth muscle actin (α-SMA) and collagen proportionate area (CPA) staining. Detail of a biopsy from a cirrhotic liver stained with Sirius red on the left and α-SMA in the right panel. Stains were quantified with digital image analysis. **B**, More activated hepatic stellate cells in severe fibrosis compared to mild or no fibrosis. α-SMA percentages were analysed between fibrosis groups with the Kruskal–Wallis test on the left; *$P < 0.01$; **$P < 0.001$. On the right, correlation between α-SMA and CPA is shown with Spearman's correlation coefficient; $Rs = 0.870$; $P < 0.001$.

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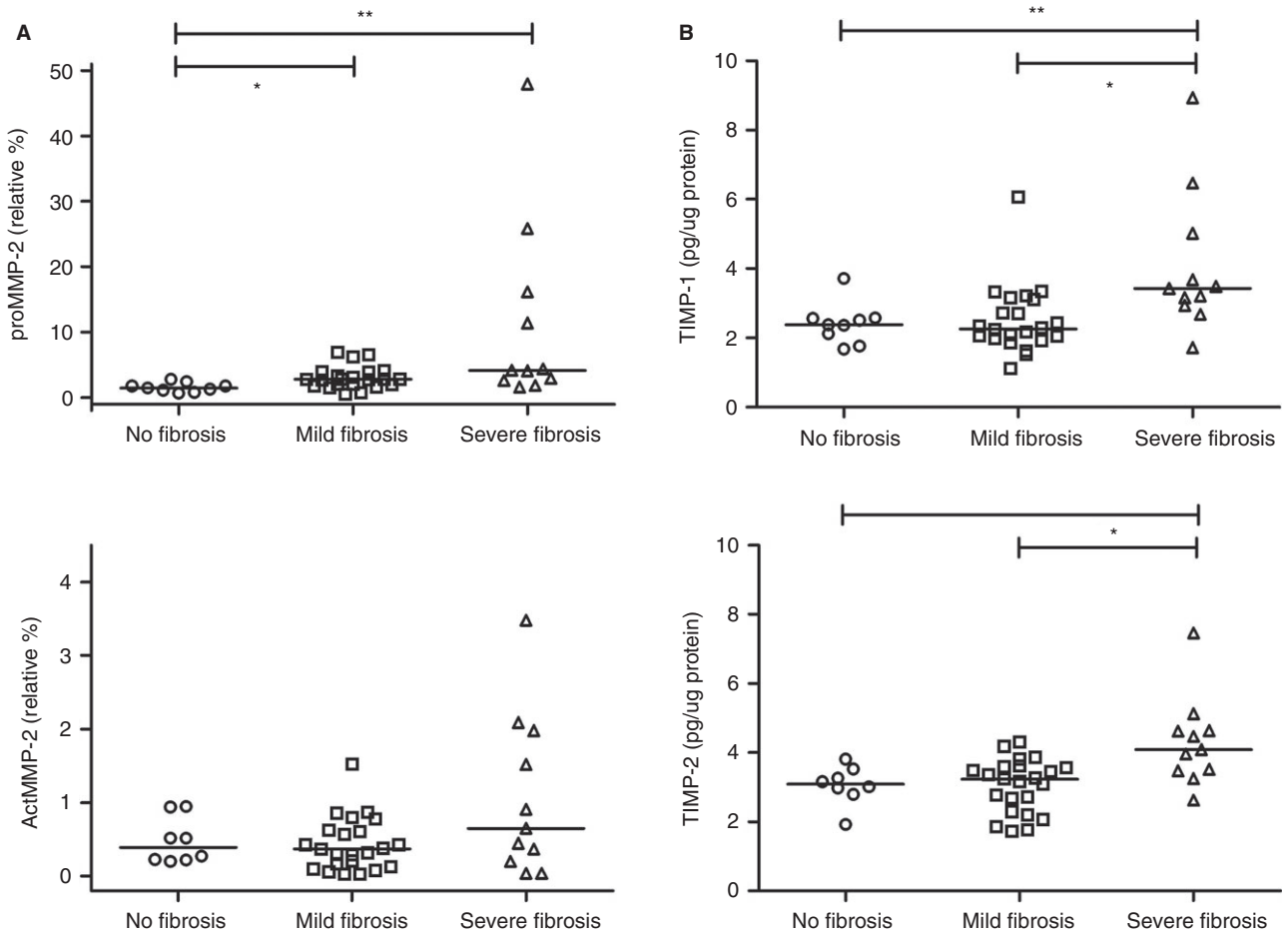

Figure 3. Matrix metalloproteinase (MMP)-2 and tissue inhibitors of MMPs (TIMPs) in fibrosis groups. **A**, ProMMP-2 and actMMP-2 levels (in relative % to reference standard) with group median (line) are shown for three fibrosis groups: no fibrosis, mild fibrosis and severe fibrosis. **B**, TIMP-1 and TIMP-2 levels (in pg/μg protein) with group median (line) are shown for three fibrosis groups. Analyses were performed with the Kruskal–Wallis test; *$P < 0.05$; **$P < 0.01$.

NAFLD is driven by activated HSCs, creating an environment which permits inhibition of matrix degradation and contributes to fibrosis accumulation. Increased activation of HSCs in the context of severe fibrosis in human NAFLD has been reported previously,[22] but data on MMP and TIMP activity were lacking. Increase of proMMP-2, TIMP-1 and TIMP-2 activity in NASH fibrosis is consistent with data obtained for other chronic liver diseases.[11,23–25]

The increase of proMMP-2 and, to a lesser extent, of active MMP-2, suggests there is still active matrix turnover in advanced NAFLD fibrosis. This is consistent with another study that quantified hepatic fibrogenesis flux rates in liver tissue and blood from NAFLD patients.[8] In experimental models, the early phase of liver injury resulted in increased levels of MMP-3, MMP-13 and, to a lesser extent, MMP-2. During the later phase of HSC activation, expression of MMP-2, TIMP-1 and TIMP-2 increased.[26] The protein concentration of TIMPs appears to be critical for the actual proteolytic activity of MMPs. Low concentrations of TIMPs mediate activation of MMP-2, while higher TIMP levels inhibit MMP-2 activation.[27] MMP-2 degrades collagen type IV (present in the basal membrane in normal liver), but MMP-2 also possesses elastase and collagenase activity *in vitro*.[9] Furthermore, there is evidence that MMP-2 and MT1-MMP (a membrane type MMP) work synergistically to degrade fibrillar collagens, and that their combination is poorly inhibited by TIMP-1.[28,29] Although we were not able to measure the activity of MT1-MMP and specific collagen subtypes, it can be speculated that MMP-2 could also have a role in degrading mature fibrosis, which consists of collagen type I, III and elastin.

Table 3. Correlations of ECM components and fibrosis staging and NAS grading

	CPA (%)	Fibrosis stage (0–4)	NAS grade (0–8)	Steatosis (0–3)	Ballooning (0–2)	Inflammation (0–3)
α-SMA	*R*s = 0.720**	0.667**	0.086	0.032	0.182	−0.011
	P = 0.000	0.000	0.587	0.841	0.248	0.943
proMMP-2	40.95**	0.647**	0.191	0.1741	0.112	0.108
	0.001	0.000	0.226	0.280	0.480	0.495
actMMP-2	0.180	0.198	−0.004	−0.024	0.124	−0.041
	0.253	0.209	0.979	0.880	0.434	0.796
proMMP-9	−0.088	0.001	0.040	−0.043	−0.078	0.211
	0.579	0.996	0.804	0.788	0.624	0.180
actMMP-9	0.033	0.041	−0.193	−0.113	−0.104	−0.104
	0.837	0.794	0.221	0.477	0.511	0.349
TIMP-1	0.471**	0.400**	0.027	−0.129	0.132	0.145
	0.002	0.009	0.866	0.414	0.405	0.358
TIMP-2	0.325*	0.482**	0.071	−0.015	0.031	0.143
	0.036	0.001	0.866	0.923	0.848	0.365

Spearman's rank (Rs) correlation coefficients and *P*-values are shown for correlations between ECM components and fibrosis staging and NAS grading. ECM, Extracellular matrix components; α-SMA, Alpha-smooth muscle actin; MMP, Matrix metalloproteinases; TIMP, Tissue inhibitors of MMP; CPA, Collagen proportionate area; NAS, Non-alcoholic fatty liver disease activity score.
*Correlation significant at <0.05 level; **Correlation significant at <0.01 level.

In clinical practice, it is important to stratify NAFLD patients based on fibrosis stage, in view of the 10–40 times fold increase in liver-related mortality among patients with progressing stages of fibrosis.[30] There is evidence to suggest that advanced fibrosis or cirrhosis may still be reversible after removal of the causal agent (hepatitis C treatment, reduction of hepatic steatosis through weight loss in NAFLD and alcohol cessation in alcoholic liver disease). Profiling ECM composition may be used to stratify patients according to fibrosis remodelling capacity. Compounds targeting ECM components could be explored further as new antifibrotic strategies in NAFLD.[31,32]

The absence of correlation between proportion of activated HSCs and AST, ALT or NASH in our study could imply that fibrogenesis is maintained independently of inflammatory stimuli. HSCs display several collagen receptors, such as integrins, that receive cytokine signals [notably transforming growth factor (TGF)-β] from ECM components, which induce differentiation and proliferation of HSCs to maintain a profibrogenic phenotype.[3,7] TIMPs have the ability to inhibit programmed cell death of HSCs.[33] A positive feedback loop with ECM components that affect HSCs promotes fibrosis progression. Conversely, this model is possibly an (over)simplification of the pathogenesis of fibrosis. NASH is a heterogeneous and multifactorial disease and insulin resistance, alcohol use, presence of other toxins, virus or cholestasis all contribute to fibrosis progression.

ProMMP-9 was the only ECM component increased in inflamed tissue. Other studies co-localised MMP-9 with inflammatory cells, such as Küpffer cells, macrophages and neutrophils.[34,35] Proinflammatory cytokines C-X-C motif chemokine ligand 8 (CXCL-8), interleukin (IL)-1β and tumour necrosis factor (TNF)-α are known substrates for MMP-9. MMP-9 increases the biological activity of these cytokines, leading to an exacerbation of inflammation. The composition of inflammatory cells was beyond the scope of this study, but further research on the interaction of immune cells, MMP-9 and fibrosis in human NAFLD may elucidate this.

Our study comes with several strengths: we had access to a well-defined cohort of NAFLD patients, encompassing the whole spectrum of NAFLD activity and fibrosis. Zymography allowed us to measure gelatinolytic activity, which resembles true proteolytic ability. Active matrix turnover in tissue is reflected more clearly with this assay than with mRNA expression or serum markers. We performed computer-aided quantitative measurements of immunohistochemical staining, minimising possible interobserver variability and lack of power to discriminate between stages, inherent to grouping and scoring. The correlation of ECM components not only to fibrosis stage, but also

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to the quantitative amount of collagen (CPA) strengthens our conclusions.

However, this study design also brings limitations. We could only examine two MMPs, as no assays are available to measure proteolytic activity of other MMPs, such as MMP-12, MMP-13, MMP-3 and MT1-MMP.[36] We could not correct for all possible confounders involved in the multifactorial fibrogenesis in NAFLD. The lack of follow-up biopsies in our cohort did not allow us to assess the predictive role of ECM components in progression or regression of fibrosis.

In conclusion, in this study the severity of human NAFLD liver fibrosis is associated with increasing α-SMA, proMMP-2 and TIMP-1 and -2. ProMMP-9 correlates with the severity of inflammation. The changed ECM composition in advanced NAFLD fibrosis suggests that inhibition of matrix degradation is maintained by activated HSCs secreting exceeding amounts of TIMPs, favouring fibrosis accumulation. MMP-2 activity in advanced fibrosis stages suggests that there is still active matrix turnover and could signal the possibility of reversibility, which should be assessed further in longitudinal follow-up studies.

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Conflicts of Interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. MMP-9 in fibrosis and inflammation.

Table S1. Core BRISQ tier 1 items to report (adapted from Moore *et al.*[37]).

Data S1. Supporting methods.

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