Matrix Metalloproteinases 2 and 9 Are Markers of Inflammation but Not of the Degree of Fibrosis in Chronic Hepatitis C

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**Key Words**

Fibrosis · Cirrhosis · Matrix metalloproteinase 2 · Matrix metalloproteinase 9

**Abstract**

**Background:** The degree of liver fibrosis and inflammation is important in patients with chronic hepatitis C (CHC) in terms of therapy as well as prognosis. To obviate the need of liver biopsy, serum markers such as procollagen I, III and hyaluronic acid have been proposed but were found to be inaccurate. Controversy still exists regarding the role of matrix metalloproteinases (MMPs) as valid markers of liver fibrosis. \textbf{Aim:} To assess liver and serum MMP-2 and -9 as markers of fibrosis and inflammation in patients with CHC. **Methods:** Thirty-five CHC patients and 8 non-hepatitis C patients with normal liver enzymes underwent liver biopsy. Activities of inflammation and fibrosis stage were determined by the Desmet score on a scale of 0–4. Serum and liver tissue MMP-2 and -9 activities were measured by zymography using substrate impregnated gels. **Results:** Patient and control groups were similar in terms of age (50.8 \pm 15.1 vs. 50.6 \pm 15.2) and male/female ratio (18/17 vs. 4/4). In serum, MMP-9 activity was increased in patients compared to controls (308 \pm 110 vs. 163.5 \pm 35, \textit{p} < 0.05). In liver tissue, MMP-9 was also higher in patients than in controls (21 \pm 4.5 vs. 17.1 \pm 5.1, \textit{p} < 0.05), whereas MMP-2 did not differ between patients and controls. Serum MMP-9 values correlated with liver histologic inflammatory grade (290.4 \pm 83 in grade 2 vs. 562.1 \pm 128 in grade 3, \textit{p} < 0.05) but not with fibrosis stage. The highest rising in serum MMP-9 levels was observed between grade 2 to grade 3 and was superior to the rising in serum transaminase levels, indicating its advantage in assessing the progression of disease activity. No correlation between liver MMP activities and liver fibrosis or inflammation was observed. **Conclusion:** Serum MMPs, in particular MMP-9, can serve as markers of disease activity rather than fibrosis stage in chronic HCV patients.

**Introduction**

Hepatic fibrosis is a wound healing or scarring process, which arises in response to liver damage [1, 2]. Hepatic stellate cells (HSC) play a central role in the pathogenesis...
of this process and are responsible for an imbalance between fibrogenesis and fibrolysis in the liver [3]. HSC increases extracellular matrix (ECM) protein synthesis and at the same time regulates matrix degradation [4]. Activation of HSC leads to release of both enzyme matrix metalloproteinases (MMPs) and several converting enzymes (MT1-MMP) that degrade extracellular matrix, and their inhibitors such as the tissue inhibitor of MMP (TIMP), which modulates the fibrolytic action [5, 6]. Liver fibrosis and cirrhosis have been believed to be irreversible. However, several studies suggest that liver fibrosis and cirrhosis are potentially a dynamic process, which may be reversible in some circumstances. Those data derived from animal models where fibrosis was induced by CCl4 [7], or from human data in response to antiviral therapy such as interferon or ribavirin [8–12]. Therefore, reliable diagnostic tests are necessary for monitoring hepatic fibrogenesis in patients with chronic liver disease. Yet, despite numerous serum assays that had been proposed for monitoring degree of liver fibrosis, none was found to be reliable and the assessment is still based on liver tissue histology [13, 14]. Most markers reflect the fibrogenetic process such as amino and/or carboxy-terminal of procollagens, non-collagenous glycoproteins or enzymes involved in fibrogenesis. In addition, the current concept is that progression of liver fibrosis may result from an imbalance between matrix degradation and matrix synthesis [15]. MMPs are a family of zinc- and calcium-dependent endopeptidases that have activity against the major components of the extracellular matrix such as collagen, gelatin, laminin, and proteoglycan core protein [17]. The MMPs can be grouped generally according to enzymatic substrate: collagenases, gelatinases, stromelysins and metalloelastases. The extracellular catalytic activity of each MMP is regulated by transcriptional activation at the level of the gene by growth factors, cytokines, and retinoids. It is also regulated by extracellular cleavage of a latent pro-enzyme to its active form as well as specific inhibition by the TIMP system [18, 19]. Therefore, serum levels of MMPs may serve as a marker of active fibrosis and help in follow-up of patients with chronic hepatitis. In this study, we selected a single etiology of liver cirrhosis, chronic hepatitis C virus (HCV) infection. We measured expression of MMP-2 and MMP-9 (gelatinase A and B, respectively), which can degrade denaturant interstitial collagens (gelatins). They are important components of the basement membrane and are considered the most relevant MMPs in hepatic fibrosis. Therefore, we measured MMP-2 and MMP-9 activity in liver tissue and at the same time their serum levels in patients with HCV and correlated the activity with their fibrosis staging on liver biopsy.

### Subjects and Methods

Percutaneous needle liver biopsies were performed in 35 patients with chronic hepatitis due to HCV. In addition, 8 HCV-negative patients served as a control group with normal or slightly elevated liver enzymes who underwent liver biopsy for the evaluation of a suspected liver mass or fatty liver and had normal liver histology. Each patient underwent routine liver function tests including aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum albumin, bilirubin, alkaline phosphatases, and anti-HCV (RIBA). Characteristics and laboratory tests of the patients are shown in table 1. Patients with elevated liver enzymes (AST, ALT > 2 x normal values) for more than 6 months and a positive HCV-RNA by PCR entered the study. The study was approved by the institutional ethical committee and each patient and control signed an informed consent form. Each patient enrolled to the study underwent ultrasound-guided percutaneous needle liver biopsy. A sample of the tissue was used for histology and another part was frozen at –70 °C and thawed before MMP activity was assessed. At the time of the liver biopsy peripheral blood was withdrawn for measuring serum liver enzymes and MMP-2 and -9 levels.

After liver biopsy, each tissue section was stained with hematoxylin-eosin and Masson’s trichrome. Histological findings were classified according to the classic classification of Desmat et al. [20]. Grading of activity: 1, minimal; 2, mild; 3, moderate; 4, severe. Staging of fibrosis: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; 3, severe fibrosis; 4, cirrhosis.

### MMP Activity

The collagenolytic activity was determined on a gelatin-impregnated (1 mg/ml, Difco, Detroit, Mich., USA), SDS-PAGE 8% gel, as previously described [21], with minor modifications. Briefly, culture media samples were separated on the substrate-impregnated gels under non-reducing conditions, followed by 30 min incubation in 2.5% Triton X-100 (BDH, England). The gels were then incubated for 16 h at 37 °C in 50 mM Tris, 0.2 M NaCl, 5 mM CaCl2, 0.02% Brij 35 (w/v) at pH 7.6. At the end of the incubation period, the gels were stained with 0.5% Coomassie G 250 (Bio-Rad Richmond CA) in methanol/acetic acid/H2O (30:10:60). The intensity of the various bands was determined on a computerized densitometer (Molecular Dynamics type 300A).

### Statistical Analysis

Data are shown as mean ± SD. Differences between groups were analyzed using the Kruskal-Wallis test for multiple comparisons. Correlations between groups were analyzed using the Pearson correlation test. p < 0.05 was considered statistically significant.
Thirty-five patients (18 male, 17 female) with chronic HCV infection confirmed by a positive HCV-PCR underwent liver biopsy to determine their disease activity and stage of fibrosis.

The HCV subjects were divided into 3 subgroups according to grade of necro-inflammatory activity: 1, 2, 3 (15 patients, 14 patients and 6 patients, respectively) as well as 3 subgroups according to stage of fibrosis: 0, 1, 2–3 (17, 10 and 8 patients, respectively). None of the patient had either grade or stage 4. Another 8 non-HCV patients (4 male, 4 female) that were evaluated for a liver mass or fatty liver and were found with no histological signs of any liver disease (grade 0, stage 0) served as a control group. All grade subgroups (Table 1) and stage subgroups (Table 2) were similar to the control group in terms of gender ratio and mean age. The histologic necro-inflammatory activity of liver disease was positively correlated with liver stage of fibrosis ($r = 0.52$, $p = 0.006$). There was no significant correlation between the level of liver MMP-2 and liver MMP-9 with histological stage or grade among HCV patients. Furthermore, no difference in liver MMP-2 activity was found between HCV patients

**Table 1. Background and laboratory tests of patients according to grade subgroups**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>Grade 1 (n = 15)</th>
<th>Grade 2 (n = 14)</th>
<th>Grade 3 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>4/4</td>
<td>8/7</td>
<td>7/7</td>
<td>3/3</td>
</tr>
<tr>
<td>Age</td>
<td>$50.6 \pm 15.1$</td>
<td>$50.4 \pm 16.1$</td>
<td>$50.1 \pm 15.3$</td>
<td>$56.6 \pm 12.2$</td>
</tr>
<tr>
<td>Liver MMP-2</td>
<td>$23.1 \pm 7.9$</td>
<td>$22.1 \pm 5.5$</td>
<td>$18.5 \pm 6.7$</td>
<td>$21.5 \pm 7.4$</td>
</tr>
<tr>
<td>Liver MMP-9</td>
<td>$17.1 \pm 5.1$</td>
<td>$20.6 \pm 6.7$</td>
<td>$19.1 \pm 5.4$</td>
<td>$21.6 \pm 13.1$</td>
</tr>
<tr>
<td>Serum MMP-9</td>
<td>$163.5 \pm 35.1^*$</td>
<td>$307.6 \pm 130.3$</td>
<td>$290.4 \pm 83.3$</td>
<td>$562.1 \pm 128.4$</td>
</tr>
<tr>
<td>Bilirubin total*, µmol/l</td>
<td>$8.33 \pm 1.7$</td>
<td>$14.96 \pm 8.5$</td>
<td>$14.62 \pm 5.1$</td>
<td>$13.43 \pm 3.4$</td>
</tr>
<tr>
<td>Bilirubin direct*, µmol/l</td>
<td>$2.89 \pm 1.7$</td>
<td>$5.95 \pm 3.4$</td>
<td>$5.44 \pm 3.4$</td>
<td>$5.1 \pm 1.7$</td>
</tr>
<tr>
<td>ALT*, IU/l</td>
<td>$49.6 \pm 30.6$</td>
<td>$81.5 \pm 57.1$</td>
<td>$141.1 \pm 118$</td>
<td>$141.2 \pm 73.3$</td>
</tr>
<tr>
<td>AST*, IU/l</td>
<td>$46.7 \pm 28.7$</td>
<td>$60.2 \pm 26$</td>
<td>$106.8 \pm 130.6$</td>
<td>$93.4 \pm 30.1$</td>
</tr>
<tr>
<td>Protein, g/dl</td>
<td>$69.2 \pm 5.7$</td>
<td>$71.6 \pm 5.1$</td>
<td>$72.7 \pm 4.2$</td>
<td>$70.2 \pm 7.8$</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>$41.5 \pm 2.4$</td>
<td>$40.6 \pm 4.3$</td>
<td>$42.3 \pm 2.4$</td>
<td>$41.2 \pm 2.3$</td>
</tr>
<tr>
<td>ALKP, IU/l</td>
<td>$69.5 \pm 17.4$</td>
<td>$47.2 \pm 25.6$</td>
<td>$57.6 \pm 15.6$</td>
<td>$61.2 \pm 49.4$</td>
</tr>
</tbody>
</table>

* $p < 0.05$.

**Table 2. Background and laboratory tests of patients according to stage subgroups**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>Stage 0 (n =17)</th>
<th>Stage 1 (n = 10)</th>
<th>Stage 2–3 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>4/4</td>
<td>8/9</td>
<td>6/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Age</td>
<td>$50.6 \pm 15.2$</td>
<td>$50.6 \pm 14.8$</td>
<td>$47.2 \pm 14.2$</td>
<td>$59.2 \pm 16.3$</td>
</tr>
<tr>
<td>Liver MMP-2</td>
<td>$23.1 \pm 7.9$</td>
<td>$21.4 \pm 5.6$</td>
<td>$19.6 \pm 5.2$</td>
<td>$20.1 \pm 4.2$</td>
</tr>
<tr>
<td>Liver MMP-9</td>
<td>$17.1 \pm 5.1$</td>
<td>$21.9 \pm 4.5$</td>
<td>$18.9 \pm 4.8$</td>
<td>$20.1 \pm 4.1$</td>
</tr>
<tr>
<td>Serum MMP-9</td>
<td>$163.5 \pm 35.1^*$</td>
<td>$342.0 \pm 121.1$</td>
<td>$305.9 \pm 81.0$</td>
<td>$397.5 \pm 331.0$</td>
</tr>
<tr>
<td>Bilirubin total*, µmol/l</td>
<td>$8.33 \pm 3.4$</td>
<td>$13.6 \pm 6.8$</td>
<td>$12 \pm 3.4$</td>
<td>$17.34 \pm 3.4$</td>
</tr>
<tr>
<td>Bilirubin direct*, µmol/l</td>
<td>$2.89 \pm 1.7$</td>
<td>$5.73 \pm 3.4$</td>
<td>$4.42 \pm 1.53$</td>
<td>$6.97 \pm 3.4$</td>
</tr>
<tr>
<td>ALT*, IU/l</td>
<td>$49.6 \pm 30.6$</td>
<td>$91.53 \pm 74.0$</td>
<td>$105.3 \pm 36.9$</td>
<td>$187.8 \pm 129.1$</td>
</tr>
<tr>
<td>AST*, IU/l</td>
<td>$46.7 \pm 28.7$</td>
<td>$63.4 \pm 36.6$</td>
<td>$72.2 \pm 21.5$</td>
<td>$153.3 \pm 28$</td>
</tr>
<tr>
<td>Protein, g/dl</td>
<td>$69.2 \pm 5.7$</td>
<td>$71.2 \pm 5.2$</td>
<td>$72.6 \pm 4.2$</td>
<td>$72.4 \pm 6.8$</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>$41.5 \pm 2.4$</td>
<td>$41.2 \pm 3.7$</td>
<td>$42.0 \pm 2.1$</td>
<td>$41.2 \pm 2.4$</td>
</tr>
<tr>
<td>ALKP, IU/l</td>
<td>$69.5 \pm 17.4$</td>
<td>$49.3 \pm 23.7$</td>
<td>$56.7 \pm 32.9$</td>
<td>$60.0 \pm 23.4$</td>
</tr>
</tbody>
</table>

* $p < 0.05$. 

Results
and controls. Although liver MMP-9 was higher in the fibrotic group than in the control group, it did not reach statistical significance (fig. 1). In contrast, the mean serum level of MMP-9 was significantly higher in the HCV group compared to the control group (344 ± 177 vs. 163.5 ± 35, p < 0.05). Moreover, serum MMP-9 was correlated with the histological grading (307.6 ± 130, 290.4 ± 83, 562.1 ± 128), for histological necroinflammation grade, 1, 2, and 3, respectively, p = 0.01) (table 1; fig. 1a). No statistically significant correlation was found between serum MMP-9 and histological stage of fibrosis (342.0 ± 121, 305.9 ± 81, 397.5 ± 331 for 0, 1 and 2–3 stage subgroups, respectively) (table 2). Nevertheless, the highest serum level of MMP-9 was detected in the group of highest degree of fibrosis stage (p = 0.02; fig. 1b). As depicted in figure 1a the highest increase in serum MMP-9 occurred between histological grade 2 and grade 3. Furthermore, this increase seemed to be more prominent than the increase in transaminases. The level of MMP-9 in the liver tissue was positively correlated to both liver MMP-2 (fig. 2a; r = 0.44, p = 0.01), and serum MMP-9 (fig. 2b; r = 0.38, p = 0.02) levels. No MMP-2 activity was detected in the serum of HCV patients as well in the controls.

Serum ALT, and AST (U/l) as well as serum bilirubin (µmol/l) levels were significantly higher in HCV patients in comparison to controls (96 ± 28 vs. 46.7 ± 28, 127.6 ± 80 vs. 49.6 ± 30.6, and 13.09 ± 4.42 vs. 8.33 ± 3.4, respectively, figure 1, p < 0.05). Finally as shown in figure 1 a the increase in serum MMP-9 levels revealed a closer correlation with histologic grading than the elevation of serum AST or ALT.

**Discussion**

Hepatic fibrosis is a wound-healing response characterized by accumulation of ECM that follows chronic but not self-limited liver injury or disease. Ultimately, hepatic fibrosis leads to cirrhosis, characterized by nodule formation and organ scarring and contraction [22].

Accurate assessment of the extent of fibrosis is essential in guiding management and predicting prognosis in...
patients with chronic liver injury [23]. Histologic assessment of a liver biopsy specimen is the gold standard for quantifying fibrosis [24, 25]. There has recently been considerable effort aimed at identifying serum markers as noninvasive measures of hepatic fibrosis. Although the accuracy and predictive value of these markers are improving, they cannot yet supplant direct analysis of liver tissue [25–27].

HCV is one of the leading causes of chronic liver disease and liver cirrhosis. Chronic hepatitis develops in over 60% of individuals after acute HCV infection, with an estimate of 50% of them that progress to liver cirrhosis [28, 29]. Yet, the pace of progression has not been clearly denoted. It is based on clinical predictors such as age, gender, and alcohol use [30, 31]. The best estimate of fibrosis progression is currently based on histopathologic assessment of inflammatory activity and fibrosis stage. Liver histology is considered the gold standard for assessing hepatic fibrosis [32, 33]. However, liver biopsy is associated with sampling error, interserver variability, and potential complications. In addition, tracking of liver disease progression requires repeated liver biopsies [33]. Therefore, laboratory tests that would predict fibrosis progression or will evaluate fibrosis score would contribute to guide therapeutic intervention in patients with HCV infection. Thus, there is a need for simple, inexpensive, and reliable noninvasive means to assess disease severity and progression in patients with HCV. Routine laboratory tests such as AST and ALT correlate weakly with disease activity (necroinflammatory scores on liver biopsy) and little or not at all with hepatic fibrosis. They may even be normal in cases of advanced liver disease [28, 29, 34]. Serum markers such as type III procollagen peptide, type IV collagen 7S domain, hyaluronic acid, laminin TGF-β have been assessed in several studies. None of these markers, however, have been found as a reliable test to determine fibrosis stage or to monitor fibrosis progression [33, 35, 36].

Liver fibrosis is a dynamic process, involving imbalance between matrix synthesis and regulated matrix degradation. It leads to the accumulation of ECM proteins, MMPs and TIMPs, which are involved in tissue remodeling during fibrotic and/or inflammatory processes [1–7, 37]. Several studies mainly in animal models of liver fibrosis found increased expression of MMPs in liver tissue [38–40]. However, only few studies were done in human which measured serum MMPs activity in chronic hepatitis and in particularly in CHV as markers for or estimating fibrosis, and aid in follow-up of patients with chronic hepatitis [41–44]. Ebata et al. [41] demonstrated higher level of MMP-2 in patients with chronic liver disease than in normal controls. Serum MMP-2 levels were also correlated with histological staging. Preaux et al. [45] showed activation of MMP-2 in fibrotic livers. Activation was inhibited by TIMP-2 but not by TIMP-1. Walsh et al. [46] found increased plasma levels of TIMP-1 and -2 with increased severity of liver disease in chronic hepatitis C. Lichtinghagen et al. [47] evaluated the role of MMP-2 and MMP-9 mRNA expression as markers of HCV disease activity. MMP-2 mRNA was expressed in liver and serum whereas MMP-9 mRNA was detected only in serum in leukocytes and was not correlated to extent of liver inflammation or fibrosis. Kasahara et al. [48] measured serum MMP-2 as well as TIMP-1 protein levels and found them (and not MMP-1) correlated to degree of liver necrosis, inflammation and fibrosis. Moreover, they found these proteins higher in non-responder patients.

In this study we evaluated the role of MMP-2 and MMP-9 as potential markers of disease activity and fibrosis stage. We measured simultaneously serum and liver MMP-2 and MMP-9 activities and correlated those to histologic necroinflammatory activity and fibrosis stage. We could identify a small group of non-HCV patients with normal liver histology who served as controls. Thus, we were able to assess whether MMP-2 and MMP-9 are markers of inflammation, fibrosis or both. In addition, we could compare serum MMP activity to liver tissue levels. Our study demonstrates that liver MMP-2 and MMP-9 are not correlated with either histological activity or fibrosis stage. In contrast, serum MMP-9 was found to be correlated with disease activity but not with fibrosis histologic stage. These results indicate that MMP-2 and MMP-9 are not valuable markers of fibrosis in HCV. However, serum MMP-9 was found to be a good predictor of disease inflammation and was even superior to serum transaminase levels. This finding may help in the assessment of HCV patients where transaminase levels may be normal despite profound disease. For hepatitis C it is clear that fibrosis progression correlates imperfectly with ALT and AST [49]. A more accurate test would involve markers directly related to fibrogenesis. Therefore, it appears from this study that serum MMP-9 may serve as a good marker to assess disease activity in HCV patients which may provide a good tool to determine whether to initiate treatment and to follow response of treatment in this group of patients.

Inflammatory and immunologic processes regulate MMP-2 and MMP-9 activity [50, 51]. These MMPs are implicated in leukocyte recruitment and transimmigration [52]. In addition, there is close interaction between...
inflammatory cytokines adhesion molecules and MMPs [53]. Generally, MMP-2 is constitutively expressed and MMP-9 is inducible by a variety of cytokines such as TNFs and interleukins (IL), while MMP-9 reciprocally enhances or downregulates the activity of chemokines and cytokines [54]. Indeed, several studies indicated that in chronic inflammation there is upregulation of MMP-9 either by TNFs or IL-1 via CD44 expressed by inflammatory cells especially by human leukocytes [55, 56]. In rheumatoid arthritis MMPs were found to have a role in the inflammation process with regulatory ability of pro-inflammatory cytokines [57].

In hepatitis C the liver damage is mainly caused by an inflammatory response to viral infection, while the fibrosis is believed to be a response to the immunologic process. Therefore, serum MMP-9 may be a marker of the inflammation and not a product of matrix degradation in the scenario of HCV. Moreover, MMP-9 may reflect inflammation and not hepatocyte damage or necrosis. Thus, it may not be surprising that we found MMP-9 activity to be a more sensitive marker of histologic grade of inflammation. Further studies are needed to look at the source of MMPs in HCV, looking at specific inflammatory cells reflecting rather the inflammatory process than fibrosis degradation.

References


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