ORIGINAL ARTICLE

Liver and serum expression of matrix metalloproteinases in asymptomatic pediatric liver transplant recipients

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SUMMARY

We related hepatic gene and serum expression of matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP) to liver histology in pediatric LT recipients. Liver biopsies and serum samples were obtained from 52 patients 10.6 years post-LT and age-matched controls for analyses of MMPs and TIMPs. Patients with fibrosis had significantly higher hepatic gene expression of MMP-2, MMP-9, MMP-14, TIMP-1, and TIMP-2 than patients without. Expression of these genes correlated with graft Metavir fibrosis stage ($r = 0.494$–0.684, $P \leq 0.006$ for all). Gene expression of MMP-1, MMP-3, MMP-8, TIMP-3, and TIMP-4 was undetectable in both patients and controls. Portal inflammation and cytokeratin 7 correlated positively with gene expression of TIMP-1. Gene expression of MMP-2, MMP-9, and TIMP-2 correlated negatively with the time of low-dose cortisone usage ($r = -0.448$ to -0.422, $P < 0.05$ for all). Serum concentrations of MMP-8 and TIMP-1 were significantly increased and MMP-9 decreased among patients compared with controls, but no correlations to graft histology or gene expression were observed. Hepatic gene expression of certain MMPs and TIMPs is increased in stable pediatric LT recipients displaying graft fibrosis, but this did not reflect to their serum concentrations. Increased hepatic gene expression of TIMP-1 correlated with graft fibrosis stage, inflammation, and chronic cholestasis.

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Introduction

High survival rates and long life expectancy following pediatric liver transplantation (LT) have moved the focus to long-term graft function and overall health. Protocol liver biopsies in asymptomatic recipients have shown surprisingly high rates of graft inflammation (26–87%), fibrosis (22–97%), and steatosis (43%) [1,2]. These alarming histological changes usually occur without alterations in the routine biochemical markers of liver injury or function, while their etiology and clinical significance are currently poorly understood. In our previous work, fibrosis correlated with portal inflammation, being less frequent among those with low-dose steroids, whereas steatosis only associated with high body mass index [1]. Inflammatory changes in patients

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with stable graft function have been attributed to chronic immunological challenge, representing a form of chronic rejection [3].

Liver fibrosis—and eventually cirrhosis—is the end-stage histological response to a chronic liver injury regardless of the initial cause. Persistent injury and associated inflammation result in an imbalance between extracellular matrix (ECM) production primarily by activated hepatic stellate cells (HSCs) and fibrinolysis by matrix metalloproteinases (MMPs) leading to accumulation of fibrosis instead of resolution and liver regeneration. Fibrotic tissue represents composition of excessive nonfunctional extracellular matrix, mainly collagen [4]. HSCs play a crucial role in fibrogenesis. In liver injury, they are activated by inflammatory cytokines such as IL-6, TNF-α, or IL-1 to proliferative myofibroblasts. Activated HSCs produce ECM components, promoting secretion of MMPs and their tissue inhibitors (TIMPs) in concert with lymphocytes [5]. MMPs are the main proteinases involved in extracellular matrix degradation, and TIMPs are their major regulators favoring fibrogenesis. Altered secretion or an imbalance between MMP and TIMP activity may lead to accumulation of ECM and progression of fibrosis [6].

We hypothesized that hepatic gene expression of MMPs and TIMPs is altered and related to graft histopathology, especially fibrosis, long term after pediatric LT in patients with stable graft function. We further hypothesized that the hepatic gene expression could be reflected by altered serum concentrations of MMPs and TIMPs. To this end, hepatic gene expression and serum expression of various MMPs and TIMPs were measured in a controlled fashion after a median follow-up of 10 years in a population-based cohort of pediatric LT recipients and related to graft fibrosis, inflammation, steatosis, and chronic cholestasis.

Patients and methods

Patients and controls

A total of 99 pediatric patients (age <18) underwent deceased donor LT in Finland between 1987 and 2007. All transplantations were performed at the Helsinki University Central Hospital, where the post-transplant follow-up of these patients is also conducted. This was a cross-sectional population-based study of all 66 survivors, of whom 52 (79%) participated at a median of 10.6 [interquartile range (IQR) 4.0–17.8] years after LT. They underwent protocol liver biopsy and serum sampling during the same hospital visit. The patients displayed stable graft function at the time of the study, as shown in Table 1.

Control blood samples were obtained from 94 day-surgery patients without evidence of metabolic, gastrointestinal or hepatobiliary diseases matched for age [18.1 (11.2–21.8) vs. 14.3 (7.5–22.5) years in patients and controls, respectively, ($P = 0.110$)] and sex (27/25 vs. 50/44 male/female distribution in patients and controls, respectively, $P = 0.884$). Core needle liver biopsies from eight patients (three females) with median age of 11.9 (7.5–15.0) years operated for cholelithiasis were used as controls for gene expression analyses. Control liver biopsies were graded normal, excluding mild (grade 1) steatosis in one specimen and mild (Metavir stage 1) fibrosis in one.

Immunosuppression

Cyclosporine (CSA), azathioprine (AZA), and methylprednisolone (MP) were used for immunosuppression after LT. If clinically indicated, CSA was switched to tacrolimus and AZA to mycophenolate mofetil (MMF). MP dose was tapered to 0.25 mg/kg/day at 2 weeks after LT and then switched to alternate-day dosing at 6 months (0.1 mg/kg/day). In most cases, MP use was discontinued when the patient reached adulthood. At the time of this study, 36 patients used CSA and 13 were on tacrolimus. AZA was used by 28 patients and MMF by 14 patients. One patient was on sirolimus monotherapy, and 33 patients used MP.

Liver biopsies and histological assessment

Liver biopsy specimens were percutaneous core needle biopsy samples taken under ultrasound guidance by an experienced pediatric radiologist. The median sample length was 18 mm (range 10–31 mm), with median of 14 (range 10–41) portal tracts in each sample. For the youngest children, the procedure was performed under general anesthesia. Two pathologists with expertise in liver histopathology, who were blinded to clinical data, reviewed the biopsy samples to reach a consensus as described previously [1]. Portal inflammation was graded according to the Banff criteria on a scale of 0–3 [(0) none, (1) mild, (2) moderate, and (3) severe]. Fibrosis was staged according to the Metavir staging on a semiquantative scale of 0–4 [(0)none, (1) mild portal fibrosis, (2) moderate scarring extending from portal areas, (3) moderate fibrosis with bridging, and (4) extensive fibrosis or cirrhosis]. The amount of macrovesicular steatosis and microvesicular steatosis

Table 1. Characteristics of patients with or without fibrosis.

	Patients with fibrosis (n = 20)	Patients without fibrosis (n = 32)	P-value
Age (years)	14.7 (9.9–25.4)	18.7 (15.1–21.2)	0.413
Gender n (male/female)	13/7	14/18	0.139
Height (cm)	154 (131–172)	160 (152–169)	0.435
Weight (kg)	52 (28–62)	53 (45–69)	0.403
BMI (kg/m²)	19.2 (17.6-23.2)	20 (17.1–24.4)	0.579
Diagnosis of liver disease			
Biliary atresia	8 (40%)	10 (31%)	
Metabolic disease*	3 (15%)	8 (25%)	
Hepatitis	4 (20%)	1 (3%)	
Hepatic malignancy	3 (15%)	3 (9%)	
PKD/congenital fibrosis	1 (5%)	4 (13%)	
Other†	1 (5%)	6 (19%)	
Surgical characteristics			
Age at LT (years)	2.0 (1.3–8.3)	3.0 (1.1–13.6)	0.585
Age at liver biopsy (years)	14.7 (9.8–25.4)	18.3 (15.3–21.1)	0.419
Whole/reduced size graft	5/15	10/22	0.632
Total ischemia time (min)	497 (403–558)	519 (434–576)	0.438
Roux-Y (yes/no) (n = 49)	15/4	22/9	0.537
Donor age (years)	17.9 (13.1–38.8)	24.7 (14.9–44.8)	0.560
Acute rejections (yes/no)	6/14	14/18	0.326
Cortisone time (years)	9.0 (4.1–12.0)	6.2 (3.1–16.0)	0.897
Laboratory data			
Bilirubin total (μmol/l)	11 (7–16)	10 (8–14)	0.792
AST (U/l)	37 (29–44)	28 (23–38)	**0.031**
ALT (U/l)	22 (14–36)	24 (14–35)	0.977
AP (U/l)	181 (106–224)	130 (94–192)	0.128
Pre-albumin (mg/l) (n = 48)	183 (159–227)	220 (176–266)	0.050
GGT (U/l)	20 (14–43)	26 (12–35)	0.858
Creatine (μmol/l)	61 (41–96)	68 (50–79)	0.631
Platelets (E9/l)	215 (156–240)	205 (165–284)	0.721
Cholesterol total (mmol/l)	3.5 (2.9–4.2)	4.1 (3.4–4.5)	**0.028**
LDL (mmol/l)	1.8 (1.6–2.2)	2.2 (1.6–2.6)	0.065
HDL (mmol/l)	1.4 (1.1–1.7)	1.4 (1.2–1.7)	0.870
Triglyceride (mmol/l)	0.9 (0.6–1.1)	0.94 (0.80–1.36)	0.161
APRI	0.37 (0.24–0.63)	0.28 (0.21–0.53)	0.220

Data are presented as median (IQR) or frequency. LT, liver transplantation; AST, aspartate transaminase; ALT, alanine transaminase; AP, alkaline phosphatase; GGT, gamma-glutamyltransferase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; APRI, AST to platelet ratio index; PKD, polycystic kidney disease.

*Diagnostic group metabolic included: familial hypercholesterolemia (1), hyperoxaluria (1), morbus Wilson (2), OTC deficiency (1), tyrosinemia (6).

†Diagnostic group other included: Budd–Chiari syndrome (1), HUS (1), extrahepatic portal vein thrombosis (1), liver failure of unknown etiology (1), MIRAS mitochondrial recessive ataxia syndrome (1), iron poisoning (1), sclerosing cholangitis (1).

was presented as the percentage of affected hepatocytes. Immunostaining was performed for cytokeratin 7 (CK-7), which is a marker of chronic cholestasis [7]. The CK-7 immunopositivity of periportal hepatocytes was graded semiquantitatively on a scale of 0–3 [(0) none, (1) mild, (2) moderate, and (3) marked]. CK-7 samples were also used to score bile ductal proliferation, which was reported on a scale of 0–2 [(0) none, (1) mild, and (2) marked].

Hepatic gene expression analyses

Gene expression was evaluated in a subset of 29 patients with sufficient biopsy material available for both histologic and RNA analyses. Prevalence of liver fibrosis was slightly higher among these 29 patients (18/29, 62%) than in the entire patient material (20/52, 38%). Liver tissue specimens were embedded in RNAlater solution (Ambion, Life Technologies, Thermo Fisher

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Scientific Inc., Waltham, MA, USA) and freezed until analyzed. RNA was extracted using the RNeasy Mini Kit (QIAGEN, Frederick, MD, USA) and integrity assessed spectrophotometrically. Gene expression of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, MMP-14, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 was analyzed in triplicate by quantitative real-time polymerase chain reaction using the Human Fibrosis RT^2 Profiler™ PCR Array (QIAGEN SABiosciences) on an ABI 7700 Sequence Detection System (Perkin-Elmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions (www.sabiosciences.com). Quantification of target gene mRNA expression was performed using the $\Delta\Delta C_t$ method and expressed after normalization to housekeeping genes and relative to control subjects.

Biochemical analyses

Serum was available for MMP analyses in 51 patients. Serum concentrations of MMP-9 and TIMP-1 were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits as earlier described [8]. MMP-8 analyses were carried out by time-resolved immunofluorometric assay (IFMA) (Medix Biochemica, Kauniainen, Finland) with the interassay coefficient of variations (CV) 7.3% and a detection limit of 0.8 ng/l. The CV% for MMP-9 and TIMP-1 were 8.8% and 13.1% and detection limits were 0.6 ng/ml and 1.25 ng/ml, respectively. The calculations of MMP/TIMP-1 ratios were performed as mol/l. Measurements of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), bilirubin, gamma-glutamyltransferase (GGT), pre-albumin, creatinine, and blood count were conducted by routine laboratory methods. Serum total and high-density (HDL) cholesterol and triglycerides were determined enzymatically using standard methods. Low-density lipoprotein cholesterol concentration was calculated. AST to platelet ratio index (APRI) was calculated as described previously by Wai *et al.* [9].

Ethics

This study was approved by the ethics committee of the Children's Hospital, Helsinki and Uusimaa Hospital District (IRB approval number 345/13/03/03/2008). All participating patients and controls (and parents in the case of minors) signed an informed consent form.

Statistical analysis

Statistical analyses were performed with SPSS 21.0 statistics software (IBM, Somers, NY, USA). Data are presented as medians and IQR, if not otherwise stated. To compare differences between groups, Mann–Whitney *U*-test was used for continuous variables. Holm–Bonferroni correction was used for multiple comparisons. Correlations were calculated with the Spearman's rho two-tailed test. The predictive values were assessed with the area under the receiver operating characteristic

Table 2. Frequency and extent of the main histological findings of protocol liver biopsies a median of 10.6 years after liver transplantation in 52 children.

Histological finding	Present (%)
Fibrosis (0–4)	20 (39)
0	32 (61)
1	16 (31)
2	2 (4)
3	2 (4)
4	0 (0)
Portal inflammation (0–3)	13 (25)
0	39 (75)
1	12 (23)
2	0 (0)
3	1 (2)
Macrovesicular steatosis (0–100%)	4 (8)
0–<5%	48 (92)
5–33%	4 (8)
Microvesicular steatosis (0–100%)	20 (39)
0–<5%	32 (61)
5–33%	15 (29)
>33–67%	1 (2)
>67–100%	4 (8)
Periportal CK-7 immunopositivity (0–3)	12 (24)
0	39 (76)
1	8 (16)
2	3 (6)
3	1 (2)
Ductal proliferation (0–2)	22 (43)
0	29 (57)
1	19 (37)
2	3 (6)

CK-7, cytokeratin 7. Fibrosis graded according to the Metavir score on a scale 0–4. Inflammation graded according to the Banff criteria on a scale 0–3. Microvesicular steatosis and macrovesicular steatosis graded according to the percentage of hepatocytes affected 0–100%. CK-7 immunopositivity was graded semiquantatively on scale 0–3. CK-7 samples were also used to measure bile ductal proliferation, which was reported on a scale of 0–2.

curve (AUROC) analysis. *P*-values less than 0.05 were considered statistically significant.

Results

Liver histopathology

Main histopathological findings among LT patients are shown in Table 2. Graft fibrosis was present in 20 (39%) patients and portal inflammation in 13 (25%). Microvesicular steatosis was observed in 20 (39%), of whom 4 (8%) also displayed macrovesicular steatosis. Ductal proliferation was present in 22 patients (43%) and CK-7 immunopositivity of periportal hepatocytes in 12 (24%).

Hepatic gene expression of MMPs and TIMPs relates with graft fibrosis

Although only minor changes in gene expression patterns between patient and controls were observed, patients with graft fibrosis had significantly higher hepatic gene expression of MMP-2, MMP-9, MMP-14, TIMP-1, and TIMP-2 than patients without fibrosis, as shown in Table 3. The increase in gene expression was most striking for MMP-9. Gene expression of MMP-2, MMP-9, MMP-14, TIMP-1, and TIMP-2 also correlated positively with the Metavir fibrosis stage, as shown in Fig. 1a–e. The strongest correlation was observed for TIMP-2. Hepatic gene expression of MMP-1, MMP-3, MMP-8, TIMP-3, and TIMP-4 was undetectable or very close to the detection limit in both patients and controls.

Inflammation, CK-7 positivity, and hepatic gene expression of TIMP-1

Patients who displayed portal inflammation had increased hepatic gene expression of TIMP-1 when compared to patients without inflammation or controls [1.42 (1.23–1.60) vs. 1.14 (0.97–1.38) $P = 0.025$ vs. 1.01 (0.85–1.19) $P = 0.018$, respectively], as shown in Fig. 2a. Grade of inflammation correlated positively with hepatic gene expression of TIMP-1 ($r = 0.421$, $P = 0.023$). Patients with CK-7 immunopositivity had increased hepatic gene expression of TIMP-1 when compared to patients without or controls [1.52 (1.16–1.79) vs. 1.13 (0.96–1.23) $P = 0.005$ and 1.01 (0.85–1.19) $P = 0.032$, respectively], as shown in Fig. 2b. CK-7 immunopositivity correlated positively with hepatic gene expression of TIMP-1 ($r = 0.554$, $P = 0.002$). Area under the receiver operating curve (AUROC) for hepatic gene expression of TIMP-1 was 0.786 (95% CI: 0.622–0.950, $P = 0.025$) to predict graft inflammation. Hepatic gene expression of other MMPs or TIMPs did not differ between patients with or without inflammation or CK-7 immunopositivity, nor correlated with them. Ductal proliferation shoved no correlations with the analyzed RNA expressions.

Serum MMP-8, MMP-9, and TIMP-1 in relation to histopathology

Patients had significantly higher serum concentrations of MMP-8 and TIMP-1 and lower MMP-9 concentration compared with controls, as shown in Table 4. Serum concentrations of MMP-8, MMP-9, or TIMP-1 were unrelated

Table 3. Hepatic gene expression of matrix metalloproteinases (MMPs) and tissue inhibitor (TIMP)-1 in controls and in patients with or without fibrosis. Data presented as median and interquartile range (IQR).

	Controls ($n = 8$)	Patients ($n = 29$)	*P*-value*	Patients without fibrosis ($n = 11$)	Patients with fibrosis ($n = 18$)	*P*-value**
MMP-2	0.98 0.84–1.01	0.84 0.60–1.04	0.530	0.52 0.43–0.84	1.03 0.79–1.88	0.002
MMP-9	0.92 0.58–1.43	1.29 0.78–2.66	0.094	0.76 0.66–2.04	2.22 0.96–4.46	0.008
MMP-14	1.05 0.71–1.22	1.06 0.80–1.41	0.605	0.88 0.69–1.00	1.22 0.98–1.46	0.009
TIMP-1	1.01 0.85–1.19	1.17 0.98–1.51	0.135	1.00 0.94–1.27	1.29 1.15–1.54	0.031
TIMP-2	1.10 0.87–1.14	0.77 0.63–0.96	0.029***	0.64 0.57–0.72	0.94 0.73–1.02	0.001

P-value calculated with Mann–Whitney *U*-test and Holm–Bonferroni correction between controls and patients * and between patients with or without fibrosis **.
***P*-value 0.029 abandoned after Holm–Bonferroni correction.

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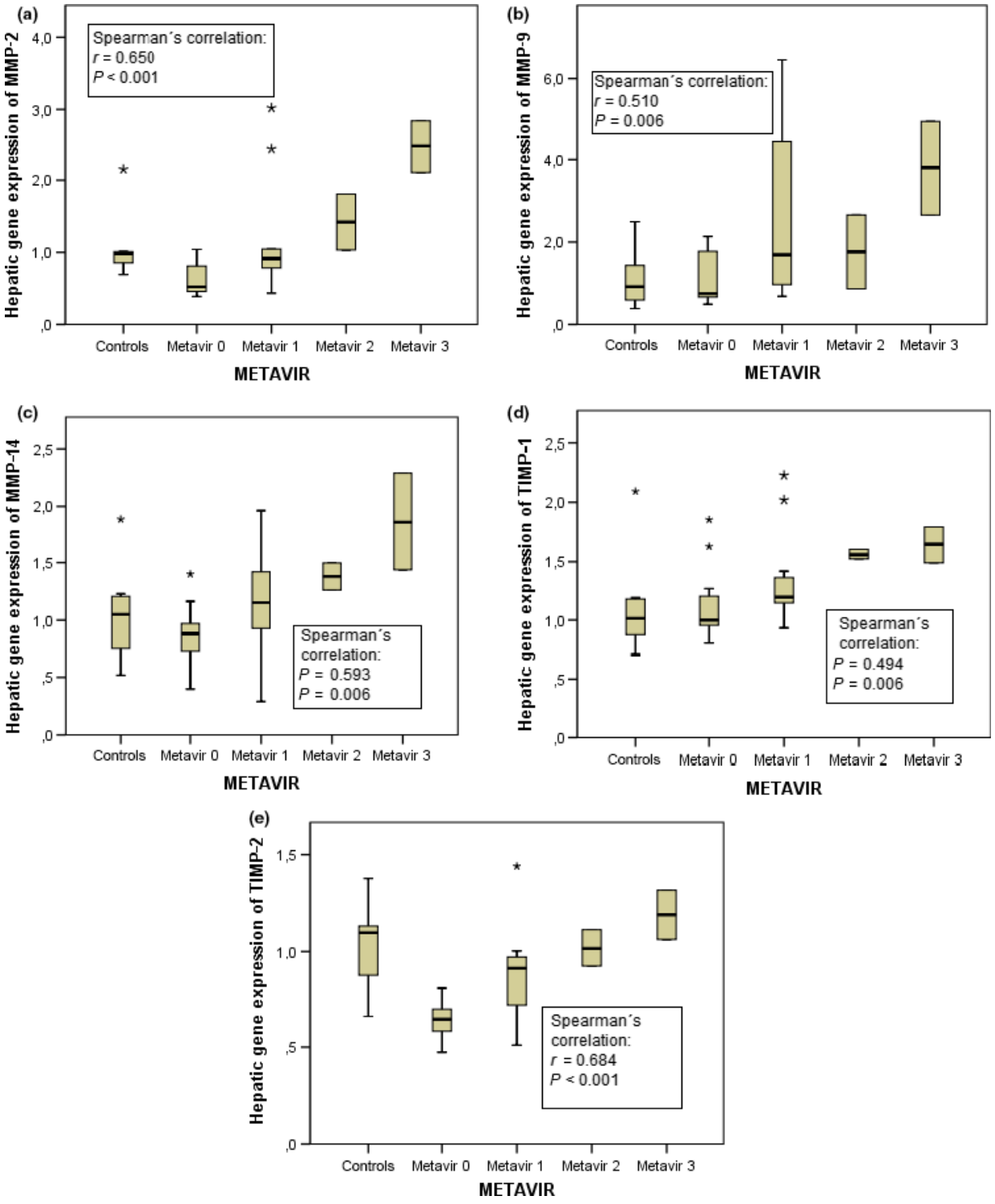

Figure 1 Hepatic gene expression of matrix metalloproteinases (MMPs) and tissue inhibitors (TIMPs) in controls and in liver transplantation (LT) patients with different stages of liver fibrosis. (a) MMP-2, (b) MMP-9, (c) MMP-14, (d) TIMP-1, and (e) TIMP-2. Spearman's rank correlation coefficient and associated *P*-value are shown for patients with different METAVIR fibrosis stages. Box plot display median (bold transverse line), interquartile range (rectangle), and range.

to respective hepatic gene expressions or histologic liver graft injury. However, patients with graft fibrosis had significantly higher serum concentrations of MMP-8 [97 (56–139) vs. 25 (12–59) $P = 0.000$] and TIMP-1 [131 (111–148) vs. 92 (75–113) $P = 0.000$] and lower MMP-9 [201 (114–300) vs. 379 (277–563) $P = 0.000$] compared

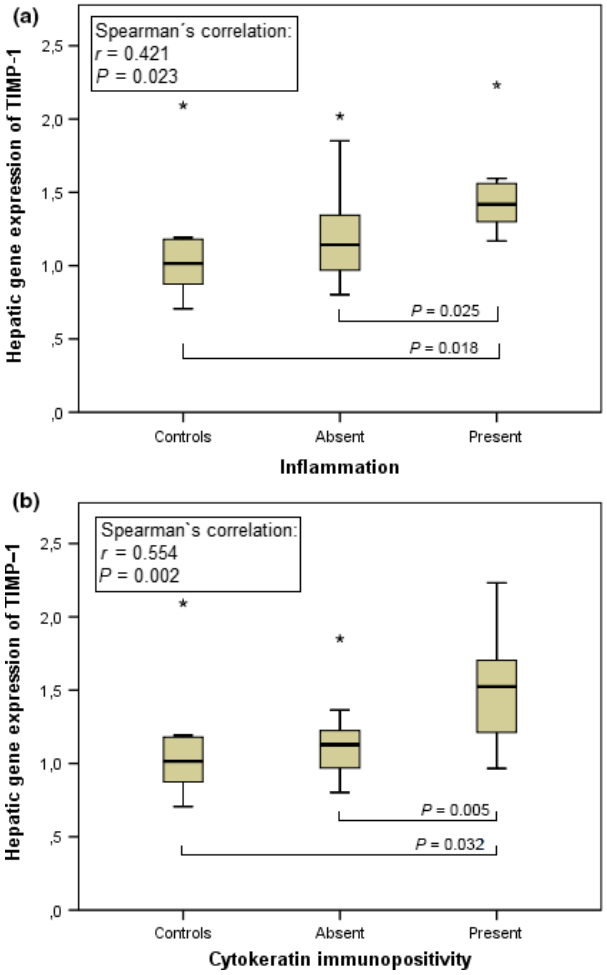

Figure 2 Hepatic gene expression of tissue inhibitor (TIMP)-1 in relation to (a) portal inflammation and (b) cytokeratin 7 positivity (chronic cholestasis) in controls and patients. Mann–Whitney *U*-test was used for comparisons between different groups. Spearman's correlation was calculated among patients. Box plot display median (bold transverse line), interquartile range (rectangle), and range.

with controls, as shown in Table 4. Serum concentrations of MMP-8 and TIMP-1 were also higher among patients with inflammation [MMP-8: 114 (35–212) vs. 25 (12–59) $P = 0.000$ and TIMP-1: 136 (112–203) vs. 92 (75–113) $P = 0.000$], CK-7 immunopositivity [MMP-8: 79 (28–139) vs. 25 (12–59) $P = 0.005$ and TIMP-1: 140 (124–164) vs. 92 (75–113) $P = 0.000$], and steatosis [MMP-8: 110 (49–162) vs. 25 (12–59) $P = 0.000$ and TIMP-1: 123 (95–142) vs. 92 (75–113) $P = 0.000$] compared with controls. Serum concentration of MMP-9 was lower among patients with CK-7 immunopositivity [256 (82–364) vs. 379 (277–563) $P = 0.011$] and steatosis [280 (148–526) vs. 379 (277–563) $P = 0.045$] compared with controls. Patients with ductal proliferation showed unaltered serum MMP-8, MMP-9, or TIMP-1 compared with controls (data not shown).

Immunosuppression and history of acute rejections

Hepatic gene expression of MMP-2, MMP-9, and TIMP-2 correlated negatively with the time of low-dose cortisone usage ($r = -0.448$ $P = 0.015$, $r = -0.444$ $P = 0.018$, and $r = -0.422$ $P = 0.022$). The number of acute rejections was unrelated to serum concentrations or hepatic gene expression of MMPs or TIMPs.

Discussion

In this cross-sectional, population-based controlled study, serum and hepatic RNA expression of various MMPs and TIMPS were measured among pediatric LT recipients with stable graft function. The major findings were that patients with graft fibrosis had increased hepatic RNA expression of MMP-2, MMP-9, MMP-14, TIMP-1, and TIMP-2, while gene expression of mostly fibrolytic MMP-1, MMP-8, MMP-13, TIMP-3, and TIMP-4 was undetectable in both patients and controls. TIMP-2 had the strongest correlation with the fibrosis stage, while the hepatic gene expression of TIMP-1 also was related to graft inflammation and chronic cholestasis (CK-7 immunopositivity). Duration of low-dose cortisone usage was inversely associated with activation of MMP-2, MMP-9, and TIMP-2 gene expression in the liver graft. Although the serum concentrations of MMP-8, MMP-9, and TIMP-1 were significantly altered after LT, no connections to graft histopathology were observed, limiting their usefulness in follow-up purposes.

Varying expression of MMPs and TIMPS occurs during both fibrogenesis and fibrolysis, and their expression varies over time. Increased expression of MMP-2, MMP-9, MMP-14, and TIMP-1 is mostly observed during accumulation of fibrotic scar or in cirrhosis [10–12]. In our study, MMP-2, MMP-9, MMP-14, and TIMP-1 gene expressions were closely related to graft fibrosis, suggesting an important role also in graft fibrogenesis after LT. Collagenases MMP-1, MMP-8, and MMP-13 are important mediators of fibrolysis, as they are mainly responsible for cleavage of fibrillar collagen. In our study, hepatic gene expression of these MMPs was undetectable, pointing to limited fibrolytic activity among these patients.

Matrix metalloproteinase-2 associates with both fibrosis and inflammation of the liver [11]. In a study on patients with cirrhosis, hepatic expression of MMP-2 was elevated compared with healthy controls [12]. Hepatic gene expression and serum levels of MMP-2 were also increased in patients with steatohepatitis compared to those with simple steatosis [13]. Patients with acute

Table 4. Serum concentrations of matrix metalloproteinases (MMPs) (ng/ml) and tissue inhibitor (TIMP)-1 (ng/ml), their molar ratios in controls and patients with or without fibrosis.

	Controls (n = 94)	Patients (n = 51)	P-value*	Patients without fibrosis (n = 32)	Patients with fibrosis (n = 19)	P-value**
MMP-8	25 12–59	93 50–159	0.000	85 39- 201	96 56–139	0.907
MMP-9	379 277–563	268 146–459	0.002	347 159–554	201 114–300	0.076
TIMP-1	92 75–113	127 111–153	0.000	127 105–155	131 111–148	0.969
MMP-8/TIMP-1	0.12 0.06–0.28	0.31 0.15–0.60	0.000	0.30 0.14–0.64	0.33 0.22–0.52	0.899
MMP-9/TIMP-1	1.33 0.80–2.02	0.59 0.32–1.03	0.000	0.77 0.34–1.26	0.41 0.28–0.88	0.147

Results presented as median and IQR. P-values calculated with Mann–Whitney U-test and Holm–Bonferroni correction between controls and patients * and between patients with or without fibrosis **.

rejection after LT had stronger immunostaining of MMP-2 compared to patients with cirrhosis or healthy donors [14]. Our results extend these observations by demonstrating an association between MMP-2 gene expression and graft fibrosis in asymptomatic patients after LT.

Mainly expressed by liver Kupffer cells and inflammatory macrophages, MMP-9 has activity against gelatins, a wide range of collagens and elastin [15]. In earlier studies, serum MMP-9 decreased in patients with chronic hepatitis and correlated inversely with fibrosis stage [16,17]. Evidence from mice supports increased hepatic expression of MMP-9 during initiation of fibrosis [11,18]. In mice with toxin-induced liver fibrosis, expression of MMP-9 was upregulated during fibrogenesis and downregulated during fibrolysis [19]. In patients with chronic viral hepatitis, serum and hepatic expression of MMP-9 decreased in those who responded to antiviral treatment [20]. In our study, hepatic gene expression of MMP-9 was increased among the patients with fibrosis, but this was not reflected by MMP-9 serum concentrations as they decreased after LT similar to patients with chronic hepatitis [16,17]. MMP-9 may be bound to the liver effectively during active fibrogenesis and inflammation leading to decreased serum concentrations.

Tissue inhibitor-1 is a major regulator of MMPs and has a key role in fibrogenesis. It is produced by HSCs [21] and also inhibits their apoptosis [22]. Serum TIMP-1 levels correlate with the degree of fibrosis in a variety of liver diseases [23–25]. In our study, hepatic gene expression of TIMP-1 was increased among patients with fibrosis compared with those without and correlated positively with the stage of fibrosis. Serum concentrations of TIMP-1 were also higher among patients with fibrosis compared with controls. TIMP-1 can promote endothelial proliferation and inflammation through MMP-independent pathways [26,27]. Accordingly, TIMP-1 expression increases in relation to histological inflammation in various inflammatory liver diseases [24,28,29]. In viral hepatitis, the liver concentration of TIMP-1 correlates with histologic inflammation and disease activity [30]. Accordingly, in our study, hepatic gene expression of TIMP-1 was higher in LT patients with inflammation or chronic cholestasis (CK-7 immunopositivity) and correlated with the stage of inflammation and CK-7 immunopositivity.

Hepatic gene expression of TIMP-2 was higher in patients with fibrosis compared with those without. It also had the strongest positive correlation to the fibrosis stage. Liang *et al.* [31] found similar results in hepatitis B patients, where serum TIMP-2 was lowest among controls and increased along with advancing stages of fibrosis. In a rat model of experimental liver fibrosis, serum levels and hepatic gene expression of TIMP-2 were elevated during liver injury and fibrosis, while the increase in serum TIMP-2 preceded upregulation of TIMP-2 expression in the liver [32]. The later finding suggests that serum MMPs and TIMPs are produced not only by the liver but also by other organs and circulating inflammatory cells. Collectively, our study supports TIMP-2 involvement in fibrosis after LT.

Adequate immunosuppression after LT is crucial. Time of low-dose cortisone usage appeared to have a favorable effect on MMP/TIMP balance, as it inversely correlated with gene expression of MMP-2, MMP-9, and TIMP-2. These findings are in line with and may partly explain our previous findings of less frequent graft portal inflammation among patients whose immunosuppression included steroids [1].

Serum concentrations of MMP-8, MMP-9, and TIMP-1 were unrelated to their hepatic gene expression or liver histopathology, suggesting that they have limited use in graft surveillance after LT. Earlier, Lichtinghagen *et al.* [33] found similar results by reporting a lack of correlation between serum MMPs and their hepatic gene expression in patients with chronic hepatitis. However, combination of other serum fibrosis markers such as amino-terminal propeptide of procollagen type III and hyarulonic acid with MMPs and TIMPs may improve their diagnostic accuracy [34].

Limitations of this study include the relatively small cohort size and cross-sectional design. Due to limited availability of biopsy material, we were unable to assess liver tissue levels, expression, or activities of MMPs and TIMPs. In addition, relatively mild fibrosis stage among our patients may have concealed some associations. This was, however, a controlled, population-based, single-center study, which provides novel evidence of altered hepatic RNA expression of MMPs and TIMPs in relation to histologic graft injury and immunosuppression in patients with stable graft function. Further studies exploring protein expression and activity of MMPs are needed to better understand their role in liver graft pathology beyond changes in gene transcription. In further studies, combination of the best markers of fibrosis could be related to the clinical course of individual patients to evaluate the diagnostic value of MMPs and TIMPs, which we are planning to perform in future studies.

Authorship

SHV and SKK: wrote the paper and performed data analysis. TIT and TAS: performed MMP analyses and wrote the paper. HJJ: designed the research and wrote the paper. MPP: designed the research, supervised data analysis, and wrote the first manuscript draft with Silja Voutilainen.

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Conflict of interest

The authors declare no conflict of interests.

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