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Serum Caspase-Cleaved Cytokeratin (M30) Indicates Severity of Liver Dysfunction and Predicts Liver Outcome

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Statistical Analysis C
Data Interpretation D
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Background: The Model for End-Stage Liver Disease (MELD) score is a well-established tool for assessing hepatic failure. The present retrospective study investigated whether serum keratin 18 (M65) and caspase-cleaved cytokeratin (M30) were associated with liver dysfunction and post-transplant graft failure.

Material/Methods: A total of 147 patients with liver cirrhosis were categorized into 2 groups according to their baseline MELD score (group I: MELD score <20, n=87, and group II: MELD score ≥20, n=60). Serum M65 and M30 levels were measured by ELISA.


Results: Cirrhotic patients had significantly higher serum M65 and M30 levels than healthy controls ($p < 0.0001$). Serum M65 was correlated with the MELD score and serum bilirubin ($p \leq 0.007$) and serum M30 was correlated with the MELD score, international normalized ratio, and serum bilirubin ($p \leq 0.001$). Group II had significantly higher serum M65 and M30 levels than group I (M65, $p = 0.025$ and M30, $p < 0.001$). Patients who lost the allograft during the first post-transplant year had significantly higher serum M30 levels than patients with a graft survival of >1 year ($p = 0.004$). In the regression analysis, serum M30 was associated with the MELD score (odds ratio [OR]=2.545, $p = 0.005$), serum bilirubin (OR=2.605, $p = 0.005$) and 1-year graft loss (OR=3.61, $p = 0.006$).

Conclusions: Our data indicate that serum M30 levels reflect the degree of liver dysfunction and can predict 1-year graft loss.

MeSH Keywords: Inflammation • Keratin-18 • Liver Cirrhosis

Abbreviations: ALT – alanine aminotransferase; AST – aspartate aminotransferase; CCL – C-C chemokine ligand; CCR – C-C chemokine receptor; CMV – cytomegalovirus; CRP – C-reactive protein; CXCR – C-X-C chemokine receptor; ESLD – end-stage liver disease; HBV – hepatitis B virus; HCC – hepatocellular carcinoma; HCs – healthy controls; HCV – hepatitis C virus; IL – interleukin; INR – international normalized ratio; IFN – interferon; LTx – liver transplant; M65 – Cytokeratin 18 (CK18); M30 – Caspase-cleaved CK18 fragments; MELD – Model for End-Stage Liver Disease; ROC – receiver operating characteristic; TGF – transforming growth factor; TNF – tumor necrosis factor

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Background

Cytokeratin 18 (CK18) or M65 is a major cytoplasmic intermediate filament protein in hepatocytes and cholangiocytes, as well as in epithelial cells and tissues [1,2]. M65 is overexpressed in most types of carcinoma, including breast, prostate, lung, colon, and ovary cancer [3]. Soluble CK18 is released from human carcinoma cells during cell death. The cytosolic pool of soluble CK18 is released during apoptosis [3]. Analyzing different forms of CK18 in patient sera identifies the mode of cell death and provides useful serum markers for predicting the clinical progression of epithelial malignancies [3–5]. The ratio of caspase-cleaved CK18 fragments (M30) to total M65 represents the balance between caspase-mediated apoptosis and non-proteolytic necrosis and can predict the clinical outcome of colorectal cancer [6].

In liver diseases, serum M30 and total M65 levels are clinically useful markers of liver fibrosis in patients with chronic hepatitis C virus (HCV) infection, acute-on-chronic hepatitis B, and hepatic and biliary inflammation in patients with chronic liver diseases [1,7–9].

There has been continued effort to improve prognostic scoring systems for the allocation of donor livers. The Model of End-Stage Liver Disease (MELD) score is a prognostic measure for hepatic insufficiency/failure and includes assessment of patient serum creatinine (Cr), serum bilirubin, and international normalized ratio (INR)/prothrombin time [10]. The MELD score predicts short- and medium-term survival in patients with liver cirrhosis [10,11] more accurately than the Child-Pugh classification [12,13]. In addition, it is associated with 3-month mortality rates in patients on the liver transplant waiting list, and is therefore useful in determining organ allocation [11]. Previous studies showed that liver disease patients with MELD scores >20 had significantly higher risk of mortality at all time points as compared to patients with MELD scores <20 [14–16].

In this study, we investigated whether serum M65 and M30 are associated with the severity of liver disease and 1-year graft loss in patients with hepatic cirrhosis. The sensitivity and specificity of these biomarkers and their predictive value in correlation to the MELD score were assessed.

Material and methods

Patients

Demographic data: The study was approved by the local ethics committee. In a retrospective study, we measured different parameters in 147 cirrhotic patients (age 51.8 ± 11.7 years, 45 females) who underwent liver transplantation (LTx) because

of end-stage liver disease (ESLD). Recipient characteristics are shown in Table 1. ESLD was caused by: alcoholic liver cirrhosis (n=45, 30.6%); congenital, autoimmune, and metabolic disorders, including cryptogenic cirrhosis, biliary disease, metabolic liver disease, autoimmune hepatitis, and amyloidosis (n=59; 40.1%); and viral hepatitis (n=43, 29.3%) (Table 1). Before surgery, we measured the severity of liver disease (MELD score) and serum levels of C-reactive protein (CRP), M65, M30, bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), INR, and albumin. To investigate viral infection status, we measured cytomegalovirus (CMV), hepatitis B virus (HBV) and HCV IgGs. Twenty-five participants were re-transplant patients and were equally distributed between the 2 groups (Table 1). Hepatic encephalopathy (51 patients) and renal insufficiency (46 patients) were significantly different between the 2 groups ($p \leq 0.005$) (Table 1). All samples were collected on the day of transplantation. Patients were classified as having either moderate (MELD score <20 (median value), group I, n=87) or severe (MELD score ≥ 20 , group II, n=60) liver disease (Table 1). We performed a “piggy-back” technique with end-to-side caval and end-to-end portal vein anastomosis for liver transplantation in all patients. We found no statistically significant differences between the 2 groups in pre-transplant body mass index (BMI) and lean body mass of recipients, operation time, cold ischemia time, and post-transplant administered immunosuppressive regimens, as well as donor age, donor sex, or CMV status of donors (Table 1). Operation time ($p=0.66$), cold ischemia time ($p=0.48$), and serum Cr at 3 ($p=0.58$), 6 ($p=0.82$), and 12 months ($p=0.48$) post-transplant were not significantly different between the 2 groups. Only 1 patient received a liver from a living donor. Combined liver-kidney transplant was performed in 6 patients (3 patients in each group: $p=0.64$). Early graft dysfunction developed in 17 of 60 patient with MELD ≥ 20 , and in 21 of 87 patients with MELD <20 ($p=0.57$). Post-operative site infection occurred in 7 of 87 and 8 of 60 patients ($p=0.30$) (Table 1). At 1 year after transplantation, 27 patients experienced graft failure (9 of 60 and 18 of 87: $p=0.38$). Thirty-three healthy controls (HCs) with no known active disease were included for comparison. Controls were free of infectious and other inflammatory illnesses.

Serum separation

Serum was collected after blood clotting in a serum separator tube by centrifugation at 4000 rpm for 15 min at 4°C. The serum was snap frozen and stored at -20°C until testing. All serum samples were thawed only once.

Determination of total CK18 (M65), M30 and neopterin levels

Serum M65 (detection limit 60 U/L and coefficient of variation <10%) and M30 (detection limit 60 U/L and coefficient of

Table 1. Recipient and donor characteristics according to MELD score.

Parameters	MELD score <20 (n=87)	MELD score ≥20 (n=60)	p Value
Age (year ±SD)	52.1±11.6	51.2±12.2	0.55
Females	26 (30%)	19 (32%)	0.82
CMV+	50 (57%)	33 (55%)	0.24
HCV+	21 (24%)	8 (13%)	0.11
HBV+	9 (10%)	3 (5%)	0.25
Liver disease (viral/alco./others)	30 (34%)/27 (31%)/30 (34%)	13 (22%)/18 (30%)/29 (48%)	0.16
Child-Pugh A/B/C category	48 (56%)/20 (24%)/17 (20%) (n=85)	7 (13%)/14 (27%)/31 (60%) (n=52)	<0.0001
Encephalopathy	24 (28%)	27 (45%)	0.03
Retransplantation	5 (6%)	11 (18%)	0.10
HCC	27 (31%)	12 (20%)	0.14
Reduced nutrition	34 (39%)	35 (58%)	0.12
Operation time, hour (mean ±SD)	5.5±1.5	5.8±1.7	0.68
Cold ischemia time, hour (mean ±SD)	9.3±2.8	9.8±2.2	0.58
BMI (kg/m ²)	26.1±4.7 (n=74)	26.7±4.4 (n=46)	0.45
Lean body mass	56.8±9.1 (n=74)	62.9±27.3 (n=46)	0.18
Immunosuppressive regimen			
Prednisolone	87 (100%)	60 (100%)	1.00
Mycophenolat-Mofetil	49 (56%)	27 (45%)	0.18
Cyclosporine A	55 (63%)	35 (58%)	0.55
Tacrolimus	30 (34%)	22 (37%)	0.79
IL-2RA	4 (5%)	2 (3%)	0.70
Donors			
Age (year ±SD)	59.0±18.0	52.8±18.4	0.03
Females	40 (46%)	32 (53%)	0.32
CMV+	50 (57%)	31 (52%)	0.49

Alco. – alcoholic cirrhosis; others – metabolic and hereditary diseases; U – units. Mann-Whitney-U, Fisher exact test, Kruskal-Wallis-Test and χ^2 tests were used to calculate p values.

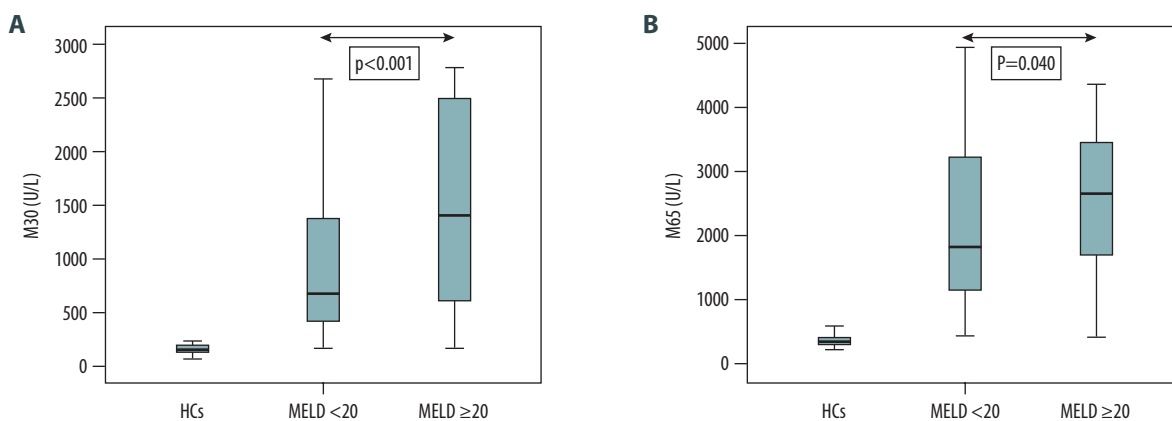


Figure 1. Increased serum caspase-cleaved cytokeratin (M30) and keratin 18 (M65) levels in patients with severe liver cirrhosis. We grouped 147 patients with cirrhosis into 2 cohorts according to their MELD scores: patients with a MELD score <20 were assigned to the moderate liver dysfunction group (group I, n=87) and patients with a MELD score \geq 20 were assigned to the severe liver dysfunction group (group II, n=60). Thirty-three healthy controls (HCs) were analyzed for comparison. Differences between the medians of parameters in the 2 groups and HCs are shown in boxplots: (A) M30 and (B) M65. P values were calculated using the Mann-Whitney U test.

variation <7%) levels were measured by ELISA (M65: cat. no. 10010, PEVIVA; M30 cat. no. 10040; PEVIVA) in HCs and cirrhotic patients. Serum from liver patients was diluted 1: 2 with the corresponding dilution buffers for M65 and M30, according to the manufacturer's protocols. Serum CRP levels were assessed in a certified laboratory at Heidelberg University Hospital.

Statistical analysis

Mann-Whitney-U, Fisher's exact, and Kruskal-Wallis tests were used to compare non-parametric continuous data, 2 groups of categorical data, and greater than 2 groups of categorical data, respectively, using the Statistical Package for the Social Sciences version 18 (SPSS, Chicago, USA). P values \leq 0.05 were considered significant. The Spearman rank correlation test was used to determine associations between variables. Receiver operating characteristic (ROC) curve analyses were performed to determine the diagnostic sensitivity and specificity of parameters.

Results

Serum M65 and M30 levels are increased in ESLD patients

We analyzed serum samples from 147 ESLD patients. Serum M65 (2294 ± 1153 U/l vs. 368 ± 108) and M30 (1096 ± 806 U/l vs. 179 ± 113) levels were higher in patients with ESLD immediately prior to liver transplantation compared with HCs ($p < 0.001$) (Figure 1).

Correlation of MELD score and immune parameters

The MELD score was positively correlated with serum CRP ($r=0.43$, $p < 0.001$), M30 ($r=0.31$, $p < 0.001$) and M65 ($r=0.23$, $p=0.006$) levels. Serum M30 was positively correlated with bilirubin ($r=0.33$, $p < 0.001$), INR ($r=0.24$, $p=0.004$), and Cr ($r=0.17$, $p=0.040$) (Figure 2).

Serum M65 and M30 levels are associated with MELD score, serum bilirubin, INR, and 1-year graft loss

Serum M65 (2156 ± 1164 U/l vs. 2563 ± 1058 U/l, $p=0.025$) and M30 (957 ± 726 U/l vs. 1490 ± 906 U/l, $p < 0.001$) were significantly lower in group I (MELD <20) than in group II (MELD \geq 20) (Table 2). Serum Cr levels in patients with hepatorenal syndrome and/or renal insufficiency were not associated with serum M65 ($p=0.07$) and M30 ($p=0.11$). This indicated no correlation of kidney function with M30 and/or M65 levels. Serum M30 and M65 levels were similar in patients with and without encephalopathy. Patients with serum bilirubin ≥ 4 mg/dl (median value) had significantly higher serum M30 ($p=0.002$) and M65 ($p=0.010$) than patients with serum bilirubin <4 mg/dl. Patients with an INR ≥ 1.3 (median value) had slightly higher serum M30 ($p=0.02$) than patients with an INR <1.3. The ratio of M30 to total M65 was significantly higher in patients with MELD scores ≥ 20 ($p=0.002$) and an INR ≥ 1.3 ($p=0.011$).

Nine patients died during the first post-transplant year and were excluded from the post-transplant graft loss analysis. Recipients with graft loss within the first post-transplant year (n=25) had significantly higher M30 serum levels than recipients

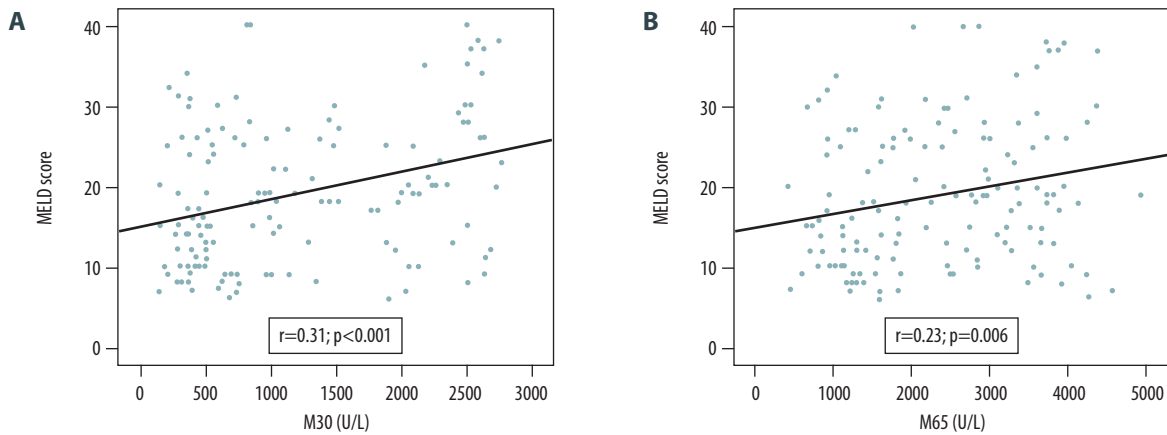


Figure 2. MELD score correlates with serum caspase-cleaved cytokeratin (M30) and keratin 18 (M65) levels. MELD scores of 147 cirrhosis patients were correlated with serum M30 (A) and M65 (B) concentrations. Spearman rank correlation test was used for statistical analysis.

Table 2. Immune parameters according to the MELD score.

Parameters	MELD score <20 (n=87)	MELD score ≥20 (n=60)	p Value
CRP (mg/L)	14±16	33±36	<0.001
M30 (U/l)	957±726	1490±906	<0.001
M65 (U/l)	2156±1164	2563±1058	0.025
M30/M65	0.45±0.29	0.55±0.24	0.002

Mann-Whitney-U test was used to calculate p values.

with >1-year post-transplant graft survival (n=113) (1702±926 U/l vs. 1091±789, p=0.004). Serum M65 and M30/M65 ratios were not significantly different between the 2 groups (p>0.05).

In our previous study of 94 patients, we showed that serum neopterin, quinolinic acid, and CRP were positively associated with the MELD score [17]. In the present study, we quantified serum CRP in more cirrhotic patients. Our current results confirm that serum CRP levels were significantly increased in patients with a MELD score ≥20 compared to patients with a MELD score <20 (30±28 mg/l vs. 15±25 mg/l, p≤0.001) (Table 2).

Sensitivity and specificity of serum M65 and M30 in patients with liver dysfunction, graft failure, and 1-year mortality

We performed ROC curve analysis to calculate the sensitivity, specificity, and cut-off values of the investigated markers to predict advanced liver failure (MELD score ≥20). Positive test results were defined as serum M65 >2200 U/l (p=0.012) and M30 >900 U/l (p<0.001). Sensitivity was 64% and 64% and specificity was 60% and 64% for M65 and M30, respectively

(Figure 3). ROC curve analysis showed a good discriminatory ratio for patients with graft loss during the first post-transplant year vs. patients with >1-year graft survival (area under the curve (AUC) 0.68, p=0.007), with 60% sensitivity and 81% specificity for a M30 cut-off of 900 U/l (Figure 3).

Regression analysis of significant parameters

Univariate regression analysis showed that serum M65 >2200 U/l (p=0.010) and M30 >900 U/l (p=0.006) was significantly associated with MELD scores ≥20. Multivariate analysis of these parameters revealed that only serum M30 (OR=2.55, confidence interval [CI]=1.30–4.97, p=0.005) was associated with liver disease activity (MELD score), high serum bilirubin (OR=2.61, CI=1.35–5.06, p=0.004), and low 1-year graft survival (OR=4.79 CI=1.29–11.93, p=0.001). M30 >900 U/l (OR=4.79, CI=1.92–11.92, p=0.001) showed higher 1-year graft loss than a MELD score ≥20 (median value) (OR=3.00, CI=1.35–6.81, p=0.006). A multivariate analysis showed that serum M30 >900 U/l (OR=3.61, CI=1.48–8.88, p=0.005) was more predictive of 1-year graft loss than a MELD score of ≥20 (OR=2.54, CI=1.00–6.57, p=0.030).

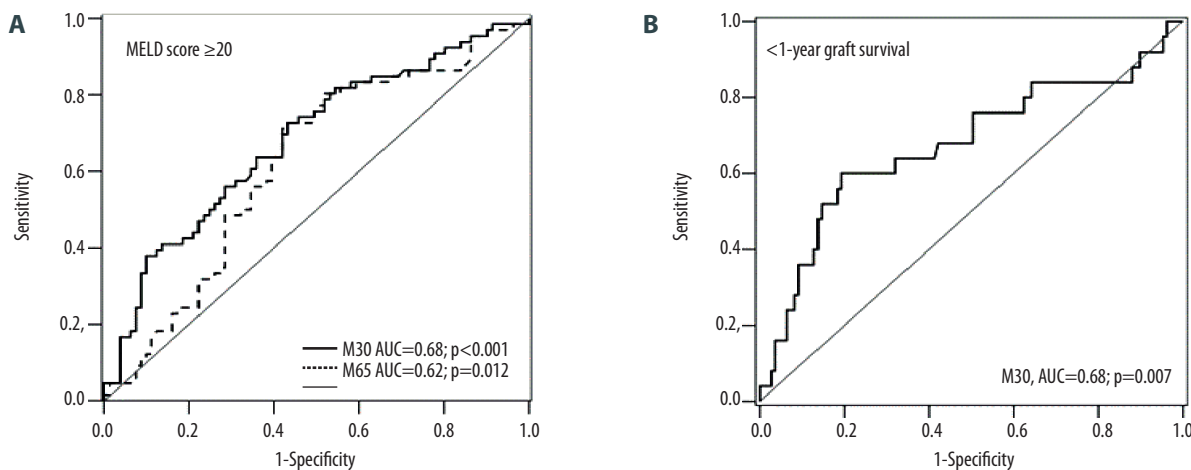


Figure 3. (A) The predictive value of serum caspase-cleaved cytokeratin (M30) and keratin 18 (M65) levels for liver disease severity (measured by MELD scores) calculated by receiver operating characteristic (ROC) curve analysis. MELD scores were assessed by ROC curve analysis by measuring the area under the curve (AUC) to assess sensitivity and specificity of M30 and M65 in patients with moderate (group I, n=87) and severe (group II, n=60) liver dysfunction. (B) Apoptotic markers in patients with 1-year graft loss (n=25).

Discussion

The MELD score was developed to predict short-term mortality of patients with transjugular intrahepatic portosystemic shunt and suggests liver disease severity [18]. Chronic liver injury involves chronic inflammation driven by overactive chemokines and cytokines that results in cirrhosis or end-stage fibrosis [19]. Cytokeratin 18 (K18) maintains hepatocyte integrity and K18 deficiency causes liver damage and dysfunction [20]. Liver fibrosis is caused by apoptosis and necrosis of parenchymal cells, which leads to recruitment of immune cells, the activation and accumulation of fibrogenic cells, and the deposition of extracellular matrix [21].

Apoptosis and necrosis are 2 major types of cell death [22]. Apoptosis is an early, chronic and temperate form of programmed cell death, whereas necrosis is acute premature cell death occurring in response to cellular injury [23]. Liver injury can be caused by different factors, such as drinking alcohol, viral infection, cholestasis, steatosis, drug abuse, and autoimmunity. These factors can stimulate immune activation. In a previous study, we showed that inflammatory biomarkers (CRP, neopterin, IL-6, and quinolinic acid) are positively associated with hepatic dysfunction in patients with liver cirrhosis [17]. Based on previous findings, we hypothesized that apoptotic and necrotic markers can predict the severity of hepatic dysfunction according to the MELD score. The objective of the present study was to investigate whether the cell death markers M65 (marker of necrosis) and M30 (marker of apoptosis) are correlated with the degree of clinical liver failure and post-transplant outcome.

Liver injury can induce necrosis or apoptosis, depending on the severity of the injury [24]. Serum M30 levels have been associated with mortality in patients with sepsis and severe traumatic brain injury [25,26]. In addition, Valva et al. reported that high serum M30 levels are associated with the pathogenesis of chronic pediatric HCV infection and the severity of steatosis [27,28].

In the present study we found a strong association of M30 serum levels with serum bilirubin and INR (2 parameters of the MELD score), which supported a correlation of M30 with liver injury but not with kidney injury. Our data demonstrate that serum M65 and M30 levels are significantly increased in ESLD patients compared with HCs ($p < 0.001$). Furthermore, a significant difference in serum M65 and M30 levels was noted between moderate (group I) and severe (group II) liver dysfunction (calculated using MELD scores) (M65: $p = 0.01$, M30: $p < 0.001$). These findings agree with previous findings that M65 and M30 are associated with hepatic injury in patients with advanced liver disease, especially non-alcoholic liver disease [29–31]. Our results also support previous findings that inflammatory cytokines/chemokines such as TNF- α , TGF- β , IFN- γ , IL-10, CCL3, CCL4, CXCR3 ligand, IP-10, CCR5 ligands, and chemokine (C-C motif) ligand 5 are released in patients with progressive liver injury [32–36] and can stimulate hepatocyte cell death through different pathways [37,38]. Although M65 and M30 have mainly been evaluated in patients with liver disease, M65 is expressed in a variety of adult epithelial organs and may play a role in carcinogenesis. M65 is a marker of necrosis and cell death during renal failure, is a response biomarker for anti-cancer drugs, and is overexpressed in breast, prostate, lung, colon, and ovary carcinomas [3,39–41].

The prognostic and predictive value of serum M30 levels has been studied in patients with hepatocellular carcinoma (HCC). Pre-transplant M30 serum levels were suggested as prognostic biomarkers of 1-year post-transplant survival in HCC patients [42]. Increased levels of M30 as a marker of apoptosis predicted non-hepatocellular carcinoma liver-related mortality in patients with alcoholic cirrhosis [20]. The predictive and prognostic value of M30 in patients with different liver diseases was studied previously [43–47]. We investigated the predictive value of pre-transplant M30 and M65 levels in cirrhotic patients with different original diseases. The results suggested that serum M30 levels of >900 U/l are more predictive of 1-year graft loss than is the MELD score.

Serum keratin has been used as tumor marker and serum M30 and M65 levels are increased in various liver diseases [20]. Mueller et al. reported that M30 and M65 are independent parameters of mortality in alcoholic cirrhosis [20]. A cascade of mediators released in the graft after transplantation can infiltrate local cells and promote inflammation and graft loss [48].

Patients with cirrhosis are faced with sarcopenia, which is associated with increased mortality and increased length of hospital stay after LTx [49]. Recent studies demonstrated that frailty and sarcopenia are related to failure to rescue after LTx and higher rates of mortality [50,51]. However, in the present study there was no difference in BMI and lean body mass of patients in the patients with a MELD score ≥ 20 compared to patients with a MELD score <20.

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Conclusions

In summary, inflammation and cell death (especially apoptosis) are prominent pathogenic parameters of liver disease. The full details of inflammation-related cell death need to be clarified in a future study. Hepatic inflammation and cell death should also be investigated further to develop novel approaches for the prognosis, prediction, and treatment of liver diseases. Pre-transplant M30 monitoring provides a serum marker which may help to distinguish liver transplant patients at risk of graft loss and for modification of immunosuppressive treatment.

Conflicts of interest

None.

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