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Evaluation of Graft Effluent High Mobility Group Box-1 (HMGB-1) for Prediction of Outcome After Liver Transplantation

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Data Interpretation D
Manuscript Preparation E
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Background: Pre-transplant assessment of the graft for liver transplantation is crucial. Based on experimental data, this study was designed to assess both nuclear high mobility group box-1 (HMGB-1) protein and arginine-specific proteolytic activity (ASPA) in the graft effluent.

Material/Methods: In a non-interventional trial, both HMGB-1 and ASPA were measured in the effluent of 30 liver grafts after cold storage before transplantation. Values of HMGB-1 and ASPA levels were compared with established prognostic parameters such as the donor risk index, balance of risk score, and Donor-Model for End-Stage Liver Disease.

Results: The early allograft dysfunction (EAD) was best predicted by recipient age (p=0.026) and HMGB-1 (p=0.031). HMGB -1 thresholds indicated the likelihood for initial non-function (1608 ng/ml, p=0.004) and EAD (580 ng/ml, p=0.017). The multivariate binary regression analysis showed a 21-fold higher (95% CI: 1.6–284.5, p=0.022) risk for EAD in cases with levels exceeding 580 ng/ml. The ASPA was lower in cases of initial non-function (p=0.028) but did not correlate with the rate of EAD (p=0.4).

Conclusions: This study demonstrates the feasibility of HMGB-1 detection in the graft effluent after cold storage. Along with conventional prognostic scores, it may be helpful to predict the early fate of a graft in human liver transplantation.

MeSH Keywords: **Immunity, Innate • Liver Transplantation • Organ Preservation**

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Background

Liver transplantation (LT) is the therapy of choice for various benign and malignant diseases. Both patient and graft survival have continuously improved over the last decades; however, prediction of early graft function still remains a major obstacle and thus it is impossible to clearly identify grafts that will sustain the recipient's life after transplantation. Due to increasing waiting list mortality, the Model for End-Stage Liver Disease (MELD) – based allocation was introduced in Germany in December 2006. Since then, liver grafts have been preferably allocated to the sickest high-MELD patients [1]. With dramatically decreased numbers of organ donors in Germany, from *1271* in 2010 to *851* in 2014, an incremental rise in the acceptance of organs from extended-criteria donors has been observed over the past few years [2]. As a consequence, the one-year graft survival after LT has dropped to a worrisome level of below 70% in Germany since 2007 [3]. Because the organ shortage problem is unlikely to be overcome in the near future, counteractive measures are focusing on the optimized utilization of available liver grafts. Predictors of graft function after transplantation are desperately needed. Donor age, which is included in the Donor Risk Index (DRI) and the Donor-MELD score (D-MELD), and elevated donor serum sodium levels have been shown to be relevant risk factors for inferior outcomes after LT [4–6]. Unlike the aforementioned risk factors that are available at the moment of graft acceptance and recipient preparation, the success of graft preservation can only be estimated by the duration of cold ischemia. Multi-visceral organ recovery and procurement carry the risk of severe organ damage, mostly via suboptimal cold perfusion and prolonged warm ischemia [7,8]. Furthermore, organ recovery-related Kupffer cell-mediated ischemia reperfusion injury (IRI) was proven to significantly impair postoperative graft function after experimental transplantation [9]. Attributed to Kupffer cell (KC) activation, the amount of arginine-specific proteolytic activity (ASPA) in the graft effluent has been shown to predict graft survival pre-clinically [10]. According to the concept of innate alloimmunity, cell and tissue injury directly affect recipient immunological response, and limitation of the initial graft damage was shown to result in better clinical outcome in renal transplantation [11–13]. Among other danger-associated molecular patterns (DAMPs), the nuclear high mobility group box-1 protein (HMGB-1) was identified as a key mediator in allograft injury and is a potential stimulus for dendritic cell (DC) maturation. HMGB-1 has been shown to correlate with IRI and to reflect surgical stress postoperatively in experimental and clinical settings [14–17]. Liu et al. demonstrated that effluent levels of HMGB-1 corresponded with the duration of cold and warm ischemia and the amount of mechanical stress applied to rat livers [18].

Since clinical data on the role of effluent measurement of ASPA and HMGB-1 is lacking, this clinical study was designed to test if the respective principles could be adapted to clinical liver transplantation. Based on the aforementioned findings, both ASPA and HMGB-1 were measured in graft effluent after cold storage to indicate KC activation and tissue injury. The objective of this clinical trial was, for the first time, to analyze the correlation of the initial graft damage after organ retrieval and subsequent cold storage with the early postoperative clinical course in human liver transplantation.

Material and Methods

For this non-interventional clinical trial, permission was obtained from the local Ethics Committee (No. S-253/2012). All study-specific activities were done in accordance with the Declaration of Helsinki (2008 version). Written informed consent for the evaluation of individual clinical data was obtained from all patients preoperatively. There was no study-specific, invasive treatment, medication, or sampling of blood or tissue specimens from any patient. All clinical data were taken from the clinical database and patient records. With respect to the "proof of principle" design, the data of 30 LT recipients and their graft effluent samples obtained from October 2012 to September 2013 were analyzed.

Graft effluent sampling

According to our center-specific, standard procedure, every LT graft was prepared for grafting and checked for damage preoperatively. At the end of this "back table" procedure, the graft was routinely rinsed with 1 liter of 4°C cold Histidine Tryptophane Ketoglutarate (HTK; Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) solution via the portal vein by the force of gravity. The first 20 milliliters emerging from the grafts' hepatic veins were captured with a sterile syringe (B. Braun Melsungen AG, Germany) and immediately frozen at –80°C.

Measurement of arginine-specific proteolytic activity (ASPA)

According to the manufacturer's instructions, H-D-Ile-Pro-Arg-pNA ×2 HCl (S-2288, Haemochrom Diagnostica, Germany) was used for the photometric measurement (Fluostar OPTIMA, BMG Labtech, Germany) of the ASPA at 405 nm. In order to avoid extended storage, which potentially affects results, the maximum storage duration at –80°C before ASPA measurement was limited to 4 months. The results are displayed in U/l.

Measurement of high mobility group box-1 (HMGB-1)

A commercially available, enzyme-linked immunosorbent assay (ELISA), designed for serum measurements of HMGB-1 (IBL International, Germany), was applied for the analysis of

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HMGB-1 concentrations in the effluent. Due to missing expectancy values of HMGB-1 concentration in the effluent, standard serial dilutions with concentrations of 80, 40, 20, 10, 5, 2.5, and 0 ng/ml HMGB-1 were prepared. Samples with values outside the standard serial dilution series were diluted further and the value was corrected accordingly. All concentrations were recorded as ng/ml.

Clinical data

The following information was taken from the clinical database and/or the patient records: patient survival, initial non-function (INF), early allograft dysfunction (EAD), infectious complications, re-transplantation, donor age, cold ischemia time, biopsy-proven rejection (BPR), and routine laboratory parameters (days 1, 3, 5, 10, 30 (±5), and 90 (±15)). INF was defined as graft loss or recipient death within 14 days after the transplant [19]. According to the definition proposed by Olthoff et al., EAD was defined as the presence of one of the following parameters postoperatively: total bilirubin ≥10 mg/dl or International Normalized Ratio (INR) ≥1.6 on day 7 and/or aspartate transaminase (AST) or alanine aminotransferase (ALT) ≥2000 U/l within the first 7 postoperative days [20]. The balance of risk score (BAR), the D-MELD, and DRI were calculated based on the baseline donor and recipient data [21]. The duration of clinical follow-up was 3 months postoperatively.

Statistics

All metric data are expressed as *median (range)* or *mean (standard error (SE))* unless otherwise stated. Statistical analyses were done with SPSS V. 21.0 (IBM, USA). Dot plots were designed with an online tool (*://biostat.mc.vanderbilt.edu/wiki/pub/Main/TatsukiRcode/Poster3.pdf*). Non-parametric testing was usually done after checking for a normal distribution with the Kolmogorov-Smirnov test. Spearman's rank correlation and univariate analyses were applied to check for correlations of preoperative parameters and effluent measurements with postoperative outcome. The Mann-Whitney U Test was used to compare means. Receiver operating characteristics (ROC) were utilized to identify factors that influenced clinical outcome parameters. After identification of relevant cut-off values for these factors and validation with Fisher's exact test, they were evaluated in a multivariate, binary logistic regression analysis.

Results

Clinical baseline characteristics

Patients with an average age of 54 years with a 2: 1 male/female ratio and a lab-MELD of 16 received full-size liver grafts, donated by deceased heart-beating 51-year- old donors after less than 11 h of cold storage (Table 1). Thirteen percent of the recipients had undergone liver transplantation before. The prognostic indices DRI, balance of risk score (BAR), and D-MELD were 1.8, 7 and 839, respectively (Table 1).

Table 1. Clinical baseline characteristics.

Donor		
Age (years)	50.5	(14–88)
Donor sodium (mmol/l)	143	(136–161)
Graft		
CIT (min)	650	(493–1020)
Recipient		
Age (years)	53.5	(24–66)
labMELD	16	(6–40)
Gender: M/F	20/10	
Previous LT: Y/N	4/26	
Prognostic indices		
DRI	1.8	(1.2–2.4)
BAR	7	(2–21)
D-MELD	839	(196–2960)

BAR – balance of risk score; CIT – cold ischemia time; D-MELD – donor-MELD; DRI – donor risk index; LT – liver transplantation; MELD – model for end stage liver disease

Table 2. Outcome.

Re-transplantation (y/n)	2/28
90-day-mortality (y/n)	4/26
INF (y/n)	4/26
BPR (y/n)	5/25
EAD (y/n)	20/10

INF – initial non-function; EAD – early allograft dysfunction; BPR – biopsy proven rejection.

Clinical outcome data

Seven percent (n=2) of cases underwent re-transplantation within 15 to 64 days. The 90-day mortality was 13% (n=4) with INF in 3 of the 4 cases. EAD and INF were 67% (n=20) and 13% (n=4), respectively, with a BPR of 17% (n=5) of all cases (Table 2).

Graft effluent high mobility group box-1 (HMGB-1) and arginine-specific proteolytic activity (ASPA)

The mean concentration of HMGB-1 and ASPA in the effluent was 995.6±290.8 ng/ml and 1516.8±162.2 mU/l, respectively.

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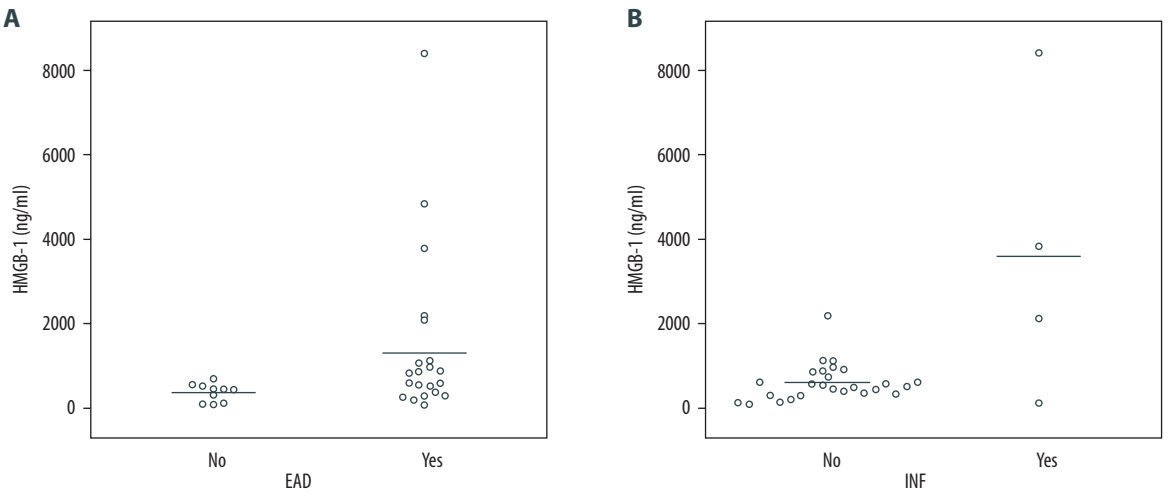

Figure 1. (**A**) Effluent HMGB-1 values as a function of early allograft dysfunction (EAD). Dots represent individual recipients and horizontal bars indicate the mean (n=30; p=.031). (**B**) Effluent HMGB-1 values as a function of initial graft non-function (INF). Dots represent individual recipients; horizontal bars indicate the mean (n=30; p=.139).

While there was a significant difference between HMGB-1 levels after cold storage in grafts with EAD 1302±421.5 ng/ml compared to 382.8±68 ng/ml without EAD after transplantation (p=0.031; Figure 1A), HMGB-1 levels for INF were 5-fold higher but did not reach a significant difference after cold storage in livers with INF after transplantation compared to grafts without INF (p=0.139; Figure 1B). No difference in ASPA was detected in grafts with INF or EAD compared to grafts without INF or EAD after transplantation. Correlation analysis revealed no strong correlation of HMGB-1 or ASPA to other clinical or laboratory outcome parameters (data not shown).

ROC analysis of prognostic indices, HMGB-1, and ASPA

In the ROC analysis, the BAR score had an AUC of 0.89% (SE: 0.06, p=0.013, 95% CI: 0.77–1.0) and was therefore the only parameter that reliably predicted 90-day mortality. For the prediction of INF, both the lab-MELD (AUC 0.75% (SE: 0.14, p=0.12, 95% CI: 0.48–1.0)) and HMGB-1 (AUC 0.74% (SE: 0.21, p=0.14, 95% CI: 0.32–1.0)) showed the largest AUC, but without reaching significance. The EAD was best predicted by both the recipient age (AUC 0.75% (SE: 0.11, p=0.03, 95% CI: 0.54–0.96)) and HMGB-1 (AUC 0.74% (SE: 0.09, p=0.03, 95% CI: 0.57–0.92)). The ROC curves for the ASPA were not predictable for any outcome parameter.

Based on the ROC analysis, HMGB-1 thresholds for the likelihood of EAD (580 ng/ml, p=0.017) and INF (1608 ng/ml, p=0.004) were identified (Figure 2A, 2B). For the recipient age, a threshold age of 48 years was identified as significantly correlating with the development of EAD (p=0.005).

Due to the low incidence rate of other endpoints, a multivariate, binary logistic regression analysis could only be done for the EAD. With the inclusion of recipient age as a continuous variable and HMGB-1 exceeding 580 ng/ml, the risk for EAD rose by 16.9% (95% CI: 0–36.8%, p=0.05) with every year of age. The development of EAD was 21 times more likely (95% CI: 1.6–284.5, p=0.02) if HMGB-1 levels exceeded 580 ng/ml.

The duration of intensive care treatment for patients with HMGB-1 levels higher than 580 ng/ml was increased from 1.8±0.37 to 4.7±1.3 days (p=0.07). For levels above 1608 ng/ml, it was 2.6±0.59 to 6±2.9 days (p=0.16).

Discussion

One of the major challenges in liver transplantation today is to find prognostic markers for graft function after transplantation. This is especially important for livers from extended-criteria donors, since this is a possible source of grafts to overcome the shortage of organs for transplantation [22]. The review of mechanisms involved in the development of IRI being associated with both DGF and INF may help to find markers that are clearly linked with the later fate of a graft. Unfortunately, at present only donor and recipient data together with an evaluation of the basic condition of the graft after cold storage are used as a standard to decide if the graft is suitable for transplantation. Additionally, fresh frozen section histological evaluation is used primarily in estimating graft steatosis [23,24]. Nevertheless, histological examination is unlikely to detect preservation damages that are below the level of cellular impairment. Molecular markers in the graft effluent, attributed

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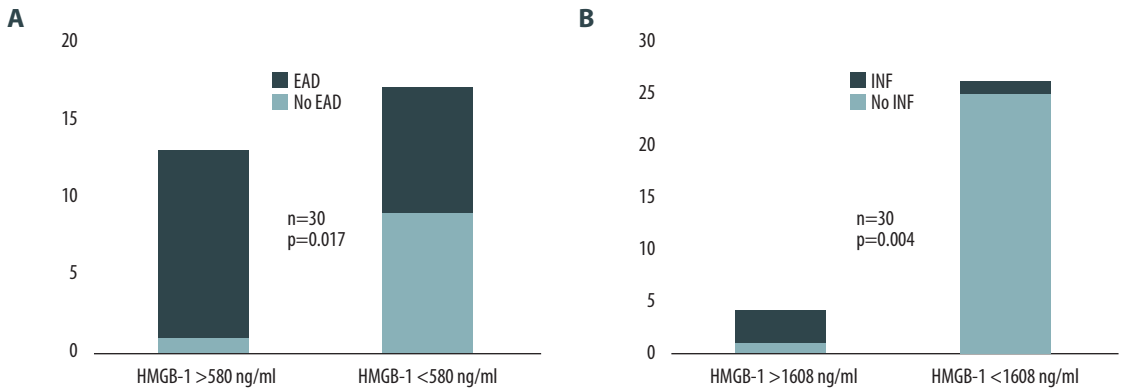

Figure 2. (**A**) Rate of early allograft dysfunction (EAD) with effluent above HMGB-1 and below 580 ng/ml. With HMGB-1 values above 580 ng/ml, only 1 out of 13 patients did not develop EAD. (**B**) Rate of initial non-function (INF) with effluent HMGB-1 above and below 1608 ng/ml. With HMGB-1 values below 1608 ng/ml, only 1 out of 26 patients developed INF.

to ischemia, hepatocyte injury, and KC activation, as well as pro-inflammatory cytokines, have therefore been extensively studied in experimental LT [10,18,25–28].

In clinical LT, measurement of effluent thrombomodulin, hyaluronic acid, and pro-inflammatory cytokines such as interleukins 1 and 6 and tumor necrosis factor-α have been shown to potentially predict clinical outcome [29–31]. Additionally, molecular markers have been shown to correlate with ischemia reperfusion injury of the graft at a very early time [32–34]. Interestingly, none of these have translated into routine clinical practice yet. HMGB-1, which is measured systemically and is found in the hepatic vein after reperfusion, has been shown to reflect hepatocellular damage in human liver transplantation [35]. Ilmakunnas et al. demonstrated that HMGB-1 was exclusively released from the graft and that its levels correlated with postoperative ALT levels in 20 LT recipients. Our results confirmed the finding that HMGB-1 is released from the graft and show that it is also correlated with clinical outcome. The occurrence of EAD in patients described here was only predicted by recipient age and HMGB-1. Surprisingly, the DRI and D-MELD failed to do so. The BAR was only predictive for 90-day mortality.

The detection of HMGB-1 in the graft effluent at a stage before the LT has begun could influence the decision-making process on whether the graft can successfully be transplanted in the future, especially if immediate laboratory or even bed-side test options were available. This is especially interesting in the context of currently emerging concepts like machine perfusion for both preservation and graft reconditioning [36–39].

Due to the small sample size, a final appraisal for the usefulness of HMGB-1 in LT cannot be made from this pilot trial. Despite correlating with EAD, HMGB-1 failed to predict INF or 90-day mortality, the latter being achieved by the BAR. The measurement of ASPA in the graft effluent did not correlate with relevant outcome parameters in this setting of clinical LT.

Conclusions

This clinical study demonstrated that the measurement of HMGB-1 in the graft effluent after cold storage in LT is feasible. Further clinical research is needed to evaluate its potential as an indicator for graft and preservation quality and as a predictor of clinical outcome.

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