Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation

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Abstract

A global rising organ shortage necessitates the use of extended criteria donors (ECD) for liver transplantation (LT). However, poor preservation and extensive ischemic injury of ECD grafts have been recognized as important factors associated with primary non-function, early allograft dysfunction, and biliary complications after LT. In order to prevent for these ischemia-related complications, machine perfusion (MP) has gained interest as a technique to optimize preservation of grafts and to provide the opportunity to assess graft quality by screening for extensive ischemic injury. For this purpose, however, objective surrogate biomarkers are required which can be easily determined at time of graft preservation and the various techniques of MP. This review provides an overview and evaluation of biomarkers that have been investigated for the assessment of graft quality and viability testing during different types of MP. Moreover, studies regarding conventional graft preservation by static cold storage (SCS) were screened to identify biomarkers that correlated with either allograft dysfunction or biliary complications after LT and which could potentially be applied as predictive markers during MP. The pros and cons of the different biomaterials that are available for biomarker research during graft preservation are discussed, accompanied with suggestions for future research. Though many studies are currently still in the experimental setting or of low evidence level due to small cohort sizes, the biomarkers presented in this review provide a useful handle to monitor recovery of ECD grafts during clinical MP in the near future.

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Introduction

Graft quality at time of liver transplantation (LT) is a major determinant of early graft performance and thereby strongly influencing graft survival and morbidity during recipient follow-up [1]. Over the last decade, grafts from extended criteria donors (ECD) had to be used increasingly for LT due to organ shortage. The quality of these grafts has been shown to be variable [2,3]. Although some ECD liver grafts turn out to function properly in recipients, their use has also been associated with impaired graft survival due to primary non function (PNF), early allograft dysfunction (EAD) and severe biliary complications like ischemic-type biliary lesions (ITBL, Fig. 1) [4,5].

Though pathophysiology between PNF, EAD, and biliary complications is assumed to differ, extensive ischemic- and preservation injury has been recognized as a shared risk factor in these entities [1,6]. Primary non-function occurs in up to 5–8% of LT's and necessitates immediate re-transplantation in all cases. Though PNF may be caused by technical failure resulting in inadequate blood flow through the graft [7], the association between unfavourable donor risk factors and PNF suggests that its cause is likely multifactorial [8]. Early allograft dysfunction is typically characterized by increased serum transaminase levels in recipients during the first postoperative week [9], but unlike PNF, liver grafts showing EAD do not always need immediate re-transplantation [10]. The most common complication associated with ischemic- and preservation injury are biliary complications. Dependent on the type of graft (donation after brain death; DBD vs. donation after circulatory death; DCD), up to 50% of recipients develop complications due to bile leakage, anastomotic strictures, ITBL, bile duct necrosis, and cast formation [11,12]. The various times of onset and different nature of biliary

Keywords: Graft dysfunction; Marker; Prediction; Biliary complications; Viability testing.

Abbreviations: LT, liver transplantation; ECD, extended criteria donors; PNF, primary non-function; EAD, early allograft dysfunction; ITBL, ischemic-type biliary lesions; DBD, donation after brain death; DCD, donation after circulatory death; MELD, model for end-stage liver disease; MP, machine perfusion; SCS, static cold storage; HMP, hypothermic machine perfusion; HOPE, hypothermic oxygenated machine perfusion; SNP, subnormothermic machine perfusion; NMP, normothermic machine perfusion; COR, controlled oxygenated rewarming; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ATP, adenosine triphosphate; HA, hyaluronic acid; sTfR, (soluble) thrombomodulin; TNF-α, tumor necrosis factor alpha; PBV, portal vein branch; miRNAs, microRNAs; HDmiRs, hepatocyte-derived miRNAs; CDmiRs, cholangiocyte-derived miRNAs; UW, University of Wisconsin solution; HTK, histidine tryptophan ketoglutarate.

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Definitions on PNF and ITBL can be found in the Supplementary data.

Estimation of the incidence of PNF, EAD, and ITBL in separate DBD and DCD grafts, incidence of EAD maintained the criteria formulated by Olthoff. Percentages represent the mean incidence ± standard error. Studies used to calculate the incidence of EAD maintained the criteria formulated by Olthoff et al. [9,10]. Definitions on PNF and ITBL can be found in the Supplementary data.

Complications suggest that they are caused by different underlying mechanisms, including surgical trauma, DCD, high donor age, prolonged ischemia time, cytotoxicity of bile salts and immune factors [6,11].

Prediction models as the donor risk index were developed to estimate the risk of graft failure in recipients and to match high-risk grafts to suitable recipients [13]. Furthermore, earlier research on the topic of predicting graft function after LT has focussed mainly on clinical characteristics from donors and recipients, including the model for end-stage liver disease-score (MELD) [14–16]. However, models that are mainly based on such characteristics are unable to assess the degree of injury that is caused by the process of graft procurement, cold preservation, and reperfusion. Moreover, the under-utilization of grafts with unfavourable donor characteristics like advanced donor age, DCD, and African race, can lead to an undesirable diminution of the donor pool [17].

Therefore, machine perfusion (MP) is increasingly being investigated as a novel technique to improve graft preservation of particularly ECD grafts. Through MP, ischemia related complications like PNF, EAD, or ITBL can be reduced or even prevented and potentially allow for expansion of the extended criteria donor pool to be utilized for LT. Other potentially beneficial features of MP consist of the possibility to add supplements during perfusion that could further benefit graft quality [18,19], or even attempt for restoration of ischemic injury [20,21]. Beside safety and technical feasibility of MP, investigators pronounce on the need of sensitive biomarkers that can distinguish poor quality grafts from those that will function properly after implantation [22,23]. Next to other well-known risk factors for impaired graft quality as illustrated in Fig. 2, the time required for ex vivo MP provides the opportunity to monitor graft quality by measurement of biomarkers in perfusates and biopsies, which could be a helpful decision tool for improving the accuracy of selecting grafts for LT. This purpose however demands for objective surrogate biomarkers that are easily obtainable at time of graft preservation and is challenged by the various techniques of MP currently investigated.

In this review, we provide an overview of potentially useful biomarkers that were identified through a systematic search of the literature [Supplementary data], in order to assess graft viability testing during various techniques of MP. Because of the limited experience with clinical MP in LT, biomarker studies regarding conventional graft preservation by static cold storage (SCS) that correlated with either PNF, EAD, or biliary complications after LT and which could potentially be applied as predictive markers during MP were also included. Finally, the pros and cons of the different biomaterials are discussed, accompanied with suggestions for future research.

**Key Points**

- The increased use of extended criteria grafts demands for more objective and sensitive biomarkers to evaluate the large discrepancy of graft quality in liver transplantation.
- Measurement of prudent biomarkers during machine preservation (MP) could be helpful in the prediction of early graft performance after LT.
- During MP, surrogate biomarkers for graft quality could help select the most optimal preservation technique before implantation.
- Research shows discriminative potential of a variety of biomarkers for graft injury and function, but requires robust validation in larger cohorts before applicable in the clinic.
- Non-invasive evaluation of biomarkers released into perfusates during MP is an attractive alternative for invasively obtained tissue biopsies.

### Different machine preservation strategies

Because of easier accessible logistics and lower costs, SCS has become the standard preservation technique in clinical practice of LT to date. The low temperature during SCS delays metabolic processes in order to restrict ischemic injury. However, especially ECD grafts seem more vulnerable for prolonged ischemia, increasing morbidity and mortality in recipients after LT. Therefore, during the last ten years, various techniques by MP have been investigated in preclinical and clinical settings in order to further optimize graft quality and thus improve outcome of ECD liver transplantation. The main differences in the setup of MP are determined by pumping-temperature, the route- and pressure of recirculating preservation solution, and whether oxygen is administered (Fig. 3). As summarized in Table 1, several studies already performed MP on human liver grafts. Hypothermic MP (HMP) without the administration of oxygen comes closest to conventional preservation by SCS, but is believed to improve preservation through continuous recirculation of solution to all segments of the liver and the removal of remnant metabolites from the graft (Fig. 4). Guarrera et al. [24] performed the first clinical series of non-oxygenated HMP in humans (n = 20) using standard criteria donors. In this study, HMP was shown to be safe and analysis of perfusates and biopsies demonstrated an attenuation of ischemic injury markers during preservation [25–27]. Furthermore, the authors suggest that HMP could have beneficial effect on the incidence of EAD and biliary complications in recipients after LT. The feasibility of HMP was also investigated by Monbaliu et al. [28], who used HMP as a screening-tool to distinguish transplantable from
non-transplantable ECD human liver grafts that were rejected for LT. Beside Guarrera et al., the second reported clinical trial using MP prior to LT is from Dutkowski et al. [29]. In contrast to Guarrera et al., this study used hypothermic oxygenated MP (HOPE) for the preservation of ECD grafts. Previous experimental studies from this group showed beneficial effects of HOPE on...

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Fig. 3. Mechanisms of various machine preservation strategies. Different techniques of graft preservation can be used to protect against ischemic injury, to recondition the graft before reperfusion or even to maintain physiology. The various techniques have different potentially protective underlying mechanisms. Via all techniques, graft quality could be evaluated through markers in tissue biopsies or perfusate analysis. The (sub)normothermic conditions also allow for the analysis and evaluation of bile. SCS, static cold storage; HMP, hypothermic machine perfusion; HOPE, hypothermic oxygenated machine perfusion; SNP, subnormothermic perfusion; COR, controlled oxygenated rewarming; NMP, normothermic machine perfusion.

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Fig. 2. Risk factors for outcome following LT. Risk factors in donors, recipients and during the transplantation and transportation procedure influencing graft quality and graft/recipient outcome.

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biliary injury and endothelial damage [30,31]. Protective mechanisms of HOPE seem to be based mainly on the down regulation of mitochondrial and nuclear activity prior to reperfusion. Moreover, the used low-pressure perfusion at 3 mmHg caused less endothelial injury compared to more physiological pressures around 8 mmHg. Notably, grafts were perfused solely through the portal vein due to practical considerations and to prevent further damaging of the usually fragile hepatic artery [32]. Reactive oxygen species that are generated during ischemia can induce injury to mitochondria, which effects appear to exacerbate after hypothermic conditions [33,34]. Some researchers believe that reconditioning of the tissue by MP at higher temperatures can prevent this [35,36]. Moreover, (sub)normothermic MP (SNP) is seen as a preferable model for viability testing because metabolic function can be judged, for instance through bile output during warm MP [37,38]. Although not yet performed in clinical LT, Op den Dries et al. performed a feasibility study of normothermic perfusion (NMP) on four discarded human donor livers, which showed no harmful effects on liver tissue after 6 h of pumping [37]. Also in large animal models, graft NMP improved survival compared to SCS [39]. Gradual rewarming in this study however did not exceed 20–25 °C because of potentially toxic effects of the preservation solution at higher temperatures.

Many experimental studies have been performed on the different techniques of MP, of which some also attempted to identify biomarkers for graft quality assessment (Table 2). One would expect that these various MP techniques require different biomarkers for the assessment of graft quality. In the next paragraphs, we highlight on the most promising biomarkers for viability testing in MP of which some have been shown also to be predictive for early graft function after clinical LT (Table 3).

### Biomarkers for viability assessment during machine perfusion

**Production and composition of bile**

Beside using bile output as a parameter for outcome after reperfusion [32], some studies also investigated whether bile production during MP is a useful indicator for graft viability and the secretory function of hepatocytes and cholangiocytes; Brockmann et al. identified bile outflow during NMP as a discriminative variable for early graft survival [35]. Op den Dries et al. [37] also demonstrated the production of bile by human liver grafts under normothermic conditions. Based on this small series, they

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**Table 1. Studies on machine perfusion of human liver grafts.**

<table>
<thead>
<tr>
<th>Study [Ref.]</th>
<th>Year</th>
<th>MP temp</th>
<th>Oxygenated</th>
<th>Pressure (mmHg)</th>
<th>Size</th>
<th>Subject Donor Transplanted</th>
<th>Markers during MP for impaired viability</th>
<th>Biomaterial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Op den Dries et al., [37]</td>
<td>2013</td>
<td>37°C</td>
<td>Yes</td>
<td>50</td>
<td>Human</td>
<td>ECD</td>
<td>↑ Enzymes, lactate levels, ↓ bile production, bile composition (γGT, bilirubine, LDH), O₂, CO₂</td>
<td>Perfusate, tissue, bile</td>
</tr>
<tr>
<td>Dutkowski et al., [29]</td>
<td>2013</td>
<td>10°C</td>
<td>Yes</td>
<td>n.a.</td>
<td>Human</td>
<td>ECD</td>
<td>↑ ALT, LDH</td>
<td>-</td>
</tr>
<tr>
<td>Guarerra et al., [24]</td>
<td>2010</td>
<td>4-8°C</td>
<td>No</td>
<td>6</td>
<td>Human</td>
<td>SCD</td>
<td>↑ AST, ALT, LDH</td>
<td>Perfusate</td>
</tr>
<tr>
<td>Guarerra et al., [25]</td>
<td>2011</td>
<td>4-8°C</td>
<td>No</td>
<td>6</td>
<td>Human</td>
<td>SCD</td>
<td>↑ ICAM-1, IL-8, TNF-α</td>
<td>Perfusate, tissue</td>
</tr>
<tr>
<td>Henry et al., [26]</td>
<td>2012</td>
<td>4-8°C</td>
<td>No</td>
<td>6</td>
<td>Human</td>
<td>SCD</td>
<td>↑ Inflammatory cytokines, adhesion molecules, oxidative markers, acute phase proteins, CD68</td>
<td>Tissue</td>
</tr>
<tr>
<td>Jomaa et al., [104]</td>
<td>2013</td>
<td>4-8°C</td>
<td>No</td>
<td>30</td>
<td>Human</td>
<td>ECD</td>
<td>↑ AST, ↑ LDH</td>
<td>Perfusate, tissue</td>
</tr>
<tr>
<td>Monbaliu et al., [28]</td>
<td>2012</td>
<td>4-6°C</td>
<td>No</td>
<td>20-30</td>
<td>Human</td>
<td>ECD</td>
<td>↑ AST, ↑ LDH</td>
<td>Perfusate, tissue</td>
</tr>
<tr>
<td>Tulipan et al., [27]</td>
<td>2011</td>
<td>4-8°C</td>
<td>No</td>
<td>6</td>
<td>Human</td>
<td>SCD</td>
<td>↑ MCP-1, ↑ IL-1Ra</td>
<td>Perfusate, serum</td>
</tr>
</tbody>
</table>

Studies on biomarkers to monitor quality of grafts obtained from standard criteria donors (SCD) or extended-criteria donors (ECD) during MP. n.r., not reported. n.a., not applied.

*These studies all derived from the trial by Guarerra in 2010.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ICAM-1, intracellular adhesion molecule 1; IL-8, interleukin 8; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte-choemoattractant protein 1; IL-1Rα, interleukin 1 receptor antagonist.
conclude that bile production during NMP is the most important parameter for viability [41], although no strong correlations could be made since these grafts were not actually transplanted. Vairetti et al. demonstrated that bile is also produced during colder SNP [36]. More importantly, this study showed that bile outflow during MP was no guarantee for improved bile flow after graft reperfusion. Boehnert et al. emphasized that evaluation of solely bile flow during MP might be biased due to the secretion of serum-like fluids from the injured biliary mucosa, which could falsely increase bile volume [23,42]. In order to correct for this bias, they measured lactate dehydrogenase (LDH) in bile as a marker for biliary epithelial injury and found its content in bile to be lower after NMP compared to SCS, while bilirubin and phospholipid concentrations were higher [23]. Impaired secretion of phospholipids gives a surplus of free bile salts which are toxic for cholangiocytes. A higher ratio of bile salts/phospholipids, rather than bile production solely, has been associated with the development of ITBL [43,44]. Also the secretion of HCO₃⁻ into bile, involved in local pH regulation, has been described as a marker for cholangiocyte function. The evidence of bile outflow or composition as a marker at temperatures below 20 °C is however marginal. Since lower temperatures shut-down metabolic cellular processes, bile parameters are probably more informative under (sub)normothermic conditions.

Liver enzyme release as indicator of hepatocyte injury

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and LDH are the most frequently studied biomarkers in liver disease. Both AST and LDH are enzymes that are mainly present in the cell cytoplasm of various tissues, including liver, and they are often used as general injury markers to monitor graft function after LT. For a more specific assessment of hepatocyte injury, ALT is often determined. In their clinical trial, Guerrera et al. found perfusate levels of AST and ALT measured during HMP to strongly correlate with post-transplant peak AST and ALT serum levels in recipients. This suggests that injury that becomes apparent after graft implantation, can already be detected during HMP. Monbaliu et al. distinguished transplantable from non-transplantable grafts based on AST levels in perfusates during HMP [28]. But also during NMP, the release of AST and ALT were predictive for recipient survival in a large animal model [35]. Moreover, hepatic enzyme release during MP strongly correlated with donor warm-ischemia time, which in turn has been associated with poor graft quality [45]. The value of enzyme release into perfusates to predict PNF and EAD has also been confirmed by clinical LT studies with conventional SCS (Table 3) [46–48].

Energetic recovery status by adenine nucleotides

Cold temperatures and the absence of oxygen supply to tissue causes the shutdown of adenine nucleotide metabolism, which causes failure of ion transport by electron pumps on the cell membrane [49]. Therefore, Minor et al. investigated whether oxygenation during MP could recover energy status by measuring the energy charge potential and adenosine triphosphate (ATP) levels in tissue [40]. At the end of various MP methods and already before reperfusion, oxygenated tissue showed a higher energy charge potential and increased ATP levels compared to cold stored livers. This study furthermore demonstrated that hypothermic conditions hampered energetic recovery compared
to (sub)normothermic conditions. In clinical LT, decreased ATP levels have been shown to increase the risk for graft PNF or EAD; Kamiike et al. [50] used expression of ATP and total adenine nucleotides in peri-transplant liver biopsies to predict graft viability, based on functional outcome within the first days after LT. Compared to other nucleotides, ATP was demonstrated to be most sensitive for ischemia, as its expression decreased faster. However, a reduction of total adenine nucleotide levels in liver biopsies was more predictive for PNF after LT than ATP levels solely. Following revascularization, good functioning grafts also showed a better recovery of ATP and total adenine nucleotide levels. These levels were inversely related to the period of warm ischemia during graft implantation. Similar studies performed by Lanir et al. [51] and Hamamoto et al. [52], confirmed lower (total) adenine nucleotide levels in biopsies that were obtained during respectively cold storage and post-reperfusion, which also correlated with the development of PNF. Moreover, Hamamoto et al. found increased levels of Xanthine in perfusates also to be associated with PNF. These findings suggest that assessing energetic recovery of grafts in tissue and perfusates might be a good predictor for graft viability during MP in both hypo- as (sub)normothermic conditions.

### Endothelial injury markers: hyaluronic acid & thrombomodulin

The absence of blood and oxygen causes ischemic- and preservation injury to cells of the liver sinusoids [53]. Hyaluronic acid (HA) is a high-molecular weight glycosaminoglycan (4–8 million kDa) formed by the cellular plasma membrane [54] and its uptake mainly occurs by sinusoidal endothelial cells of the liver sinusoids [53].

### Table 2. Studies on biomarkers that were measured during various types of MP prior to (mimicked) reperfusion in animal models.

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>MP temp °C</th>
<th>Oxygenated</th>
<th>Pressure (mmHg)</th>
<th>Size</th>
<th>Subject</th>
<th>Donor model</th>
<th>Markers during MP</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boehnert et al., [23]</td>
<td>2013</td>
<td>38</td>
<td>Yes</td>
<td>arterial 60</td>
<td>7</td>
<td>Pig</td>
<td>DCD</td>
<td>ALT, necrosis, bile volume, pO2, urea</td>
<td>Perfusate, tissue, bile</td>
</tr>
<tr>
<td>Fondevila et al., [39]</td>
<td>2011</td>
<td>36-37</td>
<td>Yes</td>
<td>40-60 8</td>
<td>18</td>
<td>Pig</td>
<td>DCD</td>
<td>AST, bilirubin, LDH, pH, pO2</td>
<td>Perfusate</td>
</tr>
<tr>
<td>Fondevila et al., [105]</td>
<td>2012</td>
<td>4</td>
<td>Yes</td>
<td>20-30 4</td>
<td>11</td>
<td>Pig</td>
<td>DCD</td>
<td>Venous/arterial flow, AST, pH, O2, Na+, K+</td>
<td>Perfusate</td>
</tr>
<tr>
<td>Jamieson et al., [21]</td>
<td>2011</td>
<td>39</td>
<td>Yes</td>
<td>85-95 n.d. 8</td>
<td>Pig</td>
<td>Steatosis</td>
<td>Bile volume, base excess, albumin, AST, ALT, steatosis, glucose, urea</td>
<td>Bile, tissue, perfusate</td>
<td></td>
</tr>
<tr>
<td>Liu et al., [45]</td>
<td>2014</td>
<td>4-6</td>
<td>Yes</td>
<td>20 3</td>
<td>36</td>
<td>Pig</td>
<td>DCD</td>
<td>pH, AST, L-FABP, ATP, redox active iron, arterial resistance</td>
<td>Perfusate</td>
</tr>
<tr>
<td>Menor et al., [40]</td>
<td>2013</td>
<td>4-20</td>
<td>Yes</td>
<td>25 4</td>
<td>24</td>
<td>Pig</td>
<td>DBD</td>
<td>Energy charge potential, ATP, AST, ALT, lactate, LPO</td>
<td>Perfusate, tissue, perfusate</td>
</tr>
<tr>
<td>Obara et al., [106]</td>
<td>2012</td>
<td>4-8</td>
<td>Yes</td>
<td>88 6</td>
<td>7</td>
<td>Pig</td>
<td>DCD</td>
<td>AST, ALT, LDH, arterial flow</td>
<td>Perfusate</td>
</tr>
<tr>
<td>Olszewski et al., [107]</td>
<td>2010</td>
<td>4-21</td>
<td>Yes</td>
<td>n.d., n.d. 30</td>
<td>Pig</td>
<td>DCD</td>
<td>Portal venous resistance, bile volume, lactate, ALT</td>
<td>Bile, perfusate</td>
<td></td>
</tr>
<tr>
<td>Perk et al., [90]</td>
<td>2012</td>
<td>37</td>
<td>Yes</td>
<td>n.d. 7-9</td>
<td>19</td>
<td>Rat</td>
<td>DCD</td>
<td>Glucose, urea, lactate</td>
<td>Perfusate</td>
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<tr>
<td>Schlegel et al., [30]</td>
<td>2013</td>
<td>4</td>
<td>Yes</td>
<td>n.a. 3</td>
<td>46</td>
<td>Pig</td>
<td>DCD</td>
<td>NADH, pCO2</td>
<td>Perfusate</td>
</tr>
<tr>
<td>Shigeta et al., [108]</td>
<td>2013</td>
<td>4-25</td>
<td>Yes</td>
<td>28 4</td>
<td>9</td>
<td>Pig</td>
<td>DCD</td>
<td>AST, LDH, HA</td>
<td>Perfusate</td>
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<tr>
<td>Varetti et al., [36]</td>
<td>2008</td>
<td>4-37</td>
<td>Yes</td>
<td>n.a., n.a. 30</td>
<td>Rat</td>
<td>DBD</td>
<td>AST, LDH, bile volume (LDH), γGT</td>
<td>Perfusate, bile</td>
<td></td>
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<tr>
<td>Varetti et al., [71]</td>
<td>2009</td>
<td>20</td>
<td>Yes</td>
<td>n.a. 6-7</td>
<td>48</td>
<td>Rat</td>
<td>Steatosis</td>
<td>AST, LDH</td>
<td>Perfusate</td>
</tr>
<tr>
<td>Xu et al., [38]</td>
<td>2012</td>
<td>39</td>
<td>Yes</td>
<td>70-80 5-8</td>
<td>12</td>
<td>Pig</td>
<td>DCD</td>
<td>ALT, bile volume, CO2, ATP, necrosis, apoptosis</td>
<td>Bile, tissue, perfusate</td>
</tr>
</tbody>
</table>

n.d., not defined.

n.a., not applied.

ALT, alanine aminotransferase; HA, hyaluronic acid; LDH, lactate dehydrogenase; L-FABP; liver-type fatty acid binding protein; ATP, adenosine triphosphate; LPO, lipid peroxides; NADH, Nicotinamide adenine dinucleotide.
liver [55]. In clinical LT, a disruption of the hepatic micro-vascular integrity by preservation injury was shown to reduce the uptake of HA from the circulation, causing levels of HA in the liver to rise, which subsequently lead to EAD [56]. Comparable studies by Bronsther et al. [57] and Rao et al. [58] provided stronger evidence for HA to be associated with PNF and diminished graft survival after LT; levels over 400 µg/L in the perfusate had a highly negative predictive value of 95%. Furthermore, these studies demonstrated a correlation between HA levels in perfusates and post-operative AST and ALT levels in recipients. In the setting of NMP, Brockmann et al. found HA levels during NMP as one of their most significant predictors for graft viability after LT in a large animal model [35]; the mean level of HA in perfusates of successful grafts was 108 ng/ml, while non-successful grafts released much higher HA levels (6087 ng/ml).

Another endothelial cell marker is Thrombomodulin (TM), which has potential anticoagulant effects if it forms a complex with thrombin. When the vascular endothelium of liver sinusoids is injured for instance by graft preservation, TM is inactivated by cleavage into smaller fragments of so-called soluble thrombomodulin (sTM) and it is subsequently released from the cell surface [59–64]. Suehiro et al. [65] found TM levels over 20 FU/ml in perfusates to be sensitive for identifying grafts with PNF or EAD after LT. These grafts showed a higher expression of TM on liver cell membranes compared to successful grafts. 

Table 3. Overview of studies investigating biomarkers during clinical LT associated with PNF, EAD or biliary complications.

<table>
<thead>
<tr>
<th>Study</th>
<th>Outcome</th>
<th>Incidence</th>
<th>Year</th>
<th>Size</th>
<th>Injury marker</th>
<th>Donor assay</th>
<th>Graft type</th>
<th>Evidence level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abraham et al., [70]</td>
<td>PNF</td>
<td>29%</td>
<td>1996</td>
<td>38</td>
<td>↑ Hepatocyte swelling, Apoptosis, hemorrhage, hepatocyte swelling and necrosis</td>
<td>Liver tissue</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Hamamoto et al., [52]</td>
<td>PNF</td>
<td>6%</td>
<td>1994</td>
<td>68</td>
<td>↓ Adenine nucleotides, ↑ Xanthine</td>
<td>Liver tissue</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Kamiike et al., [50]</td>
<td>PNF</td>
<td>20%</td>
<td>1988</td>
<td>30</td>
<td>↓ Adenine nucleotides, ↓ Bile flow rate</td>
<td>Liver tissue</td>
<td>Bile</td>
<td>4</td>
</tr>
<tr>
<td>Lanir et al., [51]</td>
<td>PNF</td>
<td>20%</td>
<td>1988</td>
<td>25</td>
<td>↓ Adenine nucleotides (ATP &lt;2 nmoles/mg)</td>
<td>Liver tissue</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Bronsther et al., [57]</td>
<td>PNF</td>
<td>9%</td>
<td>1993</td>
<td>70</td>
<td>↑ HA (&gt;400 µg/L)</td>
<td>Perfusate</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Rao et al., [58]</td>
<td>PNF</td>
<td>6%</td>
<td>1996</td>
<td>102</td>
<td>↑ HA (&gt;400 µg/L)</td>
<td>Perfusate</td>
<td>DBD*</td>
<td>2B</td>
</tr>
<tr>
<td>Berberat et al., [67]</td>
<td>PNF</td>
<td>7%/22%</td>
<td>2006</td>
<td>59</td>
<td>↑ CRP, ↓ CTGF, WWP2, CD274, VEGF, FLT1</td>
<td>Liver tissue</td>
<td>n.d.</td>
<td>3B</td>
</tr>
<tr>
<td>Khettry et al., [69]</td>
<td>PNF</td>
<td>8%/16%</td>
<td>1991</td>
<td>50</td>
<td>10%-50% hemorrhage and/or necrosis</td>
<td>Galbladder tissue</td>
<td>DBD</td>
<td>3B</td>
</tr>
<tr>
<td>Lange et al., [48]</td>
<td>PNF</td>
<td>10%/4%</td>
<td>1996</td>
<td>50</td>
<td>↑ AST, ALT, LDH</td>
<td>Perfusate</td>
<td>DBD*</td>
<td>4</td>
</tr>
<tr>
<td>Calmus et al., [68]</td>
<td>EAD</td>
<td>19%</td>
<td>1995</td>
<td>32</td>
<td>↑ Amino acids</td>
<td>Perfusates</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Cywes et al., [109]</td>
<td>EAD</td>
<td>n.d.</td>
<td>1993</td>
<td>30</td>
<td>↑ Platelet adhesion</td>
<td>Liver tissue</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Devlin et al., [46]</td>
<td>EAD</td>
<td>19%</td>
<td>1995</td>
<td>53</td>
<td>↑ AST, ALT</td>
<td>Perfusate</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Pacheco et al., [47]</td>
<td>EAD</td>
<td>21%</td>
<td>2010</td>
<td>47</td>
<td>↑ AST, ALT, LDH</td>
<td>Perfusate</td>
<td>n.d.</td>
<td>4</td>
</tr>
<tr>
<td>Suehiro et al., [65]</td>
<td>EAD</td>
<td>14%</td>
<td>1997</td>
<td>58</td>
<td>↑ TM (&gt;20 FU/ml), ↑ Sinusoidal TM staining</td>
<td>Perfusate</td>
<td>DBD*</td>
<td>3B</td>
</tr>
<tr>
<td>Brunner et al., [12]</td>
<td>Biliary complications</td>
<td>n.d.</td>
<td>2013</td>
<td>79</td>
<td>&gt;10% epithelial damage, disturbed tight junction protein architecture</td>
<td>Extrahepatic bile duct tissue</td>
<td>DBD</td>
<td>3B</td>
</tr>
<tr>
<td>Op den Dries et al., [76]</td>
<td>ITBL</td>
<td>16%</td>
<td>2014</td>
<td>128</td>
<td>Vascular injury with arteriolonecrosis, &gt;50% loss of cells in deep peribiliary glands</td>
<td>Extrahepatic bile duct tissue</td>
<td>DBD and DCD</td>
<td>2B</td>
</tr>
<tr>
<td>Farid et al., [80]</td>
<td>ITBL</td>
<td>n.d.</td>
<td>2013</td>
<td>22</td>
<td>↓ Portal vein branch-size</td>
<td>Liver tissue</td>
<td>DBD</td>
<td>3B</td>
</tr>
<tr>
<td>Hansen et al., [75]</td>
<td>ITBL</td>
<td>19%</td>
<td>2012</td>
<td>93</td>
<td>Presence of arteriolonecrosis</td>
<td>Extrahepatic bile duct tissue</td>
<td>DBD</td>
<td>2B</td>
</tr>
<tr>
<td>Verhoeven et al., [86]</td>
<td>ITBL</td>
<td>35%</td>
<td>2013</td>
<td>56</td>
<td>↑ HDmR/CDmR ratio</td>
<td>Perfusate</td>
<td>DBD and DCD</td>
<td>2B</td>
</tr>
</tbody>
</table>

ATP, adenosine triphosphate; HA, hyaluronic acid; TM, thrombomodulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; HDmR, hepatocyte-derived miRNA; CDmR, cholangiocyte-derived miRNA; DBD, donation after brain death; DCD, donation after circulatory death; n.d., not defined.

*Graft type assumed to be DBD, derived from the year of publication.
sinusoidal endothelial cells at the end of cold storage. In a smaller study performed by Sido et al., intraoperative STM levels were measured in blood to assess graft endothelial reperfusion injury [60]. After reperfusion, STM levels correlated significantly with release of liver enzymes and increased adherence of leukocytes in liver tissue. In clinical LT, however, only one study investigated TM as a predictor for outcome and graft quality [65] and no data are known on the potential use of TM as a marker for viability testing in the setting of MP.

Inflammatory markers, kupffer cells and proteolytic enzymes

Graft ischemia induces an inflammatory cascade that attracts leukocytes and neutrophils to the site of tissue injury and subsequent leakage of proteolytic enzymes, causing breakdown of cells and surrounding tissue post-reperfusion [66]. In a retrospective study that derived from the first clinical trial applying HMP for LT, Henry et al. investigated the effect of HMP on the expression of several injury markers [26]. Oxidative stress markers as hypoxia-inducible factor-1α and -1π were significantly decreased in biopsies that were taken at the end of HMP, compared to SCS grafts. Also the expression of inflammatory markers like tumour necrosis factor-α (TNF-α) were significantly lower in grafts already at time of HMP. The authors hypothesize that these pro-inflammatory factors are eliminated through the diluting effects of HMP, thereby also reducing the production of downstream chemokines and adhesion molecules like intercellular adhesion molecule-1 and P-selectin. This hypothesis was supported by the observation that at the end of HMP, less infiltrating Kupffer cells (CD68 positive) were present in tissue compared to biopsies that were taken at the end of SCS. Berberat et al. [67] found several inflammatory genes in post-reperfusion biopsies predictive of graft outcomes; high expression of TNF-α was correlated with shortened graft survival, while high c-reactive protein expression correlated with the need of interventions after LT. A linear combination of five survival genes in post-reperfusion biopsies was able to predict graft failure with a positive predictive value of 72% and negative predictive value of 96%. Calmus et al. [68] also demonstrated a strong correlation between ongoing proteolysis during SCS and EAD; increased levels of free amino acids that were released from the liver into perfusates showed good positive- and negative-predictive value (respectively 100% and 95%) for EAD in the first postoperative week. As Henry et al. and Calmus et al. show, it is feasible to measure inflammatory markers and proteolytic enzymes during cold graft preservation prior to reperfusion. However, the strongest effect on these markers usually becomes apparent after revascularization of the graft [25] and therefore it would be highly interesting to observe the predictive value of these markers in normothermic conditions. Up to now, many MP studies only investigate such markers after reperfusion [25,32].

Tissue hemorrhage and cell necrosis

The degree in which tissue is affected by graft ischemia varies and is usually reflected by histopathological changes. Xu et al. investigated these histological changes during NMP of porcine liver grafts [38]. A remarkable finding was that the degree of necrosis and apoptosis in biopsies taken after warm ischemia and subsequent cold storage, appeared to be reversed after 4 h of NMP. This not only suggests that histological evaluation at time of NMP might be a useful indicator for graft viability, it also indicates that NMP has the potential to recover ischemic damage. This has also been suggested by other NMP studies that performed histological evaluation after reperfusion [23,39]. The prognostic value of necrosis and apoptosis occurring during SCS was also evaluated in different tissues from clinical studies; Khefry et al. demonstrated extensive hemorrhage and/or necrosis of 10–50% in the donor gallbladder mucosa to have a high positive and negative predictive value for PNF and impaired graft survival, whereas vascular congestion was present in all donor gallbladders [69]. In addition, Abraham et al. identified apoptotic cells and zone 3 hemorrhage in post-reperfusion liver tissue to have good discriminative power for PNF (AUC = 0.90 and 0.77 respectively) [70].

Degree of graft steatosis

Beside DCD, steatotic livers form another important source within the category of ECD grafts that could benefit from improved preservation and subsequent graft outcome by MP. Bessems et al. [20] found improved functional parameters in steatotic rat livers after HMP compared to normal preservation by SCS. Similar beneficial effects were observed by Vairetti et al., who concluded that subnormothermic temperatures are preferred over colder temperatures for the recovery of steatotic grafts [71]. Despite exciting results on MP for optimizing the quality of steatotic grafts, these studies were not informative on potential biomarkers prior to reperfusion. However, a more recent study by Jamieson et al. measured a decrease in lipid deposits during NMP of rat livers which correlated with a reduction in the degree of steatosis [21]. Previous clinical studies showed the value of histological macro vesicular steatosis to predict graft PNF, which has been extensively reviewed earlier [72]. Dutkowski et al. [73] integrated the degree of steatosis in a balance of risk score with other risk factors for graft failure, consisting of recipient age, MELD-score, re-transplantation, cold ischemia, and donor age. This score indicates that one should be reluctant with the use of moderate to severe steatotic liver grafts (>30%) in recipients with a balance of risk-score > 9, but microvesicular steatosis has not been related to poorer outcome. Though histological scoring in steatotic grafts seems promising in the setting of both MP and SCS, in general, one should be aware for the risks of intra- and inter observer variability that hampers a standardized histological evaluation [74].

Markers for biliary injury

As previously explained, bile ducts of particularly ECD grafts have been shown to be vulnerable for ischemic injury and are responsible for a high percentage of graft loss (Fig. 1). Therefore, biliary complications are also an important outcome for several MP studies. Up to now, MP studies on human liver grafts (Table 1) have shown that MP is not harmful for bile ducts, but most studies are too small to demonstrate whether a significant benefit actually exists [24,29,37]. Schlegel et al. recently demonstrated beneficial effects of HOPE on biliary fibrosis, but no markers were investigated during HOPE on their predictive capacity for biliary injury [31]. Several clinical studies however identified markers in tissue and perfusates during SCS that were able to predict biliary complications.
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*Peribiliary epithelial damage and vascular injury*

Brunner et al. developed a bile duct damage-score based on the degree of injury in the epithelium of the extrahepatic bile duct and diminished epithelial barrier integrity measured by tight junction proteins [12]. Samples of common bile duct tissue showing more than 10% of destructed epithelium or/and subepithelial connective tissue at the beginning of cold preservation were predisposed to develop major biliary complications and diminished graft survival. Also Hansen et al. [75] scored extrahepatic bile duct specimens and found arteriolonecrosis causing mural necrosis to be the most prominent risk factor for ITBL. Similar observations were recently reported in a larger cohort studied by Op den Dries et al. [76]. Additionally, the investigators found that grafts that would develop ITBL, lost over 50% of cells within deep peribiliary glands that are located along the common bile duct and which are involved in cholangiocyte proliferation in response to injury [77,78]. Based on their findings, the authors formulated the hypothesis that ITBL results from an insufficient regenerative capacity of injured cholangiocytes by peribiliary glands, caused by arteriolonecrosis in the bile duct wall, rather than being the result of extensive epithelial injury alone [79]. Remarkably, the degree of injury in peribiliary glands did not differ between DBD and DCD grafts. Beside changes in the arterial vasculature of the peribiliary plexus, a case-control study by Farid et al. showed changes in the luminal size of the portal vein branch (PVB) in liver tissue specimens to be more pronounced after reperfusion [80]; a smaller PVB size was seen in grafts that later developed ITBL. This supports earlier findings on the importance of portal blood flow, which is responsible for approximately 40% of the blood supply in the common bile duct, for the risk to develop ITBL [81,82]. Unfortunately, differences in PVB size became apparent only after reperfusion.

**Cholangiocyte-derived microRNAs**

MicroRNAs (miRNAs) are small regulatory RNAs with high cell-type specificity and their resistance against RNase mediated degradation in different media and conditions makes them an attractive candidate for biomarker research [83–85]. Hepatocyte-derived miRNAs (HDmiRs) were identified as sensitive markers in serum for acute graft rejection and more recently, our group reported that lower levels of cholangiocyte-derived miRNAs (CDmiRs) in perfusates during SCS are predictive of ITBL in both DBD and DCD grafts [86,87]. In this study, miRNAs remained stable in University of Wisconsin (UW) and histidine-tryptophan-ketoglutarate (HTK) perfusates, also after incubation at room temperature. Preliminary data show that miRNAs can also be measured during MP (data not shown). Furthermore, HDmiRs and CDmiRs are also released into bile [88]. Interestingly, a very recent study shows that recipients developing ITBL have an altered miRNA composition in bile [89].

**Discussion**

As dynamic preservation is now entering the clinic, researchers emphasize on the need of predictive and sensitive biomarkers that are able to objectively assess graft quality during MP. Biomarkers could help to enlarge the donor pool by objectively screening liver grafts that initially would be discarded based on their predisposing characteristics. Several experimental studies already demonstrated that a combination of biomarkers measured during MP could be used as a damage index for ECD grafts [45,90]. However, since the clinical application of MP is still in its infancy, the introduction of such damage scores based on surrogate biomarkers should be studied in larger cohorts. Prospective randomized clinical trials on MP would offer the best opportunity for unbiased evaluation of potential biomarkers, provided that sampling of materials during MP is executed accurately. Moreover, such trials could also definitely answer the question which MP strategy is most capable of optimizing ECD graft quality.

The requirements for a biomarker to make it into clinical practice are that its measurement should be easy and relatively fast, with a high sensitivity and specificity for outcome. Moreover, biomarkers should be measurable in biomaterials that are available at time of graft preservation, so its discriminative capacity could be used in graft screening and allocation [91]. Biopsies from liver or extrahepatic bile duct specimens can be collected during preservation and are suitable for histological evaluation and quantification of injury based on (low) expressed biomarkers. It should however be emphasized that biopsies are obtained invasively and only represent a small part of the liver or bile duct, which could lead to incorrect interpretation when injury is unequally distributed throughout the tissue (Table 4). Moreover, inter- and intra-observer variability can hamper a standardized evaluation of histological markers. The collection of perfusates form an attractive non-invasive alternative for a variety of markers during conventional preservation and MP. Another advantage of using perfusates over tissue biopsies is that larger volumes can be collected and markers released into perfusates are believed to represent the condition of the entire liver parenchyma rather than only a small part of the liver. Limitations consist of difficulties in the normalization of markers; most MP systems use a recirculating perfusion system, in which biomarkers can accumulate. Therefore, perfusate levels of conventional biomarkers like AST could differ from standards in clinical practice. This also applies to perfusion temperature: hypothermic conditions will cause a delayed metabolism of the liver and requires an adjusted evaluation of biomarkers and cut-off values compared to normothermic, physiologic conditions.

A limitation for many biomarkers in general is that their quantification can be labour intensive and time consuming. Some techniques, for instance polymerase chain reaction, are however progressing in terms of accelerated measurements, which makes them applicable in the prolonged time-window created by MP [92,93].

In general, biomarkers can be used either to determine graft injury or graft function. Up to now, most biomarkers concern markers for injury, while bile production currently is the only marker for liver function. Robust markers of function rather than injury are however of importance, because severe ischemic injury not necessarily means that a graft will not function properly following LT. Additional markers of function could consist of substrates which do not naturally occur in the body, but are cleared by the liver. For instance the plasma disappearance rate of intravenously administered indocyanine green (PDR-ICG) or 13C-labeled methacetin (LiMAx test), which are predictive of PNF, EAD and hepatic artery thrombosis after LT [94–97]. However, results of such tests are influenced by perfusion flow rates [98,99]. Moreover, functional markers require a metabolically active liver, which can only be achieved under (sub)normothermic conditions (Fig. 4).
Beside biomarkers for injury and function, it is evident that donor- and recipient risk factors can influence outcome after LT (Fig. 2). Genetic polymorphisms in both donors and recipients have been identified that increase the risk for recipients to develop ITBL or bacterial infections after LT [100–102]. Therefore, genetic profiling could be helpful in matching donors to equivalent recipients [91]. Moreover, information on donor and recipient risk factors are usually available in an early stage of LT [103].

Concluding remarks and future directions

The limited experience of MP in clinical LT hampers the evaluation on which MP strategy is most optimal for graft quality and the evaluation of potential biomarkers for quality assessment. Another factor that hampers evaluation of biomarkers is the inconclusiveness between studies on outcome definitions; investigators maintain different criteria for comparing cohorts, making it impossible to perform a reliable meta-analysis on outcomes describing corresponding markers. More clear international guidelines on outcome definitions are therefore recommended, as was previously initiated by Olthoff et al. [9]. Comparing biomarkers during MP and conventional SCS, we can however conclude that non-invasive measurement of injury markers into perfusates and the assessment of liver function based on the production of bile are well-possible in MP. For all markers, however, one should take into account the baseline differences that can exist between donors, liver grafts, and MP techniques that influence biomarker measurements and pleas for custom criteria and cut-off values in the evaluation of biomarkers [10]. This review forms a starting point for future studies on quality assessment by biomarkers and graft screening in the changing setting of graft preservation and MP in clinical LT in the coming years.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.04.031.

References


Table 4. Materials for biomarker measurement during graft preservation. Summary on the advantages and disadvantages between the different biomaterials that can be used to assess graft quality at time of MP or during SCS prior to LT.

<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Histological evidence for graft quality</td>
<td>Invasive</td>
</tr>
<tr>
<td></td>
<td>Large amount of cells</td>
<td>Only local representation</td>
</tr>
<tr>
<td>Perfusate</td>
<td>Non-invasive</td>
<td>Timing; short before implantation</td>
</tr>
<tr>
<td></td>
<td>Larger quantities available</td>
<td>No standardized workup between LT centers</td>
</tr>
<tr>
<td>Bile</td>
<td>Non-invasive</td>
<td>Less informative during hypothermic conditions</td>
</tr>
<tr>
<td></td>
<td>Indicative for hepatocyte and cholangiocyte function</td>
<td>Smaller quantities available</td>
</tr>
</tbody>
</table>

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.
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