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

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Summary

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.

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

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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; (s)TM, (soluble) thrombomodulin; TNF-α, tumor necrosis factor alpha; PVB, portal vein branch; miRNAs, microRNAs; HDmiRs, hepatocyte-derived miRNAs; CDmiRs, cholangiocyte-derived miRNAs; UW, University of Wisconsin solution; HTK, histidine tryptophan ketoglutarate.

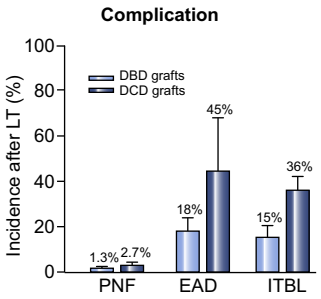

Fig. 1. Incidence of ischemia/preservation related complications after LT. Estimation of the incidence of PNF, EAD, and ITBL in separate DBD and DCD grafts, based on cohort- and case-matched studies [5,9–11,76,86,110]. Percentages represent the mean incidence ± standard error. Studies used to calculate 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

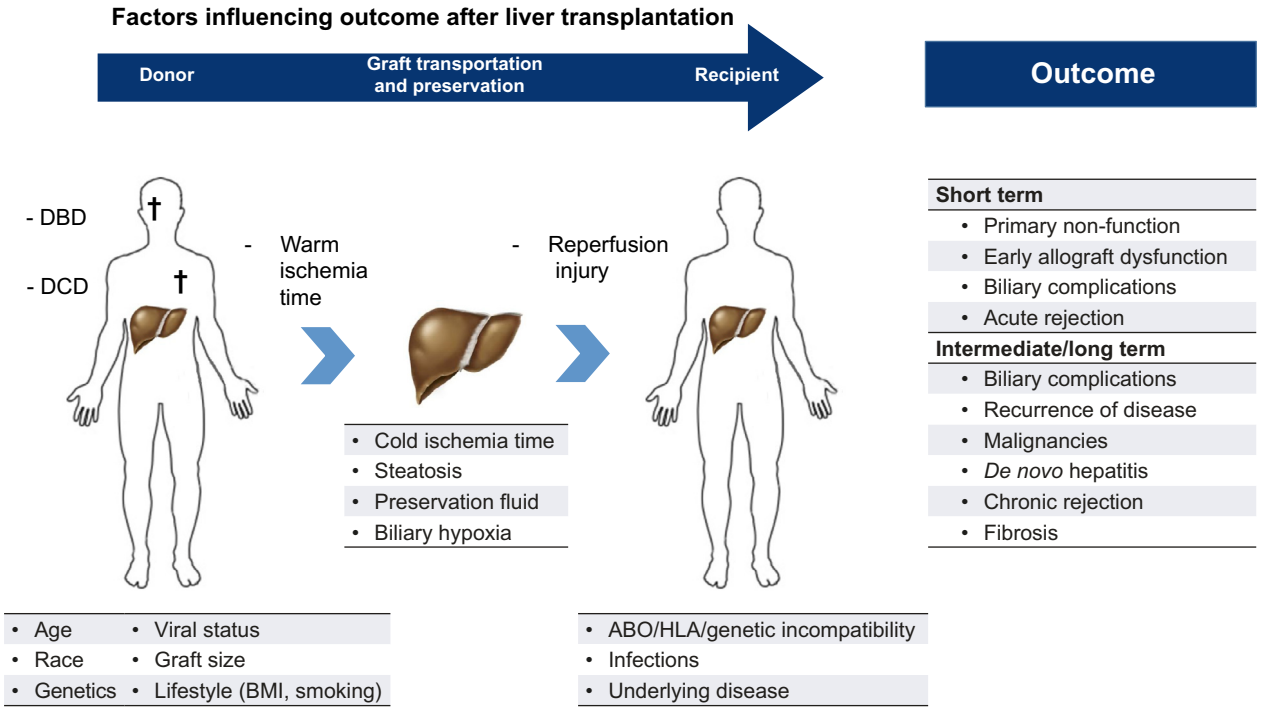

Fig. 2. Risk factors for outcome following LT. Risk factors in donors, recipients and during the transplantation and transportation procedure influencing graft quality and graft/recipient outcome.

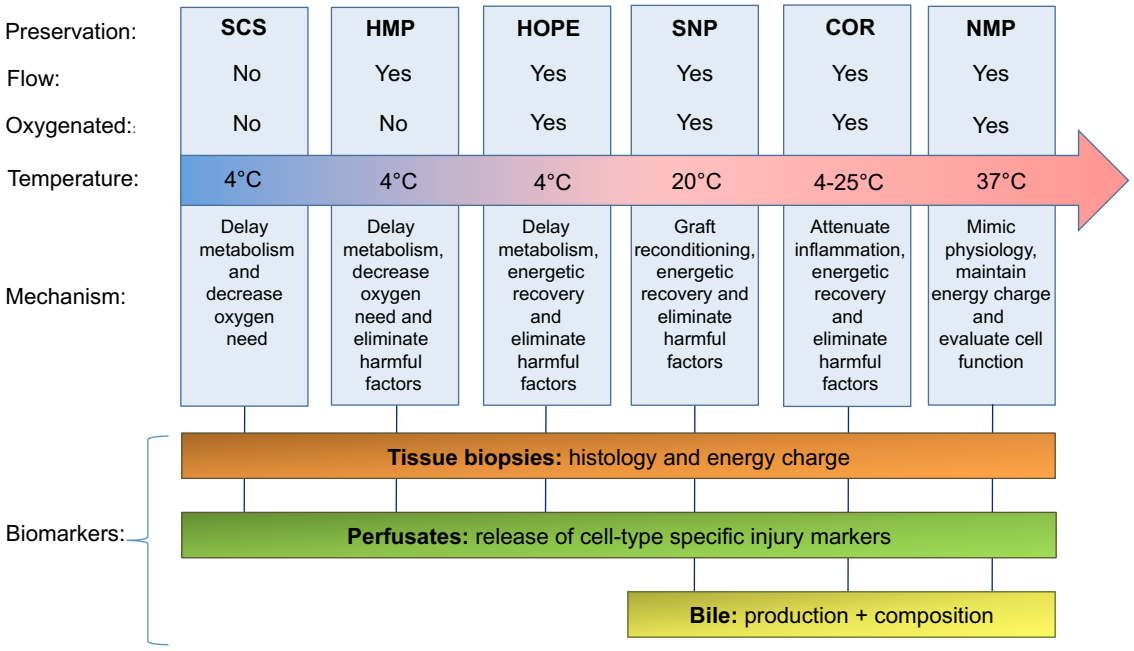

Fig. 3. Mechanisms of various machine preservation strategies. Different techniques of graft preservation can be used to protect against ischemic injury, to recondition the graft before reperfusion or even to maintain physiology. The various techniques have different potentially protective underlying 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.

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

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

Study [Ref.]	Year	MP temp	Oxygenated	Pressure (mmHg)		Size	Subject	Donor	Trans-planted	Markers during MP for impaired viability	Biomaterial
				arterial	venous						
Op den Dries *et al.*, [37]	2013	37°C	Yes	50	11	4	Human	ECD	No	↑ Enzymes, lactate levels, ↓ bile production, bile composition (γGT, bilirubine, LDH), O_2, CO_2	Perfusate, tissue, bile
Dutkowski *et al.*, [29]	2013	10°C	Yes	n.a.	<3	8	Human	ECD	Yes	n.r.	-
Guarrera *et al.*, [24]	2010	4-8°C	No	6	3	20	Human	SCD	Yes	↑ AST, ALT, LDH	Perfusate
Guarrera *et al.*, [25] *	2011	4-8°C	No	6	3	6	Human	SCD	Yes	↑ ICAM-1, IL-8, TNF-α	Perfusate, tissue
Henry *et al.*, [26] *	2012	4-8°C	No	6	3	33	Human	SCD	Yes	↑ Inflammatory cytokines, adhesion molecules, oxidative markers, acute phase proteins, CD68	Tissue
Jomaa *et al.*, [104]	2013	4-8°C	No	30	7	16	Human	ECD	No	n.r.	-
Monbaliu *et al.*, [28]	2012	4-6°C	No	20-30	7	17	Human	ECD	No	↑ AST, ↑ LDH	Perfusate, tissue
Tulipan *et al.*, [27] *	2011	4-8°C	No	6	3	n.r.	Human	SCD	Yes	↑ MCP-1, ↑ IL-1Rα	Perfusate, serum

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 Guarrera 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-chemoattractant protein 1; IL-1R α, interleukin 1 receptor antagonist.

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 [35,39]. Finally, an alternative for perfusion with constant temperature is controlled oxygenated rewarming (COR) of primarily cold stored liver grafts. Gradual increase of the MP temperature is thought to minimize re-oxygenation injury that is normally triggered by immediate rewarming of the graft, like in reperfusion and NMP. First results of COR in animal models indicate that post-reperfusion recovery is more successful in grafts that were subjected to COR compared to HMP, SNP, and SCS [40]. 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

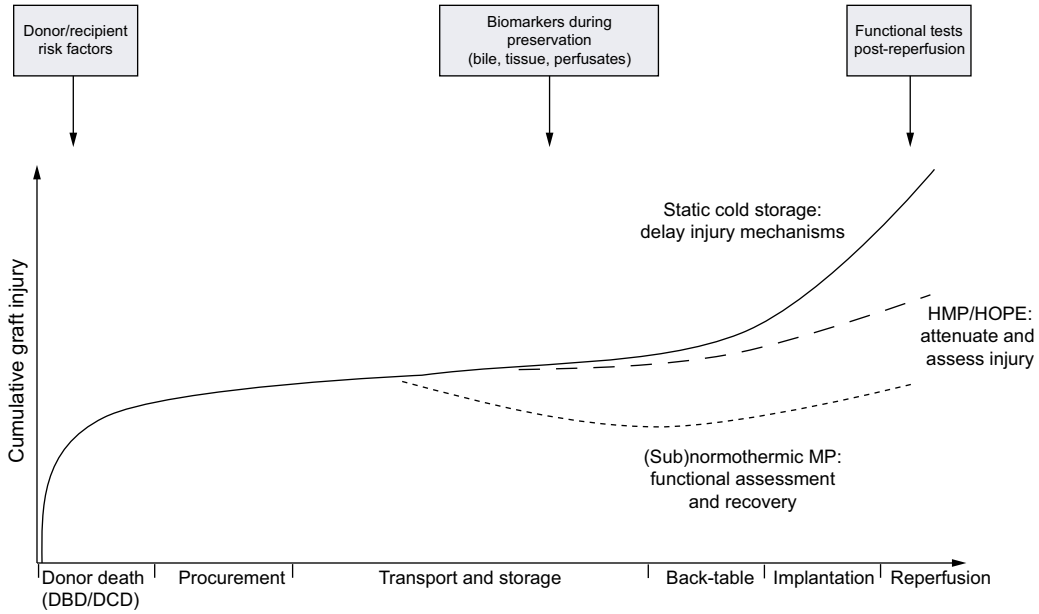

Fig. 4. Cumulative graft injury and evaluation points for graft quality. Already in an early phase of LT, known risk factors in donors and recipients could be used for deciding to accept a graft for transplantation and for which recipient. In order to determine the degree of preservation injury, biomarkers could be measured in tissue, perfusates or bile during various preservation methods. The cumulative injury of grafts that are preserved by hypothermic MP (either oxygenated or non-oxygenated) is believed to be less compared to static cold stored grafts (SCS). Mimicking normal physiological functions through (sub)normothermic MP is hypothesized to even recover injury of ECD grafts, for instance steatosis, and to provide a better assessment of graft function. After graft reperfusion, other functional tests are possible in order to monitor graft quality during follow-up.

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 HCO_3^- 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, Guarrera *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

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

Study	Year	MP temp °C	Oxygenated	Pressure (mmHg)		Size	Subject	Donor model	Markers during MP	Assay
				arterial	venous					
Boehnert *et al.*, [23]	2013	38	Yes	60	7	30	Pig	DCD	ALT, necrosis, bile volume, pO_2, urea	Perfusate, tissue, bile
Brockmann *et al.*, [35]	2009	39	Yes	n.d.	n.d.	38	Pig	DBD & DCD	Bile volume, base excess, AST, ALT, HA, portal pressure, portal venous resistance	Bile, perfusate
Fondevila *et al.*, [39]	2011	36-37	Yes	40-60	8	18	Pig	DCD	AST, bilirubin, LDH, pH, pO_2	Perfusate
Fondevila *et al.*, [105]	2012	4	Yes	20-30	4	11	Pig	DCD	Venous/arterial flow, AST, pH, O_2, Na+, K+	Perfusate
Jamieson *et al.*, [21]	2011	39	Yes	85-95	n.d.	8	Pig	Steatosis	Bile volume, base excess, albumin, AST, ALT, steatosis, glucose, urea	Bile, tissue, perfusate
Liu *et al.*, [45]	2014	4-6	Yes	20	3	36	Pig	DCD	pH, AST, L-FABP, ATP, redox active iron, arterial resistance	Perfusate
Minor *et al.*, [40]	2013	4-20	Yes	25	4	24	Pig	DBD	Energy charge potential, ATP, AST, ALT, lactate, LPO	Perfusate, tissue
Obara *et al.*, [106]	2012	4-8	Yes	88	6	7	Pig	DCD	AST, ALT, LDH, arterial flow	Perfusate
Olschewski *et al.*, [107]	2010	4-21	Yes	n.d.	n.d.	30	Rat	DCD	Portal venous resistance, bile volume, lactate, ALT	Bile, perfusate
Perk *et al.*, [90]	2012	37	Yes	n.d.	7-9	19	Rat	DCD	Glucose, urea, lactate	Perfusate
Schlegel *et al.*, [30]	2013	4	Yes	n.a.	3	46	Pig	DCD	NADH, pCO_2	Perfusate
Shigeta *et al.*, [108]	2013	4-25	Yes	28	4	9	Pig	DCD	AST, LDH, HA	Perfusate
Vairetti *et al.*, [36]	2008	4-37	Yes	n.a.	n.d.	30	Rat	DBD	AST, LDH, bile volume (LDH), γGT	Perfusate, bile
Vairetti *et al.*, [71]	2009	20	Yes	n.a.	6-7	48	Rat	Steatosis	AST, LDH	Perfusate
Xu *et al.*, [38]	2012	39	Yes	70-80	5-8	12	Pig	DCD	ALT, bile volume, CO_2, ATP, necrosis, apoptosis	Bile, tissue, perfusate

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.

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

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

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

ATP, adenosine triphosphate; HA, hyaluronic acid; TM, thrombomodulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; HDmiR, hepatocyte-derived miRNA; CDmiR, 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.

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

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 -1β 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 SCS biopsies. 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 down regulated vascular genes was superior in forecasting graft related complications, 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; Khettry *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 $\geqslant 9$, 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.

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 ^{13}C-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).

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

Biomaterial	Advantages	Disadvantages
Tissue	Histological evidence for graft quality Large amount of cells	Invasive Only local representation Risk of inter- or intra-observariability
Perfusate	Non-invasive Larger quantities available Suitable application for various types of MP	Timing; short before implantation No standardized workup between LT centers
Bile	Non-invasive Indicative for hepatocyte and cholangiocyte function Suitable application for MP	Less informative during hypothermic conditions Smaller quantities available

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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Conflict of interest

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Supplementary data

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