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The Release of MicroRNA-122 During Liver Preservation is Associated with Early Allograft Dysfunction and Graft Survival After Transplantation

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Footnotes

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CDmiR	Cholangiocyte derived micro-RNA
Cel miR-39	C.Elegans microRNA 39
CIT	Cold ischemia time
DBD	Donation after brain death
DCD	Donation after circulatory death
Erasmus MC	Erasmus Medical Center
EAD	Early allograft dysfunction
HDmiR	Hepatocyte derived micro-RNA
INR	International Normalized Ratio
ITBL	Ischemic-type biliary lesions
LDH	Lactate hyaluronate
LT	Liver transplantation
LabMELD	Model of End Stage Liver Disease
miR	Micro-RNA
RT-qPCR	Reverse transcription polymerase chain reaction
PNF	Primary non-function
reLTx	Re-transplantation
WIT	Warm ischemia time

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ABSTRACT

Introduction: Early allograft dysfunction (EAD) after liver transplantation is associated with inferior graft survival. EAD is more prevalent in grafts from donation after circulatory death (DCD). However, accurate prediction of liver function remains difficult due to the lack of specific biomarkers. Recent experimental and clinical studies highlight the potential of hepatocyte-derived microRNAs (miRNAs) as sensitive, stable and specific biomarkers of liver injury. The aim of this study was to determine whether miRNAs in graft preservation fluid are predictive for EAD after clinical liver transplantation and in an experimental DCD model.

Methods: Graft preservation solutions of 83 liver grafts at the end of cold ischemia were analyzed for miRNAs by RT-qPCR. Of these grafts 42% developed EAD after transplantation. Results were verified in pig livers (n=36) exposed to different lengths of warm ischemia time.

Results: The absolute miR-122 levels and miR-122/miR-222 ratios in preservation fluids were significantly higher in DCD grafts (p=0.001) and grafts developing EAD (p=0.004). In concordance, the miR-122/miR-222 ratios in perfusion fluid correlate with serum transaminase levels within the first 24 hours after transplantation. Long-term graft survival was significantly diminished in grafts with high miR-122/miR-222 ratios (p=0.019). In the porcine DCD model, increased warm ischemia lead to higher absolute miR-122 levels and relative miR-122/miR-222 ratios in graft perfusion fluid (p=0.009, and p=0.02, respectively). High miR-122/miR-222 ratios in pig livers were also associated with high AST levels after warm oxygenated reperfusion.

Conclusion: Both absolute and relative miR-122 levels in graft preservation solution are associated with DCD, EAD and early graft loss after liver transplantation. As shown in a porcine DCD model, miRNA release correlated with the length of warm ischemia times.

Keywords (not in the title): EAD, donation after circulatory death, graft survival

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Introduction

Due to a growing organ shortage, the use of marginal donors has increased in recent years. However, in liver transplantation (LT) the use of these extended criteria donors (ECD) has also resulted in the prevalence of early allograft dysfunction (EAD), primary non-function (PNF) and early graft loss (1-3). PNF of the liver is a life-threatening condition that is thought to be caused by microcirculation injury in the immediate postoperative period. Emergency re-transplantation of the liver is necessary because of the extreme high mortality of PNF. Hereby the prevalence of PNF has decreased over the last decades, nonetheless it occurs in 2-6% of all liver transplantations (4-6).

EAD occurs in 20-25% of all liver transplants and is less severe but remains an important determinant of graft and overall patient survival on the long term (7, 8). The definition of EAD, proposed by Olthoff et al. includes high early aminotransferase levels (alanine aminotransferase [ALT] or aspartate aminotransferase [AST]) <2000 IU/ mL within the first 7 postoperative days), cholestasis (bilirubin ≥ 10 mg/dL on day 7) and coagulopathy (INR ≥1.6 on day 7). These criteria have been mainly validated for grafts from heart beating donors (7, 9). Although the pathophysiology of EAD or PNF are not completely understood and is likely multifactorial, several risk factors for diminished graft function post-transplantation have been reported. The main factors are high donor age, donation after circulatory death (DCD) allografts, high recipient MELD score at the time of transplantation, a high degree of graft steatosis, small allograft size, long operative time and high intra-operative transfusion requirements (8, 10).

However, due to the growing demand for liver grafts, allografts which do not meet the traditional criteria for transplantation (extended criteria donors, EAD) are used more frequently (11, 12). In order to improve the quality of these EAD grafts and to reduce the post-transplantation risks for the receiver, machine perfusion as a preservation technique has received increasing attention (13-19). Machine preservation may also provide new opportunities to assess graft quality during the preservation period prior to transplantation and aid in the decision making as to transplant the organ (13-19). Reliable parameters or biomarkers for functional assessment of livers during preservation in either machine perfusion or static cold storage, are of paramount importance yet relatively unexplored (8, 20-28).

In the search for viable biomarkers, micro-RNAs (miRNAs) have gained the interest due to their low complexity, stability and organ specificity. Many studies have shown that miRNAs released in bio fluids like serum, urine and bile can be used as sensitive and accurate biomarkers (29-36). They are associated with a variety of pathologic conditions and control many cellular

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processes including tissue injury and repair (24, 29, 37-39). Therefore, circulating miRNAs might be helpful contributors in the existing decision making models to either accept or decline a potential donor liver for transplantation in order to optimize graft and patient survival.

Previous studies have reported the use of perfusates to predict graft survival and graft dysfunction by measuring lactate hyaluronate (LDH), AST and ALT levels. Our group investigated microRNAs in liver graft perfusates and proved their stability in samples up to 24h storage at room temperature (37). In this study by Verhoeven et al., an association was shown between high cholangiocyte derived miRNAs in liver graft preservation solution and ischemia type biliary lesions post-transplantation, suggesting the link between microRNA release before transplantation and post transplantation complications (40). However, no studies on miRNA biomarkers in the preservation liquid for EAD or early graft loss after liver transplantation have been reported.

This study aims to determine whether liver specific miRNAs in perfusion liquids during graft preservation are predictive of the development of EAD post-transplantation. Furthermore, the predictive value of miRNAs on long term graft survival was investigated. The hypothesis was, based on current literature on liver specific miRNAs in serum and their relation to liver function, that higher levels are correlated to damage and subsequent decreased graft function after transplantation. Due to increased levels of hepatocellular damage in DCD grafts, a correlation of high miRNA levels to longer warm ischemia times was expected. In order to verify this assumption and to minimize variations in the pre-preservation variables, we included the results of an experimental DCD porcine model in the interpretation of the results. Clinical implications of these results will be discussed.

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Patients and Methods

Explanations on study design, definition of EAD, sample collection and processing, RNA isolation, RT-qPCR, experimental design of the porcine model and statistical analyses are listed in the Supplementary data.

Results

Patient characteristics

Between April 2010 and March 2012, perfusates from 83 consecutive liver transplantations were collected at the end of cold ischemia time. Donor and recipient characteristics are shown in Table 1. Median follow-up after transplantation was 48 (SD 20) months. Thirteen patients underwent a re-transplantation after a median follow-up of 22 months (SD 24). Median graft survival was 42.2 months (SD 22.1) with a median overall patient survival of 47.2 (SD 19.6) months. Out of 83 grafts, 35 (42.2%) developed early allograft dysfunction; these included four grafts with PNF (4.8%), who were re-transplanted within seven days.

In this study, it is confirmed that graft origin is a predictor for EAD. Seven out of 48 (14.6%) of the non-EAD patients received a DCD graft while that was the case for 15 out of 35 (42.9%) in the EAD group (p=0.006). Total cold or warm ischemia time (CIT or WIT) did not differ between the non-EAD and EAD group (387 and 29 vs 426 and 32, respectively). Furthermore, receiving a graft from a male donor was associated with EAD after transplantation (p=0.03). According to the definition of EAD, recipients with delayed graft function had significantly higher AST and ALT levels in the first 24 hours after transplantation.

Relative miR-122 in biopsies and perfusates

Relative miR-122 levels in biopsies (n=34) were compared between grafts that developed EAD (n=17) and grafts with a good initial function (n=17). Individual miR-122 levels were not significantly increased in grafts with poor function post transplantation (Figure 1A, left panel). However, in these same 34 patients, relative miR-122 was significantly different in perfusates (Figure 1A, right panel).

Individual miRNAs in perfusates were analyzed in a bigger cohort of 83 patients and compared between grafts that developed EAD and non-EAD grafts (Figure 1B, left panel). Levels of miR-122 were significantly increased in grafts with poor function post transplantation. To relative

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miRNA-levels within each sample and control for variations in RNA quantities, we used the ratio of hepatocyte derived miRNA/cholangiocyte derived miRNA (HDmiR/CDmiR) of miR-122/miR-222 as previously described (37). Grafts which developed EAD after transplantation had significantly higher miRNA ratios, compared to those who did not develop EAD, due to high miR-122 levels and relative low amounts of miR-222 (Figure 1B, right panel). Relative levels of miR-222 were not significantly different between both groups (p= 0.46, data not shown).
Univariate analysis indicates high individual miRNA levels and miRNA ratios as risk factors to develop EAD (Table 2). In multivariate analysis, individual and relative high miR-122 remains important in the development of EAD, independently of the type of graft or gender of the donor. In this table, the discriminative power of miRNAs in perfusate is indicated as C-statistics and range from 0.67 to 0.74.

Relative miRNA levels in perfusates from DCD versus DBD livers

DCD livers are more prone to develop EAD than grafts from brain dead donors as is known from earlier literature and is demonstrated in our univariate and multivariate analysis. In our cohort of 83 transplanted grafts, DCD livers showed higher miRNA levels in perfusates, suggesting increased damage to the hepatocytes in these grafts. Figure 2A shows the differences in miRNA miR-122 in DBD versus DCD livers (p=0.005). Figure 2B shows the differences in miR-122/miR-222 ratios between DBD and DCD grafts that did or did not develop EAD (p=0.001). Ratios are increased irrespective of the fact whether the graft was obtained from a DCD or a DBD donor (respectively, p=0.02, p=0.001 and p=0.02). Although a clear trend was found, no significant difference was observed in length of the first WIT and miR-122/miR-222 levels (data not shown, *p*=0.07). This may be due to the lack of objective measurements of the length of warm ischemia in the clinical setting.

Increased perfusate miRNA ratios are associated with early graft loss

We found significantly higher miR-122/miR-222 ratios in perfusates of livers which were lost within the first 12 months after transplantation. Furthermore, relative high miRNA-ratios were associated with inferior graft survival on the long term (Figure 3). However, relative miR-122 levels were not significant for survival in the first year (p=0.10) or on the long term (p=0.07).

Perfusate miRNA levels correlate with post-transplant serum AST and ALT levels

To combine our observations with clinical outcome in patients post transplantation, AST (U/L) and ALT (U/L) levels 24 hours after transplantation in patient serum were correlated to miRNA

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ratios in perfusates. Figure 4 shows a significant correlation between miR-122/miR-222 ratio and AST and ALT levels in serum of recipients 24 hours after transplantation (left and right panel respectively). These correlations were also significant for miR-122 levels alone with AST, ALT, and lactate dehydrogenase (LDH) (p=0.01 and R^2=0.28, *p*=0.007 and R^2=0.30, and *p*=0.045 and R^2=0.22, respectively). The miR-122/miR-222 ratio was similarly correlated to LDH levels on day 1 (*p*=0.006, R^2=0.30, data not shown).

Validation of miR-122 in an experimental DCD model

To confirm the observation that miR-122 levels correlate with AST and can be useful as a determinant for EAD, we analyzed liver perfusates after procurement in an experimental porcine model of donation after cardiac death. This model was used to standardize the length of the warm ischemia time. Warm ischemia during normal liver procurement were mimicked by clamping the hepatic blood flow (portal vein, aorta and vena cava) in situ for various lengths of time (0, 15, 30, 45, 60 and 120 min, n=6 per group): a schematic representation of the procedure is shown in figure 5A. MiRNA levels were measured in total RNA isolated from perfusate obtained after 60 minutes of hypothermic machine perfusion. AST levels in perfusates were determined after 3 hours of warm reperfusion. Results were clustered in 3 groups: cluster 1 (0 and 15 minutes warm ischemia (WI)), cluster 2 (30 and 45 minutes WI) and cluster 3 (60 and 120 minutes WI).

Levels of miR-122 during hypothermic machine perfusion showed a significant increase in groups with more hepatocyte injury (figure 5B). MiR-122/miR-222 showed a similar increase with a marked distinction between cluster 3 and 1 and between cluster 3 and 2. During warm reperfusion, a rise in AST levels (after 3 hours) was observed in the 3 groups: 1092 U/L (SD 953.2), 1819 U/L (SD 1396), 2494 U/L (SD 2376) respectively. These findings correlated with ischemic damage on a pathological scale of liver biopsies as reported by Liu (data previously published) (41).

Levels of miR-122 in the cold perfusion liquid after 60 min and showed a significant correlation with AST levels at the end of the warm reperfusion episode/experiment (R^2=0.38, p=0.02). This correlation was even stronger between the miR-122/miR-222 ratio and AST levels (figure 5C) and was comparable to the clinical data (p=0.005, R^2 = 0.46). Furthermore, we divided the livers in 2 groups of graft with AST levels < 1500 U/L and > 1500 U/L after reperfusion, equivalent to the clinical transaminase-threshold. We saw higher miR-122 and miR-122/miR-222 levels in the samples of livers with AST levels >1500 U/L, consistent with our human data. MiR-122 as an individual marker showed similar results (Fig 5D, *p*=0.02).

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Discussion

In this study, we demonstrate that high miR-122 levels and relative high miR-122/miR-222 ratios in perfusion liquids are associated with EAD and poor graft survival after liver transplantation. As reported in several other studies, miRNA-122 was abundantly expressed in the liver and is correlated to conventional biomarkers as AST, ALT and LDH (42, 43). Relative miR-122 and ratios of miR-122/miR-222 during graft preservation are significantly higher in both DCD and DBD grafts that developed EAD than in those which had a normal post-operative organ function. Furthermore, miR-122/miR-222 ratios are significantly higher in grafts that are lost within the first year after transplantation. Elevated miR-122/miR-222 ratios are all associated with poor graft survival on the long term independently of EAD post-transplantation. We also found an association between miRNA levels in perfusates and serum AST and ALT levels in the first 24h after transplantation. Therefore, miRNAs present in the perfusion liquid might be a marker for hepatocyte injury during preservation. To support our clinical data, we used an experimental model in which porcine livers were exposed to increased degrees hepatic injury before hypothermic machine preservation. After a standardized period of warm ischemia, our results were similar to our clinical findings: miR-122 and ratios of miR-122/miR-222 were related to AST concentrations in the perfusion liquids and were higher in the groups with more severe hepatic injury. Consistent with our results, several other studies have found an increase in miRNA levels in hepatic injury. Even though the mechanism involved in the release of miRNAs is still not clear, miR-122 has been reported to have a role as biomarker in the prediction of liver function and injury, including in viral hepatitis, cirrhosis and hepatocellular carcinoma (35, 44).

MiRNA-122 in particular has been reported to be specific to the liver and therefore might be more specific to hepatocyte injury than other classic liver enzymes as AST, ALT and LDH (42, 43, 45, 46). Even though the mechanism involved in the release of miRNAs is still not clear, miR-122 has been reported to have a critical role in regulation of the lipid homeostasis by controlling cholesterol synthesis and lipoprotein in the liver (35, 44, 47-50). Furthermore, besides measurement of miR-122 levels as a biomarker in several causes of injury of the liver, it has been shown to function as a tumor-suppressor in hepatocellular carcinoma (51). Additionally, it might therefore even be the target of therapy in hepatocellular carcinoma and viral hepatitis (52, 53).

MiR-222 has not been investigated in to the same extend, but studies show that it is abundant in biliary epithelium (36). Others have also reported on high miR-222 levels in patients with primary sclerosing cholangitis and biliary atresia (54, 55).

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In the early detection of hepatic injury, miRNAs in perfusates have several advantages. Perfusates are obtained from total vascular perfusion during preservation and therefore represent the whole liver. Sampling-bias can therefore be minimized compared to tissue biopsies for example. As we showed in our data, there was a significant difference in miRNA levels in perfusates, but this difference was not observed in biopsies. This might indicate a better representation of miRNA levels in perfusates than those in biopsies due to the more complete representation or the whole organ of miRNAs in perfusates. Additionally, perfusate samples are taken in a non-invasive manner and do no further harm to the organ. Furthermore, perfusates represent graft quality prior to transplantation independent of recipient factors, where the liver is the only possible source of the released miRNAs (29, 35-38, 56). Our earlier observation that miRNAs in perfusate, like in many bio fluids, are very stable adds to the advantage of exploring miRNAs for their potential as biomarker (37).

Data of the present study are consistent with these earlier findings and provide a more specific insight in the properties of miRNAs as biomarkers. However, several limitations should be considered in this study. Although miR-122 levels in both pigs and humans were increased in liver grafts with increased hepatic injury after preservation in cold perfusion liquids, the results from our experimental study are not completely comparable to the reported clinical findings. Although both miR-122 and miR-222 are present in porcine livers, much less is known on their function and the effects of hepatic injury in pigs. Furthermore, human miRNA levels were measured in samples after an average of 6.5 hours of static cold storage and might therefore not be completely comparable to the porcine samples collected after one hour of machine perfusion. Additionally, livers in our experimental model were not transplanted after preservation, but a warm reperfusion period was performed to mimic the clinical situation. We also acknowledge the fact that isolated warm reperfusion might not be comparable with real transplantation and that 2 hours of reperfusion might not be long enough to mimic the full process of ischemia reperfusion injury and EAD. Nonetheless, we consider the hepatic injury due to warm ischemia clinically relevant and comparable to damage to the liver in the human setting, however performed in a standardized, experimental manner. Therefore, the obtained results supported our hypothesis due to similar developments in AST, hepatic injury and miRNA levels.

Lastly, it is unclear whether miRNAs in serum and biopsies differ from those in perfusate liquids, and in this study, we used only a very small selection of miRNAs which was based on the

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current literature (56, 57). Therefore, more studies regarding the properties of miRNAs and specifically those in perfusion liquids are necessary to validate our hypothesis that miRNAs in perfusates can be used as biomarkers of organ function prior to transplantation. Although miR-122/miR-222 ratios are significantly different between both groups and on a biological level related to liver damage, the predictive value of the levels is insufficient as a single biomarker. Therefore, further research is required to identify a miRNA profile that can predict which grafts are likely to develop complications post-operatively.

In conclusion, these findings identify hepatocyte specific miR-122 and the miR-122/miR-222 ratio in preservation liquids during static cold storage of liver grafts as independent factors related to EAD and graft survival. These potential biomarkers may aid in the assessment of graft function of an isolated liver graft prior to transplantation. Furthermore, our findings regarding miR-122 and miR-122/miR-222 ratios as possible biomarkers may ultimately help in the identification of therapies during graft preservation and thereby improve graft function.

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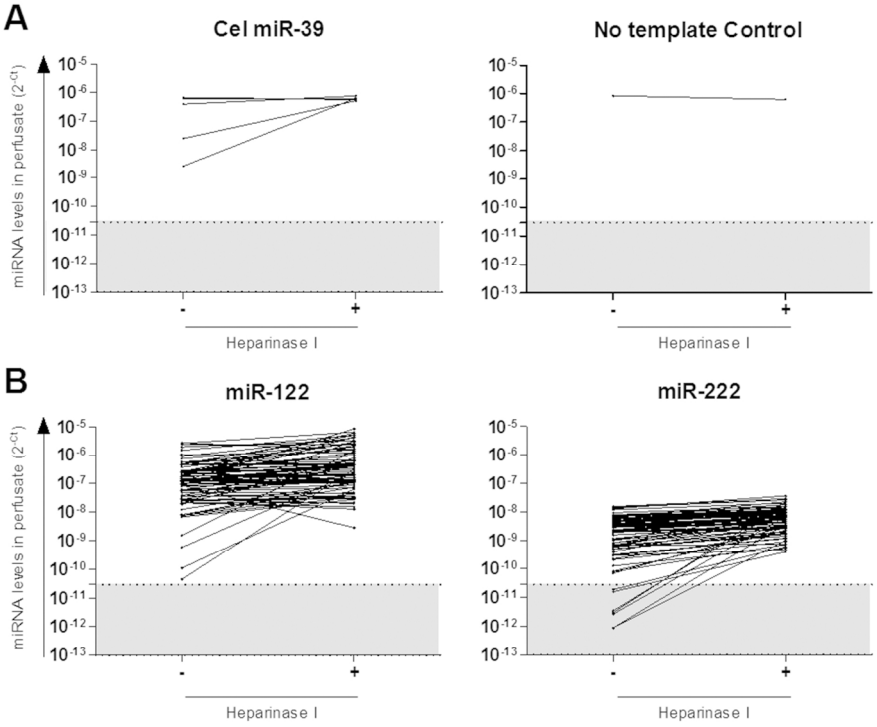

Figure 1 supplement: Heparinase I increases the number of reliably detectable miRNAs and strongly reduces the variation. (A) C-elegans miR-39 levels expressed in relative expression values without (-) and with (+) treatment of the samples with heparinase I. Unused perfusion liquid (UW) spicked with C-el miR-39 is represented in the non-template control panel. (B) Levels of miR-122 expressed in 2^-Ct values of individual microRNAs in serum samples before (-) and after (+) treatment with heparinase I. We regarded a Ct value of >35 as non-reliable as indicated by the grey area. A marked increase in reliable measurements of miRNA levels and decrease of the variable range can be observed.

182x138mm (300 x 300 DPI)

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Table 1: Patient characteristics in patients with EAD and non-EAD.

	non-EAD	EAD	Total	*p* value
	n=48	n=35	n=83	
Donor characteristics				
Demographics				
Age (mean, years) ± SD	54 (17)	49 (13)	52 (16)	0.13
Men, n (%) Women, n (%)	20 (42) 28 (58)	23 (66) 12 (34)	43 (52) 40 (48)	**0.03***
Donor Risk Index, Mean ± SD	2.9 (0.0)	3.4 (2.6)	3.2 (2.3)	0.15
BMI mean (SD)	23 (3)	24 (3)	24 (3)	0.83
Graft type				
DBD liver (n, %) DCD liver (n, %)	41 (85) 7 (15)	20 (57) 15 (43)	61 (63) 22 (27)	**0.006****
Median (SEM) cold ischemia time (min)	387 (97)	426 (121)	402 (108)	0.14
Median (SEM) first warm ischemia time (min)	16 (6)	18 (5)	17 (6)	0.25
Median (SEM) total warm ischemia time (min)	29 (10)	32 (9)	30 (10)	0.30
HTK (n, %) UW (n, %)	18 (38) 30 (62)	18 (51) 17 (49)	36 (44) 47 (56)	0.21
Laboratory results recipient at time of donation				
AST (U/L), mean ± SD	53 (33)	62 (58)	57 (46)	0.46
ALT (U/L), mean ± SD	46 (48)	47 (38)	47 (43)	0.91
AST (U/L), mean ± SD	13 (17)	9 (6)	11 (13)	0.16
Creatinine (µmol/l), mean ± SD	74 (40)	80 (53)	77 (46)	0.56
Recipient characteristics				
Demographics				
Age (mean, years) ± SD	51 (12)	50 (12)	50 (12)	0.70
Men (n, %) Women (n, %)	30 (62) 18 (38)	19 (54) 16 (46)	49 34	0.46
BMI, mean ± SD	23 (3)	24 (3)	23 (3)	0.52
Lab-MELD score, mean ± SD	26 (8)	26 (8)	26 (8)	0.65
Hepatic Artery Thrombosis (n, %)	2 (4)	5 (14.3)	7 (8.4)	0.07
Re-transplantation (n, %)	7 (14)	6 (17)	13 (16)	0.06
Median (SD) time to retransplantation (months)	37 (22)	5 (11)	22 (24)	0.30
Episode of acute rejection (n, %)	8 (17)	6 (17)	14 (17)	0.85
Median months of follow-up	48 (18)	47 (22)	48 (20)	0.77
Diagnosis at LTx (%)				
Auto-immune hepatitis (n, %)	22 (46)	15 (43)	37 (45)	0.79
Alcohol (n, %)	6 (10)	5 (14)	11 (13)	0.82
Hepatitis B/C (n, %)	11 (23)	7 (20)	18 (22)	0.75
Hepatocellular Carcinoma (n, %)	12 (25)	10 (29)	22 (27)	0.72
Other (n, %)	8 (17)	6 (17)	14 (17)	0.96
Laboratory results recipient 24h post-surgery				
AST (U/L), mean ± SD	1452 (3522)	3162.5 (2301)	2182 (3161)	**0.014***
ALT (U/L), mean ± SD	943 (1445)	1967.3 (1319)	1380 (1275)	**0.010***
AST (U/L), mean ± SD	94.5 (135.6)	90 (72)	93 (112)	0.85
Creatinine (µmol/l), mean ± SD	106 (57)	133 (86)	118 (72)	0.11
INR, mean ± SD	1.3 (0.8)	2.1 (0.7)	2.1 (0.8)	0.91

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Table 2: Univariate and multivariate analysis

	Univariate		Multivariate		
	OR	p-value (95% CI)	OR	p-value (95% CI)	C
miR-122	4.23	**0.014***	4.851	**0.015***	0.677
miR-122/ miR-222	1.88	**0.004****	7.417	**0.043***	0.742
DCD liver	3.62	**0.006****	3.673	0.119	0.620
Male donor	2.95	**0.030***	5.010	**0.040***	0.553

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