MicroRNA 29c-3p indicates advanced liver fibrosis/cirrhosis in univentricular heart patients with and without Fontan palliation

Masood Abu-Halima (masood@daad-alumni.de)
Universitat des Saarlandes https://orcid.org/0000-0001-9048-4958
Eckart Meese
Institute of Human Genetics, Saarland University Medical Center, Homburg/Saar, Germany
Mohamad Ali Saleh
Department of Pediatric Cardiology, Saarland University Medical Center, Homburg/Saar, Germany
Andreas Keller
Department of Pediatric Cardiology, Saarland University Medical Center, Homburg/Saar, Germany
Hashim Abdul-Khaliq
Center for Clinical Bioinformatics, Saarland University, Saarbruecken, Germany
Tanja Raedle-Hurst
Center for Clinical Bioinformatics, Saarland University, Saarbruecken, Germany

Research

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Abstract

Background: In patients with chronic liver disease that usually results in liver fibrosis, microRNAs (miRNAs) have been shown to be of diagnostic and prognostic value. The present study aims to identify those miRNAs in patients with univentricular heart (UVH) disease with and without Fontan palliation that may be associated with advanced liver fibrosis/cirrhosis.

Methods: A large panel of human miRNA arrays were used to determine miRNA abundance profiles in the blood of 48 UVH patients with and without Fontan palliation and 32 age and gender-matched healthy controls. The most abundantly expressed miRNAs identified by microarray analysis were correlated to prognostic scores of advanced liver fibrosis/cirrhosis and selected miRNAs validated by RT-qPCR.

Results: According to microarray analysis, 50 miRNAs were found to be significantly abundant in UVH patients of which miR-29b-3p and miR-29c-3p were significantly correlated to the model of end-stage liver disease (MELD)-Albumin and albumin-bilirubin (ALBI) score. According to ROC analysis, a MELD-Albumin score ≥ 11 was best predicted by total bilirubin (AUC 0.985, P=0.005), collapsibility index (AUC 0.952, P=0.009) and miR-29c-3p (AUC 0.941, P=0.011) and the most significant predictors of an ALBI score >−2.6 were collapsibility index (AUC 0.998, P<0.001) and albumin (AUC 0.981, P=0.006). In the multivariate analysis, miR-29c-3p turned out to be an independent predictor of advanced liver fibrosis/cirrhosis as defined by a MELD-Albumin score ≥ 11 (P=0.004) and ALBI score > −2.6 (P=0.009), respectively.

Conclusion: In UVH patients with and without Fontan palliation, miR-29c-3p seems to be an independent predictor of advanced liver fibrosis/cirrhosis and thus may be used in the risk assessment of these patients.

Background

Univentricular heart (UVH) disease is a rare and complex congenital heart disorder that is characterized by functionally single ventricle anatomy resulting in volume overload of the single ventricular chamber and systemic venous congestion. The only surgical option to improve hemodynamics in these patients is a palliative procedure called Fontan operation connecting both caval veins to the pulmonary artery and thus directing systemic venous blood return directly into the pulmonary circulation without the pulsatile function of a ventricular chamber. Depending on the pulmonary vascular resistance and diastolic function of the single ventricle, this passive venous blood flows into the pulmonary circulation results in chronic systemic venous congestion, particularly of the liver [1]. In UVH patients with and without Fontan circulation, the development of liver fibrosis due to long-standing venous congestion of the liver is a well-known and challenging complication resulting finally in liver cirrhosis and in rare cases hepatocellular carcinoma [2-4]. Thus, early detection of these complications in this cohort of patients is of diagnostic and prognostic importance. To date, liver biopsy still represents the gold standard in assessing the grade and stage of liver fibrosis. However, this technique is invasively carrying the potential risk of bleeding

complications but also sampling and interpretation errors [5]. Therefore, non-invasive diagnostic tools such as serological scoring or imaging-based methods should be used for screening and follow-up of congestive hepatopathy in these patients.

Using non-invasive methods, differentiation of reversible liver congestion from irreversible fibrosis and reliable prediction of liver fibrosis as assessed by biopsies still remains challenging. Previous studies have shown that measurement of liver stiffness using transient elastography, shear wave elastography or acoustic radiation force impulse imaging currently cannot differentiate reliably between congestion and fibrosis of the liver and thus may overestimate the extent of liver fibrosis in congestive hepatopathy [6, 7]. Moreover, biochemical assessment of liver fibrosis using laboratory scores such as APRI, Forns, FIB4 or FibroSURE score are not feasible in these patients either because these scores do not correlate well with liver biopsy findings or are not related to the presence of sinusoidal or portal fibrosis on biopsy specimens that are typical for congestive hepatopathy [7, 8]. However, the Model for End-Stage Liver Disease (MELD) excluding international normalized ratio (MELD-XI) score is a functional score indicating advanced liver disease that seems to be suitable in the risk assessment of patients with combined cardiac and hepatic dysfunction and even predicts clinical outcomes in congestive liver disease [6, 9, 10]. Recently, a new MELD-Albumin score including albumin to replace INR in the conventional MELD score has been investigated in patients undergoing tricuspid annuloplasty for severe tricuspid regurgitation revealing to be a prognostic indicator as good as MELD-XI score [11]. Moreover, the ALBI score has been shown to be applicable, robust and superior to the conventional MELD score in the evaluation of the functional status and long-term prognosis of patients with liver cirrhosis [12, 13].

MicroRNAs (miRNAs) are known to be expressed in a cell type- or tissue-specific and stage-dependent manner in chronic liver disease [14-16]. They also play a significant role in the regulation of liver fibrosis/cirrhosis representing the common end stage of most chronic liver diseases and being associated with tremendous morbidity and mortality [17, 18]. Since miRNAs are known to modulate different steps in the pathophysiology of liver fibrosis, they might be used for early detection or progression of liver fibrosis [16]. To the best of our knowledge, no data are available on the specific miRNAs that are involved in the onset or progression of liver fibrosis in UVH patients. In this study, we aimed to identify miRNAs that indicate significant liver fibrosis in UVH patients using prognostic scores of advanced liver fibrosis/cirrhosis, specifically the new MELD-Albumin and ALBI score, and to assess their predictive value in this cohort of patients.

Methods

Patients

A total of 48 consecutive UVH patients who were regularly seen in our outpatient clinic between 02/05/2015 and 18/06/2018 were enrolled in the present study. 42/48 (87.5%) patients had a complete Fontan palliation and 6/48 (12.5%) patients an incomplete or no Fontan palliation. Regarding the morphology of the dominant systemic ventricle, 32/48 (66.7%) patients had a morphological dominant

left and 16/32 (50%) patients a morphological dominant right systemic ventricle. In the left systemic ventricle UVH group, 12 patients presented with tricuspid atresia, 12 patients with double inlet left ventricle and 8 patients with pulmonary atresia with or without ventricular septal defect. In the right systemic ventricle UVH group, 8 patients had hypoplastic left heart syndrome or mitral atresia and 8 patients double outlet right ventricle with pulmonary stenosis. The mean age was 22.8 ± 10.1 years (range 11– 46 years) including 17 females and 31 males. At enrollment, a structured protocol including a 12-lead surface electrocardiogram, a physical examination, measurement of blood pressure and transcutaneous oxygen saturation at rest, two-dimensional echocardiography as well as a venous blood draw for routine laboratory parameters and blood sampling were performed. Additionally, ultrasonographic parameters of liver congestion such as the diameter of the inferior caval vein (IVC) at deep inspiration and expiration were measured to calculate the collapsibility index [19]. Clinical characteristics of the patient cohort and UVH-specific data have already been presented in a previous study [20]. Patients' specific liver parameters are illustrated in **Table 1**. Thirty-two healthy volunteers served as controls and were matched to UVH patients according to age and gender. In all volunteers, a physical examination, two-dimensional echocardiography to verify the absence of any heart abnormality, ultrasonography of the inferior caval vein to rule out liver congestion and venous blood sampling were performed on the same day. The study complies with the Declaration of Helsinki, was approved by the local ethics committee and all participants or their guardians gave written and informed consent before enrollment.

Sample preparation and RNA isolation

In all patients and controls, blood samples for miRNA detection were collected in PAXgene™ blood tubes (Becton–Dickinson, Heidelberg, Germany) shortly after echocardiographic evaluation. All PAXgene™ blood tubes were stored at room temperature for at least 24 hours to ensure complete lysis of the blood cells, then stored at -20°C for several days and finally transferred to -80°C for long-term storage until RNA isolation. Total RNA including miRNAs was isolated from blood samples using PAXgene™ Blood miRNA Kit on the QIAcube™ robot (Qiagen, Hilden, Germany) following the manufacturer's recommendations and included DNase I treatment (Qiagen). To confirm the absence of genomic DNA contamination, a conventional PCR with exon spanning primers for Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*) as previously described [21]. The concentration of isolated total RNA was measured using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, United States). RNA purity was estimated by examining the OD 260/280 and the OD 260/230 ratios. The qualities of total RNA were assessed using the Agilent Bioanalyser 2100 Eukaryote Total RNA Nano Series II (Agilent Technologies, California, United States).

Analysis of miRNAs by microarray

The miRNA abundance profile from the 48 patients with UVH and 32 age- and gender-matched healthy controls was obtained from our previously generated and uploaded raw data to the NCBI GEO database (Accession ID: GSE136547) using SurePrint™ 8X60K Human v21 miRNA platforms (Agilent Technologies) [20]. These platforms contain probes for the detection of 2,549 human miRNAs. An input amount of 100 ng of isolated RNA including miRNAs were labeled and subsequently hybridized to the miRNA microarray chip and the procedure was completed as previously described [22].

Analysis of miRNAs by RT-qPCR

Real-time quantitative PCR (RT-qPCR) validation analysis was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) and the *mi*Script PCR System (Qiagen) as previously described [23]. For the validation step, we used the same samples that were used for the microarray analysis (48 UVH patients and 32 healthy controls) [20]. Fifteen miRNAs (miR-101-3p, miR-144-5p, miR-18a-5p, miR-15a-5p, miR-17-3p, miR-96-5p, miR-18b-5p, miR-140-5p, miR-148a-3p, miR-29b-3p, miR-29c-3p, miR-210-3p, miR-125a-5p, miR-150-5p, and miR-324-3p) were chosen for RT-qPCR validation based on the significant abundance changes on the microarray. Of these miRNAs, miR-29b-3p and miR-29c-3p were significantly correlated to the prognostic scores of advanced liver fibrosis/cirrhosis (MELD-Albumin and ALBI score). For miRNA abundance level detection, an amount of 250 ng of the total RNA was converted into complementary DNA (cDNA). The resulting cDNA was then diluted to have 0.5 ng/µL input material for miRNA detection. All RT-qPCR experiments were carried out using the Liquid Handling Robot QIAgility™ (Qiagen) before performing RT-qPCR. All primer assays used in the current study were provided by Qiagen. Moreover, miRNA reverse transcription control (miRTC) (Qiagen) was performed to assess the performance of the reverse transcription reaction. The melting curve analysis was used to control the specificity of RT-qPCR products. The specificity of amplicons was further confirmed by agarose gel electrophoresis.

Data analysis

Clinical data of the patients were collected from medical records. Echocardiography and ultrasonography were performed using a Vivid™ E9 Ultrasound System (GE Healthcare, Horten, Norway). The echocardiographic loops and ultrasonographic images were stored digitally and analyzed on an Echopac server (Echopac Version 6, GE Healthcare). The collapsibility index of the IVC was calculated according to the formula: the difference of maximum and minimum diameter of the IVC divided by the maximum diameter of the IVC [19]. The formulas used for calculating MELD and modified MELD scores have been described previously [11]. ALBI score was calculated according to the equation: ALBI score = ($\log_{10}$ bilirubin x 0.66) + (albumin x −0.085) and has been previously published [24]. Echocardiographic and ultrasonographic data sets were assessed by investigators blinded to the laboratory results. Investigators of miRNA signatures were blinded to the clinical, echocardiographic, ultrasonographic and laboratory data of the patients.

Statistical analysis

DataAssist™ Software v3.0 (Applied Biosystems) was used to calculate the relative abundance changes of miRNAs by the equation $2^{-\Delta Ct}$ with RNU6B serving as an endogenous control as previously used and validated for this type of sample [22, 23, 25-30]. Clinical data were analyzed using standard statistical software (SPSS version 19; SPSS Inc., Chicago, Illinois). Continuous variables are expressed as mean ± standard deviation or median (interquartile interval) as appropriate. Differences between unpaired groups were analyzed using a Mann-Whitney-U test for continuous variables and a chi-square test (or Fisher exact test, if numbers were small) for nominal variables. Correlations were evaluated using Spearman's regression coefficient. Receiver-operating characteristic (ROC) curve analysis was used for the performance of individual parameters as well as selected miRNAs to predict advanced liver fibrosis/cirrhosis and the area under the curve (AUC) was calculated. Multivariate analysis was performed using logistic regression analysis in a stepwise forward manner to identify independent predictors of advanced liver fibrosis/cirrhosis. Variables entered into the multivariate model were those that gave statistically significant results in the univariate analysis and didn't show any multicollinearity. A two-tailed *P*-value <0.05 was considered statistically significant.

Results

Evaluation of conventional liver parameters

Conventional ultrasonographic and laboratory liver parameters according to the status of Fontan palliation are presented in **Table 1.** As compared to patients with complete Fontan palliation, patients with incomplete or no Fontan palliation had a higher NYHA class, a lower glomerular filtration rate and a lower collapsibility index of the IVC reflecting the presence of more severe global heart failure and liver congestion. Moreover, laboratory parameters of advanced liver fibrosis/cirrhosis such as total bilirubin or platelet count were also different between both subgroups. The calculated prognostic liver scores MELD-XI, MELD-Albumin, and ALBI score were significantly higher in patients without Fontan palliation indicating more advanced stages of liver fibrosis/cirrhosis.

Identification of abundant miRNAs

According to a miRNA microarray, a total of 50 miRNAs were found to be differentially abundant when comparing the samples from patients with UVH to healthy controls [20].

Out of 15 miRNAs used for the RT-qPCR validation, 12 miRNAs showed statistically significant higher abundance levels and 2 miRNAs showed statistically significant lower abundance levels in UVH patients compared with those in healthy controls **(Figure 1A)**. There was no significant difference in the abundance level of miR-342-3p (**Figure 1A)**. However, correlation analyses of significantly abundant miRNAs to prognostic scores of advanced liver fibrosis/cirrhosis (MELD-Albumin and ALBI score) identified miR-29b-3p and miR-29c-3p to be best related to these scores. Thus, these two miRNAs were further validated by RT-qPCR indicating significantly higher abundance levels in UVH patients compared to healthy controls **(Figure 1B).**

Association of miRNAs with measures of liver congestion and prognostic liver fibrosis scores

Correlation analysis revealed miR-29c-3p to be significantly related to the extent of liver congestion represented by a collapsibility index of the IVC ≤ 0.15, laboratory measures of severe liver dysfunction such as total bilirubin and platelet count as well as prognostic measures of advanced liver fibrosis/cirrhosis as indicated by the different MELD scores and ALBI score. In contrast, miR-29b-3p was only significantly related to albumin and ALBI score (**Table 2**). Relative abundance levels of miR-29b-3p and miR-29c-3p were significantly higher in patients with severe liver congestion as indicated by a collapsibility index of the IVC ≤ 0.15 (**Figure 2A**). Moreover, higher relative abundance levels of miR-29b-3p and miR-29c-3p were observed in patients with a higher MELD-Albumin and ALBI score both indicating advanced liver fibrosis/cirrhosis (**Figure 2B** and **Figure 2C**).

Prediction of advanced liver fibrosis/cirrhosis

ROC curve analysis was used to identify predictors of advanced liver fibrosis/cirrhosis as indicated by a MELD-Albumin score ≥ 11 and an ALBI score > −2.6 in all patients (**Table 3**). Here, multivariate analysis identified total bilirubin and miR-29c-3p (P<0.001 and P=0.004) as well as albumin and miR-29c-3p (P<0.001 and P=0.009) as independent predictors of advanced liver fibrosis/cirrhosis.

Discussion

In UVH patients with and without Fontan palliation, the development of liver fibrosis/cirrhosis due to chronic liver congestion is a typical late complication [2, 3]. These non-cardiac complications may limit potential heart transplantation candidacy and overall survival in this cohort of patients with advancing age. Thus, in addition to cardiorespiratory parameters, monitoring of liver status during the long-term follow-up of these patients is crucial. However, currently used methods to assess the grade or stage of liver fibrosis in these patients show significant limitations [6, 8]. Recently, specific miRNAs have been shown to be dysregulated in different liver diseases [17, 18] but are also involved in hepatic fibrosis progression [16] and thus might be used as valuable biomarkers. Therefore, our study aimed to identify those miRNAs in UVH patients with and without Fontan circulation that are associated with advanced liver fibrosis/cirrhosis and to assess their predictive value in this cohort of patients.

Using conventional ultrasonographic and laboratory parameters such as collapsibility index and different scores of liver fibrosis/cirrhosis, we were able to demonstrate abnormal liver findings in our cohort of patients that were more severe in UVH patients with incomplete or no Fontan palliation as compared to patients with complete Fontan palliation (**Table 1**). Moreover, UVH patients without Fontan palliation were significantly older and presented with a higher NYHA class reflecting more severe systolic and diastolic dysfunction of the UVH in these patients. In contrast to published data indicating Fontan physiology as the leading cause of liver morbidities, we were able to show that UVH patients without Fontan palliation may also suffer from the same liver complications in older age.

In this study, miR-29b-3p and miR-29c-3p were found to be best associated with the prognostic liver scores MELD-Albumin and ALBI score in our patients using microarray analysis. After validation by RT-qPCR, miR-29b-3p turned out to be significantly related to ALBI score whereas miR-29c-3p was best related to MELD-Albumin, MELD-XI and ALBI score (**Table 2**). It is of note that these liver scores represent advanced stages of liver fibrosis or even liver cirrhosis and also have prognostic value in patients with chronic liver and heart disease. In contrast to previous studies evaluating these miRNAs in other clinical settings, both miRNAs were upregulated in our patient cohort with or without Fontan palliation as compared to healthy controls (**Figure 1B**). However, it needs to be taken into consideration that the pathomechanism of liver fibrosis/cirrhosis after inflammatory or toxic liver damage may be different to congestive liver disease that was present in our studied patients. Moreover, higher abundance levels of both miRNAs were found in patients with a higher MELD-Albumin or ALBI score indicating advanced stages of liver fibrosis/cirrhosis (**Figure 2B** and **Figure 2C**). Furthermore, abundance levels of both miRNAs were significantly elevated in UVH patients with incomplete or no Fontan palliation reflecting the more longstanding venous congestion of the liver, more advanced stages of liver fibrosis/cirrhosis and a poorer prognosis in these patients as compared to patients with complete Fontan palliation (**Figure 2D**). Overall, both miRNAs were able to differentiate between lower and higher liver fibrosis/cirrhosis scores indicating the progression of liver pathology and worsening of prognosis.

Our results are in accordance with previous studies identifying the miR-29 member family as an indicator of advanced liver fibrosis/cirrhosis. In a previous liver biopsy study in patients with hepatitis B-associated liver fibrosis, a panel of 4 miRNAs was identified to be involved in the progression of liver fibrosis, namely miR-122, miR-146a-5p, miR-29c-3p, and miR-223 [31]. In that study, miR-122 has been shown to differentiate between various fibrosis stages (F0-F4) and thus can be used as a precise fibrosis staging biomarker, especially for the early detection of liver fibrosis. In contrast, miR-29c-3p has been shown to be only significantly abundant in advanced liver fibrosis/cirrhosis in that study and is in agreement with previous studies identifying the miR-29 family as a marker of advanced liver fibrosis/cirrhosis [32, 33].

An increasing number of studies have demonstrated the down-regulation of miR-29 in human fibrotic disorders of multiple organs by transforming growth factor ß/*SMAD3* signaling [32-34]. Moreover, miR-29 has been identified as a strong tumor suppressor rather than an oncogenic factor in most cancer studies [35] and many of these studies indicate that high miR-29 expression leads to increased survival [36, 37]. However, there are also some studies reporting that higher levels of miR-29 in plasma are associated with lower survival rates [38, 39] and that up-regulation of miR-29c may be associated with higher relapse risk in patients with acute myeloid leukemia [40]. In our study, the correlation of both miRNAs to all-cause mortality was statistically borderline (**Table 2**) what can be explained by the fact that mortality was mainly cardiac-related in this cohort of patients [20]. Nevertheless, liver fibrosis and cirrhosis are important complications increasing with age and limiting survival in UVH patients with or without Fontan palliation. In our study, the elevated abundance levels of miR-29b-3p and miR-29c-3p in UVH patients with incomplete or no Fontan palliation may indicate the potential worse overall prognosis and limited survival in these patients (**Figure 2D**).

According to ROC analysis, both miR-29b-3p and miR-29c-3p demonstrated a good performance in predicting a MELD-Albumin score ≥ 11 with an AUC of 0.911 and 0.941, respectively, but only a moderate performance for the prediction of an ALBI score > −2.6 indicating a more severe grade of liver cirrhosis (**Table 3**). In comparison, miR-29c-3p demonstrated an AUC of 0.670 for the diagnosis of liver fibrosis stage ≥ F2 and an AUC of 0.619 for the fibrosis stage F4 in patients with hepatitis B-associated liver fibrosis [31]. In the multivariate analysis, miR-29c-3p turned out to be an independent predictor of advanced liver fibrosis/cirrhosis in our cohort of UVH patients (**Table 3**).

Study limitations

This is the first study that aims to characterize signatures of miRNAs in UVH patients with and without Fontan palliation using a large panel of miRNAs for initial screening in order to identify those that may indicate advanced liver fibrosis/cirrhosis and thus might have clinical as well as prognostic impact in this cohort of patients. Since UVH disease is a rare congenital cardiac disorder, the sample size of our patient cohort is rather small. Moreover, non-invasive methods that reliably diagnose different stages of liver fibrosis in this cohort of patients are limited [6, 8]. Thus, prognostic scores of advanced liver fibrosis/cirrhosis were used for miRNA screening in the present study. The cross-sectional rather than longitudinal design of the study may limit the predictive value of the measured miRNAs. Therefore, the measured miRNAs may have limited value in detecting earlier stages of liver fibrosis in such patients. Hence, a larger cohort of UVH patients should be evaluated and liver biopsies performed to assess the diagnostic value of miRNAs in early and late stages of liver fibrosis in these patients.

Conclusion

In UVH patients with and without Fontan palliation, miR-29c-3p represents an independent predictor of advanced liver fibrosis/cirrhosis and thus may be used as a potential biomarker in the risk assessment of these patients.

List Of Abbreviations

ALBI	Albumin-Bilirubin
AUC	Area Under the receiver operating characteristic Curve
cDNA	complementary DNA
CHD	Congenital Heart Disease
CI	Confidence interval
GFR	Glomerular filtration rate
IVC	Inferior caval vein
MELD	Model for end-stage liver disease
MELD-XI	Model for end-stage liver disease without international normalized ratio
miRNA	microRNA
NS	Not Significant
NYHA	New York Heart Association
ROC	Receiver-operating characteristic
RT-qPCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
snRNA	small nuclear RNA
TA	Tricuspid Atresia
UVH	Univentricular Heart

Declarations

Ethics approval and consent to participate: Institutional Review Board approval/Ethikvotum Ärztekammer des Saarlandes: Ethical vote No. 73/09.

Availability of data and material: The data sets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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Authors' contributions: MA, performed experimental work, particularly the miRNA isolation, array experiment, RT-qPCR validation and helped in manuscript writing; EM, designed the study, coordinated the molecular biology experiment and edited the manuscript; MAS, helped in experimental work and collection of controls and in the interpretation of clinical data; AK, performed bioinformatics analysis;

HAK, designed the study and diagnosed patients; TRH, designed the study, recruited and examined controls, diagnosed patients, collected blood samples and helped in manuscript writing. All authors read and approved the final manuscript.

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Authors' information

Masood Abu-Halima, Ph.D., Institute of Human Genetics, Saarland University, 66421 Homburg /Saar, Germany. Email: masood@daad-alumni.de

Eckart Meese, Prof. Dr., Institute of Human Genetics, Saarland University, 66421 Homburg /Saar, Germany. Email: Eckart.Meese@uks.eu

Mohamad Ali Saleh, M.D., Department of Pediatric Cardiology, Saarland University Medical Center, 66421 Homburg /Saar, Germany. Email: alisaleh660@hotmail.com

Andreas Keller, Prof. Dr., Chair for Clinical Bioinformatics, Saarland University, 66041 Saarbruecken, Germany. Email: andreas.keller@ccb.uni-saarland.de

Hashim Abdul-Khaliq, Prof. Dr., Department of Pediatric Cardiology, Saarland University Medical Center, 66421 Homburg /Saar, Germany. Email: Abdul-Khaliq@uniklinikum-saarland.de

Tanja Rädle-Hurst, Prof. Dr., Department of Pediatric Cardiology, Saarland University Medical Center, 66421 Homburg /Saar, Germany. Email: tanja.raedle-hurst@uks.eu

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Tables

Table 1. Specific liver parameters of UVH patients according to the status of palliation.

Variables	All patients (n=48)	Complete Fontan palliation (n=42)	Incomplete or no Fontan palliation (n=6)	*P*-value*
Age at enrollment (years)	22.8 ± 10.1	20.5 ± 8.5	38.2 ± 4.3	<0.001
NYHA class	1.5 ± 0.7	1.4 ± 0.6	2.7 ± 0.5	<0.001
Diameter of IVC at deep inspiration (mm)	14.9 ± 4.3	14.3 ± 3.9	18.8 ± 5.6	0.038
Collapsibility index of IVC	0.27 ± 0.09	0.28 ± 0.09	0.19 ± 0.06	0.018
Collapsibility index of IVC ≤ 0.15	6/48 (12.5%)	3/42 (7.1%)	3/6 (50%)	0.020
Total bilirubin (mg/dl)	0.75 (0.60 − 0.98)	0.70 (0.60 − 0.90)	1.05 (0.80 − 2.38)	0.021
Albumin (g/l)	48.0 (44.0 − 49.0)	48.0 (44.0 − 49.0)	43.5 (37.8 − 49.0)	ns
γGT (U/l)	67.0 (40.5 − 96.0)	67.5 (45.8 − 102.0)	36.5 (22.8 − 70.8)	0.025
Platelet count (per mm^3)	193.5 (152.0 − 230.0)	200.0 (166.3 − 238.3)	147.5 (123.5 − 187.0)	0.034
Creatinine (mg/dl)	0.79 (0.66 − 0.94)	0.78 (0.64 − 0.91)	0.99 (0.78 − 1.38)	ns
GFR (ml/min)	105.9 (85.3 − 122.5)	107.8 (96.8 − 125.3)	82.4 (60.8 −92.1)	0.007
MELD-XI score	9.44 (9.44 − 10.51)	9.44 (9.44 − 9.44)	11.24 (9.44 − 15.95)	0.014
MELD-Albumin score	6.43 (6.43 − 7.40)	6.43 (6.43 − 7.17)	8.42 (7.11 − 12.89)	0.003
ALBI score	-3.25 (-3.41 − -2.97)	-3.27 (-3.42 − -3.03)	-2.79 (-3.33 − -2.36)	0.057

UVH, univentricular heart; GFR, glomerular filtration rate; MELD, model of end-stage liver disease; MELD-XI, model of end-stage liver disease without international normalized ratio; ALBI; albumin-bilirubin; ns, not significant.

Mean ± standard deviation or median (interquartile interval) are used.

* Complete compared to incomplete/no Fontan palliation subgroup.

Table 2. Correlation of miRNAs validated by RT-qPCR with liver-specific parameters and prognostic liver scores (n=48).

	miR-29b-3p		miR-29c-3p	
	r	P-value	r	P-value
Collapsibility index of IVC	-0.325	0.024	-0.359	0.012
Collapsibility index of IVC ≤ 0.15	0.336	0.020	0.340	0.018
Total bilirubin (mg/dl)	0.310	0.032	0.387	0.007
Albumin (g/l)	-0.366	0.010	-0.288	0.047
γGT (U/l)	--	ns	--	ns
Platelet count (per mm^3)	--	ns	-0.330	0.022
MELD-XI score	0.295	0.042	0.361	0.012
MELD-Albumin score	0.285	0.050	0.330	0.022
ALBI score	0.447	0.001	0.384	0.007
Death from any cause	0.283	0.051	0.276	0.058

IVC, inferior caval vein; MELD, model of end-stage liver disease; MELD-XI, model of end-stage liver disease without international normalized ratio; ALBI, albumin bilirubin; ns, not significant.

Table 3. Performance of individual liver parameters to predict advanced liver fibrosis/cirrhosis according to MELD-Albumin and ALBI score.

Prediction of MELD-Albumin score ≥ 11				
Variables	**AUC**	**95% CI**	***P*-value**	**Multivariate analysis (*P*-value)**
Total bilirubin (mg/dl)	0.985	0.952 − 1.000	0.005	<0.001
miR-29b-3p	0.911	0.828 − 0.994	0.018	ns
Collapsibility index	0.952	0.890 − 1.000	0.009	ns
Albumin (g/l)	0.930	0.842 − 1.000	0.013	ns
Platelets (per mm^3)	0.889	0.787 − 0.991	0.025	ns
miR-29c-3p	0.941	0.870 − 1.000	0.011	0.004
Prediction of ALBI score > -2.6				
Variables	**AUC**	**95% CI**	***P*-value**	**Multivariate analysis (*P*-value)**
Total bilirubin (mg/dl)	0.723	0.406 − 1.000	0.105	Not included
miR-29b-3p	0.767	0.536 − 0.999	0.052	ns
Collapsibility index	0.998	0.988 − 1.000	<0.001	ns
Albumin (g/l)	0.981	0.940 − 1.000	0.006	<0.001
Platelets (per mm^3)	0.726	0.427 − 1.000	0.102	Not included
miR-29c-3p	0.765	0.554 − 0.977	0.054	0.009

AUC, area under the curve; CI, confidence interval; ns, not significant.

Figures

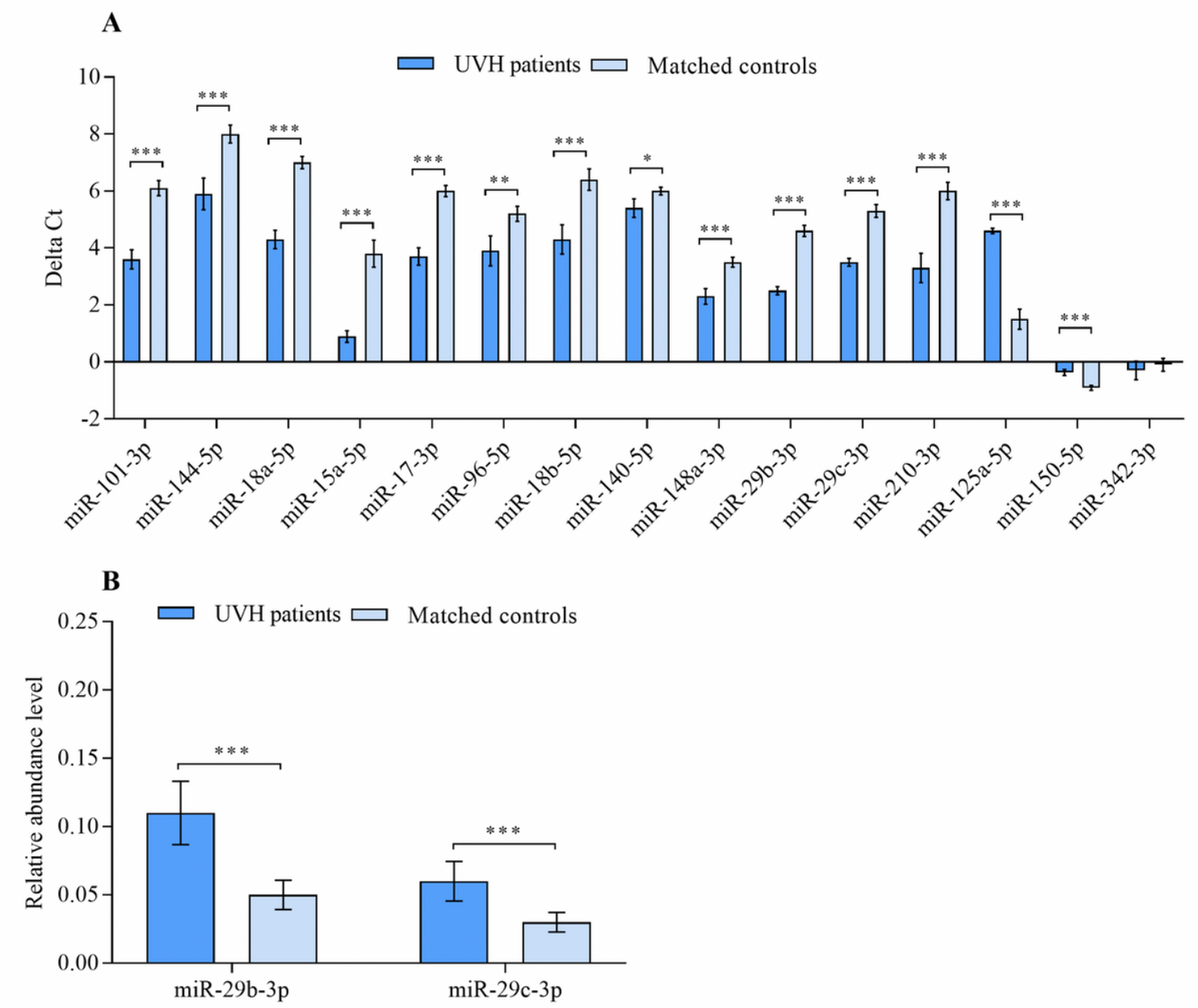

Figure 2

A) Validation of differentially abundant miRNAs in the blood of patients with univentricular heart (UVH) (n=48) compared to age and gender-matched healthy controls (n=32) as determined by RT-qPCR ($P < 0.05$). Mean ΔCt values of patients and healthy controls are illustrated with lower ΔCt values indicating higher abundance levels. B) Validation of clinically correlated differentially abundant miR-29b-3p and miR-29c-3p in the blood of UVH patients (n=48) compared to age and gender-matched healthy controls (n=32) as determined by RT-qPCR ($P < 0.05$). Mean relative abundance level (2-ΔCt) of patients and healthy controls are shown with RNAU6B as an endogenous control for normalization, un-paired-two-

tailed t-tests and mean ± standard deviation (STDV) were used to evaluate differences in abundance levels. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

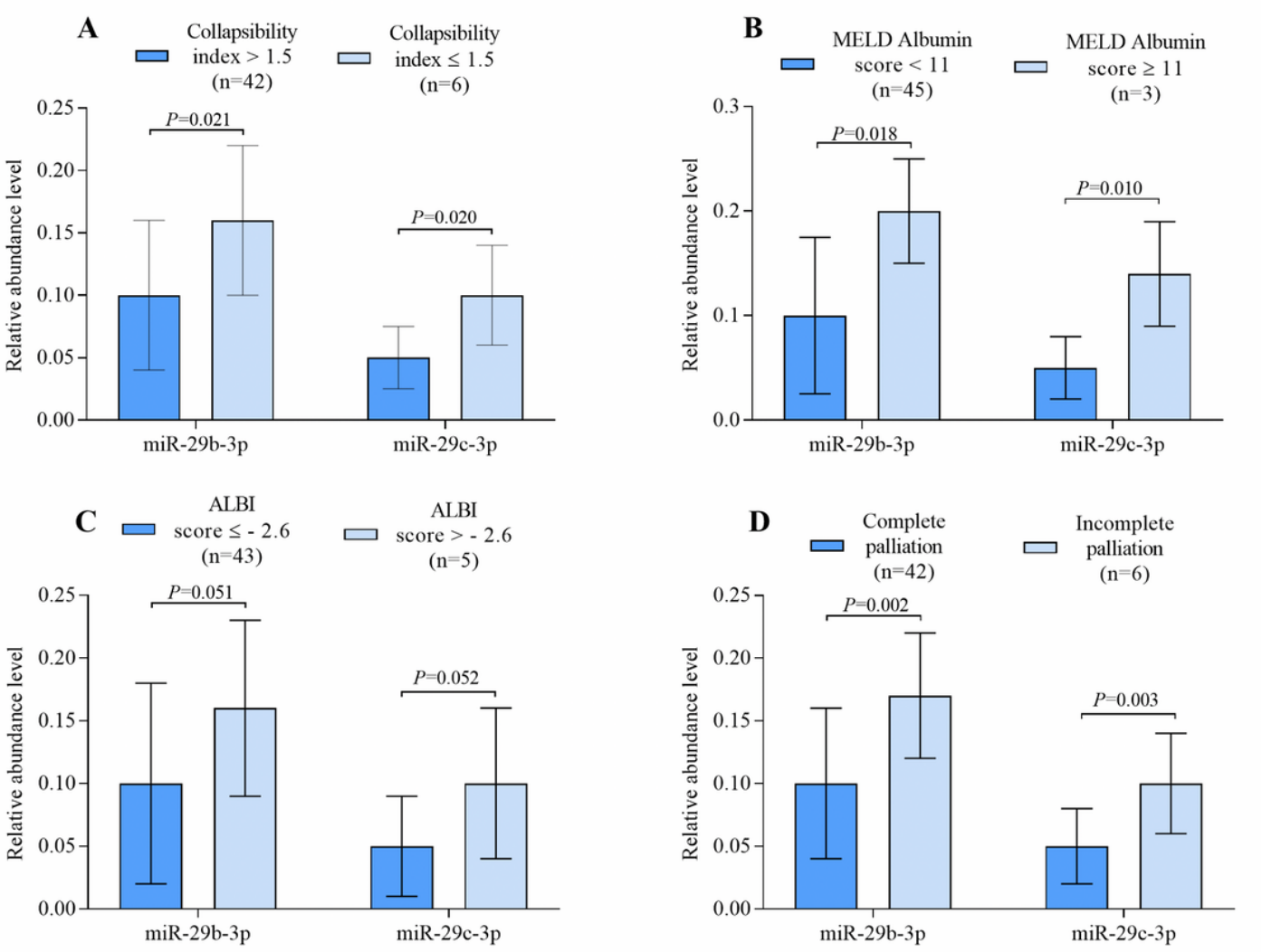

Figure 4

A) Boxplots illustrating relative abundance levels of miR-29b-3p and miR-29c-3p in UVH patients with (n=6) and without (n=42) severe liver congestion as indicated by a collapsibility index of the IVC ≤ 0.15. B) Boxplots illustrating normalized expression levels of miR-29b-3p and miR-29c-3p in UVH patients with a MELD-Albumin score < 11 (n=45) and ≥ 11 (n=3) indicating advanced liver fibrosis/cirrhosis. C) Boxplots illustrating normalized expression levels of miR-29b-3p and miR-29c-3p in UVH patients with an ALBI score ≤ −2.6 (n=43) and > −2.6 (n=5) indicating a higher degree of liver cirrhosis. UVH, univentricular heart; IVC, inferior caval vein.