MicroRNAs as potential markers of parenteral nutrition-associated liver disease in adult patients

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Short title: miRNAs as PNALD biomarkers

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Abstract

Parenteral nutrition-associated liver disease (PNALD) is a severe complication in patients completely dependent on parenteral nutrition (PN). The gold diagnostic standard, liver biopsy, is associated with significant health risk and therefore its use is limited. MicroRNAs (miRNAs) are small non-coding regulatory RNA molecules with highly tissue-specific expression and the secreted miRNAs may serve as non-invasive diagnostic biomarkers. The aim of this study was to evaluate the expression of a panel of specific miRNAs associated with liver diseases of different origin in PN-dependent adult patients in order to design miRNA panel enabling to precise monitoring of PNALD progression. Twelve PN-dependent patients with short bowel syndrome (SBS) were monitored on three/four-month basis for up to 24 months. Forty-five age- and sex-matched subjects without any known liver pathology served as controls. Specific miRNAs expression was determined by RT-qPCR using TaqMan probes (Thermofisher). Liver function test parameters were determined in certified clinical laboratories.

Six of the tested miRNAs exhibited significantly altered expression compared with healthy controls, three of them (MIR122, MIR1273g, and MIR500a) were upregulated while three were down-regulated (MIR505, MIR199a, MIR139). MIR122 positively correlated with serum AST and ALT activities while MIR1273g positively correlated with serum CRP concentration and GGT activity. MIR505, MIR199a, and MIR139 negatively correlated with serum GGT activity. Fluctuation of these parameters well paralleled serum miRNA concentrations in all patients throughout the whole observation period.

We identified six miRNAs whose serum concentrations are significantly altered in PN-dependent patients with PNALD and correlate with markers of inflammation, cholestasis or hepatic injury. Their reliability as markers of PNALD progression needs to be further evaluated.

Keywords: miRNA, parenteral nutrition-associated liver disease, biomarker

Long-term administration of total parenteral nutrition (PN) that is often associated with the development of parenteral nutrition-associated liver disease (PNALD). The origin of this pathologic condition is multifactorial with numerous contributing factors, such as sepsis, intestinal inflammation, cholangitis, cholelithiasis, bacterial translocation, short bowel syndrome, the disturbance of hepato-biliary circulation, the lack of enteral nutrition, etc. PNALD clinical manifestations – which range from steatosis, cholestasis, gallbladder sludge/stones, fibrosis, and cirrhosis – can occur separately or in combination (Drongowski et al. 2009, Luman et al. 2002). The history of PNALD in adult patients is characterized by elevated liver enzymes in association with steatosis lasting for years, followed by steatohepatitis, cholestatic hepatitis as well as fibrosis and cirrhosis (Cahova et al. 2017). The exact staging of the disease progression is necessary for the determination of the right prognosis and efficient treatment, including the indication for the intestine transplantation. It was repeatedly shown that liver tests alone are not sensitive enough for the diagnosis (Klek et al. 2016). Therefore, a liver biopsy remains the gold diagnostic procedure. Nevertheless, liver biopsy is associated with significant health risk and therefore its use is limited. There is an urgent need for seeking novel diagnostic tools for PNALD. These would help to optimize existing PN administration regimen/composition in order to delay or even prevent the development of PNALD. The aim of our study was to determine serum concentrations of selected miRNAs associated with liver pathologies of different origin in a cohort of adult PN-dependent patients in order to design miRNA panel enabling to precise monitoring of PNALD progression.

miRNAs are small endogenous RNA molecules that post-transcriptionally regulate gene expression by preferentially targeting the 3′-untranslated region of specific mRNA (Marin et al. 2014). The specific miRNA/mRNA interaction typically results in negative regulation of the expression of the protein encoded by target mRNA (Grimson et al. 2007). The occurrence

of miRNAs is not restricted into intracellular space, in contrast, they are found in extracellular body fluids like blood, milk, urine, cerebral spinal fluid, semen, saliva and bile (Shigehara et al. 2011). Extracellular miRNAs are quite stable (Gori et al. 2014). Many of the circulating miRNAs are highly tissue-specific (Ninomiya et al. 2013) and emerging evidence shows that they can serve as non-invasive diagnostic biomarkers for various diseases, including non-alcoholic fatty liver disease (Yamada et al. 2013), steatohepatitis (Jin et al. 2012), biliary diseases (Munoz-Garrido et al. 2012) or hepatocellular cancer (Gailhouste et al. 2013).

We performed an extensive computer-based search of published articles in PubMed to identify relevant studies on the usefulness of serum miRNAs as non-invasive biomarkers for the detection of liver pathologies. The used Medical Subject Headings terms and keywords were „miRNA“, “biomarker”, „liver disease“, „PNALD“, „cholestasis“ and „NASH“. We found 52 miRNAs proposed as putative biomarkers of liver injury (Table 1) that were further analyzed in a cohort of adult patients with chronic intestinal failure.

The discovery cohort consisted of 12 subjects with short bowel syndrome of different etiologies who were repeatedly monitored on three/four-month basis for up to 24 months. Underlying cause of SBS were mesenteric ischemia (n=4), Crohn disease (n=1), ulcerative colitis (n=1), Gardner syndrome (n=1), post radiation enteritis (n=3), postsurgical adhesion (n=1) and trauma (n=1). Control cohort included 45 apparently healthy age- and sex-matched subjects without any known liver pathology. Blood sample with no additives was taken between 7. – 8. a.m. in a fasting state and it was left at room temperature for 30 min. Then it was centrifuged twice for 3000 g, 15 min, 4°C, serum removed to the new tube and centrifuged again 3000g, 10 min, 4°C in order to remove any blood elements. The serum was aliquoted and stored at -80°C until analysis. miRNA extraction was performed using miRCURY RNA isolation kit – biofluids (Exiqon) with RNA Carrier MS2 10 ng/ul (Roche). miRNA detection system included specific Taqman MicroRNA Reverse Transcription kit and

TaqMan microRNA assays (Thermofisher Scientific). The PCR reaction was performed on ViiA7 Real-Time PCR system (Thermofisher Scientific). The specific miRNA expression was normalized to Stock Serum/Plasma spike-in control *Caenorhabditis elegans* MIR39 (cel miR-39-3p), 2 x 10^6 molecules per sample (Qiagen). The data are expressed as $2^{\Delta Ct}$ ($\Delta Ct = Ct_{miRNA} - Ct_{cel\ miR\text{-}39}$) and presented as a median and interquartile range. Statistical analysis was performed using the Kruskal-Wallis test. Differences were considered statistically significant at the level of $p < 0.05$. Spearman's rank correlation coefficient was used to assess the correlation between the studied variables.

SBS patients represent a highly diverse cohort with respect to the primary diagnosis, duration of PN-dependence or age. Most of the patients (11 out of 12) exhibited chronically abnormal liver function tests (Table 2). Among all miRNAs tested, six exhibited significantly altered expression compared with healthy controls. Three of them (MIR122, MIR1273g, and MIR500a) were upregulated while three were down-regulated (MIR505, MIR199a, MIR139) in SBS patients. MIR122 positively correlated with s-AST and s-ALT activities while MIR1273g positively correlated with s-CRP concentration and with s-GGT activity. MIR505, MIR199a, and MIR139 negatively correlated with s-GGT activity (Table 3). Fluctuation of these parameters well paralleled serum miRNA concentrations in all patients throughout the whole observation period.

MIR122 is highly enriched in the liver but absent in other tissues (Lagos-Quintana et al. 2002). MIR122 participates on the regulation of the expression of enzymes involved in crucial metabolic pathways in the liver including glycolysis and gluconeogenesis, carbohydrate digestion and absorption, glucagon signaling pathway, starch and sucrose metabolism, cholesterol synthesis or iron homeostasis (Joppling 2012). Several lines of evidence indicate that it functions as a tumor suppressor (Bai et al. 2009). Roderburg et al. (Roderburg et al. 2015) showed that serum MIR122 concentrations were strongly elevated in

mice after hepatic ischemia/reperfusion injury, as well as in the cellular supernatants in an *in vitro* model of hepatocyte injury, supporting the hypothesis that the passive release of MIR122 represents a surrogate for hepatocyte death in liver injury. This finding corresponds with our observation that serum MIR122 levels correlate with ALT and AST concentrations. Taken together, MIR122 levels may serve as an independent marker of ongoing liver injury and hepatic cell death.

Serum content of all other miRNAs deregulated in PN-dependent patients significantly correlated with GGT serum activity, which is a marker of cholestasis. To our knowledge, this association has not been described yet. Three of these miRNAs (MIR199a, MIR505, and MIR139) were described as tumor suppressors and their down-regulation is associated with disease progression. MIR505 is down-regulated in the serum of pancreatic cancer patients (Schultz et al. 2014) and patients with hepatocellular carcinoma (Li et al. 2015) as well as in serum of patients with primary biliary cirrhosis (Ninomiya et al. 2013). In hepatoma cell lines down-regulation of MIR505 promoted proliferation, invasion and epithelial-mesenchymal transition (Lu et al. 2016). Serum means values of MIR199a were significantly decreased among HCC patients (Kamel et al. 2016, Yin et al. 2015) and served as a predictor of HSS/HCC related hepatocellular carcinoma (Fiorino et al. 2016). MIR139 suppresses tumor growth and metastasis in hepatocellular carcinoma and its decreased serum levels may serve as biomarker of hepatocellular carcinoma (Zou et al. 2018). MIR500a promotes the progression of hepatocellular carcinoma and enhances hepatocarcinoma metastasis (Bao et al. 2018, Jiang et al. 2017, Zhao et al. 2017). The biological function of MIR1273g has not been described yet but the increased MIR1273g content was observed in mice pancreatic cancer tissue (Rachagani et al. 2015) and in human colorectal carcinoma tissue (Vishnubalaji et al. 2015). Interestingly, the expression pattern of all these five miRNAs in patients' cohorts (down MIR199a, MIR505, MIR139; up MIR500a, MIR1273g) follows the signature

characteristic of hepatocellular or pancreatic cancer. In our cohort of patients, there was only one case with diagnosed GIT-related cancer (Gardner syndrome) but our data suggest the increased risk of increased proliferation and possible malignant transformation in the liver of PN-dependent patients.

In conclusion, we identified a panel of six miRNAs differently expressed in sera of PN-dependent patients with abnormal liver function tests compared with healthy controls. These miRNAs correlated with liver injury and hepatic cell death (MIR122), cholestasis (MIR505, MIR199a, MIR139, MIR500a, MIR1273g) or inflammation (MIR1273g). This study suggests that specific miRNAs profile in serum has potential as a diagnostic biomarker of PNALD progression.

Conflict of interest

There is no conflict of interest.

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Table 1 *miRNAs identified as potential biomarkers of liver injury.*

HGNC ID		TaqMan assay ID		reference
31476	MIRLET7A	000377	liver fibrosis	10.1371/journal.pone.0048366
31479	MIRLET7b-5p	002619	APAP-induced liver injury	10.1093/toxsci/kfy200
	MIR16	000391	NAFLD, NASH	10.1371/journal.pone.0023937
31575	MIR19b	002425	liver fibrosis	10.1371/journal.pone.0048366
31586	MIR21	000397	APAP-induced liver injury liver inflammation	10.1093/toxsci/kfy200 10.1371/journal.pone.0023937
31599	MIR22	002301	liver inflammation	10.1371/journal.pone.0048366
	MIR24	000402	liver fibrosis	10.1371/journal.pone.0048366
31616	MIR29A	002112	lower circulating levels in patients with liver fibrosis	10.1002/hep.23922
31619	MIR29B1	000413		
31621	MIR29C	000587		
31625	MIR30B	000602	primary biliary cirrhosis NAFLD	10.1371/journal.pone.0066086 10.1016/j.hep.2018.08.008
31634	MIR33a	002135	primary biliary cirrhosis	10.1371/journal.pone.0066086
32791	MIR33b	002085	NAFLD	10.1016/j.hep.2018.08.008
31635	MIR34a	000426	APAP-induced liver injury liver inflammation NAFLD	10.1002/jat.3722 10.1371/journal.pone.0048366 10.1016/j.hep.2018.08.008
31648	MIR96	000186	apoptosis, necrosis	10.1080/1354750X.2018.1528631
31650	MIR99A	000435	NASH	10.4254/wjh.v6.i8.613
31495	MIR106b	000442	liver fibrosis	10.1371/journal.pone.0048366
31501	MIR122	002245	drug-induced liver injury apoptosis, necrosis oxidative stress NASH NAFLD	10.1093/toxsci/kfy200 10.1080/1354750X.2018.1528631 10.3164/jcbn.17-123 10.1016/j.cca.2013.05.021 10.1371/journal.pone.0153497
31505	MIR125	002198	NAFLD	10.1136/gutjnl-2014-306996
31514	MIR130a	000454	apoptosis, necrosis liver inflammation	10.1080/1354750X.2018.1528631 10.1371/journal.pone.0048366

31526	MIR139	001096	primary biliary cirrhosis NAFLD, NASH	10.1371/journal.pone.0066086 10.1038/ijo.2017.21
31530	MIR143-3p	002249	cholestasis	10.1093/toxsci/kfy200
32079	MIR146B	001097	NAFLD, NASH	10.1136/gutjnl-2015-309456 10.4254/wjh.v6.i8.613
31537	MIR150	002637	NAFLD, NASH	10.1136/gutjnl-2015-309456 10.1016/j.bbrc.2017.10.149
31762	MIR151a	002642	APAP-induced liver injury	10.1002/jat.3722
31549	MIR181a	000480	NAFLD progression liver cirrhosis	10.1016/j.taap.2012.04.018 10.1016/j.bbrc.2012.03.025
31554	MIR183	002269	apoptosis, necrosis	10.1080/1354750X.2018.1528631
31560	MIR190	000489	cholestasis	10.1097/MOG.0000000000000051
31562	MIR192-5p	000491	drug-induced liver injury oxidative stress NAFLD, NASH	10.1093/toxsci/kfy200 10.3164/jcbn.17-123 10.1016/j.hep.2018.08.008
31563	MIR193a	002281	APAP-induced liver injury liver inflammation	10.1002/jat.3722 10.1371/journal.pone.0048366
	MIR194	000493	APAP-induced liver injury	10.1002/jat.3722
31567	MIR196	241070_mat	apoptosis, necrosis	10.1080/1354750X.2018.1528631
31569	MIR197	474626_mat	primary biliary cirrhosis liver inflammation	10.1371/journal.pone.0066086 10.1371/journal.pone.0048366
31571	MIR199a	000498	alcoholic liver disease liver fibrosis	10.3390/ijms17030280 10.1038/nrgastro.2013.87
31579	MIR200B	002274	liver inflammation steatosis	10.1016/S0168-8278(15)31170-3 10.18632/oncotarget.9183
	MIR218a-5p	000521	cholestasis	10.1093/toxsci/kfy200
31601	MIR221	002096	liver fibrosis hepatocellular carcinoma	10.1038/nrgastro.2013.87 10.1073/pnas.0907904107
31771	MIR320-3p	002230	steatosis	10.1093/toxsci/kfy200
31868	MIR375	000564	NASH	10.1136/gutjnl-2014-306996
32053	MIR451	001141	NAFLD	10.1016/j.cca.2013.05.021
32134	MIR500a	002428	primary biliary cirrhosis	10.1371/journal.pone.0066086

32140	MIR505	002087	primary biliary cirrhosis	10.1371/journal.pone.0066086
32827	MIR571		correlates with disease stages during alcoholic or HCV-induced liver cirrhosis	10.1371/journal.pone.0032999
32828	MIR572	001614	NASH	10.3748/wjg.v18.i37.5188
32831	MIR575	001617	NASH	10.3748/wjg.v18.i37.5188
32894	MIR638	001582	NASH	10.3748/wjg.v18.i37.5188
32915	MIR659	001514	liver inflammation	10.1371/journal.pone.0048366
37316	MIR711	241090_mat	liver inflammation	10.1371/journal.pone.0048366
33658	MIR744	002324	NASH	10.3748/wjg.v18.i37.5188
33923	MIR-1224-5p	002752	oxidative stress	10.3164/jcbn.17-123
	MIR1273g	462577_mat	primary biliary cirrhosis	10.1371/journal.pone.0066086
	MIR1274B	002884	liver inflammation	10.1371/journal.pone.0048366

Table 2 *Clinical characteristics of patients.*

patient	no. of assayed samples	sex	age (yr)	SBS type	diagnosis	remnant small bowel (cm)	time on PN (month)	bilirubin total $\mu mol.l^{-1}$	bilirubin conjugated $\mu mol.l^{-1}$	AST $\mu kat.l^{-1}$	ALT $\mu kat.l^{-1}$	ALP $\mu kat.l^{-1}$	GGT $\mu kat.l^{-1}$	CRP $mg.l^{-1}$
1	6	M	36	I	1	50	8	60* (37)	32.8* (22)	1.4* (0.7)	2.6* (1.3)	4.1 * (1.4)	2.4* (1.1)	7.7 *(54.9)
2	4	F	71	I	2	100	87	28* (15)	12* (3)	0.5 (0.08)	0.5 (0.16)	2.2* (0.2)	1.9* (0.2)	0.6 (0.2)
3	1	F	52	I	3	200	84	9.4	n.d.	0.4	0.5	1.8*	0.6*	5.3
4	3	F	64	I	5	40	96	19.5* (6.3)	11.0* (2.4)	0.7* (0.2)	0.6* (0.2)	3.6* (2,3)	1.3 * 0.3)	75.8* (39.8)
5	1	M	64	I	7	?	13	19.9	11.0	0.6*	0.7*	5.1*	2.7*	2.3
6	4	F	53	I	5	120	16	7 (2.6)	3.7 (1.5)	0.7* (0.3)	0.5* (0.3)	6.5* (1.7)	1.6* (0.6)	31* (41)
7	5	F	38	II	4	15	27	8.7 (3.4)	3.4 (0.9)	0.5 (0.3)	0.7* (0.62)	1.8* (0.7)	0.3 (0.9)	3.7 (4.3)
8	4	F	68	II	1	40	88	14.6 (3.9)	7.6 (1.7)	0.5* (0.2)	0.7* (0.41)	2.2* (3.3)	2.4* (1.7)	11.4 * (19.4)
9	5	F	40	II	1	30	61	27* (18.8)	10.3* (4.5)	0.4 (0.2)	0.6* (0.19)	3.0* (1.4)	0.3 (0.2)	0.2 (0.1)
10	3	F	37	II	1	30	52	6.1 (1.9)	2.9 (0.4)	0.3 (0.02)	0.53 (0.08)	1.6 (0.1)	0.5 (0.1)	0.5 (0.4)
11	1	M	21	II	6	80	18	6.7	2.7	0.7*	0.9*	0.7	0.3	2.5
12	1	F	50	II	5	?	84	6.2	3.1	1.8*	1.1*	2.3*	0.4	13.2*
controls		29F/16M	40 (25)	N/A	N/A	N/A	N/A	7.8 (1.9)	2.8 (0.7)	0.3 (0.4)	0.4 (0.6)	0.9 (0.5)	0.2 (0.2)	1.8 (2.1)

SBS type I: end-ostomy, SBS type II: bowel in continuity. Diagnoses: 1 mesenteric ischemia, 2 Crohn disease, 3 ulcerative colitis, 4 Gardner syndrome, 5 post-radiation enteritis, 6 post-surgical adhesion, 7 trauma. AST aspartate transaminase; ALT alanine transaminase; ALP alkaline phosphatase; GGT gamma-

glutamyl transpeptidase; CRP C-reactive protein. When applicable, data are given as a median and interquartile range. Values marked with * were above the normal range in more than half samples during the observation period.

Table 3 *Content of selected miRNA in the serum of PN-dependent patients and healthy controls.*

symbol	HGNC ID	fold change	p-value	Spearman´s correlation coefficient				
				AST	ALT	ALP	GGT	CRP
MIR122	31501	1.7 (1.8)	0.024	0.685*	0.873*	0.305	0.254	0.008
MIR1273G	-	3.7 (6.9)	0.003	-0.048	-0.131	0.316	0.761*	0.531*
MIR500A	32134	2.1 (1.9)	0.049	-0.157	-0.126	-0.038	-0.463*	-0.021
MIR199A1	31571	0.3 (0.5)	2.7×10^{-5}	-0.070	-0.085	-0.246	-0.510*	-0.325
MIR505	32140	0.1 (0.1)	2.4×10^{-12}	0.336	0.250	-0.323	0.697*	0.056
MIR139	31526	0.5 (0.3)	0.025	-0.195	-0.254	-0.044	-0.599*	-0.338

Fold change is calculated as the ratio of normalized Ct values (patients vs a median of control cohort) and expressed as a median and interquartile range (IQR). p-value shows the significance of the difference between control and patient cohorts (Kruskal-Wallis test with Bonferroni correction). Spearman´s correlation coefficient: values marked by * are statistically significant at the level $p<0.05$.