



CLINICAL RESEARCH ARTICLE

MicroRNA in dried blood spots from patients with Aagaenæs syndrome and evaluation of pre-analytical and analytical factors

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BACKGROUND: Circulatory miRNAs are promising biomarkers. The feasibility of using miRNA from dried blood spots (DBS) was investigated using newborn screening cards from patients with cholestasis–lymphedema syndrome (Aagaenæs syndrome) and controls.

METHODS: Total amount of miRNA and specific miRNAs from DBS were analyzed. miRNA was also obtained from newborn screening cards in patients with cholestasis–lymphedema syndrome/Aagaenæs syndrome and in healthy newborns.

RESULTS: No differences in miRNA concentrations were found between multispotted samples and samples with one single drop of blood and between central and peripheral punches. Ten repeated freeze–thaw cycles did not significantly change miRNA levels from controls. miR-299 (1.73-fold change, $p = 0.034$) and miR-365 (1.46-fold change, $p = 0.011$) were upregulated and miR-30c (0.72-fold change, $p = 0.0037$), miR-652 (0.85-fold change, $p = 0.025$), and miR-744 (0.72-fold change, $p = 0.0069$) were downregulated in patients with Aagaenæs syndrome at birth compared to controls.

CONCLUSIONS: miRNAs were not affected by multispotting or punch location and were stable throughout repeated freeze–thaw cycles. miRNA in dried blood spots could be used to detect differential expression of miRNA in newborns with Aagaenæs syndrome and healthy controls in newborn screening cards. Dried blood spots may be a useful source to explore circulating miRNA as biomarkers.

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IMPACT:

- Circulating miRNAs can be useful biomarkers.
- miRNAs from dried blood spots were not affected by multispotting or punch location and were stable throughout repeated freeze–thaw cycles.
- Discrimination between patients and controls are allowed even with few individuals.
- Early after birth, patients with cholestasis–lymphedema syndrome exhibit miRNA profiles associated with liver fibrosis.
- This study demonstrated that newborn screening cards may be a useful source for studying miRNA as the technical variability is smaller than biological variation.

INTRODUCTION

MicroRNAs (miRNAs) are small 17–22-nucleotide noncoding RNAs that regulate gene expression at the posttranscriptional level by complementary binding with sequences in the 3′ untranslated region of mRNA targets. Some miRNA are tissue specific, and as circulatory miRNAs are stable, they may be useful biomarkers. miR-122 is the most abundant miRNA in the liver, but various circulating miRNAs have been associated with different liver diseases.¹ miRNA profiles have been associated with disease severity in non-alcoholic fatty liver disease,² acute-on-chronic liver failure,³ and post-operative liver failure.⁴ Hepatic ischemia–reperfusion injury raises miR-210, which promote hepatocyte apoptosis.⁵ miRNAs have also been used as biomarkers in liver allograft rejection,⁶ viral hepatitis,⁷ and hepatoblastoma.⁸

Circulating miRNA may be measured in whole blood, urine, serum, and plasma. One potential pitfall of using serum or plasma is that hemolysis may significantly alter the concentration of some but not all miRNAs.^{9–11} miRNA from dried blood spots (DBS) may be an alternative source for miRNA as biomarkers.¹² In newborn screening programs for metabolic diseases, biological material is often collected as DBS. This material can easily be obtained by a heel prick in newborns or a capillary sample in adults and is convenient to transport and store. In terms of this, the source is attractive for collecting screening samples from large populations both from newborns and adults. The systematic collection and high coverage in the population makes the newborn screening program valuable biobanks for research. miR-33b and miR-375 in DBS from newborn screening cards are overexpressed in children

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with high birth weight.¹³ Ponnusamy et al. extracted miRNA from DBS and found good correlation with levels from EDTA blood.¹⁴ The biological variation in miRNA from DBS in lung cancer patients exceeded the technical variation significantly.¹⁵

DBS in newborn screening programs are often collected at the local hospital and transported to a central unit for analysis. The knowledge of how miRNA is affected by factors as sampling, stability under analysis, storage, effect of freeze–thawing, and uniformity of distribution of miRNA in the blood spot is essential for the use of miRNA in DBS. Acknowledging and considering pre-analytical and analytical factors, influencing variation and technical reproducibility, will improve discriminative values of the analysis. Repeated freeze–thaw cycles have been demonstrated to significantly reduce miRNA concentrations from plasma and serum samples¹⁶ and PAXgene tubes¹⁷ but have not been examined in DBS. Pre-analytical factors like multiple blood drops in the filter paper and location of the punch have been demonstrated to affect the concentration of acylcarnitines and amino acids.^{18,19} There is no data for how such pre-analytical factors affect retrieval of miRNA from DBS. The aim of this study was to analyze pre-analytical and analytical factors affecting miRNA from newborn screening cards in order to investigate whether such analysis may be feasible for being used as biomarkers. We investigated the feasibility of miRNA from DBS by investigating the miRNA profile of patients with hereditary cholestasis–lymphedema syndrome (Aagenaes syndrome) in DBS from the newborn screening program. Aagenaes syndrome is characterized by intermittent cholestasis and lymphedema in lower and sometimes upper limbs in early childhood.^{20,21} The syndrome is inherited in a recessive pattern,^{22,23} and the mechanisms underlying the intermittent cholestasis are unknown. While some patients present early, others may have more delayed symptoms. Patients are treated with vitamins and medication or surgery²⁴ to reduce cholestasis-related pruritus. The severity of the phenotypes varies, and some children require liver transplantation. Early diagnosis might be challenging as pruritus often precedes the lymphedema.

METHODS

Sample collection

The study complies with the Helsinki Declaration and is approved by the Regional Ethics Committee (2016/2303). To test pre-analytical and analytical factors, 4 ml of EDTA blood and DBS were obtained from healthy adults ($n=8$) who had given informed written consent. Capillary blood was added to a PerkinElmer 226 Newborn Screening Card (PerkinElmer, Waltham, MA). All filter cards were inspected visually to ensure that blood had fully penetrated the filter paper. The filter cards were allowed to dry in room temperature for 18 h before storage in plastic bags at -20°C . The filter cards were acclimatized for 30 min before punches of 3.2 mm diameter were stamped out with a hole-punch device. The puncher was washed between every punch. EDTA blood was centrifuged immediately at $300\times g$ for 10 min. Plasma was aliquoted in 250 μl and stored at -80°C . To test the clinical applicability of miRNA from DBS, single 3.2 punches from the newborn screening card from healthy newborns ($n=5$) and from patients later diagnosed with Aagenaes syndrome ($n=4$) were obtained after informed written consent from parents/caregivers. DBS obtained in the newborn screening program had been stored in plastic bags at -20°C . The patients and controls were born during 2006–2011 and recruited at Oslo University Hospital from February to August 2017.

Isolation of miRNA

miRNA was extracted from one single DBS punch using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). To investigate the effect on QiAsol/chloroform ratio, a Qubit[®] 2.0

Fluorometer, with Qubit[®] MicroRNA Assay Kit, was used to measure quantity of miRNA in every sample. Three different combinations were tested; 200 μl QiAsol with 100 μl chloroform, 300 μl QiAsol with 100 μl chloroform, and 500 μl QiAsol with 200 μl chloroform. Increasing levels of QiAsol in the isolation step gave higher concentrations of miRNA (200 μl QiAsol with 100 μl chloroform vs 500 μl QiAsol with 200 μl chloroform; 181 ± 42 vs 250 ± 62 , $p=0.033$) ($n=7$). Thus 500 μl QiAsol with 200 μl chloroform was subsequently used in this study. After additions of QiAsol, the samples were incubated 15 min at room temperature and vortexed every fifth minute. When isolating miRNA from EDTA plasma, 200 μl sample was used. The miRNeasy Serum/Plasma Kit was used in accordance with the manufacturer's protocol. As the final step, all miRNAs were eluted in 30 μl nuclease-free water and stored at -80°C until use.

Quantification of miRNA with fluorometer

Quantification of miRNA in both EDTA plasma and DBS samples were performed with Qubit[®] 2.0 Fluorometer, using the Qubit[®] MicroRNA Assay Kit (Life technologies, Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's instructions. Qubit[®] working solution was prepared by diluting the Qubit[®] microRNA reagent 1:200 in Qubit[®] microRNA buffer. Four microliters of the test sample was incubated with 196 μl Qubit[®] working solution for 2 min before measurement.

TaqMan Low Density Array (TLDA)

TLDA cards were custom designed with 96 miRNAs in duplicates (Thermo Fisher Scientific, Waltham, MA) (Supplementary Table S1). Both candidates for endogenous controls ($n=8$) and miRNA that may relate to liver disease ($n=88$) were selected based on literature search.

Four microliters of RNA isolated from the newborn screening cards were reverse transcribed using custom RT primer pool followed by preamplification with custom PreAmp primer pool on a 7300 Real-Time PCR System (Applied Biosystems[™], Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. The preamplification product was diluted 1:8, and 1.13 μl of the dilution was loaded on the TaqMan Array card and run on a ViiA7 Real-Time PCR system (Applied Biosystems[™], Thermo Fisher Scientific, Waltham, MA) using the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA) with the following cycling conditions: 92°C 10 min and 95°C 1 s, 60°C 20 s (40 cycles).

Eight potential endogenous controls were selected and investigated on the custom-made TLDA card: miR-15b, miR-16, miR-24, miR-26a, miR-26b, miR-106a, miR-152, and U6. To assess the most stable miRNA for normalization of the samples across the Aagenaes group and the control group, quantification cycle (Cq) values for the eight potential endogenous controls were analyzed using the Reffinder, a web-based tool based on four different algorithms (delta Cq, BestKeeper, Normfinder, and Genorm)^{25–28}. Genorm suggested a combination of two, miR-16 and miR-15b, while the other three algorithms were rather conclusive regarding the most stable, miR-16, and the least stable miRNA, U6 (Supplementary Table S2). miR-16 was selected for normalization in the study.

Quantification of miRNA with quantitative PCR (qPCR)

The RNA samples were reverse transcribed with a fixed volume of 4 μl per sample, using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems[™], Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Individual TaqMan Assay (Thermo Fisher Scientific, Waltham, MA) for human miRNAs miR-16, miR-140, miR-210, and miR-4429 were analyzed using Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA) on a ViiA[™] 7 Real-Time PCR System, (Applied Biosystems[™], Thermo Fisher Scientific, Waltham, MA) with the following cycling program: 95°C 20 s (1 cycle) and 95°C 1 s, 60°C 20 s (40 cycles).

All samples were diluted 1:10 in nuclease-free water and analyzed in technical parallels. miR-16 was used as an endogenous control. The difference in mean Cq values of target miRNA and miR-16 (delta Cq) were used to compare miRNA expression levels in different samples.

Statistics

Data are given as mean and standard deviation. Frequency histograms were used to evaluate whether data were normally distributed. Comparisons between two groups were tested with Student's *t* test. Comparisons between multiple groups were tested with one-way analysis of variance (ANOVA). Calculations were performed with Graph Pad InStat version 03.10 for Windows (GraphPad Software Inc., La Jolla, CA) and IBM Statistical Package for Social Sciences (IBM SPSS statistics, version 21.0.1, Chicago, IL).

RESULTS

Relation between amount of material and yield of miRNA

We wanted to assess whether one 3.2-mm diameter punch would be sufficient to isolate and measure miRNA and to investigate whether there was a relationship between size of the punch and yield of miRNA. We found a significant increase in total amount of miRNA present in the sample with three punches (890 ± 222 ng/ml) compared to one punch (250 ± 87 ng/ml) (Fig. 1a), confirming a relation between the amount of punched material and yield of miRNA. This result was further validated by qPCR analysis detecting lower Cq values upon three punches for three different miRNAs (miR-140, miR-210, and miR-4429; Fig. 1c) and the endogenous control miR-16 (Fig. 1b). Correction for amount of input by relating specific miRNA to an endogenous control (miR-16) gave similar concentrations for one and three punches (Fig. 1d), demonstrating that using an endogenous control effectively corrects for differences in the amount of material analyzed.

Coefficient of variation of miR-4429 in repeated measurements of the same sample was similar in plasma 0.37% (mean Cq 33.37 ± 0.12 , $n = 8$) and in DBS 0.4% (mean Cq 33.76 ± 0.13 , $n = 8$). Coefficient of variation of miR-4429 in 5 different blood spots from the same sample had a coefficient of variation of 0.73% (mean Cq 33.74 ± 0.25).

Effect of multispotted samples and location of punch

To investigate the effect of pre-analytical factors, we wanted to determine whether there is any difference between dripping one single drop of blood directly on the filter paper vs two drops on top of each other covering the whole area. Furthermore, some compounds may not be evenly distributed in the blood spot and we wanted to assess whether the location of the punch affected the concentration of the miRNA. No differences in either punch location or one drop vs two drops on the filter card were found for total miRNA (ANOVA; $p = 0.33$; Fig. 2a), miR-16 (ANOVA; $p = 0.95$; Fig. 2b), miR-140 (ANOVA; $p = 0.18$), miR-210 (ANOVA; $p = 0.56$), or miR-4429 (ANOVA; $p = 0.23$; Fig. 2c).

miRNA from DBS and EDTA plasma

No differences between plasma and DBS were demonstrated for miR-140 ($p = 0.46$; Fig. 3a) and miR-210 ($p = 0.59$; Fig. 3b). Higher concentrations of miR-4429 ($p < 0.0001$; Fig. 3c) were found in plasma compared to DBS.

Impact of repeated freeze-thaw cycles on miRNA stability

In many clinical situations, the need for second analysis results in repeated freeze-thaw cycles of the biobank material. To validate the stability of miRNAs from DBS, the cards were freeze-thawed ten times. After one and ten cycles, miRNA was isolated from one 3.2 mm punch. No change after 10 repeated freeze-thaw cycles in the total levels of miRNA measured ($p = 0.93$; Fig. 4a) was

observed. miR-16 ($p = 0.45$; Fig. 4b), miR-140 ($p = 0.3$; Fig. 4c), miR-210 ($p = 0.42$; Fig. 4d), and miR-4429 ($p = 0.13$; Fig. 4e) were stable through 10 repeated freeze-thaw cycles.

miRNA in patients with lymphedema-cholestasis syndrome 1/ Aagenaes syndrome

To investigate whether clinical variation in miRNA in patients and controls were larger than technical variation in analysis, stored newborn screening cards were obtained from children diagnosed with congenital lymphedema-cholestasis syndrome. Blood samples at first clinical investigations at median 59 days (interquartile range 34–487) after birth revealed early liver involvement and cholestasis; international normalized ratio 1.2 (1.0–1.4), gamma-glutamyltransferase 80 (27–212) U/l, aspartate aminotransferase 237 (80–696) U/l, alanine aminotransferase 249 (46–600) U/l, total bilirubin 121 (31–178) $\mu\text{mol/l}$, and bile acids 315 (189–356) $\mu\text{mol/l}$.

One 3.2 mm punch from the newborn screening card from 4 patients with Aagenaes syndrome (3 females) and 5 healthy controls (3 females) were tested for 88 miRNA and 8 endogenous controls. Storage time for the DBS from birth to analysis was similar for controls and patients with Aagenaes syndrome (9.4 ± 1.9 vs 9.7 ± 1.9 years, $p = 0.78$). Birth weight for controls (3588 ± 252 g) and patients with Aagenaes syndrome (3839 ± 476 g) did not differ significantly ($p = 0.35$). Gestational age for the control patients were 40.3 ± 0.95 weeks, and 40.3 ± 0.48 weeks for the patients with Aagenaes syndrome ($p = 0.92$).

Sixty-six out of the 88 tested miRNA gave signal from 1 punch and 37 of these with data from all participants (all patients and controls) (Supplementary Table S1). Five out of these 37 were differentially expressed. miR-299 (1.73-fold, $p = 0.034$) and miR-365 (1.46-fold, $p = 0.011$) were upregulated and miR-30c (0.72-fold, $p = 0.004$), miR-652 (0.85-fold, $p = 0.025$), and miR-744 (0.72-fold, $p = 0.007$) were downregulated in patients with Aagenaes syndrome at birth compared to controls (Table 1). Material was available to validate the findings from the TLDA cards for two of the miRNAs with qPCR. miR-365 (1.31-fold, $p = 0.15$) and miR-299 (2.78-fold, $p = 0.04$) essentially showed the same trend as the analysis in TLDA cards.

DISCUSSION

miRNA from DBS may not only be especially attractive biomarkers in neonates as this material is available in the newborn screening program but may also be useful in screening of disease of adult patients as DBS are easy to collect, transport, and store.¹² However, before exploring miRNA from DBS as biomarkers several pre-analytical and analytical factors have to be considered. We demonstrate that miRNA can be detected from very small amounts of material by using only one small punch (3.2 mm) from DBS. Most previous studies have used either a 6-mm punch or the whole blood spot.^{13–16} There is, as expected, a relation between amount of sample material and the amount of miRNA detected. Relating the target miRNA to an endogenous control efficiently corrected for different volumes. Samples collected for the newborn screening program may be subjected to repeated freeze-thaw cycles related to requirement for re-analysis or second-tier analysis. Repeated freeze-thaw cycles lowered miRNA in PAXgene tubes, plasma and serum.^{16,17,29} In contrast, we found miRNA levels unaffected by ten cycles of freeze-thaw in DBS. This was found both for miR-16, miR-140, miR-210, and miR-4429 and for total miRNA. In this matter, miRNAs from DBS are stable and cards collected in newborn screening programs may be used as possible sources for miRNA studies.

Carnitines analyzed from multispotted samples differ from single spotted controls, especially for peripheral punches.¹⁸ This could probably be due to non-uniformity of blood distribution. For total miRNA levels, miR-16, miR-140, miR-210, or miR-4429, we did not find any difference between samples having received one or

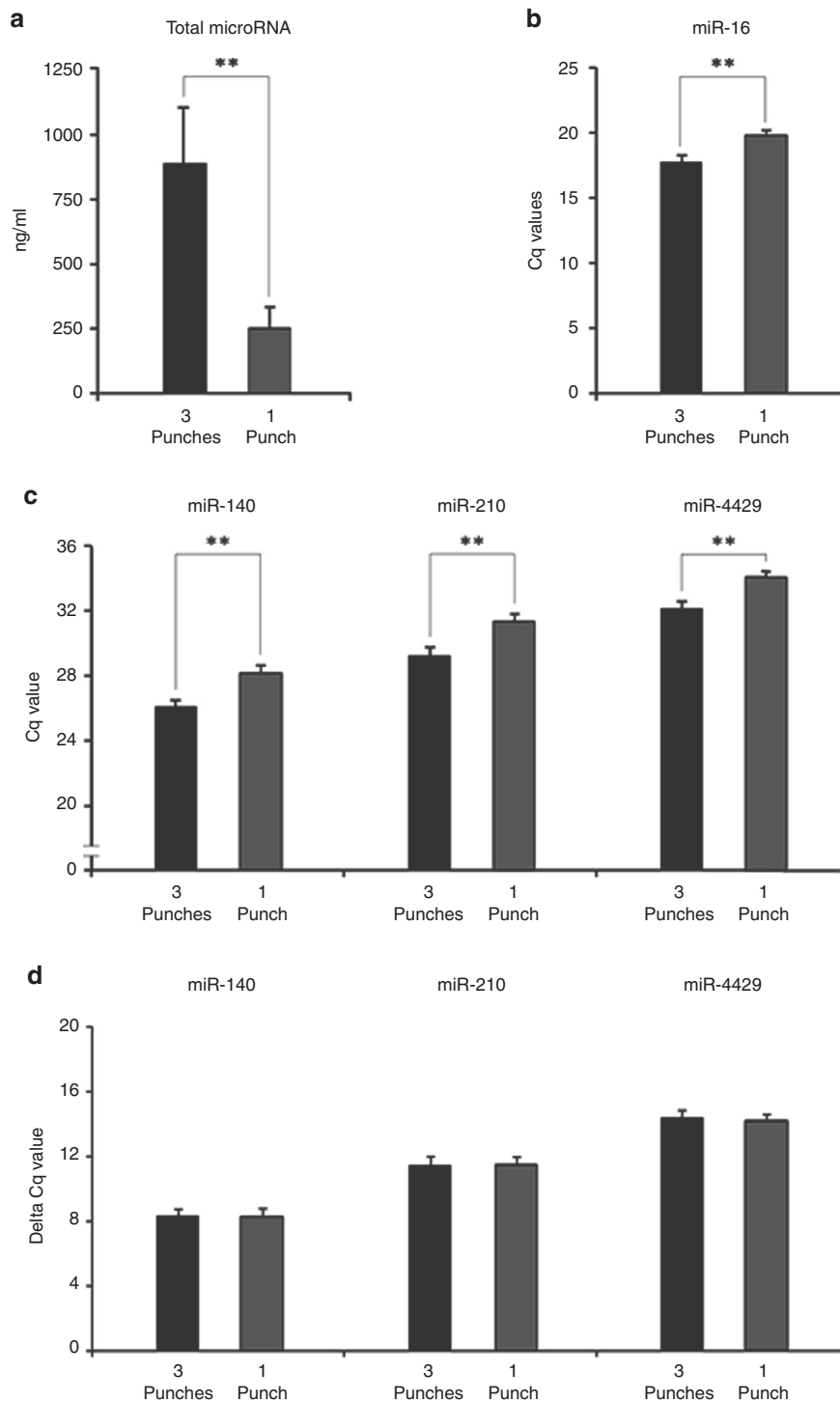


Fig. 1 Relation between the amount of material and yield of miRNA. Comparison of material from three punches (black) and one single punch (gray) measured as **a** total miRNA detected using Qubit[®] 2.0 Fluorometer with the Qubit[®] microRNA Assay Kit; **b** miR-16; **c** miR-140, miR-210, and miR-4429 (not related to endogenous control); and **d** miR-140 ($p = 0.97$), miR-210 ($p = 0.70$), and miR-4429 ($p = 0.50$) (related to endogenous control miR-16). Data are given as mean and standard deviation ($n = 8$ for all assays). Two-tailed unpaired t test was used. $**p < 0.0001$.

two droplets of blood. Thus with respect to this factor miRNA seems to be quite robust for poor sample quality. Punch location affects results of compounds as phenylalanine, leucine, acylcarnitines, and thyroid-stimulating hormone with higher concentrations in peripheral than in central punch.^{18,19,30} We did not find any differences between central and peripheral punches between total miRNA levels or with four selected miRNAs. Thus, both with

multispotting and punch location, miRNA in DBS is a rather robust method with respect to these important pre-analytical factors.

Ponnusamy et al. found significant correlations between miRNA in DBS and EDTA blood but not between the levels in DBS and plasma.¹⁴ In the current study, the expression of miR-140 and miR-210 did not differ between DBS and plasma, but higher concentrations of miR-4429 were found in plasma compared to

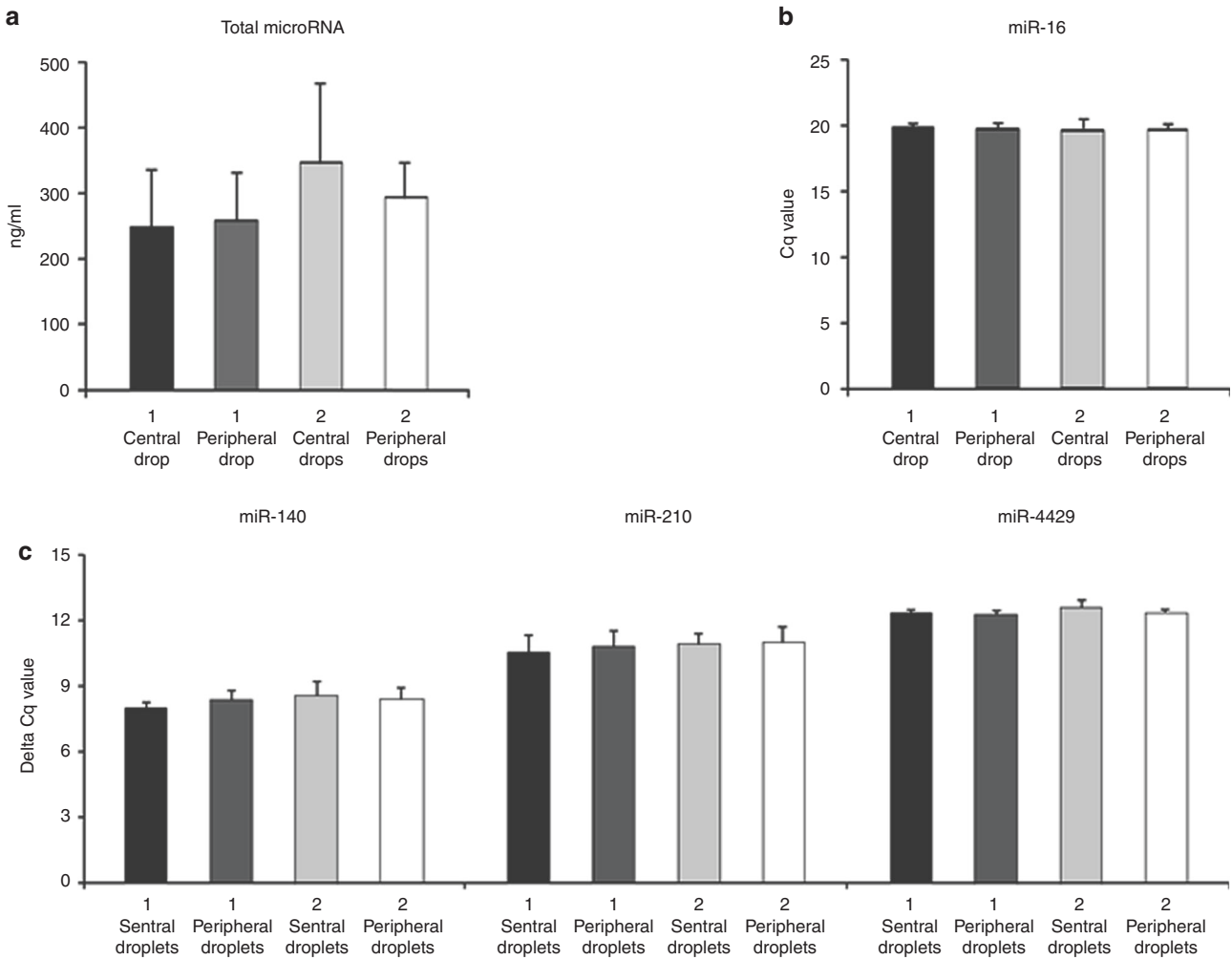


Fig. 2 Effect of multispotting samples and location of the punch. No differences in punch location (central vs peripheral punch) or multispotting (one vs two drops of blood) were demonstrated with regard to **a** total miRNA levels ($p = 0.33$), **b** miR-16 ($p = 0.95$), or **c** miR-140 ($p = 0.18$), miR-210 ($p = 0.56$), and miR-4429 ($p = 0.23$) (related to endogenous control miR-16). Data are given as mean and standard deviation ($n = 8$). Comparisons were performed with one-way ANOVA.

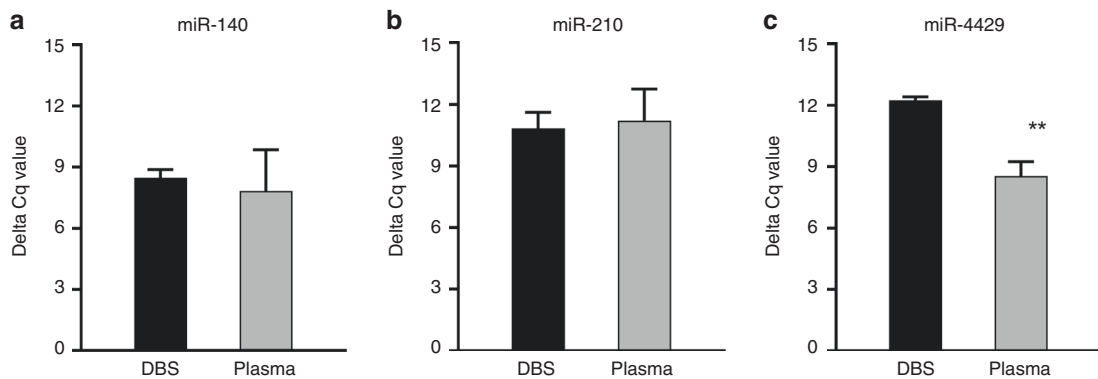


Fig. 3 Relation between miRNA from DBS and plasma. Comparison of **a** miR-140, **b** miR-210, and **c** miR-4429 from dried blood spots (DBS) (black) and plasma (gray). miRNA are related to endogenous control (miR-16). Data are given as mean and standard deviation ($n = 6$ for all assays). Comparisons were performed with two-tailed unpaired t test. $**p \leq 0.0001$.

DBS. Relation between miRNA in plasma, serum, and whole blood vary along with the differences in distribution between compartments. Some miRNA are present in high concentrations in blood cells,^{31–33} exosomes, small vesicles, and platelets.¹¹ This is

illustrated by the fact that different miRNAs in varying degrees are affected by hemolysis.^{9–11} Pre-analytical hemolysis may affect concentration of miRNA in serum and plasma. Given the differences in the expression of miRNA in erythrocytes, platelets

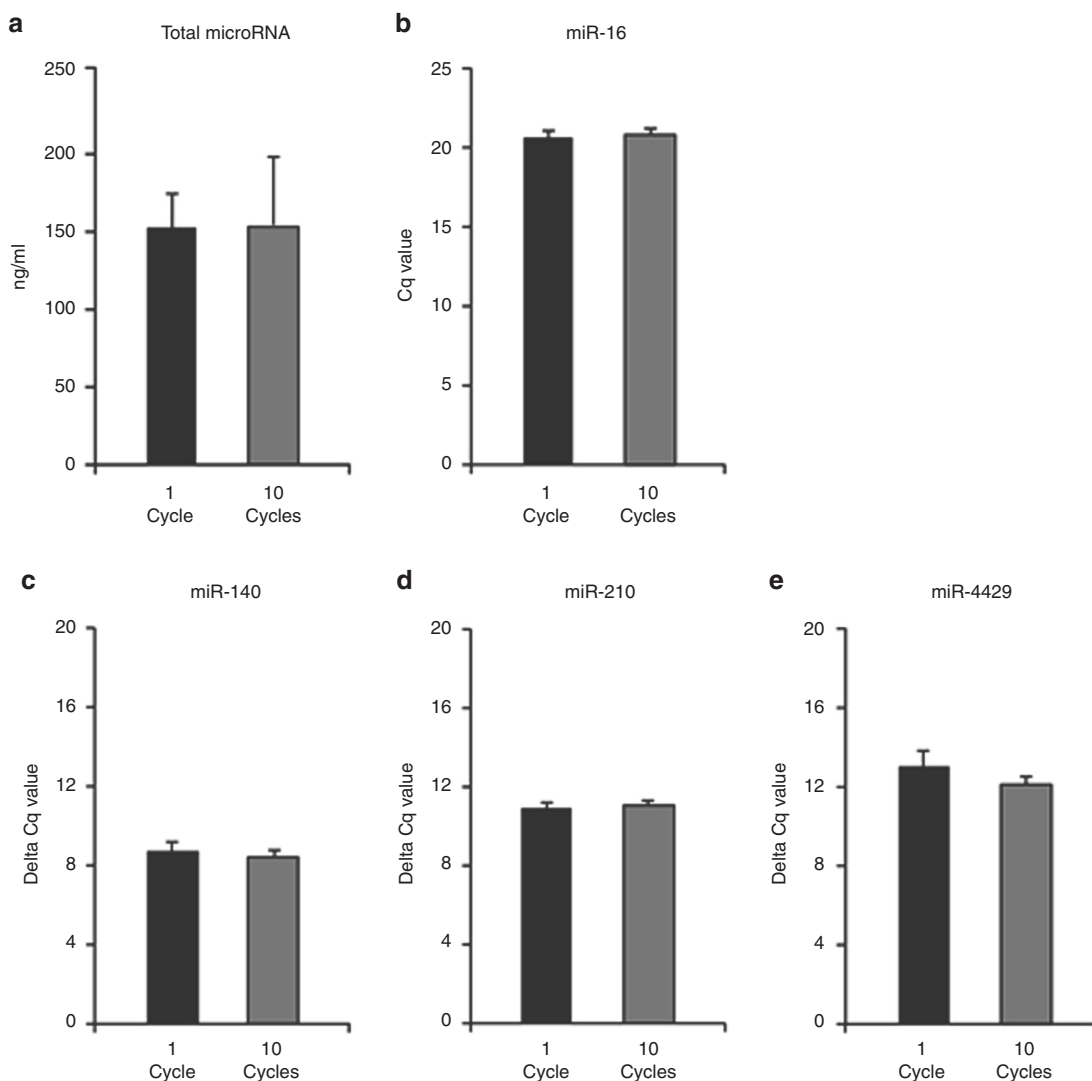


Fig. 4 Effect of repeated freeze–thaw cycles. **a** Total miRNA in dried blood spots (DBS) ($p = 0.93$) and **b** miR-16 ($p = 0.45$), **c** miR-140 ($p = 0.3$), **d** miR-210 ($p = 0.42$), and **e** miR-4429 ($p = 0.13$) in DBS after repeated freeze–thaw cycles. miRNA was isolated from one 3.2-mm punch after 1 and 10 cycles. Data are given as mean and standard deviation ($n = 6$ for all groups). Comparisons were performed with unpaired *t* test.

Table 1. Differential expression of microRNAs in dried blood spots between newborns with cholestasis–lymphedema syndrome/Aagaenae syndrome and from healthy newborns from the TaqMan Low Density Array.

	Fold change	Aagaenae group	Healthy controls	<i>p</i> value
		Mean delta Cq value	Mean delta Cq value	
miR-30c	0.72	6.627	6.154	0.004
miR-299	1.73	17.491	18.285	0.034
miR-365	1.46	14.377	14.919	0.011
miR-652	0.85	10.924	10.687	0.025
miR-744	0.72	13.320	12.848	0.007

miR microRNA, Cq quantification cycle.

and tissue, interpretation of circulating miRNA requires knowledge of the origin of the individual miRNA and especially whether miRNA are expressed in blood cells.

Aagaenae syndrome or lymphedema–cholestasis syndrome 1 is a rare disease with the combination of cholestasis in childhood and lymphedema.²⁰ Most patients experience intermittent episodes with cholestasis, elevated bile acids, and intense pruritus.^{21,22} The severity of the phenotypes varies and some are liver transplanted. During childhood and adolescence, many develop lymphedema mainly in the lower limbs, but some also in the upper limbs and thorax. The locus of the genetic changes have been mapped to chromosome 15q,²³ but neither the mechanisms underlying the lymphedema or regulating the intermittent cholestasis have been revealed. We found two upregulated (miR-299 and -365) and three downregulated miRNAs (miR-30c, -652, and -744) in newborns with Aagaenae syndrome. Sufficient material was available to validate the findings from the TLDA cards for miR-365 (1.31-fold, $p = 0.15$) and miR-299 (2.78-fold, $p = 0.04$) with qPCR. The qPCR analyses showed the same trend as the analysis in cards, although miR-365 did not reach statistical significance. miR-299 has previously been found to be upregulated in explanted livers from patients with primary biliary cirrhosis.³⁴ The patients with primary biliary cirrhosis also had downregulation of miR-30c.³⁴ Downregulation of miR-30c has been linked to activation of stellate cells and development of

cirrhosis.³⁵ Reduced circulating levels of miR-744 have been found in patients with hepatitis B³⁶ and in patients with liver cirrhosis,³⁷ and low miR-744 was associated with poor prognosis in patients with hepatocellular carcinoma.³⁸ A mouse model of fibrosis indicates a role for miR-744 in suppressing the activation of hepatic stellate cells.³⁷ Reduced levels of miR-652 has also been linked to liver fibrosis as lowered circulating levels have been found in monocytes of patients with liver cirrhosis³⁹ and in a mouse model of liver fibrosis.⁴⁰

Whether differential expression levels of these miRNAs detected at birth play a role in the development and pathophysiology of this disease or are secondary markers of the disease is yet to be explored. It is, however, interesting that patterns of miRNA previously linked to cholestasis and liver fibrosis are present at this very early stage immediately after birth. This study demonstrates that miRNAs in DBS are stable and analyses can be performed with low technical variability compared with biological variability to allow discrimination between controls and patients with disease even though the number of patients was limited. Some of the preloaded miRNAs were not detected in all patients, and this may be due to the use of only one punch. Increasing the amount of punched material will lower the detection limit and increase the number of miRNA detected using premade cards. Our approach with a limited number of miRNAs on a card is a first screening method. In order to get a complete profile of miRNA in lymphedema–cholestasis syndrome, miRNA sequencing could be performed.

A substantial amount of laboratory errors are due to pre-analytical factors.^{41,42} Using DBS reduces these factors since there is no centrifugation or pipetting steps, and anticoagulants and additives like heparin that may directly interfere with the PCR analysis are avoided.^{16,43} While miRNA decreases after a few freeze–thaw cycles in serum, plasma, and PAXgene tubes,^{16,17,29} we found that miRNA is stable even after 10 freeze–thaw cycles in DBS. The lack of effect of multispotting and punch location and the stability after isolation makes analysis of miRNA in DBS feasible.

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AUTHOR CONTRIBUTIONS

M.A.-Å.: contributed to study design, analysis and data collection, contributed to writing, approved the last version of the manuscript. M.M.: contributed to study design, analysis and data collection, statistical analysis, contributed to writing, approved the last version of the manuscript. R.A.: contributed to study design, statistical analysis, drafted manuscript, approved the last version of the manuscript.

ADDITIONAL INFORMATION

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Consent: Written informed consent was obtained from participants or parents/caregivers.

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