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ORIGINAL RESEARCH ARTICLE

MicroRNA profiling as a novel diagnostic tool for identification of patients with inflammatory and/or virally induced cardiomyopathies

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Abstract

Aims MicroRNAs (miRNAs) might be used as prospective biomarkers for the identification of unexplained heart failure caused by a viral and/or inflammatory process. The aim of this study was to identify and to evaluate prognostic miRNAs in serum of patients with inflammatory heart diseases diagnosed by endomyocardial biopsies.

Methods and results After TaqMan® OpenArray® screening of 754 unique circulating miRNAs in serum of biopsy-proven patients [184 patients with inflammatory and/or virally induced myocardial diseases (DCMi), 25 patients with dilated cardiomyopathy (DCM), and 25 healthy donors], we identified seven miRNAs of interest ($P < 0.05$). These data have been verified by single qRT–PCR assays in other biopsy-proven patients (159 patients with viral and/or inflammatory myocardial diseases, 46 patients with DCM, and 60 healthy donors). The expression of let-7f, miR-197, miR-223, miR-93, and miR-379 allowed us to differentiate between patients with a virus and/or inflammation and healthy donors ($P < 0.05$) with the specificity over 93%. Based on the expression of miR-21 and miR-30a-5p, we could sort out patients with DCM from all other study groups ($P < 0.05$) with the specificity over 95%.

Conclusions This miRNA profile provides for the first time a new non-invasive diagnostic perspective to identify patients with intramyocardial inflammation and/or viral persistence only from single serum sample, independently of prescribed therapy and time of symptoms onset. It allows the early finding of those patients relevant for myocardial biopsy for exact diagnosis and further proscription of causal aetiology-driven specific treatment.

Keywords MiRNA; Virus; Inflammation; Myocarditis; DCM

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Introduction

Endomyocardial biopsies (EMBs) are the gold standard[1] for identification of causative factors in patients with unexplained heart failure (HF) as a prerequisite of an aetiology-driven treatment.[2–5] Nevertheless, EMB is used infrequently because of missing non-invasive, clear defined diagnostic criteria.[6]

MicroRNAs (miRNA) are small, 19–23 nucleotides long non-coding RNAs that bind to complementary sequences on the 3′ untranslated region of target messenger RNAs (mRNA) post-transcriptionally regulating mRNA expression.[7] It is already known that miRNAs are involved in cardiac differentiation,[8] proliferation,[9] apoptosis[8] as well as in myocardial injury,[10] and inflammation.[11] Because of their stability in the circulation, miRNA profile has emerged as prospective biomarker for many human diseases, in particular in cardiovascular diseases,[12–15] providing novel molecular insight and new therapeutic strategies to treat diseases.

Cardio-enriched miRNAs play a crucial role in cardiac development[16] and have been associated with the development of dilated cardiomyopathy (DCM).[17]

Despite their promise, miRNAs still have not entered the clinical routine scenario, mainly because of a lack of large

cohort studies,[15] or several challenges related to technical aspects, miRNAs normalization, drugs interaction, and quality reporting of statistical multivariable analysis of the miRNAs observational studies.[18]

Several data indicate that miRNA profiling in serum samples might be a diagnostic marker in specific inflammatory heart disease, beyond being a biomarker of disease course and survival.[19] Therefore, in this retrospective study that was designed to ascertain differential miRNA patterns in human serum samples, miRNA expression levels of biopsy-proven patients with viral/inflammatory myocardial diseases and DCM were compared with healthy controls.

The aim of the study was to identify clinically relevant novel diagnostic markers of HF in form of miRNAs for the selection of patients who require EMBs as a prerequisite of exact diagnosis and causal specific treatment.

Methods

Study patients

This retrospective study composed of two parts (screening and validation) evaluated blood serum samples (500 μL) of 414 patients in total with clinically unexplained HF (343 patients with inflammatory and/or virally induced heart diseases diagnosed by EMB and 71 patients with DCM diagnosed by EMB) based on different disease entities sent to the CAP-accredited laboratory IKDT (Institute for Cardiac Diagnostic and Therapy Berlin, Germany). The patients complained about symptoms of HF with fatigue, reduced physical capacity or dyspnoea on exertion, and cardiac dysfunction. Patients with coronary artery disease (CAD), other possible causes of myocardial dysfunction (e.g. valvular heart disease, hypertension, restrictive, or constrictive heart disease) diagnosed by angiography and echocardiography, and concomitant chronic inflammatory disease (e.g. rheumatological disorders) were excluded from this study.

Left ventricular ejection fraction was determined by echocardiography. Therapy at the time of serum samples taking was not known as we received only blood or serum samples and EMBs for diagnostic evaluation.

Blood serum samples (500 μL) of 85 healthy donors have been used as a control group.

Endomyocardial biopsy analysis

Histological and immunohistochemical staining for assessment of inflammation

We diagnosed patients based on EMB as follows:

- An active myocarditis was diagnosed according to the Dallas criteria[20] as a histological evidence of inflammatory infiltrates of >14.0 lymphocytes/mm^2, including >7.0 CD3 + lymphocytes/mm^2 according to the European Society of Cardiology guidelines[21] within the myocardium associated with myocyte degeneration and necrosis.
- A borderline myocarditis was diagnosed with histological evidence of inflammatory infiltrates within the myocardium without myocyte degeneration and necrosis.
- Inflammatory cardiomyopathy (DCMi) was diagnosed by evidence of intramyocardial inflammation association with cardiac dysfunction.
- The diagnosis DCM was made on morphological and functional characterization with significantly impaired left ventricular ejection fraction and/or dilated left ventricle (*Table 1*). EMBs from DCM patients were negative for histologically or immunohistologically detected inflammation and for the detection of cardiotropic viral genomes.

Patients with a genetic form of cardiomyopathy were excluded from the study due to missing data.

Used antibodies and immunohistological staining procedure and evaluation of EMBs can be found elsewhere.[3,22]

Table 1 Clinical data, ejection fraction, and histological findings of all patients

Characteristic	Inflammatory and/or virally induced cardiomyopathy patients	DCM patients	Healthy controls	All patients
Number	343	71	85	499
Age, mean (years)	49 ± 29	55 ± 11	44.5 ± 16.5	53 ± 25
Male sex (%)	268 (78)	38 (53)	27 (31)	333 (67)
LVEF (%), mean ± SD	48 ± 19	28 ± 9	—	46 ± 19
EF < 55% (%)	60	100	0	—
LVEDD (mm), mean ± SD	55.7 ± 21.3	66.3 ± 15.7	—	58.3 ± 23.7
Fibrosis (%)	No fibrosis[a]—83% Low fibrosis[b]—17%	No fibrosis—86% Low fibrosis—14%	—	—

DCM, dilated cardiomyopathy; EF, ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; SD, standard deviation.
[a]Up to 3% of connective tissue.
[b]5-15% of connective tissue.

Detection of viral genomes

DNA and RNA were extracted from frozen heart muscle tissue probes. RT–PCR was performed for the detection of enteroviruses (including Coxsackievirus), adenovirus, parvovirus B19 (B19V), and human herpesvirus type 6 (HHV6) (*Table 2*) using methods published previously.[23,24] In addition, DNA was extracted from peripheral blood cells to exclude a systemic infection with B19V and HHV6. As a control for successful extraction of DNA and RNA from heart muscle tissue, oligonucleotide sequences were chosen from the DNA sequence of the GAPDH gene.

The clinical and EMB-based molecular virological and immunohistochemical data of all study patients are summarized in *Tables 1* and *2*.

MicroRNAs isolation

Ten millilitres of patients' blood have been collected using BD Vacutainer and centrifuged for 10 min by 10 000–2000 g by 4°C. Serum (approximately 4 mL serum) was collected in tubes and immediately frozen by −80°C for further miRNA analyses.

MicroRNAs were obtained from 500 μL of frozen (−80°C) patients' serum using mirVANA™ PARIS™ RNA and Native Protein Purification Kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions.

MicroRNA reverse transcription, pre-amplification, and expression analysis using TaqMan® OpenArray and qRT–PCR

Total RNA including miRNA fraction was initially reversely transcribed to cDNA using Megaplex stem-loop RT primer (Thermo Fisher Scientific, Waltham, MA, USA) for Human Pool A and B in combination with the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) for low content samples. The entire procedure for quantification of miRNAs is described elsewhere.[25] Hsa-miR-30a-3p was used as reference miRNA used for data normalization.

Table 2 Immunohistological analysis of endomyocardial biopsy (median) of all patients with unexplained heart failure

	Diagnosis	CD3 + cells/mm^2	CD45RO + cells/mm^2	LFA-1 + cells/mm^2	Mac-1 + cells/mm^2	HLA-1% area fraction
Inflammatory myocardial disease patients	Active myocarditis (MCA)	58.78	104.18	138.27	219.23	9.43
	Borderline myocarditis	20.65	73.92	29.92	48.09	10.59
	DCM with inflammation (DCMi)	16.08	44.18	39.08	53.50	7.40
Virally and/or inflammatory myocardial disease patients	Adenovirus (ADV)	0.00	0.00	—	—	8.05
	Enterovirus (Coxsackie virus)	2.12	16.19	11.04	20.82	6.83
	Human herpesvirus 6 (HHV6)	2.80	19.13	8.65	25.35	6.72
	Parvovirus B19 (B19V)	3.86	29.31	10.15	23.69	6.80
Dilated cardiomyopathy (DCM)		2.85	10.69	7.85	18.05	5.37

The calculated objects were related to the unit heart area (mm^2) or % area fraction.

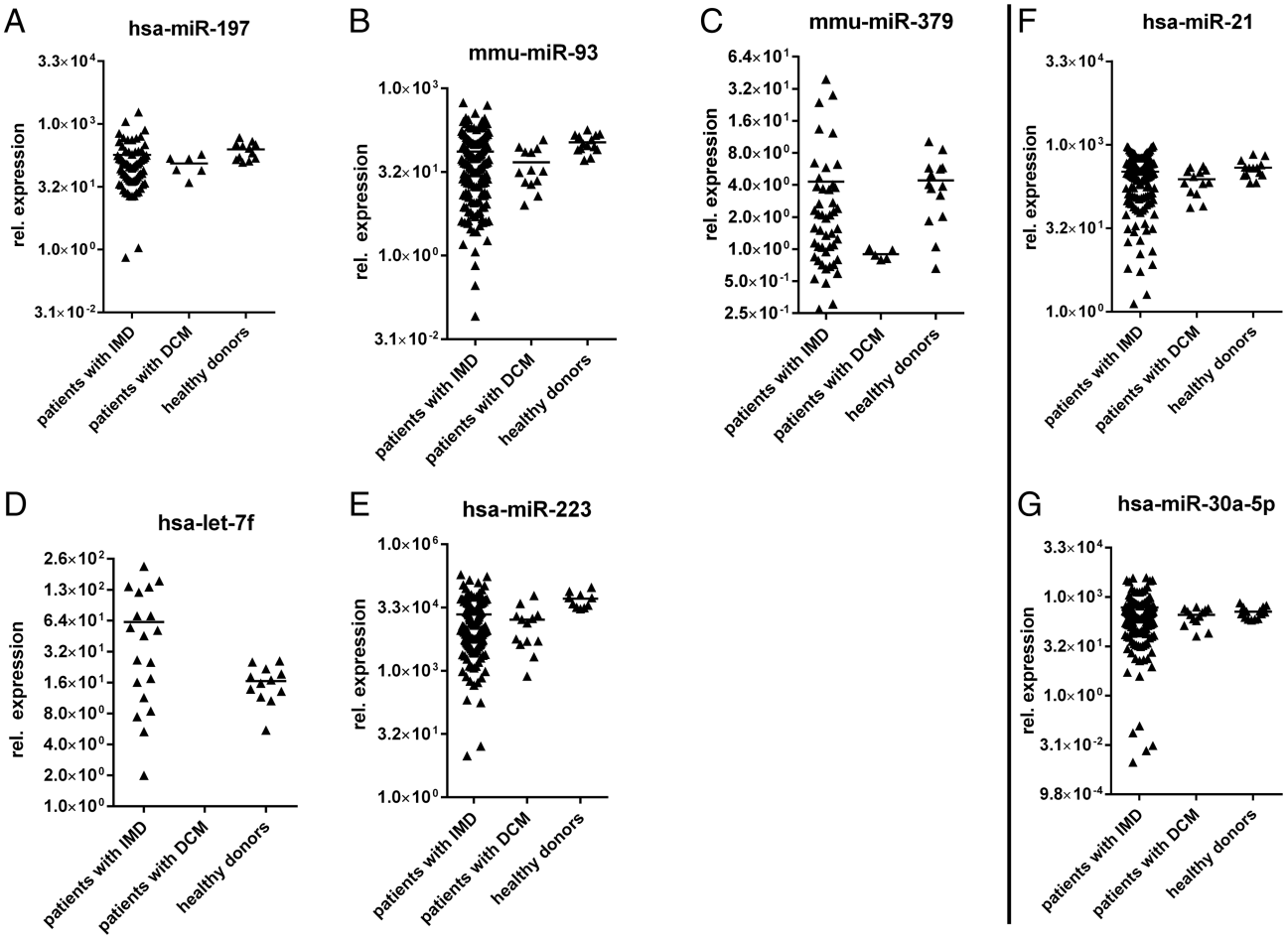
Table 3 Number of screened samples for microRNA analysis sorted by diagnosis

		Number of serum samples				
Diagnosis by endomyocardial biopsy		With inflammation	Without inflammation	With virus	Without virus	Total
Inflammatory myocardial disease patients	Myocarditis	29	0	5	24	29
	DCM with inflammation (DCMi)	22	0	0	22	22
	Borderline myocarditis	8	0	4	4	8
Virally and/or inflammatory myocardial disease patients	Adenovirus (ADV)	2	7	9	0	9
	Enterovirus (Coxsackie virus)	21	60	81	0	81
	Human herpesvirus 6 (HHV6)	8	4	12	0	12
	Parvovirus B19 (B19V)	10	13	23	0	23
Total						184
Dilated cardiomyopathy (DCM) patients		0	25	0	25	25
Healthy blood donors		0	25	0	25	25

Table 4 Number of validated samples for microRNA analysis sorted by diagnosis

Diagnosis by endomyocardial biopsy		Number of serum samples				
		With inflammation	Without inflammation	With virus	Without virus	Total
Inflammatory myocardial disease patients	Myocarditis	35	0	0	35	35
	DCM with inflammation (DCMi)	32	0	0	32	32
	Borderline myocarditis	8	7	0	15	15
Virally and/or inflammatory myocardial disease patients	Adenovirus (ADV)	2	16	18	0	18
	Enterovirus (Coxsackie virus)	3	21	24	0	24
	Human herpesvirus 6 (HHV6)	2	11	13	0	13
	Parvovirus B19 (B19V)	2	20	22	0	22
						159
Total						
Dilated cardiomyopathy (DCM) patients		0	46	0	46	46
Healthy blood donors		0	60	0	60	60

FIGURE 1 Normalization of data using geometrical mean of all measured microRNAs (miRNAs). (A–E) MiRNAs of patients with inflammatory and/or viral cardiomyopathies (IMD); (F, G) miRNAs of dilated cardiomyopathy (DCM) patients. IMD, inflammatory myocardial disease.



Screening of samples for microRNA profile identification

MicroRNAs from serum samples of 184 patients with biopsy-proven inflammatory and/or virally induced heart muscle diseases, 25 patients with DCM, and 25 healthy subjects (in.vent Diagnostica GmbH, Hennigsdorf) were measured using TaqMan® OpenArray® (*Table 3*). This allowed us to measure the expression of 754 unique circulating miRNAs (Human MicroRNA Panels A and B; https://www.thermofisher.com/order/catalog/product/4470187#/4470187) in each sample to find out the miRNAs of interest.

Validation experiments

After identification of miRNA profile consisting of eight miRNAs with TaqMan® OpenArray®, the results have been confirmed in single assays of qRT–PCR in serum of another 159 patients with biopsy-proven inflammatory and/or virally induced diseases, 46 DCM patients, and 60 healthy donors (*Table 4*).

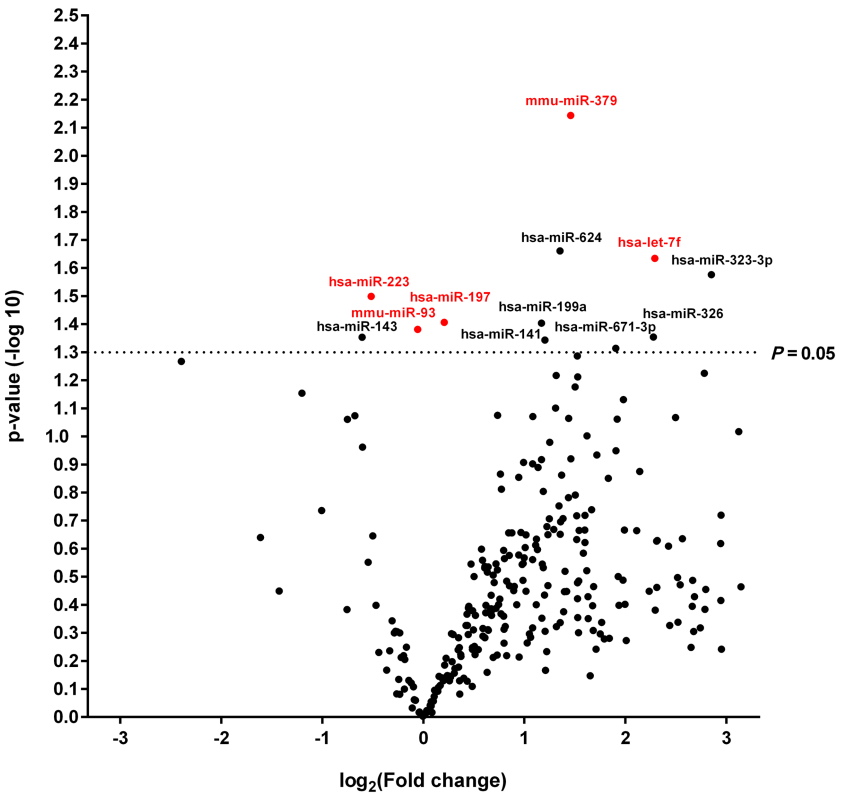
Ethical approval

The study was performed within the CRC Transregio 19 and was approved by the local ethics committees of the participating clinical centres as well as by the committees of the respective federal states. The study complies with the Declaration of Helsinki. An informed written consent was obtained from each study patient.

Statistical analysis

To normalize the data, we first performed the normalizationo using geometrical mean of all measured

FIGURE 2 Volcano plot: patients with inflammatory and/or virally induced myocardial disease (including dilated cardiomyopathy) vs. healthy donors. Comparisons of expression of 323 microRNAs (miRNAs) assessed in OpenArray analysis isolated from serum of patients with inflammatory and/or virally induced myocardial diseases (n = 184) or dilated cardiomyopathy (n = 25) and healthy controls (n = 25). The volcano plot displays the relationship between fold change and significance between the two groups, applying a Student's t-test. The y-axis depicts the negative log 10 of P-values of the t-tests (the horizontal slider at 1.3 corresponds to a P-value of 0.05; a higher value indicates greater significance), and the x-axis is the difference in expression between the two experimental groups as log2 fold. Highlighted are five abundant miRNAs (let-7f, miR-197, miR-223, miR-379, and miR-93) significantly expressed in all study groups (*Tables 3* and *5*).



miRNAs.[26] However, we have not detected any significant differences between the groups by any expressed miRNA (*Figure 1*).

Table 5 Significantly deregulated microRNAs in serum samples of patients with inflammatory and/or virally induced myocardial disease detected by the OpenArray® screening

MiRNA	Fold change	*P*-value (−log 10)
mmu-miR-379	2.75	2.14
hsa-miR-624	2.55	1.66
hsa-let-7f	4.90	1.63
hsa-miR-323-3p	7.22	1.58
hsa-miR-223	0.70	1.50
hsa-miR-197	1.15	1.41
hsa-miR-199a	2.25	1.40
mmu-miR-93	0.96	1.38
hsa-miR-326	4.84	1.35
hsa-miR-143	0.66	1.35
hsa-miR-141	2.30	1.34
hsa-miR-671-3p	3.74	1.31

MiRNA, microRNA.

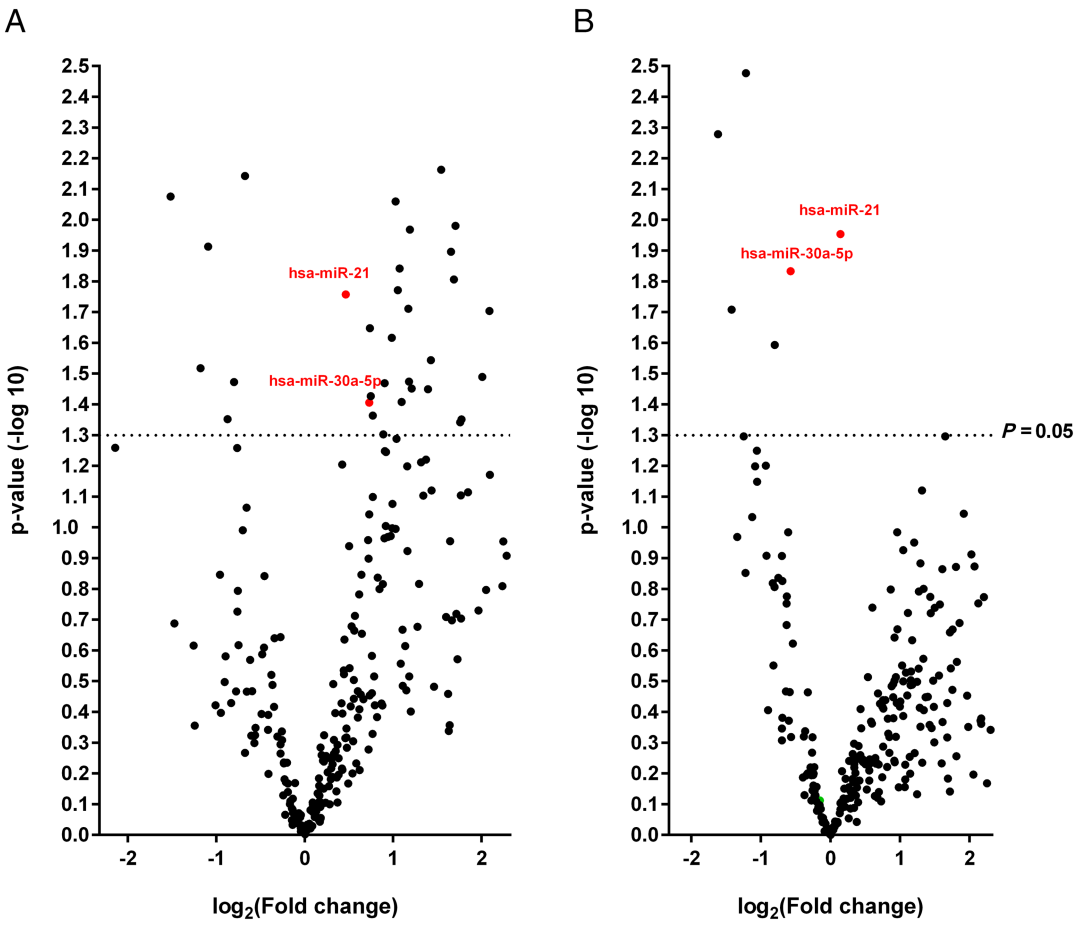
Thus, it has been detected that *Ct* values of U6 (*Ct* = 16.86 ± 2.75) and hsa-30a-3p (*Ct* = 23.83 ± 1.17) have not changed in all study groups, so hsa-30a-3p has been used as reference miRNA for data normalization.[27,28]

All log-transformed expression data were analysed and represented with GraphPad Prism 7 (GraphPad Software, La Jolla, USA) log-transformed to create a normal distribution. The Student's *t*-test and one-way ANOVA were used to compare miRNA expression levels between all study groups. Multivariate regression analysis has been used to exclude the influence of gender, age, and ejection fraction (EF) on the expression levels of each miRNA.

The receiver operating characteristic curves were plotted for every single miRNA, and the areas under the curve were calculated to prove their value and diagnostic accuracy.

All data were presented as single values with mean, with a significance level of $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, $****P < 0.0001$.

FIGURE 3 Volcano plot: patients with dilated cardiomyopathy (DCM) vs. healthy donors (A) and vs. patients with inflammatory and/or virally induced myocardial disease (B). Comparison of 323 microRNAs (miRNAs) assessed in OpenArray analysis isolated from serum of DCM patients (n = 25), patients with inflammatory and/or virally induced (n = 184) myocardial disease, and controls (n = 25). Highlighted in green are two abundant miRNAs (miR-21 and miR-30a-5p) significantly expressed in DCM group (*Tables 3* and *6*).



Results

Serum samples were obtained from patients with biopsy-proven inflammatory and/or virally induced cardiomyopathies (n = 343), DCM (n = 71), and corresponding healthy controls (n = 85) (*Tables 1* and *2*). All data were generated in the same laboratory to facilitate comparative data analysis.

Identification of microRNAs differentially expressed in patients with unexplained heart failure using TaqMan® OpenArray® analysis

First, we performed miRNA expression studies (screening) with TaqMan® OpenArray® (*Table 3*). Missing miRNA expression data were excluded from the study.[29] Based on the expression levels of 323 differently expressed miRNAs measured in duplicate, only 12 miRNAs (*Figure 2* and *Table 5*) were significantly deregulated [P (−log 10) $>$ 1.3] in all groups of patients with heart diseases (n = 184) including DCM (n = 25) compared with healthy donors (n = 25) (*Table 3* and *Figure 2*).

As indicated in *Figure 3* and *Table 6*, only two miRNAs (has-miR-21 and has-miR-30a-5p) allowed us to distinguish DCM patients from all other study groups.

Confirmation of differentially expressed microRNAs using qRT–PCR

To confirm the significant changes in miRNA expression detected by TaqMan® OpenArray®, 15 miRNAs (12 miRNAs from *Table 5*, 2 miRNAs from *Table 6*, and hsa-miR-30a-3p) were assessed by qRT–PCR using independent sets of another patients with inflammatory and/or virally induced cardiomyopathies (n = 159), DCM (n = 46), and control healthy subjects

Table 6 Significantly deregulated microRNAs in serum samples of dilated cardiomyopathy patients detected by the OpenArray® screening compared with healthy donors (left side) and patients with inflammatory and/or virally induced cardiomyopathies (right side)

MiRNA	Fold change	P-value (−log 10)	MiRNA	Fold change	P-value (−log 10)
hsa-miR-101	3.22	1.81	hsa-let-7f	0.33	2.28
hsa-miR-106b	2.31	2.73	hsa-miR-195	0.41	2.94
HSA-MIR-1180	3.38	1.34	hsa-miR-21	1.10	1.95
hsa-miR-125b	0.57	1.47	hsa-miR-30a-5p	0.67	1.83
hsa-miR-130a	2.26	1.47	hsa-miR-422a	0.37	1.71
hsa-miR-130b	1.87	1.47	hsa-miR-590-5p	0.43	2.48
hsa-miR-132	1.66	1.65			
hsa-miR-140-3p	3.26	1.98			
hsa-miR-141	2.75	2.60			
hsa-miR-146b-3p	4.25	1.70			
hsa-miR-155	2.69	1.54			
hsa-miR-185	1.98	1.62			
hsa-miR-18a	1.97	2.81			
hsa-miR-21	1.38	1.76			
hsa-miR-223	0.29	2.51			
hsa-miR-23a	2.25	1.71			
hsa-miR-296	2.10	1.84			
hsa-miR-30a-5p	1.66	1.41			
hsa-miR-30d	2.04	2.06			
hsa-miR-320	4.13	5.62			
HSA-MIR-320B	7.64	4.01			
hsa-miR-323-3p	5.69	1.70			
hsa-miR-335	2.14	1.41			
hsa-miR-363	2.31	1.45			
hsa-miR-365	0.47	1.91			
hsa-miR-455	4.02	1.49			
hsa-miR-486-3p	10.16	1.86			
hsa-miR-502	3.42	4.30			
hsa-miR-502-3p	3.42	1.35			
hsa-miR-574-3p	0.63	2.14			
hsa-miR-579	2.92	2.16			
hsa-miR-624	2.28	1.97			
hsa-miR-885-5p	0.35	2.08			
hsa-miR-92a	2.07	1.77			
hsa-miR-942	1.68	1.43			
hsa-miR-99a	0.44	1.52			
mmu-miR-134	2.63	1.45			
mmu-miR-140	0.55	1.35			

Only two microRNAs (miRNAs) (hsa-miR-21 and hsa-miR-30a-5p) have been significantly different in dilated cardiomyopathy patients compared with healthy or inflammatory and/or virally induced subjects.

(n = 60) (*Table 4*). Only five miRNAs (let-7f, miR-197, miR-223, miR-379, and miR-93) showed statistically significant deregulation in patients with inflammatory and/or virally induced cardiomyopathies, and two miRNAs (miR-21 and miR-30a-5p) were significantly deregulated in patients with DCM compared with healthy controls and patients with inflammatory and/or virally induced cardiomyopathies (*Figure 4*).

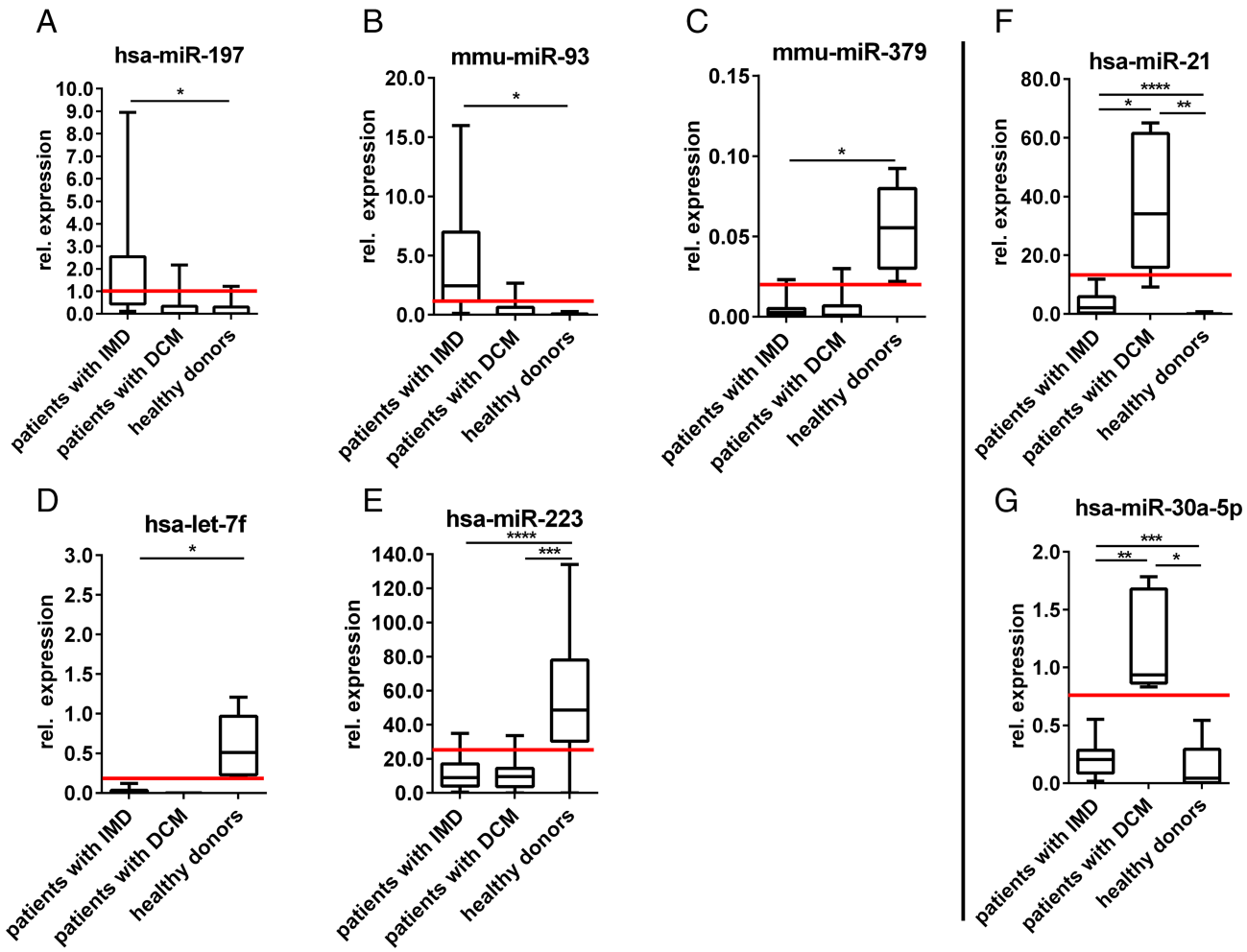
Expression level

As the achieved results have to be used further in the diagnostic, it was necessary to set the expression level for each miRNA in three study groups (*Table 7*). Given their significantly different levels in patient serum, these seven miRNAs (referred to hsa-miR-30a-3p) may have potential as diagnostic biomarkers (*Figure 4*).

Diagnostic value of microRNAs for dilated cardiomyopathy and cardiac diseases

To discriminate between the patients with inflammatory and/or virally induced cardiomyopathies, DCM patients, and healthy controls (diagnostic value), the receiver operating characteristic curves were plotted for every single miRNA in confirmation of qRT–PCR that was expressed at a significantly higher level in patient serum (*Figure 5*). Despite the overlaps in the expression levels (*Figure 4*), the areas under the curve ranged from 0.904 to 1 in all of miRNAs, proving that these

FIGURE 4 Confirmation of differentially expressed microRNAs (miRNAs) in patients with inflammatory myocardial disease (IMD), dilated cardiomyopathy (DCM), and healthy subjects using qRT–PCR. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$. According to single measurements of relative expression referred to hsa-miR-30a-3p of each patient, it was possible to plot expression level limits for each miRNA (red lines). These boundaries can be used further in the diagnostics to distinguish between healthy donors and patients with IMD (A–E) as between healthy donors, patients with IMD, and patients with DCM (F, G).



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circulating miRNAs are of particular interest for cardiac disease, DCM detection, and diagnosis.

Influence of ejection fraction, gender, and age on the microRNA expression

We evaluate the role of miRNAs in the classification of patients with inflammatory myocardial diseases and DCM.

The relationship between the expression levels of circulating miRNAs and cardiac function, gender, and age was further studied. In seven selected miRNAs, we have not detected any significant dependence of EF (*Figure 6*), gender (*Figure 7*), and age (*Figure 8*) on the miRNA expression level.

Discussion

In the present study, we identified for the first time a miRNA profile from patient's serum samples, which matches the criteria for different inflammatory and/or virally induced cardiomyopathies. The expression of let-7f, miR-197, miR-223, miR-93, and miR-379 allowed us to differentiate between patients with a virus or/and inflammation and healthy donors with average specificity of about 93%, while based on the expression of miR-21 and miR-30a-5p, we could distinguish between patients with DCM, inflammatory, and/or virally induced cardiomyopathies and healthy controls with specificity of 95%. This new approach is of high clinical relevance to clarify the indication of taking EMB from patients with suspected inflammation and/or viral heart muscle disease

Table 7 Relative expression level limits in three study groups based on *Figure 3*

	Relative expression levels						
Diagnosis	hsa-let7f	hsa-miR-197	mmu-miR-93	mmu-miR-379	hsa-miR-223	hsa-miR-21	hsa-miR-30a-5p
Patients with inflammatory/virally induced cardiomyopathies	0–0.2	>1	1–10	>0.005	0–30	0–12	0–7
Patients with DCM	0–0.2	0–1	0–3	0–0.005	0–20	>12	>7
Healthy donors	>0.2	0–1	0–1	0–0.005	>30	0–1	0–1

DCM, dilated cardiomyopathy.
The relative expression of each microRNA is referred to hsa-miR-30a-3p used as housekeeping microRNA.

FIGURE 5 Diagnostic value of microRNAs (miRNAs) for dilated cardiomyopathy and unexplained heart failure presented in receiver operating characteristic curves with area under the curve (AUC) compared with each other. As receiver operating characteristic curve illustrates the diagnostic ability of a binary classifier system as its discrimination threshold is varied, it is created by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity). AUC is equal to the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. (A–E) Heart failure patients against healthy donors; (F, G) dilated cardiomyopathy patients against the rest.

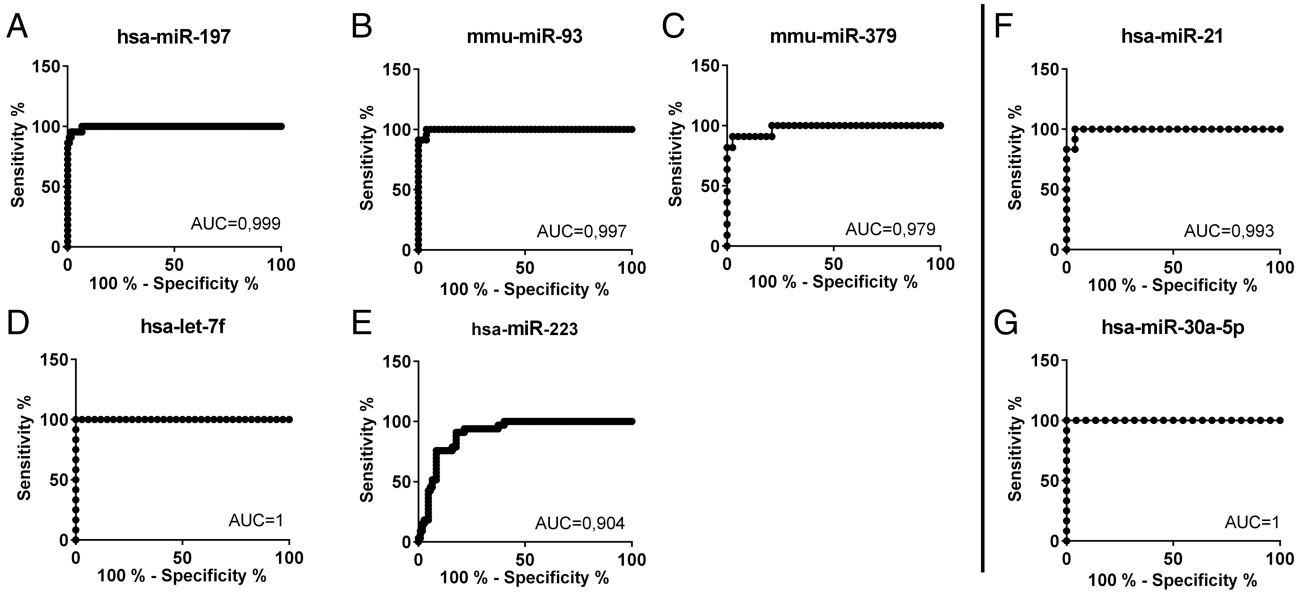
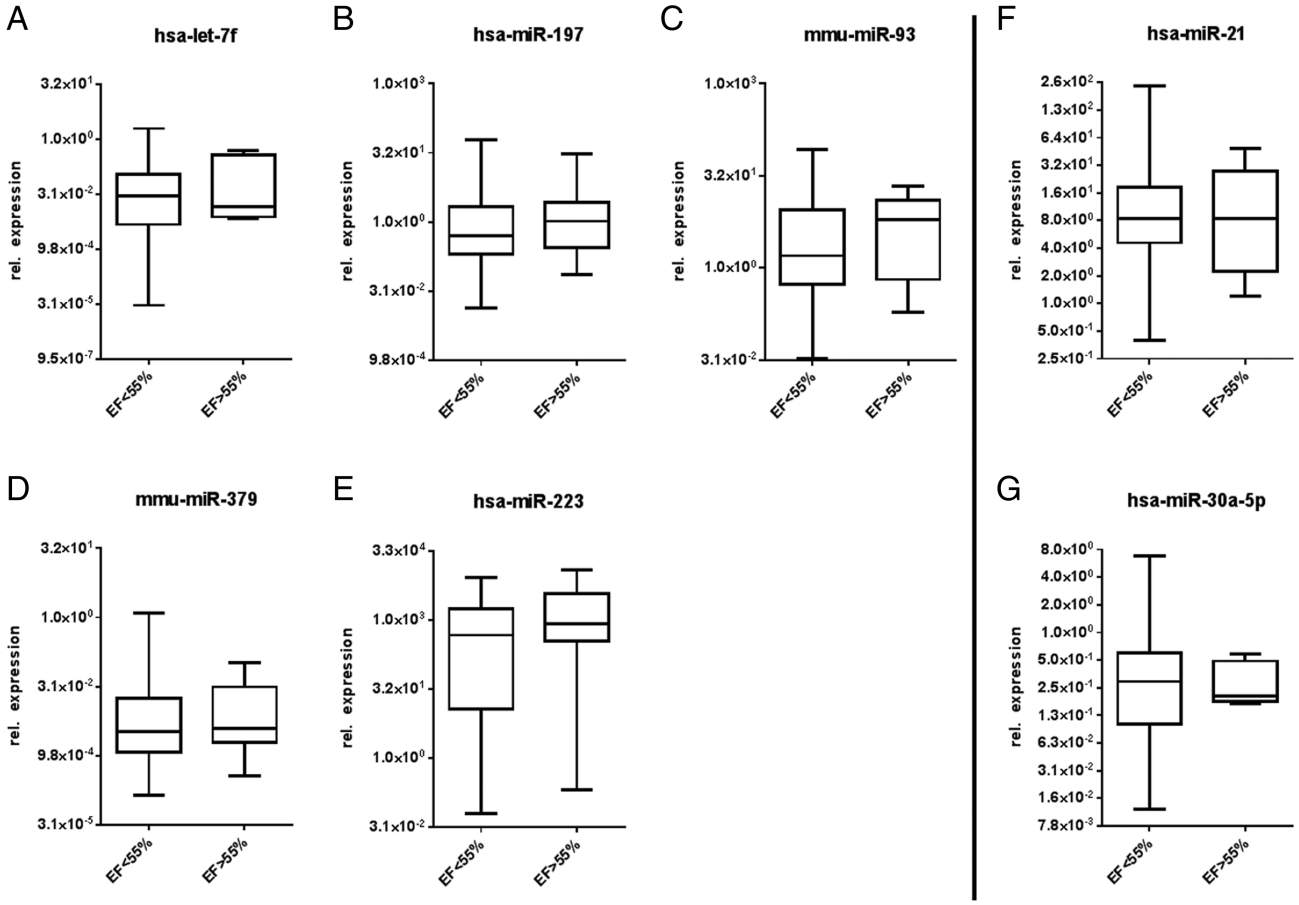


FIGURE 6 Influence of ejection fraction (EF) (<55%, >55%) on the miRNA expression. (A–E) MiRNAs of patients with inflammatory and/or viral cardiomyopathies; (F, G) miRNAs of DCM patients.



for identification of causative factors (inflammation and/or virus), getting a clear diagnosis and biopsy-proven causal treatment. The main message of this study is that accurate molecular signatures in serum essentially improve the clinical detection of patients with inflammatory/viral diseases as a prerequisite for the targeted diagnostics by taking EMBs, which are the gold standard for correct diagnosis.

Moreover, in this study, we showed that EF, gender, and age have no significant influence on the expression level of miRNAs in this setting, meaning that the miRNA profile can identify patients with inflammatory and/or virally induced cardiomyopathies by the expression level of particular miRNAs regardless of other factors.

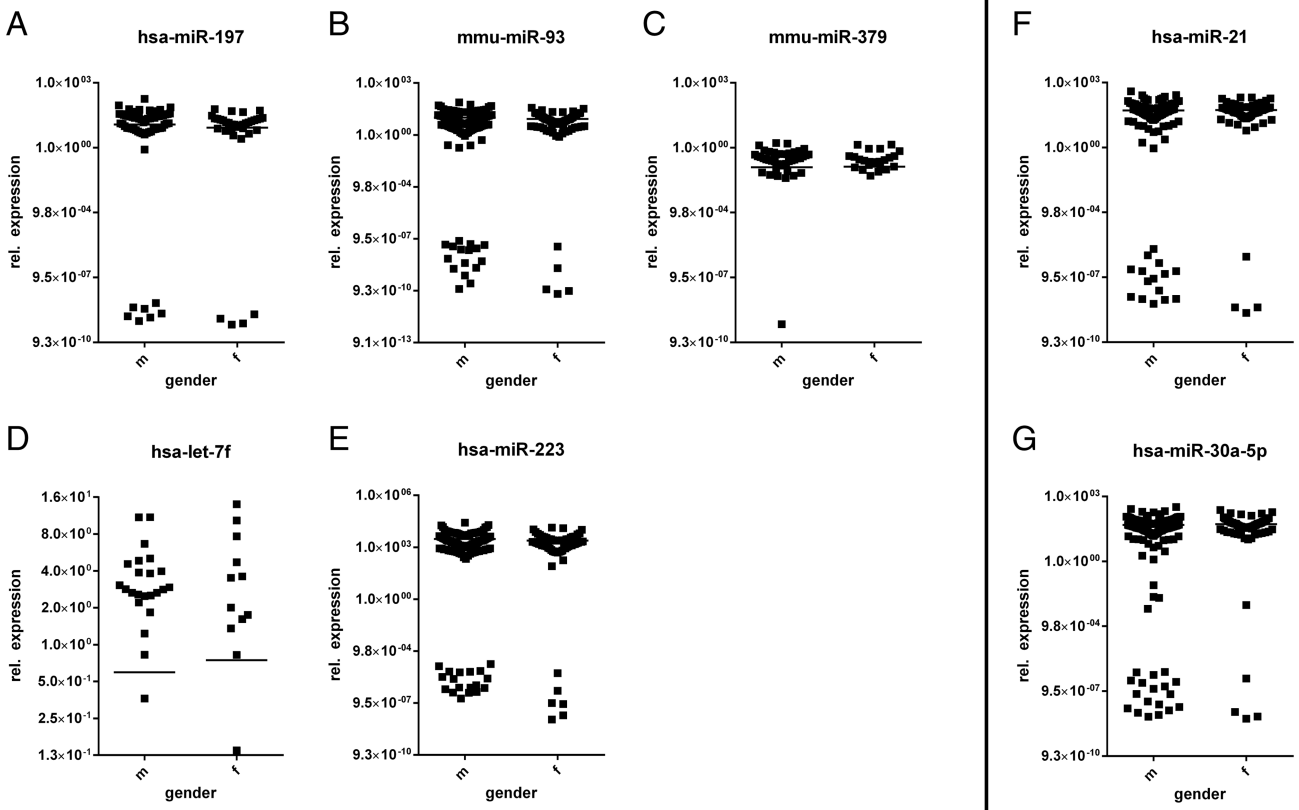
Investigations of other microRNAs in cardiovascular diseases

As miRNAs are highly investigated, their dysregulation has been widely reported in cancer and different cardiac and vascular diseases.[30,31] However, all investigations have been performed on the research level so far. MiR-208 production was detected exclusively in the heart being a candidate biomarker of myocardial injury.[32] Plasma concentrations of miR-208 increased significantly ($P < 0.0001$) after isoproterenol-induced myocardial injury and showed a similar time course to the concentration of cTnI, a classic biomarker of myocardial injury.[32]

MicroRNA-155 plays a crucial role in haematopoietic lineage differentiation, immunity, inflammation, viral infections, and vascular remodelling, which is linked to cardiovascular diseases such as CAD, abdominal aortic aneurysm, HF, and diabetic heart disease.[33] The results, however, are not consistent.

Declining levels of circulating miRNAs, including miR-18a, miR-27a, miR-30e, miR-26b, miR-199a, miR-106a, and miR-652, are found in patients with HF. Reductions in circulating miRNAs let-7i, miR-18b, miR-18a, miR-223, miR-301a, miR-652, and miR-423 have been reported within 48 h after acute HF admission and are associated with an increased risk of 180 days of mortality. MiR-21 is up-regulated and miR-1 is down-regulated in patients with symptomatic HF.[34]

FIGURE 7 Influence of gender on the microRNA (miRNA) expression. (A–E) MiRNAs of patients with inflammatory and/or viral cardiomyopathies; (F, G) miRNAs of dilated cardiomyopathy patients.



MicroRNAs profile in cardiovascular diseases

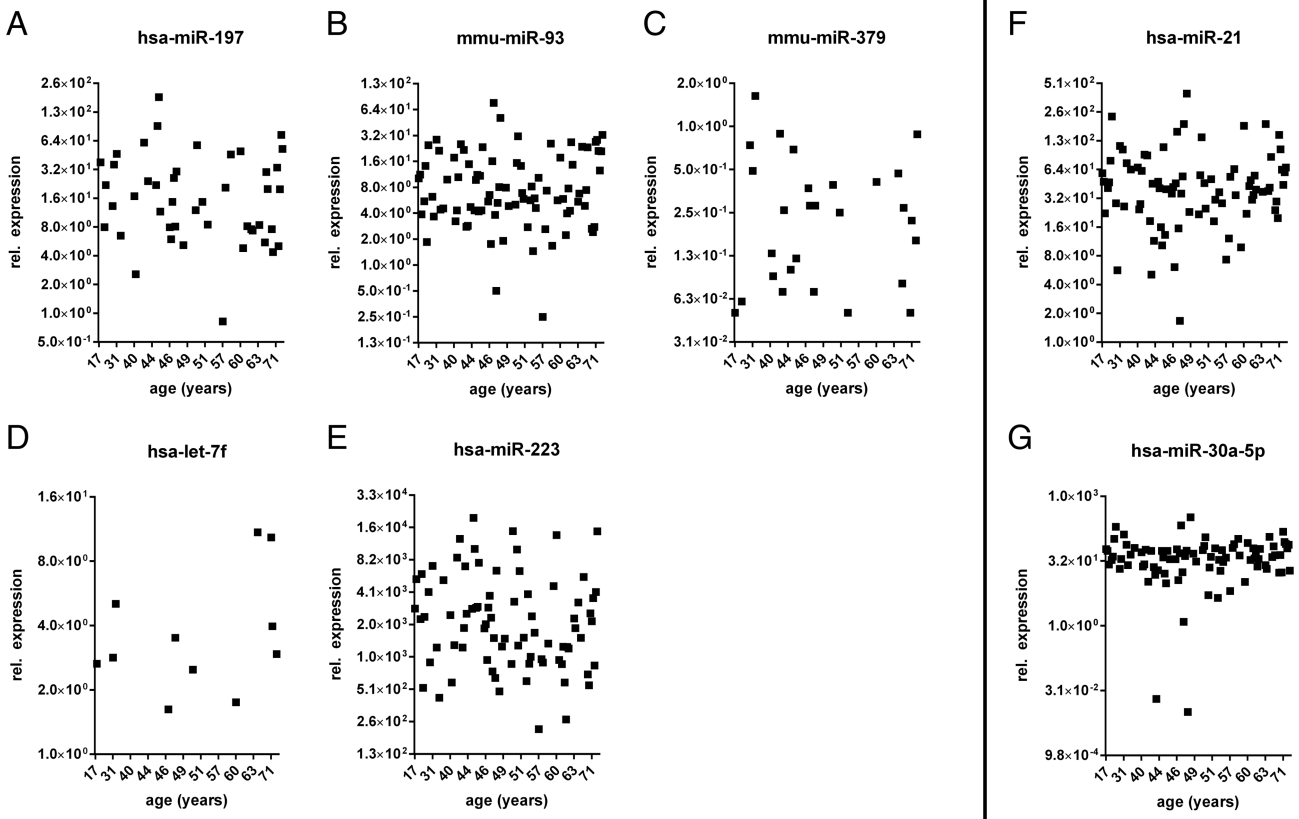
Let-7 was subsequently found as the first known human miRNA. The human let-7 family of miRNAs contains now 12 members that become the most studied miRNAs in development, stem cell biology, aging, and metabolism.[35] The aberrant expression of let-7 members has been revealed in cardiovascular diseases, such as heart hypertrophy, cardiac fibrosis, DCM, myocardial infarction, arrhythmia, angiogenesis, atherosclerosis, and hypertension.[36] The circulating let-7b is suspected to be the biomarker of acute myocardial infarction and let-7i, the biomarker of DCM,[36] and, as stated during our investigations, let-7f is suspected to be the biomarker of inflammatory and/or virally induced heart diseases.

MiR-197 represents a molecule that is associated with a wide range of pathologic conditions—from non-neoplastic diseases (e.g. myocardial infarction) to major human malignancies (e.g. cancers).[37] The dysregulation of miR-197 has been already reported in human heart disease[38] that confirms our results achieved in patients with unexplained HF.

MiR-223 expression is a biomarker and therapeutic target in cancer and inflammation.[39] MiR-223 is deregulated in damaged smooth, skeletal, and cardiac muscles. Many studies have demonstrated a possible role of miR-223 in vascular smooth muscle cells and in cardiomyocytes: miR-223 is proposed to play a protective and anti-inflammatory role, and its expression is dysregulated in cardiovascular diseases such as vascular calcification, acute myocardial infarction, and diabetic and non-diabetic HF.[40] Thus, from our experiments, we can confirm a crucial role of miR-223 in patients with unexplained HF.

MiR-197 and miR-223 predict cardiovascular death in patients with symptomatic CAD[41] and are involved in endovascular inflammation and platelet activation and have been described as biomarkers in the diagnosis of CAD.[42]

FIGURE 8 Influence of age on the microRNA (miRNA) expression. (A–E) MiRNAs of patients with inflammatory and/or viral cardiomyopathies; (F, G) miRNAs of dilated cardiomyopathy patients.



MiR-93 is elevated in the blood of CAD patients.[43] Similar to human CAD, miR-93 is elevated in both ventricle tissue and blood in mice and is secreted from cardiomyocytes cultured under hypoxia. Interestingly, miR-93 inhibits apoptosis and protects cardiomyocytes from ischaemia/reperfusion injury. In another type of ischaemic disease like stoke and peripheral arterial disease, miR-93 shows long-term protective effects via enhancing angiogenesis.[43] In our research, we confirmed the influence of pathological cardiac conditions on the miRNA expression.

MiR-379 may be a novel biomarker for the diagnosis of acute myocardial infarction, as plasma miR-379 level was significantly decreased in patients with acute myocardial infarction compared with healthy donors[44] and increased in patients with unexplained HF that has been detected during our experiments.

MiR-21 is one of the most intensively studied miRNAs in recent years.[45] Due to the critical functions of its target proteins in various signalling pathways, miR-21 has become an attractive target for genetic and pharmacological modulation in various disease conditions. MiR-21 has been found to be up-regulated in many pathological conditions including cancer and cardiovascular diseases[46] that has been confirmed in our experiments by DCM patients.

MiR-30a-5p was significantly elevated on admission in those patients who developed left ventricular dysfunction and HF symptoms 6 months after acute myocardial infarction: a bioinformatics analysis indicated that miR-30a-5p may regulate genes involved in cardiovascular pathogenesis[47] and up-regulated in patients with DCM as stated by our experiments.

We think that miRNA profile consisted of let-7f, miR-197, miR-223, miR-93, miR-379, miR-21, and miR-30a-5p normalized by miR-30a-3p has great clinical potential in the future. These accurate molecular signatures in serum essentially improve the clinical detection of patients with inflammatory diseases of the heart.

Study limitations

Further in-depth studies have to be conducted to demonstrate the relevance of miRNA profiling in clinical routine practice and to investigate the more value of differential miRNAs.

It has to be mentioned that results might vary across the source material (i.e. serum, plasma, and tissue), the disease process including other forms of cardiomyopathies being investigated, and platforms (PCR devices, software, and thresholds). There is a huge number of factors that may alter the miRNAs level between cohorts and studies including methodology and therapy of the patients, requiring careful and critical evaluation when interpreting findings and comparing result from different groups.

Conclusions

Application of miRNA profiling in blood samples is a clinical option to early identify patients who have unexplained HF based on virus positive/negative inflammatory myocardial disease. This blood-based miRNA screening will reduce the disproportion of underdiagnosed cardiac patients by strong indication for an EMB to diagnose the origin of heart muscle disease.

This is the first study investigating the miRNA expression based only on EMB diagnostics.

Clinical perspectives

This study provides miRNA expression profiling in patient serum in which we could find a distinct pattern of differentially expressed miRNAs for identification of different inflammatory and/or virally induced heart muscle diseases. The analysis of miRNA expression profile is of high clinical relevance to clarify the indication for EMB in unexplained HF to get a reliable diagnosis and improve the prognosis by direct therapeutic implications for the patients. Thus, according to our data, the cost-effective expression analysis of miRNA profile in the serum at the time of diagnosis can be used as a novel molecular tool that will contribute to a more optimized clinical management for patients with unexplained HF. However, it has to be mentioned that it is not replacing EMB, as EMB is the only diagnostic tool for establishing aetiological diagnosis (viral or immune-mediated) in myocarditis and DCMi.[3] An incomplete diagnosis may provide an incomplete picture of the disease, leading to misinterpretation and possibly incorrect treatment decisions. MiRNA profile just determines which patient requires an EMB, as a prerequisite for an aetiology-driven diagnosis and specific, causal, personalized therapy.

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Conflict of interest

None declared.

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