Altered Expression in Patients with Heart Failure of Circulating MicroRNAs Related to Lipoprotein Metabolism

Running Head: Circulating miRNAs in Heart Failure

Priscila O. Carvalho, Fatima R. Freitas, Ana Elisa M. Martinelli, Bruna M. O. Silva, Milena N. C. Curiati, Roberto Kalil-Filho, Antonio C. Pereira Barretto and Raul C. Maranhão

1. Lipid Metabolism Laboratory, Heart Institute (InCor), Medical School Hospital, University of São Paulo, São Paulo 05403-000, Brazil
2. Santa Marcelina Hospital, São Paulo 08270-070, Brazil
3. Prevention and Rehabilitation Service, Heart Institute (InCor), Medical School Hospital, University of São Paulo, São Paulo 05403-000, Brazil
4. Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo 05508-000, Brazil

Abstract: Objective: This study aimed to investigate, for the first time, the expression of circulating miRNAs (microRNAs) related to lipoprotein metabolism in patients with HF (heart failure). Methods: Twenty patients with HF and 10 controls without HF were included. BNP (brain natriuretic peptide), a marker of HF severity, plasma lipid parameters and the expression of circulating miRNAs were determined. Key findings: Total, LDL-, non-HDL- and HDL-cholesterol, triglycerides, and apo A-I did not differ between both groups, but apo B was lower in the HF group compared to controls ($p = 0.007$). In respect to miRNAs, miR-33a, miR-144, miR-125, miR-30c, miR-122, miR-26a, miR-185, miR-758 and miR-106b were higher, from ten- to 25-fold, and miR-10b was lower about 4-fold, in HF group compared to controls. In HF patients a negative correlation between miR-26a and BNP, the marker of disease severity, was found ($r = -0.552; p = 0.041$). Conclusions: Plasma levels of miRNAs involved in HDL and LDL metabolism regulation were strikingly changed in HF patients. The negative correlation between miR-26a and BNP values may suggest the possibility of the rise of a novel biomarker or therapeutic target in HF.

Key words: Lipids and lipoprotein metabolism, HDL (high-density lipoprotein), LDL (low-density lipoprotein), HF, BNP; miRNAs, miR-26a.

1. Introduction

The World Health Organization estimated that there were twenty-six million subjects worldwide suffering from HF (heart failure) in 2014, with mortality rates equivalent to those of cancer [1]. The growing prevalence of HF may reflect the increase in the aged population and the improvements in the treatment of patients with acute cardiovascular diseases leading to higher prevalence of chronic cardiomyopathy and HF development in those patients [2].

The metabolism of plasma lipoproteins is altered in HF, and higher HDL-cholesterol concentrations in the plasma (HDL-C) have been associated with longer survival in HF [3, 4]. HDL has several protective functions, such as the anti-oxidative, vasodilatory, anti-inflammatory, anti-thrombotic, anti-apoptotic, and anti-infectious [5-10]. These functions can be independent of HDL-C, and it is now consensual that they should be evaluated to assess the full protective
Altered Expression in Patients with Heart Failure of Circulating MicroRNAs Related to Lipoprotein Metabolism

capacity of the HDL fraction. Recently, it has been described a novel function for HDL, as carrier of small non-coding RNAs, namely miRNAs (microRNAs) [11]. MiRNAs downregulate the gene expression by degrading mRNA (messenger RNA) or by inhibiting translation, thereby blocking protein synthesis [12, 13]. MiRNAs are present in tissue and body fluids and circulating miRNAs have been emerged as a new class of biomarkers for several diseases, including cancer, diabetes, obesity and cardiovascular diseases [14-16].

MiR-423-5p was the first circulating miRNA described as a diagnostic predictor of HF [17], followed by miR-126 [18]. However, miR-126 lacks specificity for HF, since it is also increased in chronic coronary artery disease [19] and diabetes [20]. Plasma levels of miR-21, miR-126 and miR-423-5p were reportedly increased in patients with acutely decompensated HF and again decreased upon successful clinical HF compensation [21].

A vast number of circulating miRNAs have been shown to participate in the regulation of the HDL metabolism and reverse cholesterol transport, such as miR-33, miR-758, miR-10b, miR-26, miR-106b, miR-144, miR-125, miR-185 [22]. On the other hand, miR-122 and miR-30c have been described as influential in the metabolism of LDL [23]. However, for the best of our knowledge, none of the miRNAs related with lipoprotein metabolism was explored in studies on HF. Thus, this study was aimed to investigate whether the expression of circulating miRNAs that are involved in lipoprotein metabolism and reverse cholesterol transport is changed in patients with HF.

2. Methods

2.1 Study Subjects

For this study, twenty patients with HF (age 66 ± 12 years, 11 males and 9 females) and 10 patients without HF (controls, age 61 ± 10 years, 5 males and 5 females) were selected from the Outpatient Clinic of the Heart Institute at the University of São Paulo Medical School and Santa Marcelina Hospital. Patients with HF had stable systolic left ventricular ejection fraction < 40% as determined by echocardiography. Exclusion criteria were severe hypertension (> 180/110 mmHg), renal and hepatic failure, hypothyroidism and recent surgery. Among the HF patients, etiology was malignant hypertension in 7, dilated cardiomyopathy in 5, ischemic heart disease in 5, myocarditis in 2 and idiopathic in one. Among the control patients, all ten had diagnosis of ischemic heart disease.

The investigation conforms to the principles outlined in the Declaration of Helsinki. The research protocol was approved by the Ethics Committee of the University of São Paulo Medical School (number 9194/12) and all participants signed a written informed consent.

2.2 Biochemical Assay

The blood samples were collected after 12 h fasting. Commercial enzymatic colorimetric methods were used to determine triglycerides and total cholesterol (Roche, Mannhein, GER). HDL-C was measured by the same method used for total cholesterol after apolipoprotein B-containing lipoprotein precipitation with magnesium phosphotungstate. LDL-C was determined by the Friedewald equation [24] and non-HDL-C concentration was determined by the following equation: total cholesterol minus HDL-C. Apolipoproteins A-I (apo A-I) and B (apo B) were measured by rate turbidimetry (Roche, Mannhein, GER). Brain Natriuretic Peptide (BNP) was measured by the immunoassay (ELISA) (Abcam Inc., Cambridge, UK).

2.3 Circulating MiRNAs Expression Analysis

Expression of circulating miRNAs involved in lipoprotein metabolism was performed by quantitative real-time PCR (qRT-PCR).

An aliquot of 200 µL of the plasma with EDTA was incubated with 1 mL of Qiazol (Qiagen, California,
USA) for 5 minutes at room temperature. After that, 5 uL was added into the plasma samples of a “pool” from synthetic miRNAs. This pool was composed by three known Caenorhabditis elegans miRNAs (cel-miR-39, cel-miR-54 and cel-miR-238) purchased from Sigma (Sigma Aldrich, Missouri, USA), in a final concentration of 5 fmol/uL. These cel-miRNAs were used as normalization controls to correct for technical variations in RNA recovery [14].

Total RNA extraction was performed using MiRNeasy Serum/Plasma kit (Qiagen), according to manufacturer’s instructions. The oligonucleotides sequences and I.D. number of miRNAs selected for this study were described in Table 1.

RT (reverse transcription) reactions are performed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems by Thermo Fisher Scientific Inc., California, USA). RT products were submitted to a pre-amplification reaction, prior to the qRT-PCR step to potentially enhance sensitivity, using the TaqMan Pre-Amp Master Mix (2×) kit (Applied Biosystems), according to manufacturer’s instructions. RT pre-amplified and diluted (1:8) products were submitted to qRT-PCR in duplicate for each of the three synthetic C. elegans miRNAs across all samples in an experimental set. Reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems). A volume of 0.5 µL of each sample was added in 5 µL of TaqMan Universal PCR Master Mix II no UNG 2× and 0.5 µL of TaqMan MicroRNA Assay 20× (Applied Biosystems), and 4 µL of ultrapure water to generate a PCR product of 10 µL of a total volume. Data were analyzed with SDS Relative Quantification Software version 2.2× (Applied Biosystems), with the automatic Ct setting for assigning baseline and threshold for Ct determination.

The relative quantification was performed calculating the median of all the C. elegans synthetic miRNAs Cts, considering all the samples. For each sample, it was calculated a normalization factor by subtracting the mean C. elegans synthetic miRNAs Ct of the sample of interest from the median value obtained in previous step. The normalization factor was added to the raw Ct value obtained for each assay [25]. Finally, the equation \(2^{-\Delta\Delta C_t}\) was applied to calculate the relative expression of targets miRNAs in HF samples versus the mean of controls [26].

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Assay ID*</th>
<th>Mature miRNAs oligonucleotides sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-33a</td>
<td>002135</td>
<td>GUGCAUUGUAGUUGCAUUUGCA</td>
</tr>
<tr>
<td>hsa-miR-26a-1</td>
<td>002443</td>
<td>CCUAUUCUUGUACAUUGACAG</td>
</tr>
<tr>
<td>hsa-miR-758</td>
<td>001990</td>
<td>UUGUGUGCCUGUCCACUACC</td>
</tr>
<tr>
<td>hsa-miR-10b</td>
<td>002218</td>
<td>UACCCUGUAGAACCAGAAUUGUG</td>
</tr>
<tr>
<td>hsa-miR-106b</td>
<td>000442</td>
<td>UAGAGUGAGACAGUGACAGAUG</td>
</tr>
<tr>
<td>hsa-miR-122</td>
<td>002245</td>
<td>UGGAGUGUCAAAUGGUGUUG</td>
</tr>
<tr>
<td>hsa-miR-30c</td>
<td>000419</td>
<td>UGUAACAUCCUCACUCUCAGC</td>
</tr>
<tr>
<td>hsa-miR-185</td>
<td>002271</td>
<td>UGGAGAGAAAGCGAGUCCUGA</td>
</tr>
<tr>
<td>hsa-miR-144</td>
<td>002676</td>
<td>UACAGUAUAGAUGAUCU</td>
</tr>
<tr>
<td>hsa-miR-125a</td>
<td>002198</td>
<td>UCCUGAGACCCUUAAACUGU</td>
</tr>
<tr>
<td>cel-miR-39</td>
<td>002000</td>
<td>UCACGGGUGUAAACUGGCUUG</td>
</tr>
<tr>
<td>cel-miR-238</td>
<td>000248</td>
<td>UUGUGUCAUGGACAGUAGAUG</td>
</tr>
<tr>
<td>cel-miR-54</td>
<td>001361</td>
<td>UACCGGUAACAUCAUAACUGGAG</td>
</tr>
<tr>
<td>cel-miR-39**</td>
<td>UCACGGGUGUAAACUGGCUUG</td>
<td></td>
</tr>
<tr>
<td>cel-miR-238**</td>
<td>UUGUGUCAUGGACAGUAGAUG</td>
<td></td>
</tr>
<tr>
<td>cel-miR-54**</td>
<td>UACCGGUAACAUCAUAACUGGAG</td>
<td></td>
</tr>
</tbody>
</table>

hsa—Homo sapiens; cel—Caenorhabditis elegans. * TaqMan microRNA assay purchased from Applied Biosystems, Thermo Fisher Scientific Inc. (USA); ** purchased from Sigma-Aldrich (St. Louis, MO).
2.4 Statistical Analysis

Statistical analysis was conducted using SPSS 19.0 statistical software (SPSS® Advanced Statistics, IBM Corporation, Illinois, USA). Shapiro-Wilk normality test was applied across the data to verify the Gaussian distribution. Data were compared using the Student’s t-test for Gaussian distribution data and the Mann-Whitney U test for those with non-Gaussian distribution. In all analysis, parameters were considered significantly different when \( p < 0.05 \). Correlations between miRNAs expression and biochemical parameters were assessed using Spearman’s rho correlation test.

3. Results

3.1 BNP Levels and Plasma Lipids

Table 2 shows BNP levels and plasma lipids. BNP levels, as expected, were higher in patients with HF \( (p < 0.001) \) compared to those without the disease. Regarding the concentrations of total cholesterol, LDL-C, HDL-C, non-HDL-C and triglycerides, no differences were found between both groups. Apo B serum concentration was lower in HF patients compared to controls \( (p = 0.007) \), but Apo A-I was equal.

3.2 Expression of Circulating MiRNAs

Expression distribution of circulating miRNAs related to lipoprotein metabolism is shown in Fig. 1. The expression of all analyzed miRNAs but miR-10b was higher in the HF group than in the controls.

In respect to the miRNAs that have been associated to HDL metabolism and reverse cholesterol transport, the expressions of miR-33a, miR-144, miR-125, miR-26a, miR-185, miR-758 and miR-106b were remarkably higher, from ten- to 25-fold in the plasma of HF patients than in the plasma of controls. In contrast, the expression of miR-10b, also related with HDL metabolism, was about 4-fold lower in HF than in controls. As to the miRNAs described as related with LDL metabolism, miR-122 and miR-30c expressions were markedly higher than those of controls, both by eleven-fold (Fig. 1).

3.3 Correlation Analyses

In the correlation analysis performed with the data of the HF patients, in which data of all analyzed miRNAs were plotted against the biochemical parameters, the only significant finding was a negative correlation between miR-26a and BNP values \( (r = -0.552; p = 0.041) \). Triglycerides, total cholesterol, HDL-C, LDL-C, non-HDL-C, apo A-I and apo B values

<table>
<thead>
<tr>
<th>Table 2</th>
<th>BNP levels and plasma lipids of the group of patients with heart failure (HF) and of the controls without HF.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls ( (n = 10) )</td>
</tr>
<tr>
<td>BNP (pg/mL)</td>
<td>54 ± 23</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>168 ± 64</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>218 ± 59</td>
</tr>
<tr>
<td>non-HDL</td>
<td>186 ± 65</td>
</tr>
<tr>
<td>LDL</td>
<td>153 ± 63</td>
</tr>
<tr>
<td>HDL</td>
<td>32 ± 14</td>
</tr>
<tr>
<td>Apolipoproteins (mg/dL)</td>
<td></td>
</tr>
<tr>
<td>A-I</td>
<td>142 ± 17</td>
</tr>
<tr>
<td>B</td>
<td>129 ± 38</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. BNP: brain natriuretic peptide; LDL: low-density lipoprotein; HDL: high-density lipoprotein.
Altered Expression in Patients with Heart Failure of Circulating MicroRNAs Related to Lipoprotein Metabolism

Fig. 1  Box plots distribution showing the expression of 10 circulating miRNAs in HF. Circulating miRNAs were determined on plasma from 20 subjects with heart failure (HF) and 10 subjects without HF (Controls). MiRNAs were reverse-transcribed into cDNA using miRNA-specific stem-loop primers for each miRNA to be analyzed: miR-26a, miR-30c, miR-125, miR-122, miR-144, miR-33a, miR-758, miR-185, miR-106b and miR-10b. cDNAs were submitted to a pre-amplification reaction prior to the quantitative real time PCR (qRT-PCR). The qRT-PCR was performed using TaqMan detection method and the quantitative data were normalized relative to the synthetic exogenous miRNAs controls corresponding to three known Caenorhabditis elegans miRNAs: cel-miR-39, cel-miR-54 and cel-miR-238. It was calculated the median of all the C. elegans synthetic miRNAs Cts, considering all the samples. For each sample, it was calculated a normalization factor by subtracting the mean C. elegans synthetic miRNAs Ct of the sample of interest from the median value obtained in previous step. The normalization factor was added to the raw Ct value obtained for each assay. Finally, the equation \(2^{-\Delta\Delta Ct}\) was applied to calculate the relative expression of targets miRNAs in HF samples versus the mean of controls. Data were expressed as log10 values. The middle line indicates the median statistic, the bottom and top of the box show the 25th and 75th percentiles, the lower and upper whiskers show the minimum and maximum values of the data after detecting outliers (circles). The differences in expression levels between the two groups were determined using the Mann-Whitney \(U\) test.

were not significantly correlated with none of the analyzed miRNAs (data were not shown).

4. Discussion

In this study on circulating miRNAs related with lipoprotein metabolism in HF, it was remarkable that the expression of all the 10 tested miRNAs was altered manifold in the HF patients. The expression of nine of these miRNAs, namely miR-33a, miR-144, miR-125, miR-26a, miR-185, miR-758, miR-106b, miR-122 and miR-30c, was upregulated and, downregulated in miR-10b only.

The first described miRNA regarding lipoprotein metabolism was the miR-33 family that consists of miR-33a and miR-33b, encoded within the introns of the sterol regulatory element-binding proteins (SREBP) 2 and 1 genes, respectively [27-29]. SREBP genes regulate the intracellular cholesterol levels and lipid homeostasis [30]. The miR-33 family was also reported to regulate the hepatic expression of ATP-binding cassette transporter A1 (ABCA1) and plasma levels of HDL-C [22, 31, 32]. MiR-33a is more conserved across the species than miR-33b, and is by far more studied [27, 28, 31]. Indeed, there are other miRNAs that regulate ABCA1 expression, namely miR-758, miR-26, miR-27, miR-106b, miR-144, miR-145 and miR-10b [22, 32, 33]. ABCA1 is an integral cell-membrane protein that is crucial in reverse cholesterol transport, by exporting phospholipids and cholesterol from the cells to lipid-poor apolipoproteins, including apo A-I, which is a critical step for HDL biogenesis [34, 35].
The fact is that, in our HF patients, the overexpression of miR-33a, miR-144, miR-758, miR-26a and miR-106b suggests that the ABCA1 expression may be downregulated, since all these miRNAs have been associated with post-transcriptional repression of the ABCA1 proteins. Consequently, the first step of reverse cholesterol transport, which consists in the uptake of cholesterol from the cells by HDL, would be impaired in HF patients. A recent study has shown that miR-33a expression in cardiac tissue of patients with dilated cardiomyopathy was downregulated so that it was associated with worsened cardiac function [36]. Our results in which miR-33a level was found markedly elevated in the plasma of the HF patients are thus suggestive that perhaps the uptake of the circulating miR-33a by the dilated heart is diminished.

In respect of miR-10b, it was the only miRNA that had lower expression in the plasma of our HF patients compared to the controls. MiR-10b is synthetized in the intestine, differently from the other miRNA studied here, that are synthetized in the liver. In patients with chronic HF it has been reported the existence of intestinal edema and of increased bacterial population [37-39]. As the intestinal mucosa of HF patients is damaged, it can be postulated that the expression of miRNA of intestinal origin was compromised and accounts for the diminished levels of miR-10b found in the plasma of our HF patients. Inflammatory states, such as that occurring in non-alcoholic steatohepatitis patients may elicit diminished serum levels of miR-10b [40]. As a background chronic inflammatory status also occurs in HF patients, it is possible that inflammation could also account for the low expression of miR-10b, in conjunction with the high levels of tumor necrosis factor-α, interleukin 6 and interleukin 8 that reportedly occur in HF [41-43].

Regarding HDL metabolism, in the final step of the reverse cholesterol transport, the scavenger receptor class B type I (SR-BI) plays a crucial contribution shuttling the HDL cholesteryl esters to the liver for excretion in the bile. Thus, SR-BI functional deficiency causes reduction of cholesterol removal from the body and contributes to atherosclerotic plaque formation [44, 45]. The importance of SR-BI regulation by miRNAs, such as miR-125a and miR-185, has been investigated and it was shown that the overexpression of those miRNAs suppresses SR-BI protein levels and HDL-C uptake [46, 47]. Our current finding that the plasma levels of both miR-125a and miR-185 were higher in HF patients compared to controls suggests that the reverse cholesterol transport could be affected in HF patients.

In this study, we also evaluated the expression of miRNAs related to LDL metabolism, namely miR-30c and miR-122. Little is known about how miRNAs regulate LDL-C levels. MiR-122 was the first identified miRNA related to hepatic function and LDL metabolism and it is the most abundant miRNA in the hepatic tissue [48]. Antisense oligonucleotide inhibition of miR-122 hepatic expression in mice and non-human primates resulted in reduction of the LDL-C and triglyceride levels [49, 50]. However, the high expression of circulating miR-122 observed here was not accompanied by alterations in the plasma lipids of our patients.

In addition to miR-122, miR-30c was also associated to LDL metabolism by regulating MTP (microsomal triglyceride transfer protein), a crucial protein in the intracellular assembling of the apo B-containing lipoproteins. Overexpression of miR-30c lowers the plasma cholesterol and reduces lipidemia and atherogenesis in apo E (-/-) mice [51, 52]. In the current study, we found in the HF patients both high expression of circulating miR-30c and low concentration in the plasma of apo B, which should be expected from the effects of this miRNA described in the animal experiments. Interestingly, LDL-C and non-HDL cholesterol were not different, but apo B stands for number of non-HDL lipoprotein particles in the circulation, whereas cholesterol is prone to
variations in lipoprotein composition.

It is worthwhile to point out that, despite the fact that all miRNAs analyzed here showed marked differences between HF and controls, only miR-26a had a significant correlation with BNP. The participants of this study showed extreme variation of the BNP values, from 177 to 3,350 pg/mL. As BNP is the most important biochemical marker of HF severity, this implies that our patients had from the mildest to the most severe presentations of HF. The finding of the negative correlation between miR-26a and BNP values may suggest that the aggravation of HF is related with decrease of miRNA expression. This would result in stimulus for the ABCA1 function and increase in cholesterol efflux from the cells to the HDL fraction, i.e., increase in the reverse cholesterol transport.

As a limitation of the study, the number of participant patients was small and non-significant trends for lower levels of triglycerides and total cholesterol could be statistically confirmed by the inclusion of a larger number of participant subjects. In this regard, the control group was constituted of subjects without HF but with ischemic heart disease. In ischemic heart disease, risk factors such as total and triglycerides tend to be higher than in other diseases that also lead to HF. In fact, apo B, the protein label of LDL and other apo-B containing lipoproteins was significantly lower in HF than in the controls. In case the comparisons were made with healthy subjects as controls, somewhat different results could eventually be found.

5. Conclusions

In conclusion, the results of this study, which was the first to approach the issue of miRNAs related to plasma lipids in HF, show that the plasma levels of miRNAs involved in HDL and LDL metabolism regulation are profoundly changed in HF patients. Among our findings, the negative correlation between miR-26a and BNP, a well-established marker of HF severity, may bear important pathophysiological implications to be explored in further investigations.

Conflict of interest

The authors have no conflicts of interest to declare.

Funding

This study was supported by the State of São Paulo Research Support Foundation (FAPESP, São Paulo, Brazil; grant number 2014/03742-0). Dr. Raul Maranhão has a Research Carrier Award from the National Council for Scientific and Technological Development (CNPq, Brasilia, Brazil). Ana Elisa M. Martinelli had a doctorate scholarship from Coordination for the Improvement of Higher Education Personnel (CAPES, Brasilia, Brazil).

References


Altered Expression in Patients with Heart Failure of Circulating MicroRNAs Related to Lipoprotein Metabolism


