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Statins differentially modulate microRNAs expression in peripheral cells of hyperlipidemic subjects: A pilot study

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ABSTRACT

Aim: Although statins are considered a cornerstone for the treatment of high cholesterol levels due to their powerful cholesterol-lowering effects, response to drug administration is still one of the main pitfalls of statin treatment. So far, the reasons underlying this undesired outcome are still poorly understood, but recently, various studies have suggested that miRNAs may be involved. Therefore, we aimed at evaluating the effect of short-term low-dose treatment with 2 statins on miRNAs expression in patients with hypercholesterolemia.
Methods: A total of 40 hypercholesterolemic (HC) subjects following 1 month of atorvastatin (10 mg/day; n = 20) or simvastatin (10 mg/day; n = 20) were included. Multiple available boinformatic algorithms (TargetScan, miRanda, DianaLab, MicroCosm and PicTar) were employed to select miRNAs regulating genes involved in cholesterol metabolism and statin response. Differential miRNAs expression was determined in peripheral cells using the miScript® miRNA PCR Array platform. Pathways involving differentially expressed miRNAs were explored using the Ingenuity Pathway Analysis software.
Results: Atorvastatin repressed miR-29a-3p, miR-29b-3p, miR-300, miR-33a-5p, miR-33b-5p and miR-454-3p in HC subjects. On the contrary, simvastatin did not show any effect on miRNAs expression. Network analysis indicated that atorvastatin-modulated miRNAs regulate key cholesterol genes (*ABCA1*, *HMGCR*, *INSIG1*, *LDLR*, *LPL*, *SCAP* and *SREBF1*). Further subgroups analyses showed that miR-106b-5p, miR-17-3p and miR-590-5p were repressed in HC subjects within the lower quartile of atorvastatin response (lower LDL-C reduction), while the expression of miR-106b-5p, miR-17-3p and miR-183-5p was higher in the upper quartile of simvastatin response (higher LDL-C reduction) ($p < 0.05$).
Conclusion: We show that a miRNAs-mediated epigenetic mechanism is differentially affected by statins therapy in vivo, which could be implicated in the variable response to these drugs. Further studies are necessary to disclose their particular role in the cholesterol-reduction response to statins.

1. Introduction

Cholesterol homeostasis is regulated by two pathways, the sterol response element binding proteins (SREBPs) (Brown and Goldstein, 1997) and the liver X receptors (LXRs) transcription factors (Peet et al., 1998). However, microRNAs (miRNAs), a novel class of post-transcriptional regulators, have emerged as pivotal for cholesterol control. These molecules comprise a large family of short endogenous non-coding RNAs (ncRNAs) that target the 3′-untranslated region (3′-UTR) of messenger RNAs (mRNAs) through complementary base pairing with the miRNA “seed”, a region with a length of 2–8 nucleotides, controlling post-transcriptional gene expression via translational repression or mRNA degradation (Bartel, 2004). As miRNAs are known to affect > 30% of protein-coding genes, they are known to be deeply involved in almost every biological pathway, regulating processes such as cellular division and apoptosis, metabolism, intracellular signaling, immune response and cellular movement (Filipowicz et al., 2008). Multiple studies have linked these molecules to cholesterol levels regulation, but only a few miRNAs have appeared as major controllers of lipid homeostasis (Fernandez-Hernando et al., 2011; Goedeke et al., 2014; Rotllan and Fernandez-Hernando, 2012). For instance, miRNA-122 is highly expressed in the liver, representing about 70% of all hepatic miRNAs. In this tissue, silencing of miRNA-122 revealed a vital function in the regulation of lipid metabolism, shown by a decrease in plasma

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cholesterol levels in experimental animals (Krutzfeldt et al., 2005), and increased hepatic fatty acid oxidation along with decreased cholesterol and fatty acid synthesis (Esau et al., 2006). Additional key players are miRNA-33a and miRNA-33b, encoded by introns of *SREBF2* and *SREBF1* genes, respectively. Several reports have implicated these miRNAs into lipid metabolism, mainly by targeting sterol transporters (Horie et al., 2010; Marquart et al., 2010; Najafi-Shoushtari et al., 2010; Rayner et al., 2010).

Lately, it has been firmly established that low-density lipoproteins (LDL) cause atherosclerotic cardiovascular disease (ASCVD), showing a consistent dose-dependent relationship between LDL-C levels and cardiovascular risk (Ference et al., 2017). Therefore, LDL-C lowering becomes fundamental, and to date, inhibitors of 3-hydroxymethyl-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase, commonly known as statins, correspond to the most widely used medication for treating hypercholesterolemia. The extensive clinical use of these drugs is supported not only on their lipid-lowering power, but they also reduce the risk for coronary artery disease (CAD) and 20 to 30% of deaths and major cardiovascular events (LaRosa et al., 1999; Ross et al., 1999), along with several beneficial cholesterol-independent pleiotropic effects (Satoh et al., 2015). However, clinical trials have also shown large interindividual variation following statin therapy (Fox et al., 2007), which is a main concern in clinical practice, especially when important LDL-C reductions are needed. A large meta-analysis showed that several factor were associated with response variability (e.g., sex, age, smoking status, body weight, diet, and physical activity), but with a rather small contribution. Interestingly, authors conclude that an important issue contributing to statins variability is non-adherence, which could be probably the main reason underlying the wide variation in response to lipid-lowering agents (Boekholdt et al., 2014). Very recently, it was demonstrated that most of statin variability is observed when employing low-doses, e.g. atorvastatin 10 mg and simvastatin 10–20 mg, which were associated with higher rates of suboptimal responses (Karlson et al., 2016b). Also, authors showed that low baseline LDL-C and younger age were associated with a suboptimal response, and that Black and Hispanic race were all weak predictors of suboptimal response. Authors also mention the contribution of genetic polymorphisms in genes involved in the transport, uptake, and metabolism of statins, but without quantifying the magnitude of this effect. On the contrary, Postmus et al. (2014) did quantify the genetic contribution, estimating that around 5% of statin response is explained from a genetic perspective. Consistent with this issue, multiple genome-wide association studies (GWAS) have demonstrated an interesting, but modest genetic contribution to variability in response to statin medication (Barber et al., 2010; Chasman et al., 2012; Deshmukh et al., 2012; Postmus et al., 2014), hence, a better understanding is still lacking, as additional pathways could be implicated, and miRNA-mediated epigenetic modulation could represent a plausible mechanism. Consequently, we evaluated the expression profile of miRNAs in peripheral cells of subjects with elevated cholesterol levels undergoing two different short-term low-dose statins treatments.

2. Material and methods

2.1. Subjects

A total of 40 hypercholesterolemic (HC) subjects diagnosed according to the NCEP criteria (Grundy et al., 2004) were randomly selected at the University Hospital of the University of Sao Paulo (HU/USP). Patients went through a washout phase of 4 weeks, with indications of a low-fat diet, following recommendations of the American Heart Association (AHA) (Chahoud et al., 2004). After this period, subjects with LDL-C > 160 mg/dL were treated with 10 mg/day of atorvastatin (n = 20) or simvastatin (n = 20) during 1 month. Subjects with diabetes, hepatic, renal or kidney disease, endocrine disorders and malignant pathologies were excluded from the study. Additionally, patients undergoing concomitant lipid-lowering treatment or any medication known to affect lipoprotein metabolism at the time of screening e.g. cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, hydroxymethylglutaryl-coenzyme A reductase inhibitors, β-blockers, thiazide diuretics, diphenylhydantoin, cis-retinoic acid, ascorbic acid, estrogens, progestins, anabolic steroids, hydrocortisone, fish oil capsules, or thyroxin, were also excluded.

Information on age, body mass index (BMI), gender, menopause status, hypertension, obesity, tobacco/alcohol consumption, physical activity, family history of CAD and simultaneous medication was registered. The lipid profile was obtained to evaluate the response to both treatments. Creatine kinase (CK) and alanine aminotransferase (ALT) enzymes were analyzed to identify any undesired adverse effect caused by the lipid-lowering therapies. The Ethics Committee of the University Hospital of Sao Paulo (Protocol #699/06) and the School of Pharmaceutical Sciences of the University of Sao Paulo (Protocol #426/07) approved the study protocol. All subjects gave their written informed consent to participate from this investigation.

2.2. Biochemical analysis

Blood sampling was performed via direct venous puncture after a 12-h overnight fast into vacutainer tubes without anticoagulant to determine serum lipid levels and apolipoproteins AI (apoAI) and B (apoB), before and after statins treatments. For RNA extraction, two additional samples were obtained in tubes containing Ethylenediaminetetraacetic acid (EDTA) at the same times aforementioned. Total cholesterol, HDL cholesterol (HDL-C) and triglycerides (TG) were measured by routine enzymatic-colorimetric methods. LDL cholesterol (LDL-C) was calculated using the Friedewald equation, when TG levels were not above 400 mg/dL. ApoAI and apoB were determined by nephelometry, while ALT and CK activities were measured by kinetic methods.

2.3. Selection of miRNAs

Since commercial kits containing cholesterol- and statin-related miRNAs were lacking at the time of the experimental study, we first selected twenty-eight genes strongly implicated in both cholesterol homeostasis and statins response (Table S1). Then, a bioinformatics approach was used to determine the regulatory miRNAs predicted to target the 3-UTR of the 28 genes listed, using a combination of five freely available algorithms e.g. TargetScan (Friedman et al., 2009), miRanda (John et al., 2004), DianaLab (Maragkakis et al., 2009), MicroCosm (formerly miRBase) (Griffiths-Jones et al., 2008) and PicTar (Krek et al., 2005). Initially, miRNAs recognized by all bioinformatics tools were registered. Later, we narrowed down miRNAs selection to those simultaneously identified by at least three algorithms predicted to interact with the 28 candidate genes previously identified. In addition, we carried out a review of the literature to search for additional miRNAs related to cholesterol homeostasis and statin treatment genes, to finally select 84 miRNAs for downstream analyses.

2.4. RNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll-hypaque density gradient centrifugation. Afterwards, these cells were submitted to total RNA extraction with TRizol (Invitrogen-Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration and purity was assessed through the A260/280 ratio using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then, RNA quality was determined by the RNA integrity number (RIN) using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). An A260/280 ratio between 1.8 and 2.0, and RIN > 8 were considered suitable for subsequent analyses.

2.5. Reverse transcription

The miScript II RT kit (SABiosciences, Qiagen, USA) was used for the reverse transcription reaction, using total RNA that contains miRNAs as the starting material for cDNA synthesis. 5× miScriptHiSpec Buffer was used to specifically convert mature miRNAs into cDNA. The reaction mix contained 250 ng template RNA, 5× miScriptHiSpec Buffer, 10× miScriptNucleics Mix, RNase-free water and miScript Reverse Transcriptase (RT) Mix in a final volume of 20 µL. RT mix was then incubated for 60 min at 37 °C, and 5 min at 95 °C to inactivate RT and then placed on ice. The mix was diluted with 200 µL of RNase-free water and immediately submitted to real-time PCR analysis.

2.6. Expression profile of miRNAs

To determine differential miRNAs expression, we used the Custom miScript® miRNA PCR array plate (Cat. No. 331231) (SABiosciences, Qiagen, USA), which was set up including our previously selected 84 mature miRNAs, 6 endogenous controls (RNU6-2, SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A), 3 RT controls (miRTC), and 3 positive PCR controls (PPC). The PCR reaction mix (25 µL) was prepared with 2× QuantiTect SYBR Green PCR Master Mix, 10× miScript Universal Primer, RNase-free water and template cDNA. Then, the reagents were added to the Custom miScript® miRNA PCR array. The activation of HotStarTaq DNA Polymerase was performed at 95 °C for 15 min, following forty cycles at 94 °C for 15 s, 55 °C for 30s and 70 °C for 30 s, using a 7500 fast real-time PCR equipment (Life Technologies, Carlsbad, CA, USA). PCR assay specificity was verified by melting curve analysis. Baseline and threshold were set automatically and cycle threshold (Ct) values were exported to an Excel sheet (version 1997–2003), and uploaded to the web-based software (http://pcrdataanalysis.sabiosciences.com/mirna) for data analysis. Quality control data was rigorously reviewed in order to assess PCR reproducibility, RT efficiency and genomic contamination. The expression levels of all miRNAs were calculated by the ΔΔCt method using the Ct arithmetic mean of the endogenous controls for normalization.

2.7. Regulatory pathways

To establish the biological pathways of the differentially expressed miRNAs, results obtained from the miRNA profiling were submitted to Ingenuity Pathway Analysis software (IPA®, QIAGEN, Redwood City, CA, USA), for network associations and posttranscriptional targets regulation.

2.8. Statistical analysis

Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Continuous variables are described as mean ± SD and compared by *t*-test. Gaussian distribution was assessed by Shapiro-Wilk normality test ($\alpha = 0.05$). Lipid-lowering therapy response was evaluated using the LDL-C as endpoint. Lipids values before and after statins therapies were evaluated by paired *t*-test. Differences in categorical variables were compared by Chi-square test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Clinical data, plasma lipids and statin response

Table 1 displays clinical, laboratory and anthropometric data of the 40 HC subjects. Both groups were comparable, finding a difference only in the higher proportion of subjects with family history of coronary artery disease (CAD) in the simvastatin group ($p = 0.034$). Lipid levels before and after atorvastatin and simvastatin treatments are shown in Table 2. As expected, both treatments significantly improved the lipid profile in the patients. However, neither atorvastatin nor simvastatin had a significant effect on HDL-C or apoAI. Even though both drugs reduced triglycerides and VLDL-C, only simvastatin lowered these lipid fractions significantly.

Table 1
Clinical and demographic characteristics of HC patients.

Parameter	Atorvastatin (n = 20)	Simvastatin (n = 20)	p-Value
Age, years	57.4 ± 8.4	58.3 ± 8.6	0.71
Gender (women), %	59	63	0.09
Menopause, %	79	79	1.00
Family history of CAD, %	36	68	0.03
Hypertension, %	36	63	0.07
Systolic pressure, mm Hg	125.5 ± 16.8	132.1 ± 17.3	0.22
Diastolic pressure, mm Hg	81.7 ± 13.3	79.2 ± 10.3	0.49
Obesity, %	23	23	1.00
BMI, kg/m^2	28.0 ± 3.3	27.6 ± 3.7	0.71
Physical activity, %	36	59	0.13
Smoking, %	31	50	0.22
CK, U/L	137 ± 82	130 ± 57	0.75
ALT, U/L	21 ± 11	24 ± 9	0.49

Number of subjects in parenthesis. Continuous variables are presented as mean ± SD and compared by *t*-test. Categorical variables were compared by chi-square test. Obesity: IMC > 30 kg/m^2. Hypertension: systolic and diastolic pressure > 140 and 90 mm Hg, respectively, or use of antihypertensive medication. Physical activity: exercise of 2 h per week. Smoking: cigarette consumption > 2 per week. ALT: alanine aminotransferase; BMI: body mass index; CAD: coronary artery disease; CK: creatine kinase; HC: hypercholesterolemic.

Table 2
Lipid profile of HC patients treated with statins.

Parameter (mg/dL)		Atorvastatin (n = 20)	Simvastatin (n = 20)	p-Value
TC	Basal	275.5 ± 27.6	263.4 ± 26.1	0.16
	Treatment	200.4 ± 25.0	208.3 ± 26.2	0.33
	% change	-27.0 ± 8.1***	-20.4 ± 10.8***	0.03
LDL-C	Basal	182.3 ± 20,5	174.5 ± 25.2	0.29
	Treatment	116.3 ± 21.5	124.3 ± 23.7	0.27
	% change	-36.1 ± 9.8***	-28.0 ± 14.0***	0.04
HDL-C	Basal	57.9 ± 9.8	56.7 ± 13.7	0.76
	Treatment	55.1 ± 8.2	56.6 ± 15.6	0.69
	% change	−4.0 ± 9.5	−1.2 ± 19.5	0.56
VLDL-C	Basal	33.9 ± 11.1	31.5 ± 12.2	0.52
	Treatment	28.2 ± 10.9	26.3 ± 8.8	0.55
	% change	-11.4 ± 36.8	-11.0 ± 22.6**	0.97
TG	Basal	169.6 ± 56.0	157.8 ± 61.0	0.52
	Treatment	141.2 ± 53.9	132.0 ± 44.1	0.55
	% change	-11.4 ± 36.8	-11.0 ± 22.6 **	0.97
ApoAI	Basal	144.1 ± 21.2	149.2 ± 28.7	0.52
	Treatment	150.5 ± 18.4	148.8 ± 37.6	0.85
	% change	3.1 ± 12.3	-1.2 ± 12.4	0.28
ApoB	Basal	147.6 ± 28.4	120.8 ± 18.8	< 0.01
	Treatment	108.1 ± 22.3	90.2 ± 15.3	< 0.01
	% change	-38.9 ± 29.6***	-25.3 ± 10.2***	0.06

Number of subjects in parenthesis. Continuous variables are presented as mean ± SD and compared by *t*-test. TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein cholesterol; TG: triglycerides; ApoAI: apolipoprotein AI; ApoB: apolipoprotein B; *p < 0.05; **p < 0.01; ***p < 0.0001.

3.2. LDL-C lowering response to statins

Even though exploring a relatively small cohort, the distribution of the LDL-C lowering by both statins was highly variable (Fig. S1). An identical 10 mg/day dose in all subjects showed LDL-C reductions

ranging from 51% to 10% (Δ = 41%) for atorvastatin-treated patients, while simvastatin showed LDL-C reductions ranging from 54% to 6% (Δ = 48%). Considering this marked variability, we further compared the extremes of each statin treatment, e.g. patients located within quartiles of response (lower and higher LDL-C reduction after statin treatment), in order to explore miRNAs expression (Fig. S1). Thus, extreme quartiles of atorvastatin response were composed by patients showing LDL-C reductions < 22% and > 46%, while subjects having LDL-C reductions < 12% and > 46% were included in the extremes quartiles of simvastatin response.

3.3. Statin effect on miRNAs expression

Clustering of miRNAs expression from PBMC following atorvastatin treatment is shown in Fig. S2. In atorvastatin-treated patients, 10 out of 84 miRNAs (miRNA-206, miRNA-653, miRNA-613, miRNA-183-5p, miRNA-129-5p, miRNA-544a, miRNA-205-5p, miRNA-519d, miRNA-122-5p and miRNA-758-3) showed poor expression in PBMC (Ct > 32) and 2 miRNAs (miRNA-384 and miRNA-367-3p) were undetectable (Ct > 35) in the samples (data not shown). From the 72 miRNAs available for further analysis, only six were found deregulated (miRNA-29a-3p, miRNA-29b-3p, miRNA-300, miRNA-33a-5p, miRNA-33b-5p and miRNA-454) (Fig. 1), and significantly repressed after treatment (Fig. 2). Conversely, 10 mg/day of simvastatin during 4 weeks had no significant impact on any of the 84 miRNAs selected for profiling (Fig. S3). Additional clustering of the patients according to quartiles of response showed that miRNAs miRNA-17-3p, miRNA-106b-5p and miRNA-590-5p were down-regulated in individuals with low-response to atorvastatin (LDL-C reduction < 22%) compared with high-responders (LDL-C reduction > 46%) (Table 3). In the simvastatin group, miRNA-17-3p, miRNA-106b-5p and miRNA-183-5p were up-regulated in patients with high-response to simvastatin (LDL-C reduction > 46%) vs. low-responders (LDL-C reduction < 12%) (Table 3).

3.4. Regulatory pathway analysis

IPA was used to explore the interaction network between deregulated miRs and their 3-UTR mRNA targets. Fig. 3 shows that miRNAs significantly modulated after atorvastatin treatment have interaction sites complementary with the 3-UTR of important genes involved in cholesterol metabolism (*HMGCR*, *LDLR*, *ABCA1*, *SCAP*, *INSIG1*, *LPL*, *SREBP1*).

4. Discussion

This study evaluated the effect of short-term low-dose atorvastatin and simvastatin treatment on 84 cholesterol- and statins-related miRNAs in patients with hypercholesterolemia. We show that atorvastatin represses in vivo the expression of six miRNAs (miRNA-29a-3p, miRNA-29b-3p, miRNA-300, miRNA-33a-5p, miRNA-33b-5p and miRNA-454).

A major finding derived from this study is that miRNA-33 was downregulated after atorvastatin treatment. To date, it has been well established that inhibition of cholesterol synthesis by statins activates several genes involved in the cholesterogenic pathway, including *SREBP* genes, which would consequently favor miRNA-33 expression. In fact, Niesor and colleagues reported that incubation of THP-1 cells (which are frequently used as in vitro homologues of PBMC) with atorvastatin, was associated with a significant increase in miRNA-33 expression, together with lower *ABCA1* and *ABCG1* mRNA levels (Niesor et al., 2015), findings supported by a preceding report (Genvigir et al., 2010). However, using the same cellular model evaluated in our study, Arazi and colleagues indicated that atorvastatin could reduce rather than increase SREBF1a expression in patients having low baseline levels (Arazi et al., 2008). Also, additional differences could exist due to dissimilar cellular models are being assessed, as a previous report of our group showed a differential miRNA signature in liver cells induced with different statins (Zambrano et al., 2015). Although controversial, statin-mediated miRNA-33 downregulation in PBMC is

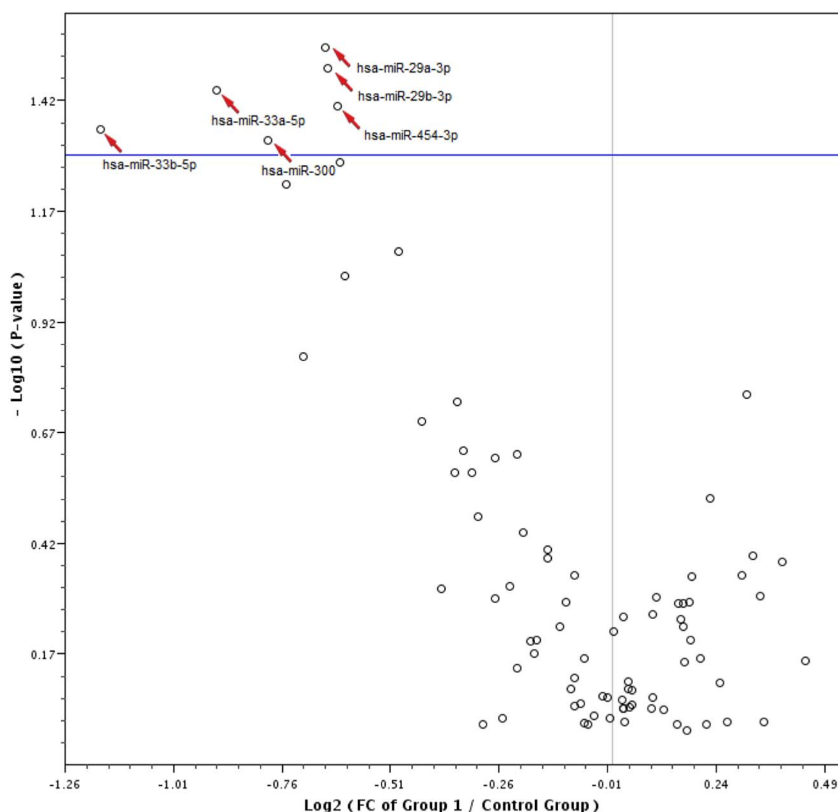

Fig. 1. Volcano plot of deregulated miRNAs in PBMC of HC subjects following atorvastatin therapy. Points above the blue line correspond to $p < 0.05$. FC: fold change; PBMC: peripheral blood mononuclear cells; Control Group: patients before atorvastatin therapy; Group 1: atorvastatin-treated patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

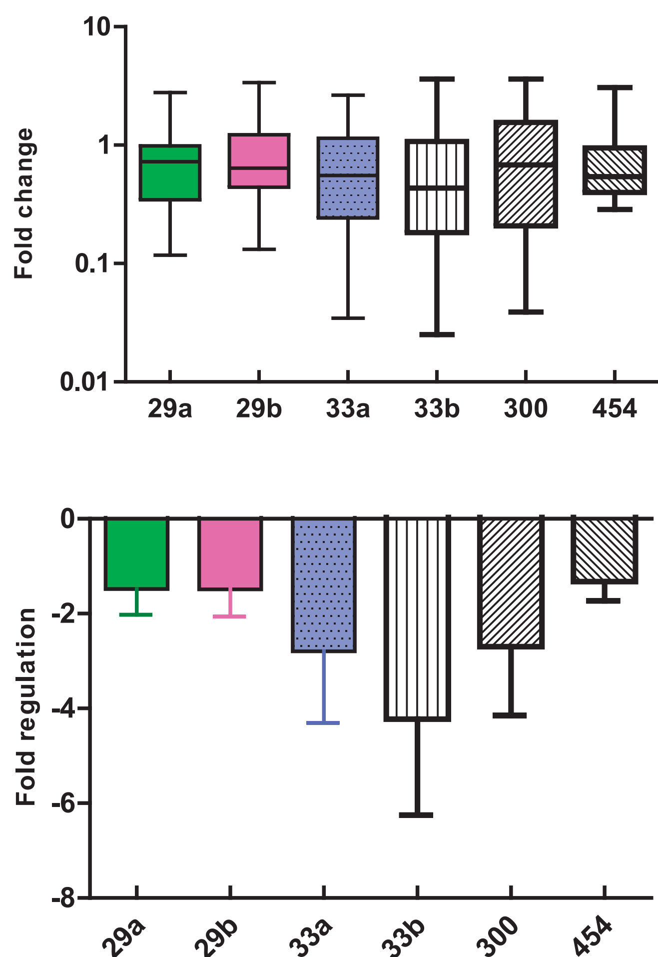

Fig. 2. Fold change and fold regulation of miRNAs differentially expressed in PBMC of HC patients after atorvastatin treatment. Fold change ($2^{-\Delta\Delta Ct}$) mean ± SD expression values from basal and after 4 weeks of atorvastatin treatment were compared by *t*-test (n = 20, $p < 0.05$); PBMC: peripheral blood mononuclear cells.

plausible, especially considering the undetermined and complex interactions existing in vivo, for instance, the hyperlipidemic nature of the patients. Interestingly, a recent study performed in subjects with metabolic syndrome (MetS), a disease encompassed by several features such as hyperglycemia, hypertriglyceridemia, low HDL-C, and hypertension, among others, has shown that plasma miRNA-33 expression is positively correlated with levels of fasting blood glucose, and that this miRNA was increased in subjects after treatment with atorvastatin and pitavastatin (Chen et al., 2016). However, differences exist between that study and ours, as we did not evaluate circulating miRNAs. Moreover, to date, there is no information available correlating circulating miRNAs to any specific type of cellular miRNAs, such as PBMC. As the exact cellular release mechanism of miRNAs continue to be largely unknown, we cannot firmly establish that circulating miRNA-33 levels originate from PBMC only. Besides, it is also likely that hyperlipidemia, hyperglycemia or many of the several MetS characteristics have changed the regulation of cholesterol synthesis, including and possibly affecting miRNA-33 and its response to statin treatment. In a less complex model, a report from 2014 showed that miRNA-33a levels are elevated in the sera of hyperlipidemic subjects compared to normolipidemics (Simionescu et al., 2014), which would explain why statins can repress miRNA-33 expression in hypercholesterolemic patients normally having higher miRNA-33 levels. Regarding to miRNA-29a and miRNA-29b, they have been associated with modulation of oxidized LDL (oxLDL) and CVD (Chen K.C. et al., 2011; Chen T. et al., 2011), but the role portrayed by miRNA-300 and miRNA-454 in cholesterol metabolism has not been identified yet.

On the other hand, our results indicate that patients undergoing the same 10 mg dose of simvastatin did not show deregulated miRNAs in peripheral cells. This result could be due to simvastatin is less potent than atorvastatin (Dansette et al., 2000), which is reflected on different studies demonstrating the higher disruptive power shown by atorvastatin than simvastatin on both gene expression (Leszczynska et al., 2011) and miRNAs profiles (Zambrano et al., 2015) in liver cells. However, we must also consider that, in terms of LDL-C reduction, equal doses of different statins are not equipotent (Karlson et al., 2016a); therefore, we cannot exclude that using an equivalent 20 mg/day simvastatin dose could have shown a deregulatory effect on miRNA expression in the patients evaluated.

A significant amount of data clearly demonstrates the wide inter-individual variability in response to statins, a scenario that up to this day has been poorly understood. Our results also come to support the highly variable LDL-C reduction observed following both atorvastatin (10% to 51%) and simvastatin (6% to 54%) treatments. Taking the extremes of statins response, data showed that 3 miRNAs (miRNA-17-3p, miRNA-106b-5p and miRNA-590-5p) were repressed in atorvastatin-treated patients having LDL-C reductions < 22%, while miRNA-17-3p, miRNA-106b-5p and miRNA-183-5p were up-regulated in simvastatin-treated patients with LDL-C reduction > 46%. Although interesting, miRNAs expression in the extremes of response to different statins must be interpreted with caution, as the subgroup analyses were performed on the basis of an already limited population, therefore, some bias may have been introduced. Nevertheless, among our main

Table 3
PCR array expression of miRNAs deregulated in PBMC from HC patients after statin treatments.

Groups	miR	Fold change	95% CI	Fold regulation	p-Value
Atorvastatin, total group	hsa-miR-29a-3p	0.6321	(0.39, 0.88)	−1.5821	0.02
	hsa-miR-29b-3p	0.6388	(0.35, 0.92)	−1.5655	0.03
	hsa-miR-300	0.5767	(0.26, 0.89)	−1.7340	0.04
	hsa-miR-33a-5p	0.5314	(0.17, 0.89)	−1.8819	0.03
	hsa-miR-33b-5p	0.4415*	(0.10, 0.78)	−2.2648*	0.04
	hsa-miR-454-3p	0.6444	(0.37, 0.92)	−1.5519	0.03
Atorvastatin, low-response group	hsa-miR-106b-5p	0.5393	(0.21, 0.87)	−1.8542	0.04
	hsa-miR-17-3p	0.3885	(0.20, 0.58)	−2.5738	< 0.01
	hsa-miR-590-5p	0.3410	(0.08, 0.60)	−2.9323	0.03
Simvastatin, high-response group	hsa-miR-106b-5p	1.5987	(1.01, 2.19)	1.5987	0.03
	hsa-miR-17-3p	1.7154	(0.98, 2.45)	1.7154	0.04
	hsa-miR-183-5p	2.1926	(0.65, 3.73)	2.1926	0.03

Low-response to atorvastatin: LDL-C reduction < 22%. High-response to simvastatin: LDL-C reduction > 46%. *MiRNA average Ct was relatively high (> 30) in one (basal or atorvastatin) test sample and it is reasonably low in the other sample (< 30). These data mean that the miRNA expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result. This fold-change result may also have greater variations if p value > 0.05, therefore, the number of biological replicates may be it is not enough to assure valid results for this miRNA.

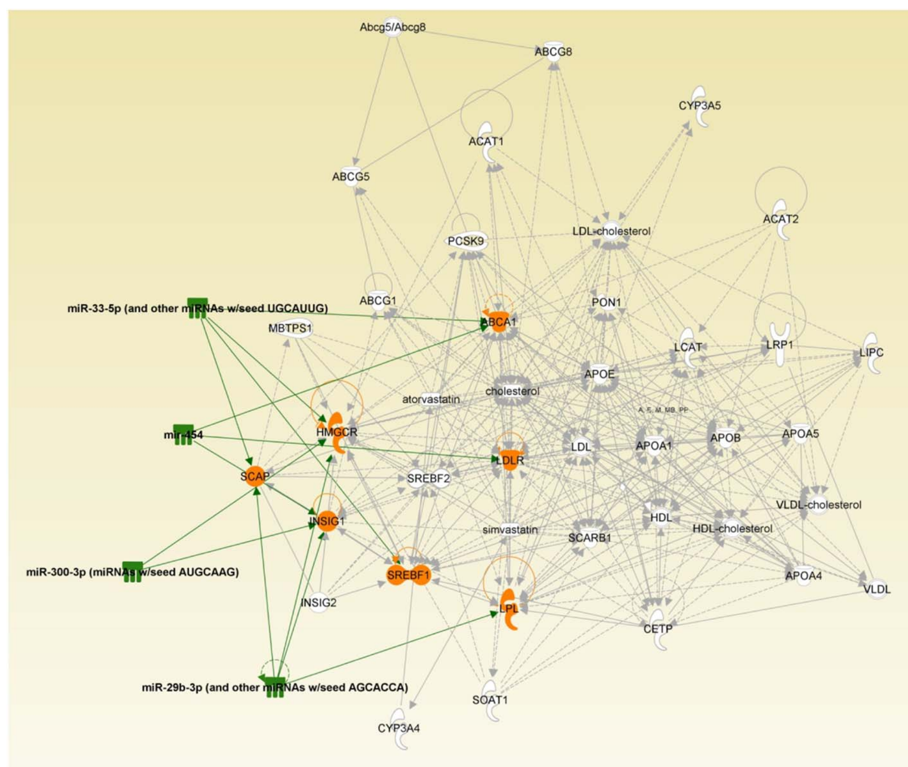

Fig. 3. Predicted interaction of deregulated miRNAs with their target mRNAs following statin treatment in HC subjects. Data were analyzed using IPA software. In green are miRNAs repressed following atorvastatin treatment. *ABCA1*: ATP-binding cassette, sub-family A, member 1; *HMGCR*: 3-hydroxy-3-methyl-glutaryl-CoA reductase; *INSIG1*: Insulin-induced gene 1; *LDLR*: low-density lipoprotein receptor; *LPL*: lipoprotein lipase; *SCAP*: SREBP cleavage-activating protein; *SREBF1*: Sterol Regulatory Element-Binding Transcription Factor 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

finding, IPA software showed feasible interactions between miRNAs and important genes related to cholesterol metabolism (Fig. 3), for instance, the interaction between miR-454, a miRNA that has not been previously related to cholesterol metabolism, and *ABCA1*. The *ABCA1* gene plays an essential role in the reverse cholesterol transport (RCT) pathway, and has been experimentally validated as a target of miRNA-33 (Horie et al., 2010; Marquart et al., 2010; Najafi-Shoushtari et al., 2010; Rayner et al., 2010), miR-758 (Ramirez et al., 2011) and miRNA-144 (Ramirez et al., 2013). In addition, *ABCA1* was also reported to be regulated by miRNA-291b-5p (Ramirez et al., 2011), but paradoxically, instead of post-transcriptional repression, this miRNA determined higher *ABCA1* expression. Since only one miRNA can regulate > 100 mRNAs (Friedman et al., 2009), it is highly possible that further miRNA-mediated epigenetic regulation is taking place, making cholesterol control more intricate than previously recognized. This last scenario seems very likely when considering recent reports demonstrating that, for instance, the *LDLR* is modulated by multiple miRNAs, such as miRNA-185 (Jiang et al., 2015), miRNA-27a (Alvarez et al., 2015) and miRNA-148a (Goedeke et al., 2015), this last regulating *ABCA1* as well (Goedeke et al., 2015), a contradictory finding considering the dissimilar pathways in which *LDLR* and *ABCA1* are involved.

Finally, some limitations must be considered for a better interpretation of the results. First, while miRNA target prediction and bioinformatics tools can relate miRNAs to their targets, we did not include target mRNA expression data, which could help clarify the biological meaning of our results, especially in the case of newly reported interactions. Secondly, although we successfully found deregulated miRNAs following statins treatments, the restricted quantity of miRNAs included in the array prevented us to have a clearer image of statin effect on miRNAs expression. Thus, we cannot exclude other miRNAs not considered in this study, to be implicated in statins therapy, especially considering that we have identified miRNAs not been previously related to lipid-lowering treatment that are deregulated by statins. As only a fraction of the 2588 mature miRNAs annotated in the latest miRBase 21 release (June 2014) have been characterized in terms of their biological role, additional studies comprising a larger cohort and assessing functional studies e.g., miRNAs antisense silencing or transfection, and also incorporating a control group i.e., normolipidemic subjects, are needed to corroborate our findings and elucidate the function that miRNAs may play upon cholesterol-lowering drugs. Nevertheless, differential miRNAs expression observed in quartiles of response to statins could constitute a first preliminary result towards disclosing additional mechanisms involved in lipid-lowering treatment.

5. Conclusion

In summary, our results show that statins differentially affect the expression of miRNAs in vivo. Further studies including functional assays are needed for a better understanding of the miRNA-dependent pathways and mechanisms involved in the response to statins, which may lead to the therapeutic manipulation of miRNAs with the aim of reducing the variability of statin response.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2018.02.007.

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Conflicts of interest

None.

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