ESC European Society of Cardiology
Cardiovascular Research
doi:10.1093/cvr/cvaa039

High miR-133a levels in the circulation anticipates presentation of clinical events in familial hypercholesterolaemia patients

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Received 11 May 2019; revised 17 November 2019; editorial decision 31 January 2020; accepted 10 February 2020

Time for primary review: 29 days

Aims

Presentation of acute events in patients with atherosclerosis remains unpredictable even after controlling for classical risk factors. MicroRNAs (miRNAs) measured in liquid biopsies could be good candidate biomarkers to improve risk prediction. Here, we hypothesized that miRNAs could predict atherosclerotic plaque progression and clinical event presentation in familial hypercholesterolaemia (FH) patients.

Methods and results

Circulating miRNAs (plasma, exosomes, and microvesicles) were investigated by TaqMan Array and RT-qPCR assays. Patients with genetic diagnosis of FH and healthy relatives from the SAFEHEART cohort were included. A differential signature of 10 miRNA was obtained by comparing two extreme phenotypes consisting of FH patients suffering a cardiovascular event (CVE) within a 8-year follow-up period (FH-CVE, $N = 42$) and non-FH hypercholesterolaemic relatives from the same cohort, matched for age and treatment, without CVE during the same period (nFH-nCVE, $N = 30$). The validation studies included two independent groups of patients with FH background (discovery group, $N = 89$, validation group $N = 196$), developing a future CVE (FH-CVE) or not (FH-nCVE) within the same time period of follow-up. Of the 10 miRNAs initially selected, miR-133a was significantly higher in FH-CVE than in FH-nCVE patients. Receiver operating characteristic analysis confirmed miR-133a as the best microRNA for predicting CVE in FH patients (0.76 ± 0.054; $P < 0.001$). Furthermore, Kaplan–Meier and COX analysis showed that high plasma miR-133a levels associated to the higher risk of presenting a CVE within the next 8 years (hazard ratio 3.89, 95% confidence interval 1.88–8.07; $P < 0.001$). *In silico* analysis of curate biological interactions related miR-133a with target genes involved in regulation of the cell-membrane lipid-receptor LRP6 and inflammatory cytokines (CXCL8, IL6, and TNF). These predictions were experimentally proven in human macrophages and endothelial cells transfected with agomiR-133a.

Conclusion

Elevated levels of miR-133a in the circulation anticipate those FH patients that are going to present a clinical CVE within the next 2 years (average). Mechanistically, miR-133a is directly related with lipid- and inflammatory signalling in key cells for atherosclerosis progression.

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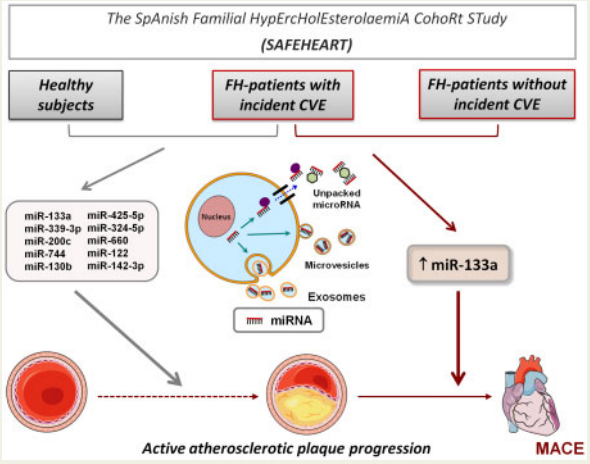
Graphical Abstract



Keywords Atherothrombosis • Cardiovascular disease • MicroRNAs • Extracellular vesicles • Cardiovascular risk factor

1. Introduction

Dyslipidaemia is an independent risk factor for the progression of atherosclerotic cardiovascular disease (CVD). Heterozygous familial hypercholesterolaemia (FH) patients have 3- to 13-fold higher atherosclerotic risk than those without this genetic disease and have premature presentation of cardiovascular events (CVEs).[1,2] Indeed, FH patients, due to their lifelong exposure to high low-density lipoprotein (LDL) cholesterol levels, have an early development of atherosclerotic plaques and, ultimately, premature CVE presentation.[3] Up till now the use of lipid-related markers in combination with classical risk factors in conventional risk estimation algorithms has led to only slight improvement in CVD event prediction. MicroRNAs (miRNAs), small non-coding RNAs (22-nucleotides) involved in post-transcriptional regulation of gene expression, have been implicated in CVD pathophysiology.[4] Recently, miRNAs have been found in a remarkably stable form in circulating blood and other body fluids either as conjugates with lipoproteins, leaked into plasma RNA-binding proteins or packed into extracellular vesicles (microvesicles/exosomes).[5] Exosomes are small vesicles (20–200 nm) formed by the inward budding of cellular compartments[6,7] and secreted by merging with the cell membrane. Exosomes, loaded with unique RNA and protein cargo, have a wide range of biological functions, including cell-to-cell communication and signalling by transferring phenotypic traits from the parent cell.[8] The biogenesis, release, and uptake of exosomes are tightly regulated processes governed by diverse signalling mechanisms, which can be altered in CVD.[9]

miRNAs have been proposed as prognostic biomarkers and their utility in precision medicine represents a promising biomedical tool that needs to be fully investigated.[10,11] miRNA signatures could lead to the development of minimally invasive diagnostics and next-generation therapies within the next few years. In addition, changes in miRNA levels may be a surrogate marker of cellular alterations due to homeostatic imbalances, cell activation, and cell death. Indeed, the early changes induced by hypercholesterolaemia (high LDL levels) on the arterial wall causing damage might contribute to abnormal release of miRNAs, in the same manner as the release of microvesicles before the existence of clinically relevant atherosclerosis.[2,12]

Another characteristic of FH is the high heterogeneity in the presentation of clinical events,[13] even between siblings receiving the same treatment regimes. These differences in clinical premature disease presentation are of unknown cause and could well be due to differential epigenetic regulation. Here, we have hypothesized that miRNAs could be predictors of atherosclerotic plaque progression and clinical event presentation in FH patients and performed a discovery proof of concept study to investigate miRNA profiles in different subgroups of FH- and non-FH relatives of the SAFEHEART cohort[1,14] by a non-targeted approach to miRNA discovery.

2. Methods

2.1 Clinical population and study design

This is a prospective study with a follow-up of 8 years of FH patients from the SAFEHEART cohort. The SAFEHEART cohort is an open multicentre, nationwide, long-term, prospective cohort study consisting of molecularly defined heterozygous FH patients and their non-FH relatives.[1,14] The study design refers to a discovery- and a validation phase (*Figure 1*). Each one of the two phases has used independent patient groups of the SAFEHEART cohort (FH discovery group and FH validation group). In total, 327 patients were included in this study.

2.1.1 Discovery phase

(a) *miRNA profiling*: Aimed to identify (TaqMan Array) the differential exosomal miRNA signature between patients with extreme phenotypes ($N = 6$/each) . This refers to: (i) Genetically diagnosed FH patients who did not present any clinical manifestation of CVD before inclusion but suffered a CVE within a 8-year follow-up [median (interquartile range, IQR) period for CVE: 2.0 (1.0–4.2)] after entering the SAFEHEART

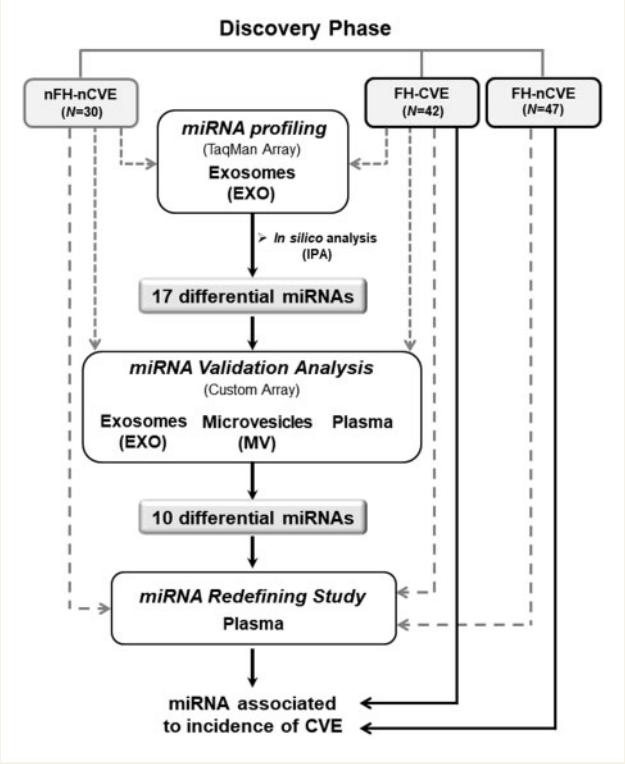


Figure 1 Schematic diagram representing the study design with 119 subjects of the SAFEHEART cohort. FH-CVE, familial hypercholesterolaemia patients with cardiovascular event; FH-nCVE, familial hypercholesterolaemia patients without cardiovascular event; nFH-nCVE, healthy subjects non-FH with secondary hypercholesterolaemia and without cardiovascular event.

cohort study (FH-CVE) and (ii) subjects of the SAFEHEART cohort with secondary hypercholesterolaemia (negative genetic testing of FH) and without CVE presentation before entry nor within the same follow-up time-frame (nFH-nCVE) (see Supplementary material online, *Table S1* for anthropometric and clinical characteristics). (b) *miRNA validation*: The differential miRNA signature was analysed by real time-PCR in exosomes (EXO), microvesicles (MV), and plasma of larger subgroups of nFH-nCVE subjects ($N = 30$) and FH-CVE patients ($N = 42$) with similar anthropometric and lipid profile and clinical characteristics as per the groups included in the miRNA profiling phase (Supplementary material online, *Table S2*). (c) *Redefining study: association with incident CVE*: This part of the study focused on patients with FH genetic background. Thus, FH-CVE ($N = 42$) was compared with FH patients without events in the same time period FH-nCVE ($N = 47$) patients. Supplementary material online, *Table S2* provides information on anthropometric variables, lipid profile, cardiovascular risk factors, and lipid-lowering therapy (LLT) for both FH study groups.

2.1.2 Validation phase

The selected miRNA(s) were investigated in an independent subgroup of FH patients (FH validation group) with/without incident CVE within a 10-year follow-up [median (IQR) period for CVE: 3.0 (1.0–7.0)] after entering the SAFEHEART cohort (Supplementary material online, *Table S3*, FH-nCVE = 100; FH-CVE = 96).

2.2 Clinical characteristic and follow-up of study patients

FH cases had positive genetic diagnostic of FH and clustering of additional cardiovascular risk factors. FH patients fulfilled the WHO criteria and both FH- and nFH patients were receiving stable LLT, before the inclusion in the SAFEHEART cohort, following updated clinical practice guidelines.[15]

Major acute CVEs (MACE) in the FH-CVE groups included sudden death, fatal and non-fatal myocardial infarction (MI), unstable angina (UA), and cerebrovascular accident, as shown in the Supplementary material online, *Tables S2* and *S3*, MI and UA were the most frequent CVE in both groups (FH discovery group: 42.9% MI, 23.8% UA, FH validation group: 56.3% MI, 38.5% UA). Through a yearly scheduled standardized phone call to the cohort members, the co-ordinating centre gathered relevant information regarding changes in lifestyles, medication, and CVEs. Medical histories, hospitalization records, and communication with personal physicians were all used to obtain information about CVD events on follow-up. In the event of death, a copy of the death certificate was provided.

Informed consent was obtained from all patients, and the ethics committee of the Investigación Clínica Fundación Jimenez Diaz approved the study protocol (n°: 01/09). The study was conducted according to the Declaration of Helsinki and the results of the study are presented in accordance with STROBE guidelines.

2.3 Blood sampling for analysis

At entry in the SAFEHEART cohort, venous blood was withdrawn after 10–14 h fasting from the cubital vein without tourniquet using a 20-gauge needle into serum and EDTA-anticoagulated tubes under standardized conditions for biochemical, genotyping, and miRNA analysis, respectively.[16] All samples were processed identically and within the first two hours after extraction. miRNA analysis was performed in plasma (EDTA anticoagulated blood) centrifuged at 1500 *g* for 20 min at room temperature to obtain the platelet-free plasma. Plasma samples were tested with a cell counter for the absence of residual cells after centrifugation. Aliquots were generated from all samples, snap-frozen in liquid nitrogen and stored at -80°C until analysis.

2.4 Cell culture and miRNA transfection studies

In vitro miR-133a transfection studies were performed in human monocyte-derived macrophages (MAC), obtained from leucocytes-rich buffy coats from healthy donors (Barcelona Blood Bank), and in immortalized human microvascular endothelial cells 'HMEC-1' (ATCC) that expresses the typical molecular markers of endothelial cells such as CD31, CD36, ICAM, CD44, and retain the morphological, phenotypic, and functional characteristics of human microvascular endothelial cells.[17] MAC and HMEC-1 were cultured as described in previous studies of our group.[18–20] In short, peripheral blood mononuclear cells (PBMN) were platted to obtain cellular density of 2×10^6 monocytes/mL. Adherent monocytes differentiate into macrophages after 7 days in culture (MAC).[18,19] HMEC-1 were used at the density 1.5×10^6 cells/mL.[20]

MAC and HMEC-1 were transfected for 48 h with agomiR-133a (5 nM, MSY0000427, Qiagen), antagomiR-133a (50 nM, MIN0000427, Qiagen), or the corresponding negative controls (5 nM agomiR-NC 1027280, 50 nM antagomiR-NC 1027271; Qiagen) that were used as controls for non-sequence-specific effects, according to the

manufacturer's instruction. All the transfection procedures were effectively maintained for at least 72 h.[18–20]

2.5 Lipid analysis and LDL-mutation characterization

For lipid profile, total cholesterol, triglycerides, and HDL-c were measured by standardized enzymatic methods; serum LDL-c concentration was calculated using the Friedewäld formula[21] and lipoprotein (a) levels were measured using a turbidimetric method. Genetic diagnosis of FH was made using a DNA-microarray (LIPOCHIP).[22] Mutations were classified as receptor negative or receptor defective, depending on their functional class.

2.6 Extracellular vesicle isolation and RNA extraction

Microvesicles (MV) and exosomes (EXO) were purified from plasma with two serial filtration processes using filters of 200 nm and 20 nm to obtain MV and EXO, respectively. RNA from EXO and MV fractions and from plasma was obtained with the ExoMir kit (Bio Scientific), as described by the providers.[23,24] Total RNA from MAC and HMEC-1 were extracted using the mirVana miRNA isolation Kit (AM1561, Thermo Fisher Scientific).

RNA concentration was determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and purity was checked by calculating the A260/A280 ratio. All samples were spiked-in with 25 fmol of cel-miR-39 prior to RNA extraction for normalization.

The exosome fraction was characterized by flow cytometry analysis of tetraspanins (CD63, CD81) (ExoS-25-P81, Inmunostep; Supplementary material online, *Figure S1A*) and the size and concentration of the exosomes contained in EXO defined by Nanoparticle Tracking Analysis (NTA) using a Nanosight and NTA 3.1 Build 3.1.46 software (Supplementary material online, *Figure S1B*).

2.7 miRNA profiling and validation analysis

For miRNA profiling, total RNA (100 ng input) from EXO was analysed by the low-density TaqMan® Array Human MicroRNA A Card v2.0. This array focuses on highly characterized miRNAs[25] and contains 377 miRNAs, 3 endogenous (RNU6, RNU48, and RNU44), and 1 negative control (ath-miR-159a) that does not amplify in human samples.[18] In brief, miRNA was transcripted to cDNA with MegaPlex Reverse Transcription primers pool-A followed by a pre-amplification step and amplified by using the TaqMan Array, according to the manufacturer's instruction (Applied Biosystems). Only miRNAs with expression levels below 32 cycles were accepted.

In the FH discovery group, miRNA expression levels were analysed by quantitative PCR with the 'Taqman miRNA Assay' using Custom TaqMan Array MicroRNA cards.

In the FH validation group, plasma miR-133a was analysed with a single assay and the 'Taqman Advanced miRNA Assays' that uses a universal reverse transcription methodology for the synthesis of cDNA (differing from the 'TaqMan miRNA Assays' based on miRNA-specific primers).

miRNA and mRNA gene expression in the cell culture studies were analysed by single assays as previously described.[26] The assays used in miRNA- and gene expression analysis are listed in Supplementary material online, *Table S4* [miRNAs were identified by the reference code provided by Applied Biosystems and the accession number according miRbase (http://www.mirbase.org/)].

Cel-miR-39 and PIK3C2A were used as internal controls to normalize the expression levels of miRNAs and genes, respectively, by using the ΔCt, according the equation $2^{-(Ct\ [target]\ -\ Ct[endogenous])}$. Data were analysed by SDS 2.4, RQ Manager 1.2.1 and DataAssits v3.0.1 software.

2.8 Target gene prediction and integrated analysis by databases

Potential target genes candidates were defined by using databases such as TargetScan, miRmap, miRWalk (3UTR, CDS, 5UTR), PACCMITCDS, PACCMIT3UTR, and Ingenuity Pathway Analysis (IPA; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/). Target genes considered were those with were highly predicted probability to be regulated by miRNA, according to their search algorithms (Supplementary material online, *Table S5*). IPA Core analysis was used to describe the biological functions/diseases. Molecular interactions in canonical pathways were predicted by Molecule Activity Predictor (MAP). Specifically, MAP predicts the upstream and/or downstream effects by activation (up-regulation) or inhibition (down-regulation) of molecules in the network, given one or more neighbouring molecules with 'known' activity. Predicted networks are based on the IPA Knowledge Base, a repository of curated biological interactions and functional annotations supported on existing literature.

Furthermore, RNAhybrid was used to assess the robustness of the binding between the miRNAs and their target genes, through measurement of the minimum free energy (MFE). MFE is represented as negative real values. Thus, the greater miRNA binding to mRNA candidate genes is reflected by the lower free energy (highly negative MFE values), which indicate higher likelihood that the interaction between the miRNA and its target gene occurs.[18,27]

2.9 Statistical analysis

The sample size estimation for the profiling study was calculated using the JavaScript provided in http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html for power/sample size calculation when two independent groups are compared[19,28] assuming a standardized fold change of 1.5 and a loss of 0% patients. A sample size of at least six per group would yield 93% power with a α of 0.05. All data are presented as median (interquartile range), except when indicated. An initial descriptive analysis was provided using number of cases and percentages for qualitative variables and mean ± SEM for quantitative variables. Frequencies of qualitative variables were compared between groups by using the χ^2 analysis. Continuous variables were test for normal distribution with the Kolmogorov–Smirnov test. Mean and median values of quantitative variables were compared with multiple comparisons by analysis of variance (ANOVA/ANCOVA) and Student's *t*-test and the non-parametric test, Mann–Whitney *U* test and Kruskal–Wallis or the paired-Wilcoxon test in the cell culture studies. When significant, Bonferroni *post hoc* analysis was used to assess intergroup differences. Spearman correlation coefficients were calculated to describe the relationship between quantitative variables. The power of plasma miRNAs to discriminate FH patients with incident CVE was analysed by c-statistics through the receiver operating characteristic (ROC) curve. The area under the curve (AUC) along with its 95% confidence interval (CI) was calculated for each miRNA. Kaplan–Meier survival (free of major CVE) plots and COX regression analysis were performed to evaluate the value of the studied miRNAs for predicting incident major CVEs in FH. Kaplan–Meier curves performed after including the study variables in a logistic rank analysis. The hazard ratio (HR) in the COX regression analysis was obtained using a cut-off value

obtained from c-statistics for the best sensitivity and specificity. The proportional hazard assumption was evaluated by the Shoenfeld residuals test. Propensity score matching was used to balance the comparison groups and to designate the pairs of cases and controls. Propensity scores were matched using the MatchIt statistical package in R. Independent variables were adjusted by SAFEHEART risk score (SRsc-5y)[29] to create the match. The variables were selected by using a statistical criterion ($P < 0.05$). The statistical analysis was carried out using StatView 5.0.1, SPSS statistics version 25 and R version 3.5.2. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Exosomal miRNA profiling

In the discovery phase, we comparatively analysed levels of 381 miRNAs in EXO-fraction of FH-CVE and nFH-nCVE patients, with extreme phenotypes to identify the miRNA signature that better differentiate the two groups. One hundred thirty miRNAs were consistently detected in the EXO-fraction. As shown in *Figure 2*, only miRNAs exceeding 1.5-fold change (log_2: >+0.58-fold or <-0.58) between FH-CVE and nFH-nCVE groups were considered for further analysis. This refers to 42 miRNAs, 21 with increased levels (log_2 change >+0.58-fold) and 21 with decreased levels (log_2 change <-0.58-fold).

Focusing on the 42 differentially expressed EXO-miRNAs and by performing *in silico* IPA Core analysis, we identified the top 10 cell functions (and/or disease) directly related to the atherosclerotic process and selected those miRNAs with annotations for these functions. As reported in Supplementary material online, *Table S6*, 17 of the 42 EXO-miRNAs were related to the top 10 functions and those were selected for further analysis.

3.2 miRNAs in exosomes, microvesicles, and plasma of FH-CVE and nFH-nCVE subjects during follow-up

The differential miRNA-signature (17 miRNAs) derived from the miRNA profiling studies was validated in EXO-samples of FH-CVE ($N = 42$) and nFH-nCVE ($N = 30$) patients using a Custom array. Differences between FH-CVE and nFH-nCVE patients reached statistical significance in 10 miRNAs (*Figure 3*). Nine exosome-associated miRNAs (miR-130b, miR-133a, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744) had higher levels in FH-CVE than in the nFH-nCVE group. In contrast, miR-122, showed lower levels in exosomes from FH-CVE patients (see *Figure 3*).

The 10 miRNAs with statistically different detection levels in EXO of FH-CVE and nFH-nCVE patients were further analysed in MV-fraction (0.2–1.0 μm) and plasma of the same patients (Supplementary material online, *Table S7*). The pattern of miRNA changes in the EXO-fraction was similarly found in plasma, while MVs showed some differences (compare *Figure 3* with Supplementary material online, *Table S7A* and *B*). Specifically, plasma miRNAs, including miR-130b, miR-133a, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744 were significantly higher in FH-CVE than in nFH-nCVE patients (Supplementary material online, *Table S7A*, $P \leq 0.001$ for differences in all miRNAs), whereas results in MV (Supplementary material online, *Table S7B*) demonstrated a different miRNA processing. In contrast, miR-122 that was significantly decreased in EXO-fraction of FH-CVE patients was unchanged in plasma, but it was significantly reduced in MV. Interestingly, total levels of plasma-miRNAs significantly correlate with those in the exosomes for the 10 analysed miRNAs (Supplementary material online, *Table S7C*), significant correlation between levels of plasma-miRNAs and MV-miRNAs was not found for the analysed miRNAs. These results support the concept of taking miRNAs in plasma as representative of the EXO-fraction content.

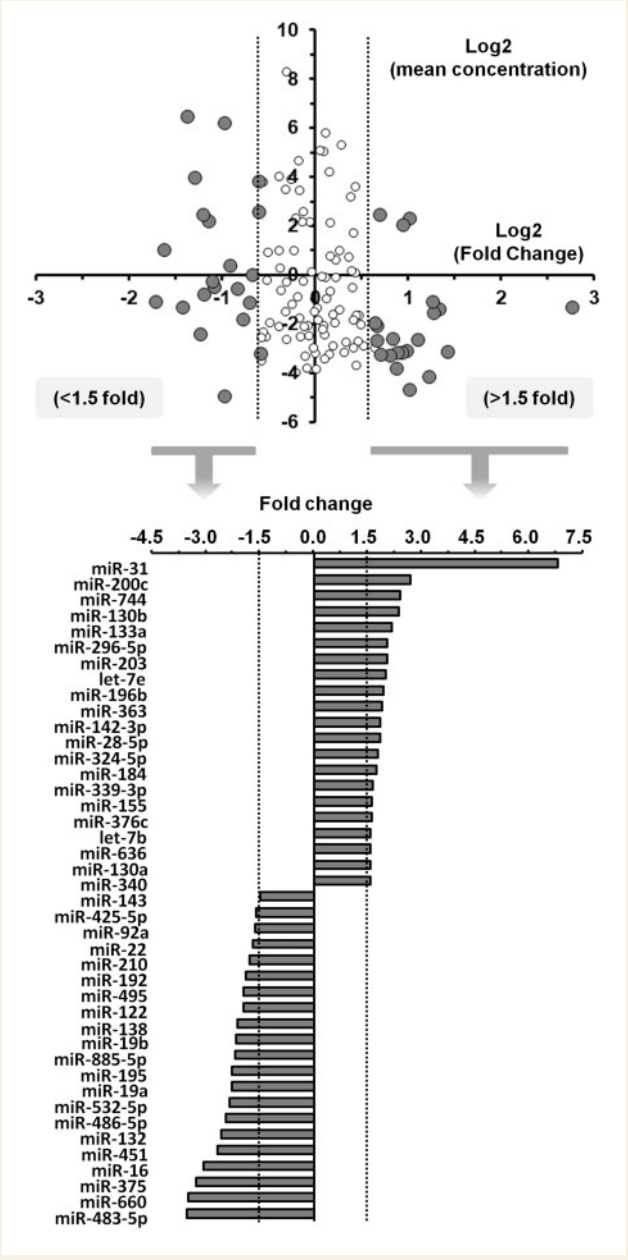


Figure 2 miRNAs profiling. EXO-miRNAs were analysed in FH-CVE ($N = 6$) and nFH-nCVE ($N = 6$) groups by low-density TaqMan Array Human MicroRNA A Card. miRNAs profiling is shown as dot plots in graph cartesian. *X*-axis represents log_2 (fold change) and *Y*-axis represents log_2 (mean concentration). Threshold for miRNAs with changes in concentration level of 1.5-fold {greater than 0.58, [log_2 (1.5)] and lesser than 0.58 [log_2 (1/1.5)] are shown in black dots.

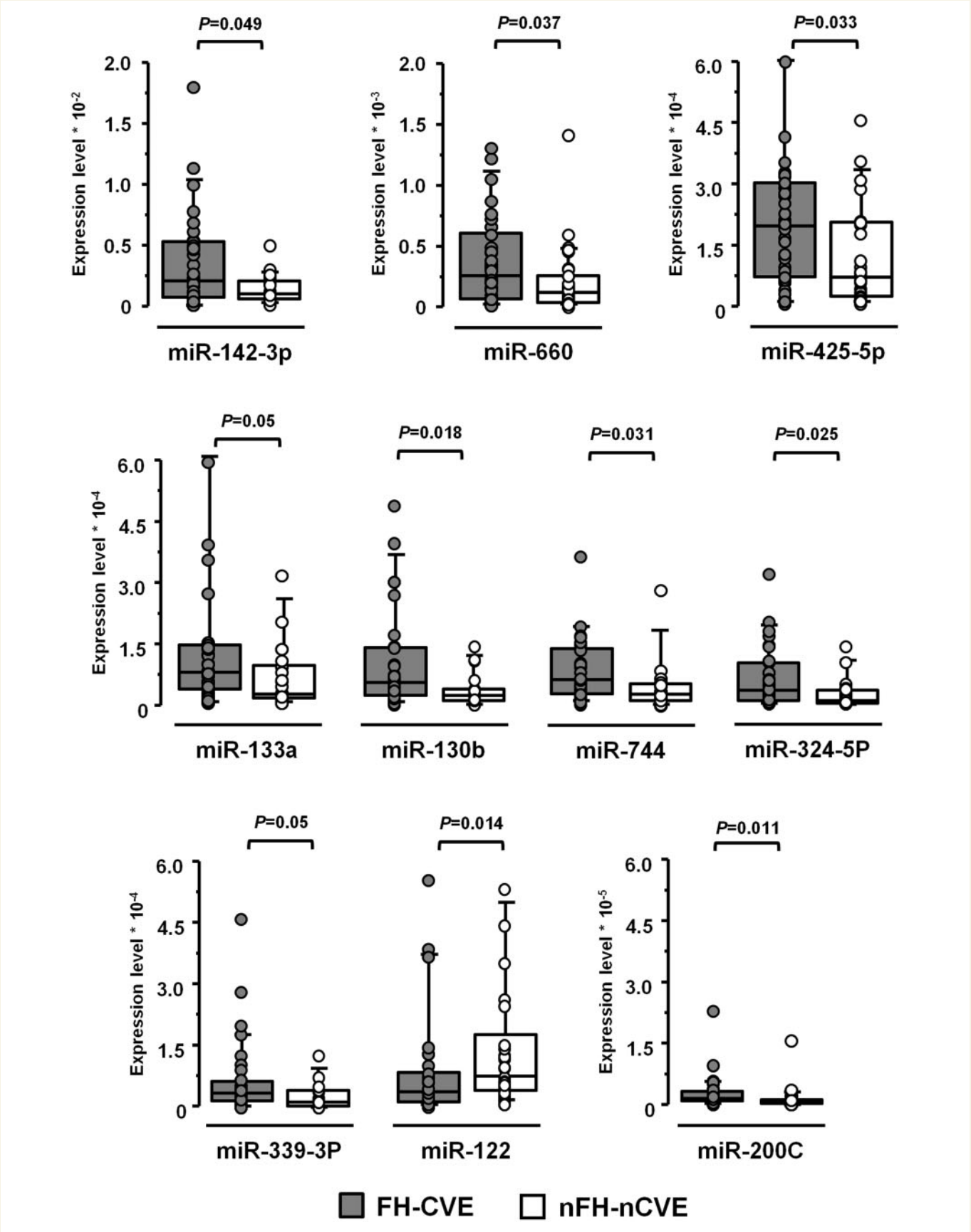


Figure 3 miRNAs validation. EXO-miRNAs were analysed in FH-CVE ($N = 42$) and nFH-nCVE ($N = 30$) using a TaqMan custom array. miRNAs levels are shown as box plots for the FH-CVE and nFH-nCVE patients. *P*-values were calculated by the Mann–Whitney test.

3.3 Plasma levels of miR-133a associates to CVE-presentation in FH patients

In order to redefine the miRNA signature associated to disease progression and incident CVE-presentation in FH patients, we comparatively analysed plasma levels of the top-10 selected miRNAs in the FH-CVE patients ($N = 42$) and in FH-nCVE, a group of FH patients who did not present MACE during the 8-year follow-up period ($N = 47$). Levels of the mature form of the microRNA miR-133a (MIMAT0000427, hsa-miR-133a-3p; Supplementary material online, *Table S4*) reached a highly significant difference between both FH groups ($P < 0.001$ for FH-CVE vs. FH-nCVE, *Figure 4*).

Differences in miR-133a plasma levels remained statistically significant after adjustment for the cardiovascular risk burden (number of cardiovascular risk factors beyond FH; $P = 0.019$ for differences by ANOVA analysis using cardiovascular risk burden as covariable) and for the type of statin ($P = 0.028$). To identify whether miR-133a values were dependent on plasma lipid parameters or high-sensitivity C-reactive protein (hs-CRP), the FH discovery group ($N = 89$) was categorized in two subgroups, using the median miR-133a value (0.636) as cut-off point. The mean values for the lipid variables, such as LDLc, HDLc, TG, and Lp(a), did not differ among the low- and high- miR-133a groups, suggesting that plasma changes on miR-133a occurred irrespectively of changes in the lipid pattern. Furthermore, the group below and above the median miR-133a value did not differed on plasma hs-CRP levels, biomarker of systemic inflammation (Supplementary material online, *Table S8A*). Plasma levels of the other eight miRNAs (miR-130b, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744), significantly increased in FH-CVE when compared with nFH-nCVE, but they did not show any significant difference with the FH-nCVE group, suggesting additional roles in the regulation of the effects of hypercholesterolaemia in the cardiovascular system (*Figure 4*).

3.4 LDLR mutation in FH patients

The profile of LDLR mutations in the FH patients of the SAFEHEART cohort included in this study and the predicted effects of the different mutations on LDLR functionality are listed in Supplementary material online, *Table S9*. Patients with mutations entailing a shift reading frame or mutations entailing an early stop codon leading to lack of protein were included in the FH null group ($N = 27$; FH-CVE: 14 and FH-nCVE: 13), whereas the FH non-null group ($N = 62$; FH-CVE: 28 and FH-nCVE: 34) refers to patients with mutations on the promoter, mutations entailing an amino acid change, mutations with a splicing change, and other mutations inducing the production of a mutated protein. FH non-null and FH null groups did not differ in the level of plasma-miR-133a (1.92 ± 0.37 vs. 1.04 ± 0.21; $P = 0.153$) nor in the level of the other nine differential miRNAs (data not shown). miR-133a levels were increased 2.1-fold ($P = 0.044$) in plasma from non-null FH patients suffering a CVE during follow-up. A similar trend (1.62-fold), although did not reach statistical significance, was found in FH patients with FH null mutation.

3.5 miR-133a plasma levels and prognosis of CVE

To assess whether the levels of miRNAs in plasma associate with actively evolving atherosclerotic disease in FH patients and future CVE, c-statistics analysis was performed. Thus, AUCs were calculated for the top-10 plasma miRNAs individually (*Figure 5A*). Three miRNAs (miR-133a, miR-339-p, and miR-200c) had statistically significant AUC values for predicting incident CVE. Among them, miR-133a showed the highest AUC [0.76 ± 0.054 (95% CI 0.66–0.87), $P < 0.001$] and a cut-off of 0.673 with 73% sensitivity and 72% specificity to discriminate FH patients with MACE presentation during follow-up. A panel combining the three miRNAs with statistically significant ROC curves gave an AUC of 0.72 ± 0.057 (95% CI: 0.61–0.83) (*Figure 5A*) that did not improve the readout of miR-133a (DeLong test $P > 0.05$). The ROC curves for miR-133a and the 3-miRNA signatures are shown in *Figure 5B*. Furthermore, to contextualize the given AUC value of miR-133a, C-reactive protein (CRP) was also tested with C-statistics. Compared with miR-133a and the 3-miRNA-panel (see *Figure 5A*), CRP showed the lowest AUC value [0.644 ± 0.062 (95% CI 0.52–0.77), $P = 0.027$] (graphic not shown), although no significant differences between AUC curves were evidenced.

In addition, to exclude any potential bias related to the FH background, we also analysed the sensitivity/specificity of the top-10 miRNAs to differentiate FH patients within a general population (FH and non-FH genetic background). As shown in the Supplementary material online, *Table S10*, miR-133a did not discriminate between FH- and non-FH groups [0.55 ± 0.082 (95% CI 0.38–0.71), $P = 0.577$], suggesting that the strength of miR-133a to identify FH patients with incident CVD events was not biased by the FH background. It is to notice that miR-660 showed the highest AUC to differentiate FH patients [AUC 0.92 ± 0.030 (95% CI 0.86–0.98), $P < 0.001$], whereas did not show any power to identify incident CVD events [AUC 0.58 ± 0.066 (95% CI 0.45–0.71), $P = 0.247$], as shown in *Figure 5A*.

3.6 Relation of plasma miR-133a with time to clinical cardiovascular event

Kaplan–Meier plot analysis for the relation between plasma miR-133a levels at entering the SAFEHEART cohort (blood sampling) and the incidence rate of MACE as a function of time revealed that miR-133a levels >0.673 (cut-off value obtained from c-statistics) were associated with a significantly higher incidence rate of CVEs (shorter time free of CVE events) within the 8-year follow-up (*Figure 6*).

By using the Cox proportional hazards regression model, the HR, calculated using a miR-133a cut-off value of 0.673, as for the Kaplan–Meier plot, was estimated at 3.89 (95% CI 1.88–8.07), $P < 0.001$ for the same follow-up period, suggesting that FH patients with high plasma miR-133a levels have a >3-fold risk to suffer a CVE-event within the 8 years after sampling for measurement of the miR-133a.

Furthermore, 5-year risk of incident CVE in FH patients was calculated according the recently published SAFEHEART risk score (SRsc-5y) that includes variables such as gender, age, weight, height, tobacco consumption, arterial hypertension, previous CVE, LDL cholesterol, and Lp(a).[29,30] The SRsc-5y had a median (LQ–HQ) value of 2.1 (0.98–4.17) and 3.74 (2.15–8.92) for the FH-nCVE and FH-CVE groups, respectively ($P < 0.001$),

In order to minimize potential bias derived from any relevant variable affecting CVE presentation in FH patients (variables included in the SAFEHEART risk score) or the sample size, we performed additional conditional logistic regression analysis (Cox regression model) using balanced groups obtained with the propensity score matching for the SAFEHEART risk score ($N = 37$/group). Results based on miR-133a cut-off value of 0.673 (sensitivity: 73% and specificity: 72%) for CVE gave an HR of 17 (95% CI 2.26–127.7; $P = 0.006$), further supporting the power of miR-133a to identify FH patients with active disease progression and high risk of incident CVEs.

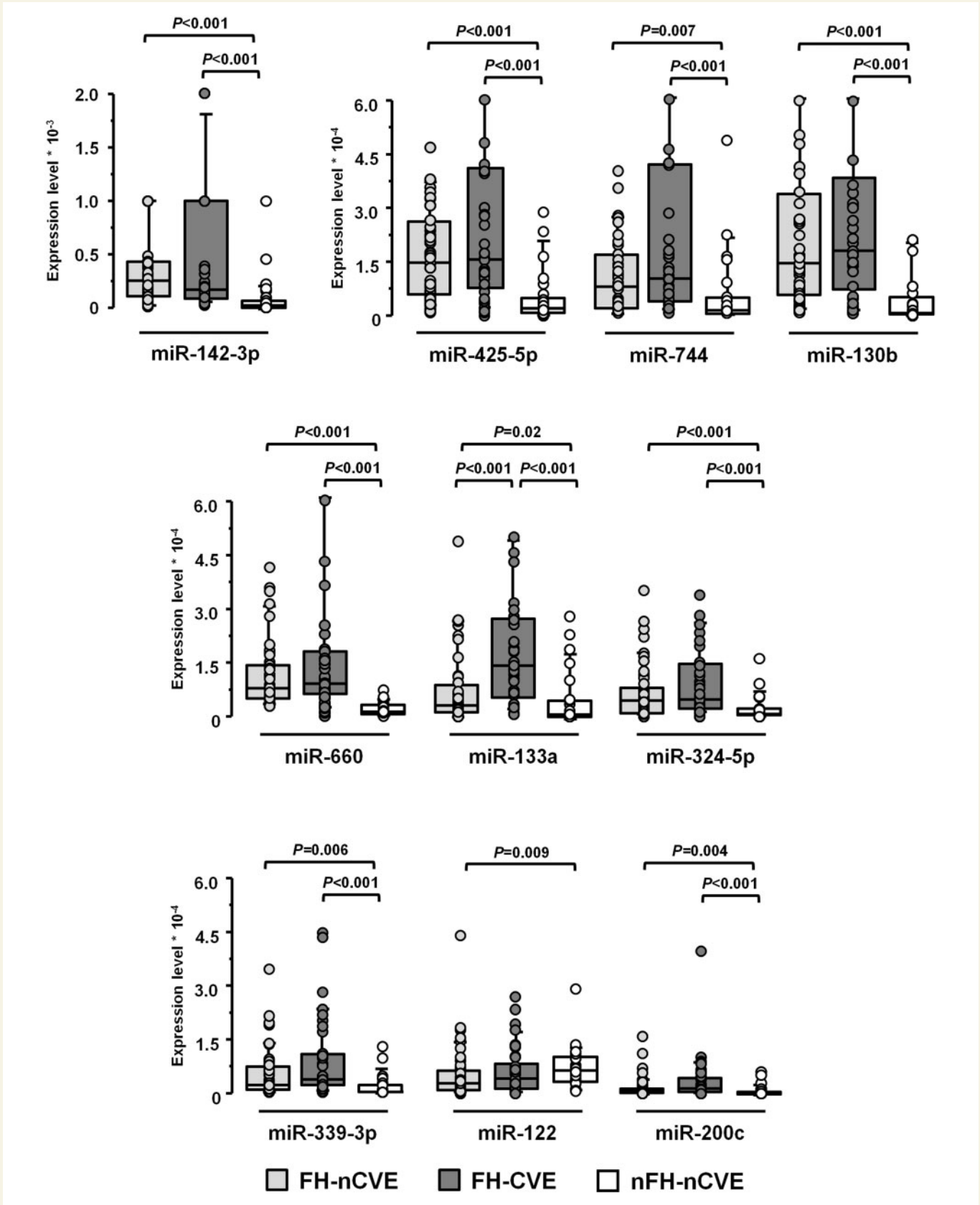


Figure 4 miRNAs redefining study in plasma. The miRNAs selected were analysed in the FH-CVE ($N = 42$), nFH-nCVE ($N = 30$), and FH-nCVE ($N = 47$) groups by the TaqMan custom array. Data are shown as box plots. *P*-values were calculated by the Mann–Whitney test and corrected by the Bonferroni test for multiple comparisons.

A

miRNAs	AUC ± SE (95% CI)	*P*-value
miR-133a	**0.76 ± 0.054 (0.66 - 0.87)**	**<0.001**
miR-339-3p	**0.65 ± 0.062 (0.52 - 0.77)**	**0.025**
miR-200c	**0.63 ± 0.064 (0.5 - 0.75)**	**0.050**
miR-744	0.62 ± 0.063 (0.5 - 0.75)	0.06
miR-130b	0.6 ± 0.064 (0.48 - 0.73)	0.115
miR-425-5p	0.6 ± 0.065 (0.47 - 0.73)	0.133
miR-324-5p	0.59 ± 0.064 (0.46 - 0.71)	0.190
miR-660	0.58 ± 0.066 (0.45 - 0.71)	0.247
miR-122	0.53 ± 0.067 (0.4 - 0.66)	0.653
miR-142-3p	0.52 ± 0.067 (0.39 - 0.65)	0.789
3-miRNA signature	**0.72 ± 0.057 (0.61 - 0.83)**	**0.001**

B

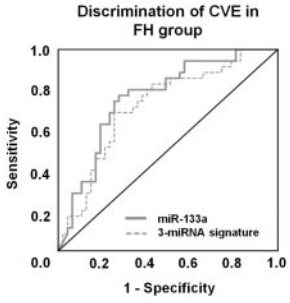


Figure 5 Discriminatory power of miR-133a for CVE. (*A*) The area under the ROC (associated receiver operating characteristic) curve (AUC) was calculated for plasma miRNA levels in FH population. (*B*) ROC curves are shown for miR-133a (continuous line) and the 3-miRNA signature (discontinuous line).

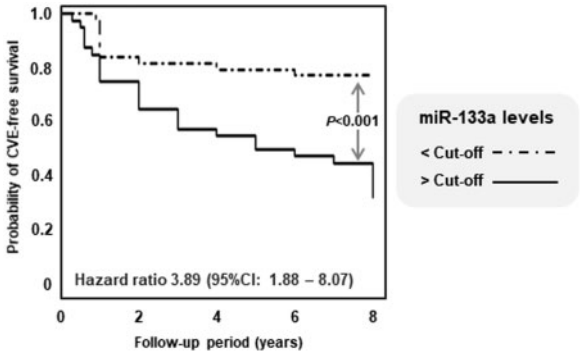


Figure 6 Kaplan–Meier survival curve analysis. FH patients were classified according to plasma miR-133a value of 0.673, as cut-off obtained from ROC analysis, during follow-up period of 8 years. Unadjusted Cox regression analysis shows the hazard ratio and *P*-value. Log-rank test: *P*-value between the divergent curves is shown.

3.7 miR-133a plasma levels in an independent group of FH patients with/without incident CVE of the SAFEHEART cohort (FH validation group)

The relationship between miR-133a plasma levels and incident clinical events was further replicated in an independent group of FH patients of the SAFEHEART cohort (FH validation group), based on a similar study design as for the FH discovery group and with 10 years of follow-up after entering the SAFEHEART cohort. Due to the current lack of standardized methods to extract and measure plasma miRNAs and to minimize the effect of the sample analysis procedure in our results, this replication study was performed with a recently developed TaqMan technology consisting in a universal reverse transcription (RT) step that incorporates a 3′ poly(A) tail and 5′ adapter sequence to amplify all mature miRNAs present within a sample (see Section 2). MiR-133a was successfully amplified in plasma of the FH subjects and those suffering a CVE within 10-year follow-up had significantly higher levels than FH subjects who did not present MACE during a similar follow-up period (FH-CVE, $N = 96$ vs. FH-nCVE, $N = 100$: 1.15 ± 0.16 vs. 0.71 ± 0.11, $P = 0.028$). In addition, as in the FH discovery group, mean values of LDLc, HDLc, TG, Lp(a), and hs-CRP according to the median value of miR-133a (0.464, as cut-off) did not show statistically significant difference (Supplementary material online, *Table S8B*). On line with this result, FH subjects suffering an event within the 5-year period ($N = 68$) had two-fold higher miR-133a plasma levels at entering the cohort than those subjects free of CVE ($N = 26$) during the same follow-up period (0.96 ± 0.14 vs. 0.38 ± 0.08, $P = 0.029$).

3.8 miR-133a effects on target genes and biological insights: *in silico* analysis

To investigate potential target mRNAs and signalling pathways affected by changes in miR-133a, we performed *in silico* analysis based on five different databases (Supplementary material online, *Table S5*). As summarized in Supplementary material online, *Figure S2*, we identified 923 potential target genes with a binding site for miR-133a. Sixty-one of these genes were included within biological functions (or disease), associated to CVE presentation (tissue injury, CVD, and inflammatory disease/inflammatory response) according to the IPA-Core Analysis. Moreover, 42 of the 61 genes were included in three independent networks associated with 'Cardiovascular disease' by IPA (Supplementary material online, *Figure S3*). The minimum hybridization free energy (MFE) values of these genes were calculated to screen optimized miR-133a-mRNA interactions. Nineteen genes were identified by their conservative binding site for miR-133a. The top 5 target genes with the lower MFE values (<-25 kcal/mol) and consequently the highest predicted binding capacity to miR-133a were selected for further *in silico* analysis (Supplementary material online, *Table S11*).

As shown in *Figure 7A*, the results of the *in silico* analysis suggest that a decreased expression of the transcription factors FOXL2 and DNAJB6 and the membrane receptor CD130, direct target genes of miR-133a were linked to the up-regulation of molecular components with a role in atherosclerosis progression, such as soluble ligands of the Wnt-mediated pathway (WNT1, WNT3A, and WNT5A), the low-density lipoprotein receptor-related protein 6 (LRP6), the cytokine receptor ILR6 and the inflammatory cytokines CXCL8, tumour necrosis factor (TNF), and interleukin (IL)6. According to the results of the IPA-analysis, FOXC1 and SYNCRIP do not participate in any established interactional network.

3.9 Gene expression regulation by high miR-133a levels in MAC and HMEC-1 cells

To further investigate the IPA-predicted mechanistic network linked to miR-133a regulation, we conducted cell culture studies based on miR-133a transfection in human monocyte-derived MAC and HMEC-1 cells. Analysis of mRNA expression focused on IPA-predicted genes such as PPARGC1A, DKK1, CTNNB1, IL6R, presumably regulated by miR-133a molecular targets, and mRNA expression of genes linked with miR-133a through signalling pathways involved in lipid metabolism and inflammatory response, according the *in silico* analysis. Non-stimulated MAC and HMEC-1 cells expressed very low levels of miR-133a (Supplementary material online, *Figure S4*), suggesting a limited gene-expression regulation mediated by miR-133a in these cells under physiological conditions. Human monocyte-derived MAC exposed to agomiR-133a resulted in a

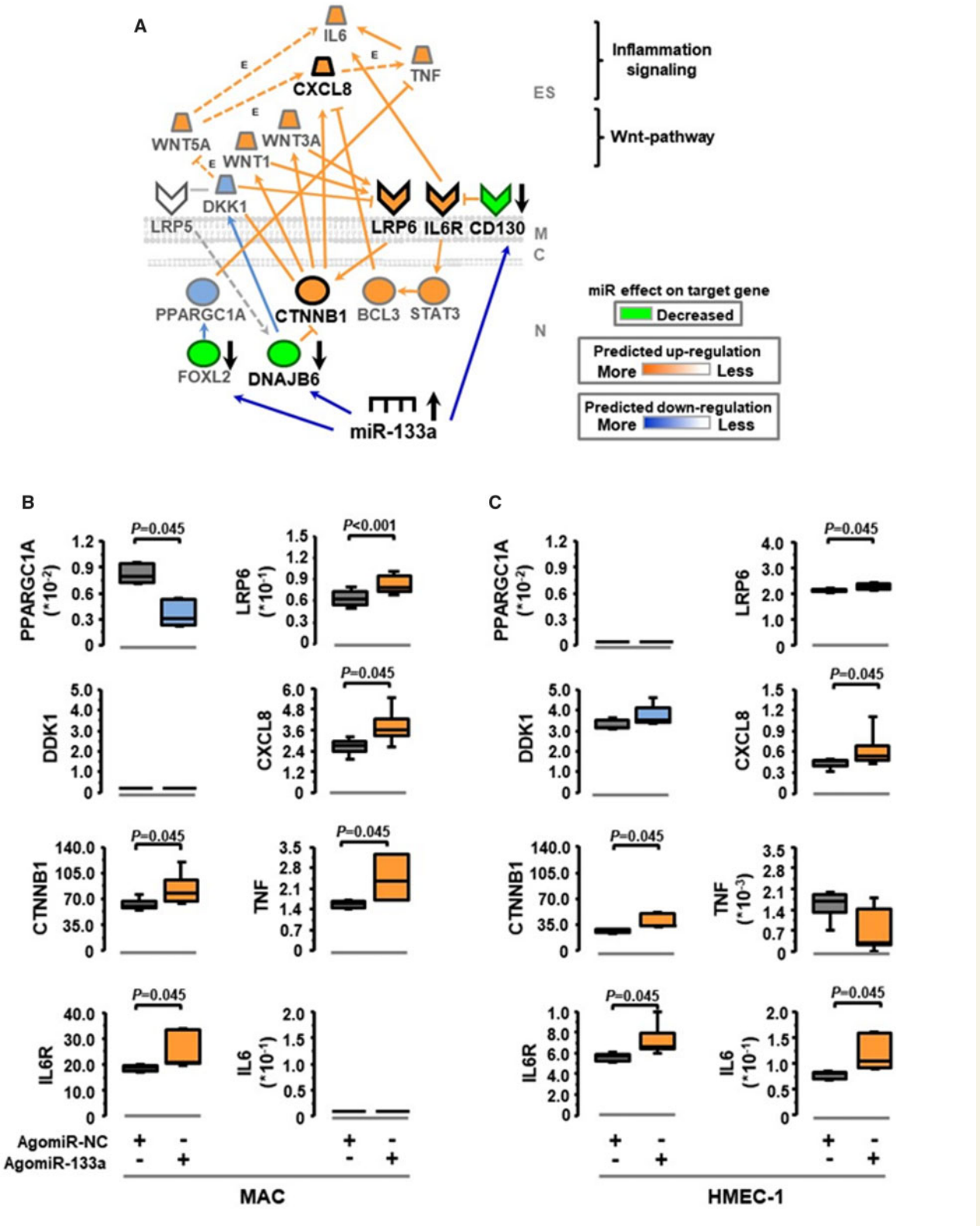


Figure 7 *In silico* analysis and gene regulation by miR-133a. (*A*) *In silico* analysis (IPA platform) for miR-133a network including FOXL2, DNAJB6, and CD130 (miR-133a target genes). Predicted gene expression is shown in orange (up-regulation) or blue colour (down-regulation) by MAP analysis. Green colour refers to the effect of miR-133a on the target genes. Molecules are shown according their cell distribution: ES (extracellular space), M (membrane), C (cytoplasm), N (nucleus). Direct molecular interactions are displayed as whole-line. Indirect interactions are given as dotted lines: E (expression). Gray line refers to low predictive power by the *in silico* analysis. (*B*) Gene expression levels in MAC and (*C*) HMEC-1 transfected with agomiR-133a or agomiR-NC ($N = 4$). Box plots represent of expression levels in duplicates by real-time PCR. In bold, genes regulated by miR-133a in MAC and HMEC-1. MAC, human monocyte-derived of macrophage; HMEC-1, human dermal microvascular endothelial cell line. *P*-value shows statistical significance.

significant increase in the cellular level of miR-133a compared to cells treated with agomiR-NC (Supplementary material online, *Figure S4*, $P < 0.001$). The increase in intracellular miR-133a went along a statistically significant increase in IL6R- and CTNNB1-mRNA expression, a decrease in PPARGC1A mRNA expression, and the up-regulation of the membrane receptor LRP6, and the inflammatory chemokines TNF and interleukin-8 (CXCL8) (*Figure 7B*). DDK1 and IL-6 mRNA expression levels in human MAC were below the detection level independently of the amount of miR-133a present in the cells (*Figure 7B*). Similarly, HMEC-1 transfection with the agomiR-133a resulted in a significant increase in IL6R and CTNNB1 mRNA expression (*Figure 7C*). DDK1mRNA was consistently expressed in HMEC-1, but its levels did not significantly differ between cells transfected with the agomiR-133a or the agomiR-NC, as control (*Figure 7C*). Furthermore, mRNA expression of LRP6, CXCL8, and IL6 was significantly increased in response to the agomiR-133a, whereas changes in TNF-mRNA levels were not statistically significant. PPARGC1A expression was below the detection limit both in agomiR-133a and agomiR-NC transfected cells (*Figure 7C*).

4. Discussion

Heterozygous FH patients, have diffuse atherosclerosis and high risk for premature MACE,[29,31,32] due to the lifelong elevated plasma cholesterol levels consequence of presenting an autosomal-dominant disorder caused by mutations in the LDL receptor.[33] These patients, however, have a high heterogeneity in the presentation of clinical disease, even between siblings. Therefore, other local and systemic genetic and molecular factors may exert significant influences to induce the progression of atherosclerosis into plaque complication/rupture and clinical event presentation. In the present study, we have used a non-targeted approach to miRNA discovery and investigated plasma liquid biopsies in different subgroups of FH- and non-FH patients from a well-characterized cohort of FH patients (SAFEHEART study—NCT02693548), in order to identify those miRNAs (single or signature) that better highlight those FH patients with active atherosclerotic plaque progression that are going to present and acute event.

Here, by top-down functional genomics and *in silico* analysis, we have identified a differential signature of 10 miRNAs (9 up-regulated, 1 down regulated) in blood of FH patients that were going to have a MACE episode within the next 8 years compared with subjects with secondary hypercholesterolaemia (without FH genetic background) and no incident CVE in the same period of time. Further inclusion in the analysis of a FH group with no evidence of incident CVE (FH-nCVE) served to identify the mature form of miR-133a (hsa-miR-133a-3p) as the best miRNA predictor of atherosclerosis progression and clinical CVE presentation in FH patients. Specifically, we demonstrated for first time, that baseline plasma levels of miR-133a (samples collected at time of inclusion in the SAFEHEART cohort) were higher in FH patients presenting a MACE within the next 8 years compared with levels in FH patients without incident CVE in the same period of time. A larger prospective study once all subjects with genetic diagnosis of FH will have completed 10-year follow-up from time of entering the SAFEHEART cohort will provide a better understanding on the power of miR-133a as a prognostic biomarker for CVD risk and patient stratification.

MicroRNAs, circulating in the bloodstream and extracellular space, are protected from degradation by specialized packaging in extracellular vesicles or RNA-binding protein.[34] Our results show that miRNAs measured in plasma are more representative of the exosome miRNA content that of that found in microvesicles of size 0.2–1.0 µm. It is plausible to think that advanced clinically relevant plaques release more exosomes; however, we cannot extrapolate from the data here presented that the increase in plasma miRNA-133a found in FH patients is due to increased released of exosomes in FH. This is a matter for further investigation. Interestingly, we have recently reported high levels of cMV in FH patients with progressive atherosclerotic lesions and incident CVEs[35] and had previously shown that cMV levels associate with atherosclerotic plaque burden (measured by CMR) in FH patients even when their LDL cholesterol levels were controlled by LLT.[2,12]

All FH patients included in the study were under high level of LLT according to guidelines[36,37] during more than 1 year before the baseline samples were collected. In addition, none of the patients were receiving LDL/Lp(a) apheresis therapy at time of inclusion in the SAFEHEART cohort nor before entering the study cohort. Therefore, differences in plasma levels of miR-133a between FH patients with and without incident CVE do not apparently relate to changes in lipid haemostasis, as suggested by Dlouha *et al.*[38] in FH patients undergoing LDL/Lp(a) apheresis. Indeed, we have here provided evidence of no affectation on miR-133a of the plasma levels of LDL, HDL, triglycerides, or Lp(a). Although abundant literature links miRNA regulation to lipid metabolism, the effect of LLT on miRNA expression levels has been less studied. Regarding miR133a, an experimental study using hyperlipidaemic- and hyperglycaemic mice models and cell culture approaches has suggested a statin-dependent inhibition of miR-133a expression in the vascular wall of hyperlipidaemic- or hyperglycaemic animals as well as in cytokine-stimulated endothelial cells.[39] To our knowledge, only a pilot study has dealt with modulation of miRNA expression by statin therapy in hypercholesterolaemic patients and suggested that this is dependent on the type of statin employed after short-term low-dose treatment.[40] In our study, patients were treated long-term with high-dose statins according to the guidelines, therefore the effect of statin treatment vs. non-statin could not be tested, but instead, the effect of the statin type could be analysed. The differences in miR-133a levels between FH-nCVE and FH-CVE groups remained statistically significant after including the type of statin treatment as covariable in the analysis.

Previous studies on miR-133a have reported the association of high levels with ST-elevation MI.[41–45] Those studies mainly focused in the post event period (acute phase), time in which miR-133a was consistently elevated compared with plasma levels in healthy controls, patients with coronary heart disease without AMI, or patients with other CVDs. In this regard, plasma miR-133a levels post-MI have shown to correlate with clinical features relevant for patient outcomes such as infarct size, microvascular obstruction, and reperfusion injury and to associate with a worse outcome within 6 months of the onset.[42] Up to now, however, studies had failed to prove the prognostic value of miR-133a in high risk STEMI patients after adjustment for traditional clinical markers.[41,42]

To our knowledge, our study demonstrates for first time that plasma levels of miR-133a predict MACE in patients with FH background and high cardiovascular risk years before its presentation. Indeed, plasma levels of miR-133a were elevated in FH patients several years preceding sudden death or an acute ischaemic event, suggesting its potential utility in terms of improving risk stratification and prognosis in FH patients with atherosclerotic plaque progression. Supporting this novel finding for miR-133a, Kaplan–Meier curves and Cox proportional hazards regression analysis showed that FH patients with high levels of miR-133a are at >3-fold higher risk of presenting a MACE within 8-year period. The fact that this differential miR-133a pattern was also found after performing additional statistical testing consisting of conditional logistic regression

analysis using balanced groups, obtained with the propensity score matched for the SAFEHEART risk score, strengthens the value of our findings regardless the pathophysiological pattern of other biological and variables affecting CVE presentation. From our study, we cannot exclude or prove the value of miR-133a for highlighting progression of atherosclerosis to clinical events in the general population or in patients with cardiovascular risk factors different to hypercholesterolaemia. In fact, the idea of using miR-133a to assess risk disease progression has been recently explored in patients with myocardial fibrosis[46] and in patients with stable coronary artery disease.[47] Although the prognostic value of miR-133a in these studies was linked to the necessity of performing myocardial biopsies or coronary catheterization, a major challenge for investigating the potential use of miR-133a as predicting biomarker in the clinical practice, these previous results are online with our present findings in plasma and exosomes of specific subgroups of FH patients. Thus, our findings and those of others warranted the need of performing further prospective studies in larger groups and longer follow-up periods to prove the value of miR-133a as diagnostic tool to discriminate patients with incident clinical CVEs within or beyond the FH background, or in patients with other CVD comorbidities and either in asymptomatic populations.

In silico analysis based on the Ingenuity Pathway Analysis (IPA) data repository of curate biological interactions revealed that miR-133a presumably targets genes involved in regulation of the cell membrane lipid-receptor LRP6 and cytokine-induced inflammation (IL6R, IL6, CXCL8, and TNF), findings that were supported by agomiR-133a transfection studies in cell culture of human monocyte-derived macrophages and microvascular endothelial cells, relevant cell components for plaque composition and evolution, independently of the FH background. Nowadays, increasing evidence points out to an association between dysfunction of the microvascular and epicardial endothelium during coronary atherosclerosis development in humans.[48] Similarly, coronary microvascular endothelial dysfunction is associated with characteristics of plaque vulnerability in epicardial atherosclerotic coronary arteries.[49] Thus, the miR-133a mediated proinflammatory profile, characterized by high levels CXCL8 and the miR-133a signalling mediator β-catenin (CTNNB1), in microvascular endothelial cells might contribute to the active progression of atherosclerotic plaques in FH patients.

FH patients, although being treated as per guidelines, have increased levels of extracellular microvesicles originated from inflammatory cells in plasma[2] as well as monocyte-derived macrophages with inflammatory phenotype[26] and an up-regulated expression of members of LDL receptor family, such as LRP5 and LRP6.[19] Several studies, including those of our group have shown a link between the LRP family and molecular targets of the Wnt pathway in the regulation of signalling processes and functions of cells that account for plaque progression and stability.[50–52] Here, we further expand our previous findings and provide evidence of a direct link between LRP6 and miR-133a in human monocyte-derived macrophages and microvascular endothelial cells through a signalling pathway mediated by Wnt in agreement with the IPA prediction. Further studies are needed to determine if the relevance of these pathways is higher in FH monocyte-macrophages since FH patients have higher rates of disease progression.

In summary, miRNAs that circulate in the bloodstream can be taken up by distant cells and exert cell-to-cell communication; therefore, they have the potential of regulating gene expression simultaneously in different tissues and cells. In the clinical context, there is a need of specific biomarker-based prognostic tests to identify changes in CVD progression that end up in a CVE presentation in FH patients. In this scenario, we described for the first time that FH patients with active atherosclerosis leading to the presentation of an acute event have increased plasma levels of miR-133a years before the onset of the event. In addition, we have demonstrated that through miR-133a patients at risk have exacerbated inflammatory responses. Taken together, our findings indicate the potential value of measuring plasma levels of miR-133a for prognostic risk stratification in patients with FH. Over the past few years, microRNAs (miRNAs) have also emerged as potential drug targets for preventing active atherosclerosis progression. In this respect, methods for selectively modulating the miRNA function *in vivo* through the delivery of inhibitory oligonucleotides or of novel molecule that could interfere with the candidate miRNA could represent novel innovative therapeutic approach for treating CVD.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

The authors acknowledge to Dr Javier Crespo (Cardiovascular-Program ICCC; Research Institute Hospital Santa Creu i Sant Pau, IIB-Sant Pau, Barcelona) for his help in flow cytometry analysis, to Dr Hernando del Portillo (ISGlobal/CRESIB Barcelona Centre for International Health Research) for his support in nanoparticle tracking analysis and, to Andreu Ferrero for his support in the statistical analysis. The authors are indebted to the patients for their valuable contribution and willingness to participate. R.E. is a postdoctoral fellow contracted by the Spanish National Network CIBER (Cardiovascular area) of the Institute of Health Carlos III. The authors thank Fundación Jesus Serra and Fundación de Investigación Cardiovascular, Barcelona, for their continuous support.

Conflict of interest: none declared.

Funding

This work was supported by the Spanish Ministry of Economy and Competitiveness of Science [PNS2016-76819-R to L.B.] and the Institute of Health Carlos III, ISCIII [FIS PI16/01915 to T.P., Red Terapia Celular TerCel—RD16/0011/0018 to L.B.]; cofounded by FEDER 'Una Manera de Hacer Europa'. Secretaria d'Universitats i Recerca del Departament d'Empresa i Coneixement de la Generalitat de Catalunya [2017 SGR 1480].

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Translational perspective

The present study in patients with familial hypercholesterolaemia (FH) shows that epigenetic markers can allow the identification of those patients that are going to present an acute clinical event within the next 2 years (average). There are currently few prognostic biomarkers able to identify subjects at risk of developing major acute cardiovascular events. Here, by using a non-targeted approach of miRNA-discovery, we show for first time that plasma levels of miR-133a have prognostic value to predict incident cardiovascular events in patients with FH treated as per guidelines. Future studies with larger independent cohorts are needed to validate the prognostic value of miR-133a in the general population.