

## Research article

## A miRNA signature linked to high lipoprotein (a) and coronary calcification in familial hypercholesterolaemia

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## ABSTRACT

**Background:** High lipoprotein(a) [Lp(a)] levels are associated with increased coronary artery calcification (CAC) in familial hypercholesterolaemia (FH) patients. However, mechanisms linking high Lp(a) with CAC remain poorly understood. In this study, we have performed a bioinformatics and system biology analysis to identify miRNAs and their target genes involved in Lp(a)-associated atherosclerotic lesion and coronary calcification in FH patients.

**Methods:** Patients with a genetic diagnosis of FH ( $n = 24$ ) from the SAFEHEART were included in the study. Plasma miRNA signature was obtained using Affymetrix miRNA microarrays from patients with FH grouped using an Lp(a) cut-off of ( $>50$  mg/dL) and presence or absence of coronary artery calcification [CCS(+) or CCS(-)]. *In silico* analyses were performed to identify potential miRNA target genes.

**Results:** Forty-two miRNAs had  $> 1.5$ -fold difference in their detection levels when grouped by Lp(a) [FH-Lp(a)  $> 50$  ( $n = 9$ ) vs FH-Lp(a)  $< 50$  ( $n = 15$ )]. Among these, 9 miRNAs were associated with CCS(+) (miR-1228-5p, miR-3940-5p, miR-1237-5p, miR-3196, miR-6765-5p, miR-6786-5p, miR-4486, miR-6821-5p and miR-1908-5p). *In silico* analysis, identified 68 target genes of these 9 miRNAs in lipid and atherosclerosis pathways (KEGG code: hsa05417). Network analysis revealed seven target genes (AKT3, APAF1, BCL2L1, TRAF6, MYD88, STAT3, and CASP9) with stronger interactions and higher binding probability for the nine-miRNA signature, mainly linked to lipid metabolism, inflammation and calcification processes.

**Conclusion:** Our results identify a miRNA signature that regulates atherosclerotic processes associated with high Lp(a) levels and CAC in asymptomatic FH patients. These findings offer new insights into the underlying mechanisms and highlight potential therapeutic targets.

## 1. Introduction

High levels of lipoprotein (a) [Lp(a)] and low-density lipoprotein cholesterol (LDL-C), as seen in familial hypercholesterolaemia (FH), are genetically determined and strongly associated with atherosclerotic cardiovascular disease (ASCVD). Notably, elevated Lp(a) levels are more commonly observed in FH patients compared to non-FH individuals [1, 2]. Moreover, the presence of both high Lp(a) and FH appears to significantly increase the risk of ASCVD [3,4]. Measuring Lp(a) could

therefore offer valuable insights for more precise stratification of ASCVD risk in FH patients, especially in cascade screening programs [5].

Lp(a) is a low-density lipoprotein (LDL)-like particle with an additional protein, apolipoprotein(a), covalently bound to apolipoprotein B-100. Lp(a) levels above 50 mg/dL are linked to an increased risk of cardiovascular disease (CVD) [6]. In particular, Lp(a) levels above this threshold in patients with FH correlate with reduced cardiovascular-free survival [3] and a higher estimated risk of developing ASCVD [7]. Despite treatment with lipid-lowering therapies (LLT) recommended by

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guidelines, subclinical atherosclerosis remains prevalent among FH patients [8]. Additionally, high Lp(a) levels are associated with a greater coronary calcium score (CCS) in asymptomatic FH patients [9]. In the general population, asymptomatic individuals with elevated Lp(a) are more likely to develop coronary calcification, with a 31 % probability for a coronary artery calcium (CAC) score > 0 and a 29 % probability for a CAC score > 100.

Atherosclerosis is a chronic inflammatory disease, induced by elevated lipids and the continuous interaction between molecular and cellular components of the blood and the arterial vessel wall [10]. Proposed mechanisms by which Lp(a) may increase the risk of ASCVD include lipid oxidation and deposition of oxidized phospholipids, prothrombotic effects, promotion of proinflammatory responses and calcification [6,11]. However, the precise pathogenic mechanisms of Lp(a) and its molecular interactions with LDL metabolism in individuals at high cardiovascular risk remain poorly understood.

MicroRNAs (miRNAs) are small non-coding RNAs involved in post-transcriptional regulation [12] of molecules involved in various pathophysiological processes, including familial hypercholesterolaemia (FH) [13], dysregulated lipid metabolism [14], and cardiovascular calcification [15]. There is growing evidence highlighting the critical role of miRNA involvement in CVD, particularly in atherosclerosis [16].

Circulating plasma miRNAs have been proposed as potential diagnostic and prognostic biomarkers of CVD [17]. In our previous studies involving FH patients, we have demonstrated that plasma miRNAs are associated with the presence of calcified plaques in coronary arteries [18] and with predicted cardiovascular events [13]. However, there is currently a lack of information on the relationship between plasma miRNAs, atherogenic Lp(a) concentrations (>50 mg/dL), and coronary artery calcification in asymptomatic FH patients.

In this study, we conducted a bioinformatics and system biology analysis to identify the miRNA signature associated with atherogenic Lp(a) levels in FH patients with subclinical atherosclerosis, differentiating those with and without coronary calcification.

## 2. Material and methods

### 2.1. Clinical population

The study included 24 patients (12 men and 12 women) with genetic diagnosis of heterozygous familial hypercholesterolaemia (FH) from the SAFEHEART cohort [18], a nationwide, real-world, multicentre, long-term prospective study [19]. The study included two groups based on Lp(a) levels: > 50 mg/dL ( $n = 9$ , 44 % women) and < 50 mg/dL ( $n = 12$ , 53 % women). As shown in [Supplementary Table S1](#), groups did not differ significantly in age [Lp(a) > 50: 50 (48–56) years vs Lp(a) < 50: 44 (42–54) years,  $p = 0.16$ ], BMI [25 (23–27) vs 24 [22–28] Kg/m<sup>2</sup>,  $p = 0.633$ ], or traditional CV risk factors (diabetes mellitus, hypertension, smoking, obesity). At the time of entering the cohort, none of the participants had clinical evidence of cardiovascular disease. However, 22 % of participants in the high Lp(a) group and 47 % in the low Lp(a) group reported a family history of premature CVD ( $p = 0.231$ ). All participants had been receiving lipid-lowering therapy, in accordance with clinical guidelines, for more than 1 year before entering the study [20]. Coronary computed tomographic angiography revealed calcified coronary arteries in 78 % of FH patients with Lp(a) > 50 mg/dL compared with 33 % of those with Lp(a) < 50 mg/dL ( $p = 0.035$ ). Median Lp(a) levels were 76.4 (65.3–102.9) mg/dL in the high group and 13.9 (7.1–22.1) mg/dL in the low group ( $p < 0.001$ ). Other lipid parameters, high-sensitivity CRP, and fasting glucose did not differ significantly between groups.

Excluded criteria were pregnancy, sepsis, inflammatory disorders, a history of cancer or clinical cardiovascular events. The study was approved by the local ethics committee of the Investigación Clínica Fundación Jiménez Díaz (protocol number: 01/09) and conducted in accordance with the Declaration of Helsinki. Informed consent was

obtained from all participants.

### 2.2. In silico study design

#### 2.2.1. miRNA profiling analysis

In a first stage, the study design ([Fig. S1](#)) compared the plasma miRNA profiles of two FH subgroups with high and low Lp(a) levels (cut-off: 50 mg/dL). This analysis was based on previously generated miRNA datasets obtained by high-throughput profiling with the Affymetrix GeneChip microRNA 4.0 and stored in the NCBI Gene Expression Omnibus (GEO) (GSE178126), as reported [18]. The GSE178126 dataset refers to the miRNA profile associated with calcified plaque, determined by the coronary calcium score (CCS) [CCS(+): score > 100 vs CCS(-): score 0].

A new analysis (GSE265997), derived from GSE178126 dataset, was performed to identify a plasma miRNA signature associated with atherogenic Lp(a) concentrations and the presence of coronary calcification. This analysis focused on Lp(a) levels, using the cut-off of 50 mg/dL was aimed to identify differentially expressed miRNAs associated with atherogenic Lp(a) concentrations [FH-Lp(a) > 50 mg/dL ( $n = 9$ ) vs FH-Lp(a) < 50 mg/dL ( $n = 15$ )]. The identified miRNA signature was further explored using bioinformatic tools to predict target genes, functional characteristics, and regulatory networks.

#### 2.2.2. Target gene prediction

Differentially detected miRNAs were identified and their annotations validated in miRBase v22.1 (<https://www.mirbase.org/>), a registry of published miRNA sequences that includes both stem-loop and mature structures.

Potential target genes with a high predicted probability of being regulated by the selected miRNAs were obtained using the miRWalk search algorithm (<http://mirwalk.umm.uni-heidelberg.de/>), a publicly comprehensive resource that predicts miRNA binding sites across complete gene sequences.

In addition, the miRWalk was also used to assess the robustness of the miRNA-target gene interactions by calculating the minimum free energy (MFE), expressed as a negative real value in kcal/mol; lower (more negative) MFE values indicate stronger miRNA-target gene binding [13].

#### 2.2.3. Integrated analysis of gene and miRNA functions

Genes associated with lipid metabolism and atherosclerosis were obtained from the KEGG database (<https://www.genome.jp/pathway/hsa05417>) and analysed together with the identified miRNA target genes.

Tissue-specific expression patterns of the selected miRNAs and their target genes were obtained from publicly available resources: the miRNA Tissue Atlas ([https://ccb-compute2.cs.uni-saarland.de/mirnatissueatlas\\_2025](https://ccb-compute2.cs.uni-saarland.de/mirnatissueatlas_2025)) and the Genotype-Tissue Expression (GTEx) portal (<https://www.gtexportal.org/home/>). The miRNA tissue atlas and GTEx are comprehensive sources to study human miRNA/gene expression profiles across a wide range of human tissues.

Enrichment analysis of target genes were performed using ShinyGO v0.82 (<http://bioinformatics.sdstate.edu/go/>), focusing on Gene Ontology (GO) terms in biological processes, molecular functions and signalling pathways (Reactome database). The analysis is based on Ensembl gene ID and STRING-db protein IDs. Functional enrichment was assessed using the hypergeometric test, with false discovery rate (FDR) correction by the Benjamini-Hochberg method, and fold enrichment was used to quantify the magnitude of gene representation.

#### 2.2.4. Interaction network of miRNAs and target genes

Genes associated with atherosclerosis and their molecular affinities by protein-protein interaction were analysed using STRING Protein query and MCODE, both integrated into the Cytoscape v3.10.3 platform (<https://cytoscape.org/>). Cytoscape is a network analysis platform

software that provides a basic set of functions for data integration, visualization, and molecular profiling through multiple applications and different database connections.

2.3. Statistical analysis

Initial descriptive analysis was provided using number of cases (*n*) and percentages for qualitative variables and medians [Interquartile

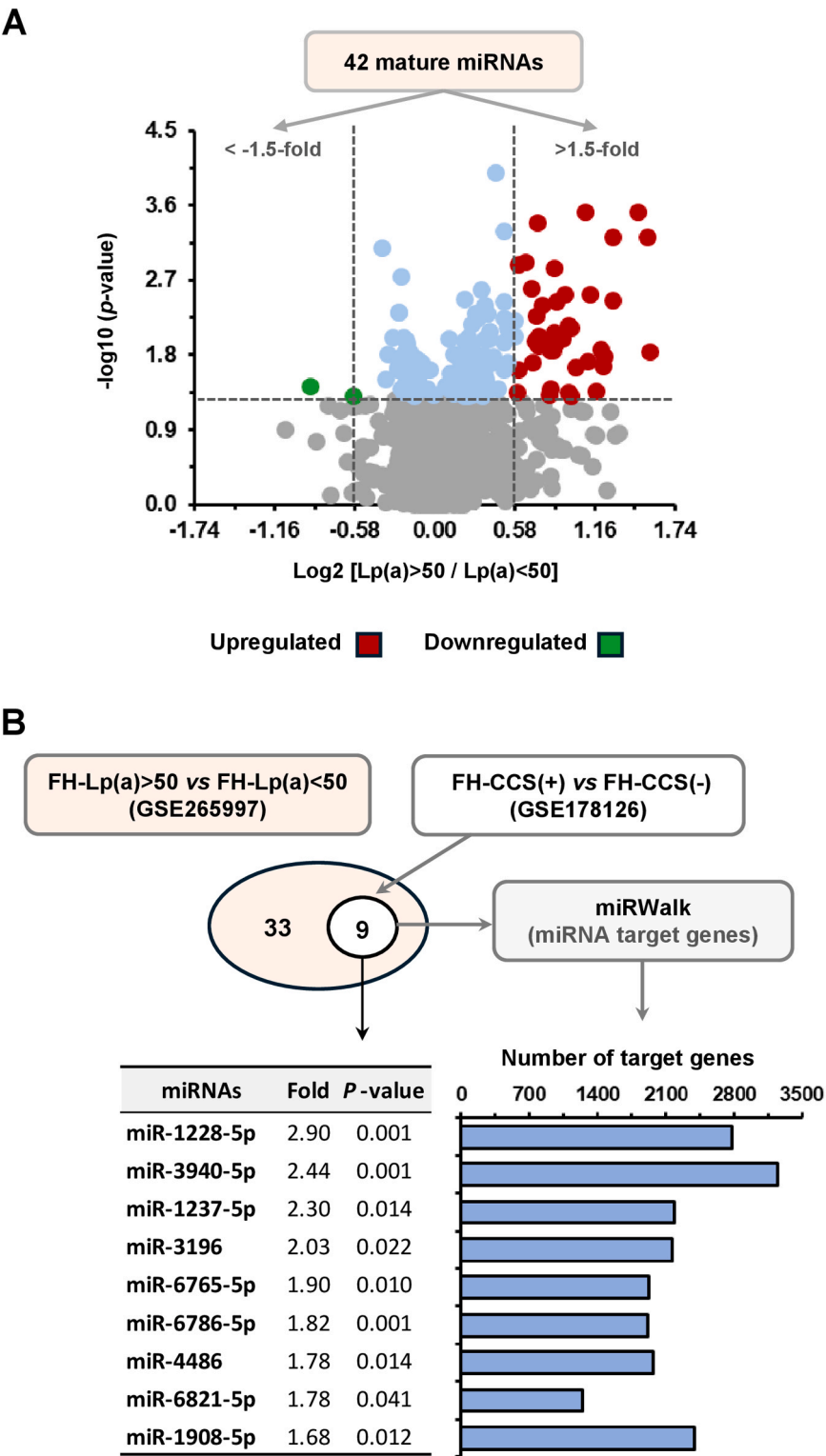


**Fig. 1.** Affymetrix microarray analysis. Circulating plasma miRNAs analysed by microarray in FH patients [GSE265997: FH-Lp(a)> 50 mg/dL (*n* = 9) vs FH-Lp(a)< 50 mg/dL (*n* = 15)]. **(A)** Results are shown in volcano plot for mature miRNAs. Dots refer to fold change (log2) for FH-Lp(a)> 50 / FH-Lp(a)< 50 related to Student's *t* test *p*-values (-log10). miRNAs with *p*-values > 1.30 [-log10(0.05)] are indicated. Differential changes of 50 % (greater than -0.58, [log2(1/1.5)] or > 0.58, [log2(1.5)]) and values of *p* > 1.30 were selected for additional studies. **(B)** Nine differential miRNAs for GSE265997 analysis were also found in GSE178126 dataset, presence vs absence of coronary calcium score [FH-CCS(+) vs FH-CCS(-)]. Number of miRNA target genes were obtained by miRWalk database.

range: IQR (Q1–Q3)] for quantitative variables. Differences between groups for frequencies of qualitative variables such as sex, cardiovascular risk factors and drug therapy were compared by chi-squared test and quantitative variables were compared by Student's *t*-test and the non-parametric test Mann-Whitney U. The statistical analysis was carried out using StatView 5.0.1 and SPSS statistics version 29.  $p < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. miRNA profiling in FH patients with high Lp(a) levels

In a previous study, we identified 4603 miRNAs (pre-mature and mature forms) in the plasma of FH patients ( $n = 24$ ) using the Affymetrix microRNA 4.0 system [18]. In the present study, we re-examined the complete plasma miRNA dataset, stratifying patients by Lp(a) levels with a cut-off of 50 mg/dL [GSE265997; high Lp(a),  $n = 9$ ; low Lp(a),  $n = 15$ ].

Of the 4603 circulating miRNAs, 285 showed significantly different detection levels in plasma ( $p < 0.05$ ) between individuals with high- and low- Lp(a) levels, with corresponding to mature forms. Among these, 42 mature miRNAs exhibited  $> 1.5$ -fold differences in expression (40 upregulated, 2 downregulated) in the high- versus the low- Lp(a) group (Fig. 1A; detailed information in Table S2).

Genomic distribution analysis of the 42 differential miRNAs revealed that six miRNAs (miR-6805-5p, miR-3940-5p, miR-4530, miR-6789-5p, miR-150-5p and miR-1227-5p) were located on chromosome 19, while none were mapped to chromosomes 1, 4, 5, 8, 12, 18, or sex chromosomes X and Y. These findings suggest a genomic pattern of epigenetic regulation by miRNAs in FH patients with high Lp(a) (Table S2).

Comparison of the 42 differential miRNAs associated with high plasma Lp(a) with those of the differential miRNA signature previously reported by our group for coronary calcium status [CCS(+)] vs [CCS(-)] in FH patients (GSE178126 dataset) [18], identified nine miRNAs (miR-1228-5p, miR-3940-5p, miR-1237-5p, miR-3196, miR-6765-5p, miR-6786-5p, miR-4486, miR-6821-5p and miR-1908-5p) that were significantly upregulated in patients with both Lp(a)  $> 50$  mg/dL and CCS(+) (Fig. 1B and Table S2). These nine miRNAs (9-miRNA signature) were selected for further *in silico* analysis.

#### 3.2. Target genes for the 9-miRNA signature associated with high-Lp(a) / CCS(+) in FH patients

*In silico* analysis using the miRWalk algorithm (binding site: 3UTR, binding probability score = 1 and class: protein-coding) was performed to identify potential target genes regulated by the nine differential miRNAs. A total of 9590 predicted target genes were obtained, with five miRNAs each having more than 2000 target genes (Fig. 1B). Focusing on these target genes, we explored molecular interactions and signalling pathways related to lipid metabolism and atherosclerosis in the FH-Lp(a)  $> 50$  mg/dL group. Based on the KEGG database and analysed through Cytoscape, 129 target genes were identified within the “lipid and atherosclerosis” pathway (hsa05417).

Further analysis using STRING (species: *Homo sapiens*, network type: full STRING network, confidence cut-off: 0.7 and additional interactors: 0) revealed that 68 genes (52.7 %) corresponded to target genes of the 9-miRNA signature within the hsa05417 pathway (Fig. S2). Of the nine miRNAs, miR-1908-5p, miR-1228-5p and miR-3940-5p had  $\geq 20$  target genes each, whereas miR-1237-5p, miR-3196, miR-4486, miR-6765-5p, miR-6786-5p and miR-6821-5p had  $\leq 17$  target genes (Table S3).

To refine the analysis, Minimum Free Energy (MFE) was used as a predictor of miRNA-mRNA interaction stability. Among the 68 targets initially identified, we selected those with the greatest thermodynamic stability (lowest MFE values) for each miRNA (top 10), and that also

presented multiple binding sites (MBS  $\geq 2$  miRNAs) for the 9-miRNA signature. This approach identified 39 different target genes with MFE values  $< -23.5$  Kcal/mol, 28 of which had MBS  $\geq 3$  miRNAs (Table S3). As shown in Fig. 2, these subsets of target genes formed two clusters: one associated with caspase pathways (CASP3, CASP6, CASP8, CASP9, APAF1 and BCL2L1) and another with inflammatory signalling (IL1B, IRAK4, TLR4 and MYD88).

#### 3.3. Features of functions for the 9-miRNA target genes

We investigated the tissue expression patterns of the 9 miRNAs and their 39 selected target genes. For miRNAs, we used the ncRNA pattern tool from the miRNA Tissue Atlas database, while for target gene expression, we utilized the GTEx portal. As shown in Fig. 3A, 78 % of the miRNAs were expressed in whole blood and their target genes expressed in lymphocytes (23 %), whole blood (18 %), and arterial vessel wall (13 %).

The 39 target genes were analysed using the ShinyGO database to classify them according to Reactome canonical pathways and Gene Ontology (GO) terms for biological processes (BP) and molecular functions (MF) (Table S4 and Fig. 3B). Cytokine signalling emerged as the top Reactome pathway, with the lowest FDR value (2.16E-16) and the highest number of genes involved (21), contributing to 41–54 % of GO\_BP, including stress response, cell communication, and immune system processes. Innate immunity showed similar involvement, associated with 41–54 % of GO\_BP and 21 genes. For GO\_MF, the most represented categories were enzyme binding (41 %), catalytic activity (28 %), signalling receptor binding (33 %), protein kinase binding (26 %), and cytokine receptor binding (23 %, most of which were associated with cytokine signalling GO\_BP). The TLR4 cascade displayed the highest fold enrichment (43.72), involving 12 target genes with 26–31 % participation in GO\_BP and 13–23 % in GO\_MF. Overall, these analysis highlight cytokines signalling and innate immunity as the two main functional processes regulated by the 9-miRNA signature.

#### 3.4. Molecular interactions of the 9-miRNA target genes

Network analysis using STRING and MCODE (Cytoscape bioinformatics tools) revealed that 67 % of the 39 target genes were interconnected through protein-protein interactions, forming three clusters. Cluster 1 included 15 genes (score 8.3), Cluster 2 included 8 genes (score 6.3) and Cluster 3 included 3 genes (score 3.0), with MAPK14, APAF1 and ATF6 serving as the respective seed molecules for Cluster 1, Cluster 2 and Cluster 3 (Fig. 4).

Further *in silico* analysis using Reactome tool from ShinyGO database revealed that Cluster 1 was associated with immune system pathways (14 genes), cytokine signalling (FDR: 2.81E-14) and signalling by interleukins (fold enrichment: 27.63) as the main Reactome categories. The target genes of Cluster 2 were related to apoptosis (8 genes, FDR: 9.70E-15), particularly its intrinsic pathway (fold enrichment: 286.01). Cluster 3 did not reach statistically significant associations with any signalling pathway (Fig. 4). Based on these results, the 23 genes from Cluster 1 and Cluster 2 were selected for further *in silico* analysis.

#### 3.5. Target genes of the 9-miRNA signature related to Lp(a) regulation and calcification

To elucidate potential interactions between our 9-miRNA signature targets and established genes in Lp(a)-related and vascular calcification pathways, we performed an additional *in silico* protein-protein interaction using Cytoscape platform.

Using STRING, with a confidence cut-off of 0.7, we identified 81 apo(a)-related genes and an additional 81 genes directly associated with a group of 12 calcification-related genes that we had previously reported [18]. As shown in Fig. S3, these two gene sets were linked to the 23 target genes included in Cluster 1 and 2, with FN1 emerging as the

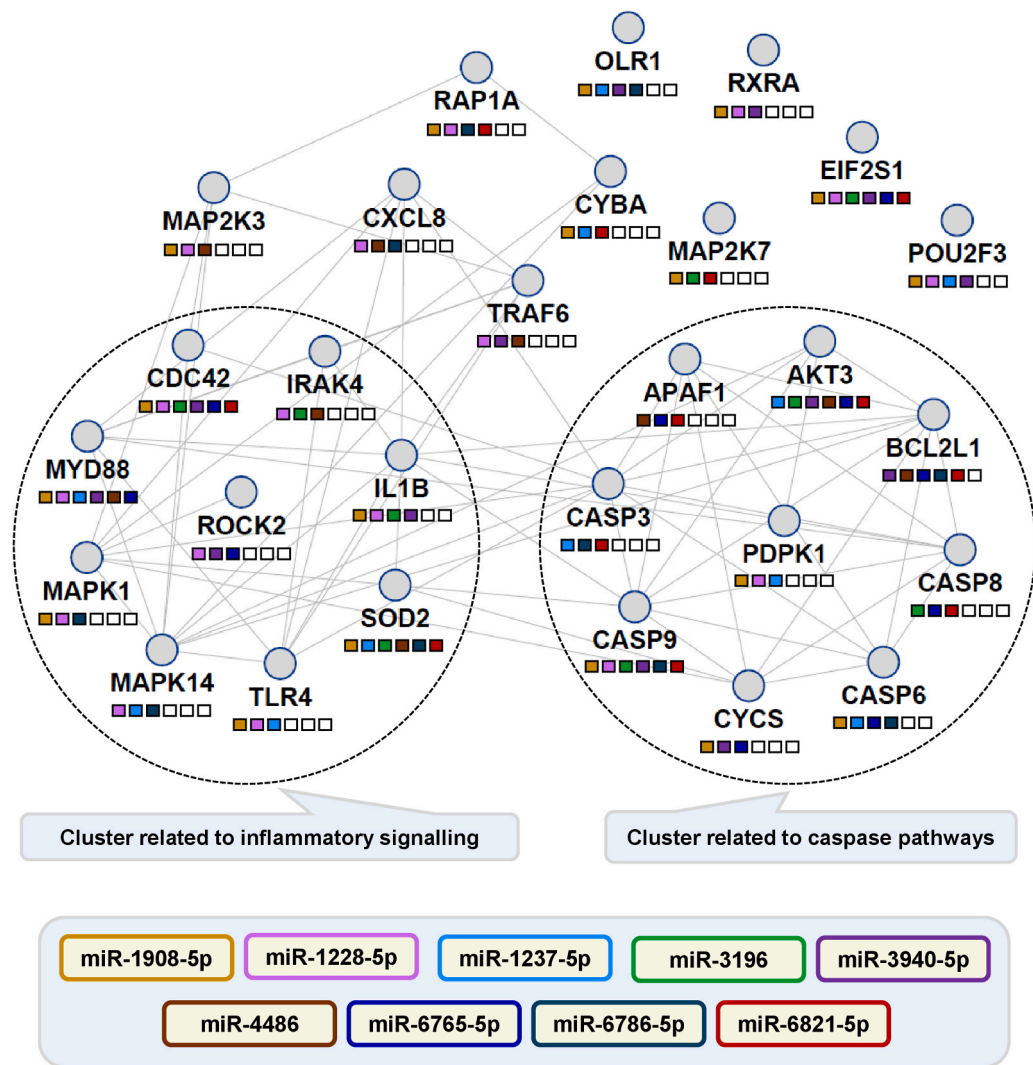


**Fig. 2.** The 9 miRNA target genes within lipid and atherosclerosis pathway. The molecular interactions obtained by STRING (Cytoscape platform) between target genes with  $\geq 3$  multiple binding sites for the specific miRNAs (miR-1228-5p, miR-1237-5p, miR-1908-5p, miR-3196, miR-3940-5p, miR-4486, miR-6765-5p, miR-6786-5p and miR-6821-5p). Colour pattern in each target gene indicates the binding sites of the miRNAs.

primary connector between Lp(a) and the calcification process. First-neighbour analysis revealed interactions among the 23 target genes with 29 genes related to Lp(a) and 45 genes of pro-calcification (Fig. S3 and Fig. 5). Predicted binding sites for miRNA-target interactions (Table S3 and Fig. 5) indicated strong regulatory potential based on MFE values for miR-6821-5p with AKT3 (MFE:  $-37.1$  Kcal/mol), miR-6765-5p with APAF1 (MFE:  $-34.6$  Kcal/mol), and miR-6786-5p with BCL2L1 (MFE:  $-32.5$  Kcal/mol). Additional significant interactions were observed for miR-1228-5p, miR-1237-5p, miR-3940-5p, miR-3196, miR-1908-5p, and miR-4486 with their respective targets (MYD88, STAT3, CASP9 and TRAF6), all with MFE values below  $-28$  Kcal/mol. These findings suggest a complex regulatory network of the 9-miRNA target genes associated with Lp(a) and coronary calcification, in which miR-6821-5p shows the strongest predicted binding capacity.

4. Discussion

Lp(a) contributes significantly to ASCVD through its high content of oxidized phospholipids, which exert pro-inflammatory effects [21], and through potential prothrombotic effects via apolipoprotein(a) [11,22]. Elevated Lp(a) levels ( $>50$  mg/dL) are an independent predictor of ASCVD in familial hypercholesterolaemia (FH) patients, even under

lipid-lowering therapy (LLT) [3,8], and are associated with higher coronary calcium scores (CCS) and atherosclerotic lesion progression [9]. In this hypothesis generating discovery study, we compared two groups of FH patients without prior clinical coronary artery disease. The groups did not differ significantly in any of the variables included in the SAFEHEART risk equation for cardiovascular risk prediction in FH, except for Lp(a) levels [23]. Notably, FH patients with Lp(a)  $> 50$  mg/dL showed a trend toward higher 5-year CVD risk scores, although this did not reach statistical significance. In addition, a positive association has been reported between CCS and Lp(a) in population studies among people who have not yet developed a coronary event [24]. Building on these clinical observations, our study applied an *in silico* systems biology approach to identify a distinct profile of plasma miRNAs associated with elevated Lp(a) levels and coronary calcification in FH patients.

Plasma miRNAs, which are known to modulate lipoprotein metabolism and atherosclerosis [13,25,26], have been associated with FH and ASCVD risk, but their relationship with elevated Lp(a) levels is poorly understood.

Our *in silico* analysis of a non-targeted plasma miRNA profile of patients with genetic FH [18] and different LP(a) levels identified a distinct 9-miRNA signature (miR-1228-5p, miR-3940-5p, miR-1237-5p, miR-3196, miR-6765-5p, miR-6786-5p, miR-4486, miR-6821-5p and

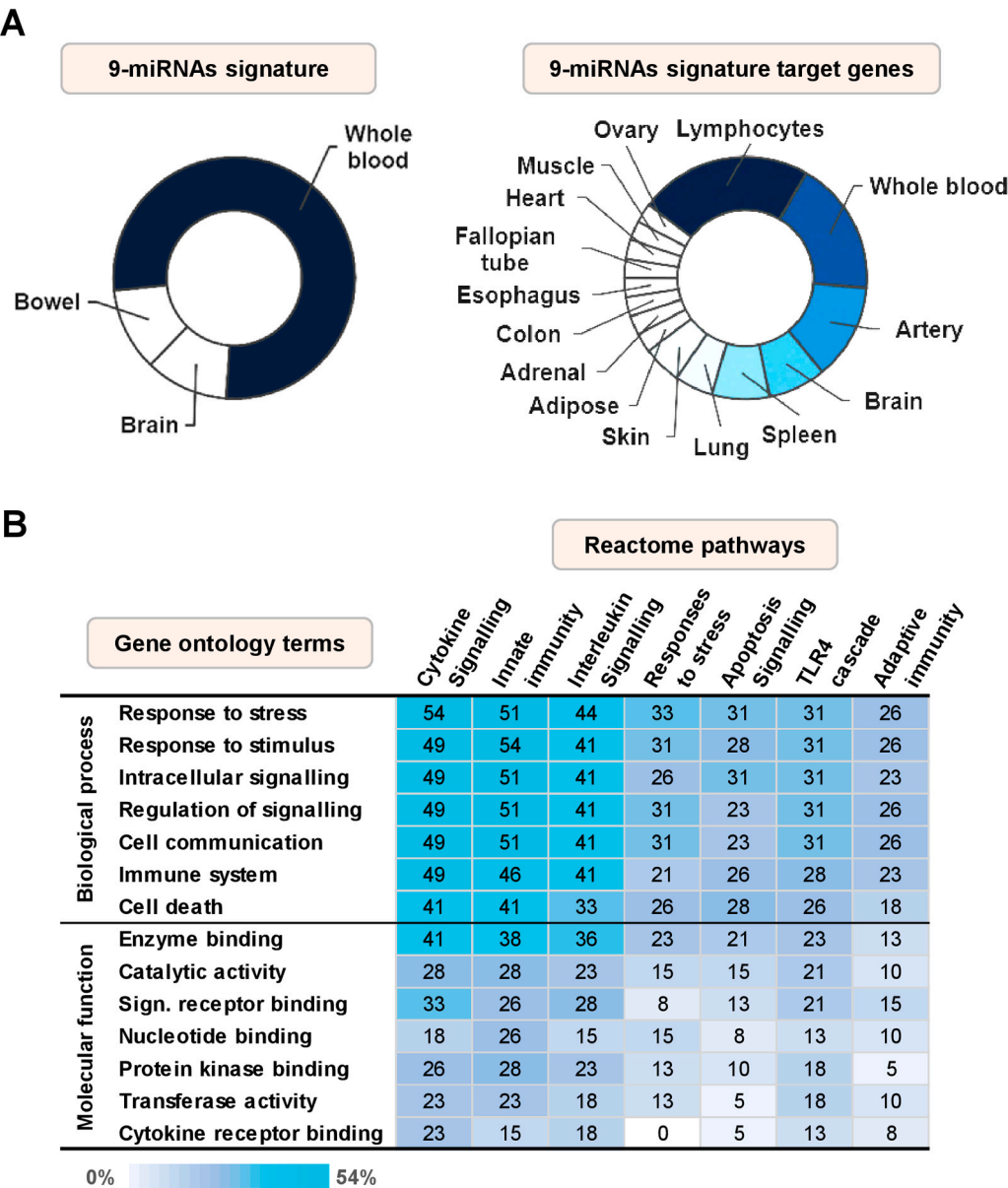


**Fig. 3.** Features of functions of 9-miRNA target genes. **(A)**Tissue origin of the 9 miRNAs and their target genes was obtained by miRNA tissue atlas- and GTEx portal-database. **(B)** Relationship between Reactome pathways and gene ontology (GO) terms for biological process and molecular functions obtained by ShinyGO platform. The participation of the GO terms with reactome pathways is indicated in percentage and colour intensity.

miR-1908–5p) associated with Lp(a) levels > 50 mg/dL and coronary calcification (Agatston score >0). These findings suggest a common cluster of epigenetic regulators at the interplay between Lp(a) and coronary calcification. Bioinformatics and system biology analysis of the 9 miRNAs and their target genes revealed FN1 (encoding fibronectin), as the mayor molecular connector among genes related to high Lp(a) levels and coronary calcification. Fibronectin, a key extracellular matrix (ECM) component, regulates cell adhesion, migration, growth, and differentiation [27], and is expressed in areas with atherosclerotic plaque [28]. FN1 has also been reported to be highly expressed in calcified aorta in experimental models of vascular calcification [29]. Moreover, recent studies have shown that the apo(a) exhibits proteolytic activity on fibronectin, potentially altering vascular integrity [30]. Our bioinformatic *in silico* study, highlights multiple potential FN1-mediated gene-gene interactions associated to Lp(a) and calcification processes that are regulated by the 9-miRNA signature, providing valuable information for preventing or treating atherosclerosis in individuals with high Lp(a) levels.

The 9-miRNA signature identified in this study includes components that have been linked to key biological processes associated with atherosclerosis. These include inflammatory regulation (miR-6786–5p) [31], neurological functions (miR-1228–5p and miR-1237–5p) [32,33], thrombotic complication (miR-6765–5p)[34], cell survival (miR-3196 and miR-4486)[35,36], modulation of immune responses (miR-3940–5p) [37] and lipid metabolism (miR-1908–5p) [38]. *In silico* analysis also revealed that the potential target genes of the 9-miRNA signature are involved in regulating the lipid and atherosclerosis pathways, particularly cytokine and apoptosis signalling. Together, these findings suggest a functional link between the miRNA signature, elevated levels of Lp(a) and calcified plaques in FH patients. This provides new insights into the epigenetic regulation of atherosclerosis in this high-risk population.

Our analysis of miRNA-target genes, focusing on those with the lowest MFE values identified genes associated with Lp(a) metabolism and pro-calcification processes. These include AKT3, APAF1, BCL2L1, TRAF6, MYD88, STAT3 and CASP9. These genes, regulated by the 9-

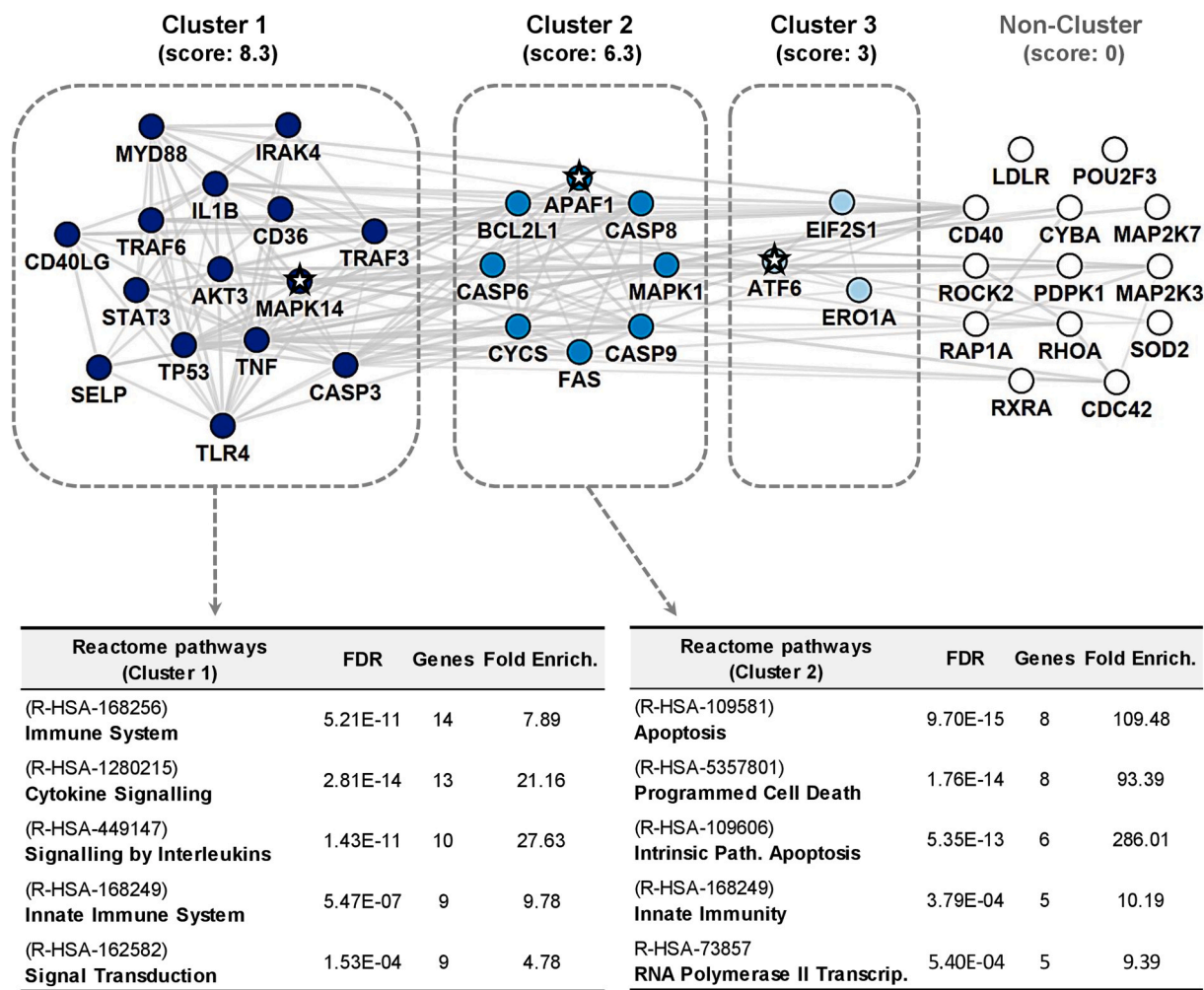


**Fig. 4.** Molecular network analysis of 9-miRNA target genes. Molecular interactions and clustering algorithm for the target genes obtained by STRING Protein query and MCODE, both bioinformatics tools of Cytoscape platform. Seed clusters are indicated with a star symbol. Network connections are defined by protein-protein interaction and clusters by scoring. Reactome pathways for Cluster 1 and Cluster 2 are sorted by number of target genes and related to FDR values and Fold enrichment.

miRNA signature associated with Lp(a) > 50 mg/dL and calcified plaques in FH, are involved in critical cellular processes, including cellular response (AKT3), apoptosis (APAF1, BCL2L1 and CASP9), inflammatory processes via the TRAF protein family (TRAF6), IL-1 and TLR signalling via the adapter protein (MYD88), and the STAT family in response to cytokines (STAT3).

Atherosclerotic plaque formation and inflammation have been related to myeloid differentiation factor 88 (MYD88) gene, which lays a key role in Toll-like receptor (TLRs) signalling [39] by interacting with IRAK4 and signalling to NF-κB and MAPK [40].

Studies in atherosclerosis-prone mice have shown that genetic deficiency of MYD88 or lack of TLR4 significantly reduces the area of aortic plaque [39]. In calcific aortic valve disease, MYD88 upregulation has been associated with increased pro-inflammatory cytokine secretion, calcified nodule formation, and elevated levels of pro-calcifying genes [41]. Moreover, the myddosome complex, which is formed by MYD88 and interacts with TRAF6, triggers inflammatory responses by activating STAT3 signalling [42]. These findings emphasise the significance of MYD88 and its associated pathways in the context of Lp(a)-related atherosclerosis and calcification in FH patients.

The miRNA signature associated with Lp(a) targets several caspase genes (CASP6, CASP8 and CASP9), which are involved in apoptotic pathways [43]. Among them, CASP9, a key component for apoptosome activation [44], contains binding sites for 66 % of the components of the

differential miRNA-signature in FH patients with high Lp(a) levels. CASP9 expression varies across atherosclerotic lesion types, being higher in fatty streaks than in unaffected intima, initial lesions, and lipofibrous plaques [45]. This suggests differential CASP9 regulation associated with the lipid and atherosclerosis pathways in FH patients with high Lp(a) levels and coronary calcification.

Previous studies in murine models have demonstrated the important role of AKT3 in atherosclerosis progression [46,47]. AKT3 deficiency has been associated with larger atherosclerotic lesions, increased macrophage infiltration, and foam cell formation [46]. Similarly, AKT3 knockdown in human monocyte-derived macrophages induces cholesterol accumulation, which contributes to foam cell formation [47]. Furthermore, AKT3 expression has been linked to calcification processes in endochondral ossification [48]. Together, these findings highlight the potential significance of AKT3 regulation by our identified miRNA signature in the context of Lp(a)-associated atherosclerosis and calcification in FH patients. This suggests that downregulation of AKT3 by the miR-6821-5p, the miRNA predicted to interact most strongly with the 9-miRNA signature, could decrease the atherosclerotic progression towards calcified plaque in FH patients with elevated Lp(a).

Lipid-lowering therapies such as statins and PCSK9 inhibitors are known to modulate microRNA expression, thereby influencing pathways involved in cholesterol metabolism and atherosclerosis [49,50]. In our study population, all patients were receiving statin therapy, whereas

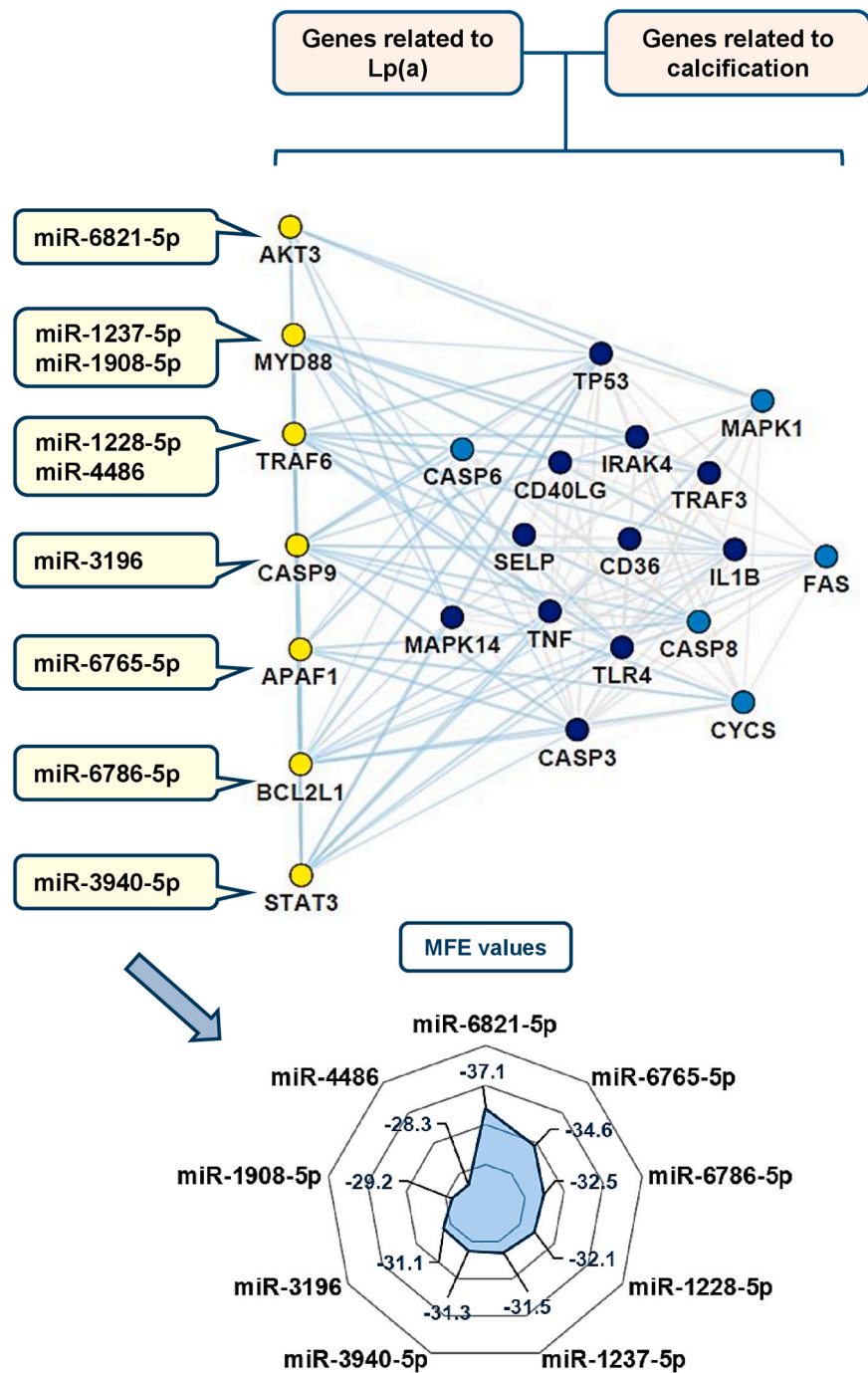


**Fig. 5.** Relationship of 9-miRNA target genes and genes related to Lp(a) and calcification. Molecular interactions of the miRNA target genes associated to Lp(a) and calcification process obtained by STRING Protein query. Targets genes highlighted in yellow indicate the miRNA-target gene interactions with strong regulatory potential based on MFE. Radial graphs show the minimum free energy (MFE) obtained by miRWalk for the 9 miRNAs. MFE is expressed as a negative real value in kcal/mol, more negative MFE values indicate a higher likelihood of miRNA-target gene binding.

none had been treated with PCSK9 inhibitors, which may modulate miRNAs profiles differently [51,52]. Statins have been reported to affect both circulating and cellular miRNAs, including let-7d-5p and miR-92a, which regulate pathways involved in inflammatory responses, lipid homeostasis and vascular endothelial function [51,53,54]. By comparison, PCSK9 inhibitors have been associated with distinct miRNA changes, notably an increase in the expression of miR-483-5p, which is linked to the regression of atherosclerotic changes and reductions in Lp (a) levels in high-risk patients [52,55]. Therefore, we cannot exclude the possibility that the miRNA profiles observed in our study reflect statin-driven modulation in the presence of elevated Lp(a), while the

potential additional effects of PCSK9 inhibitors on miRNA regulation remain to be elucidated in future studies.

Taken together, these considerations highlight both the novelty and the exploratory nature of our findings. However, this bioinformatics and system biology study, designed to be hypothesis-generating, had certain limitations, primarily due to its sample size. Nevertheless, our findings provide a rationale for further studies in a larger FH population, encompassing patients with diverse Lp(a) levels and varying degrees of atherosclerotic plaque severity.

In summary, consistent with previous research identifying distinct miRNA signatures associated with familial hypercholesterolemia and

cardiovascular risk, we identified a nine-plasma miRNA signature in asymptomatic FH patients with elevated Lp(a) levels (>50 mg/dL) and evidence of coronary calcification. This represents a novel epigenetic axis linked to atherogenesis in this high-risk population. Prior studies using LDL/Lp(a) apheresis and pharmacologic lipid lowering therapies have shown modulation of miRNAs involved in cholesterol homeostasis, inflammation, and endothelial dysfunction, including miR-92a, miR-21, miR-126, miR-185, and miR-33b [56]. Here, using a systems biology approach, we extend these findings and highlight a new set of predicted target genes associated with high Lp(a) and positive coronary calcium scores in FH patients. Taken together, our results suggest an epigenetic axis linking elevated Lp(a) with vascular damage and may support the development of novel biomarkers and therapeutic strategies for individuals with FH. Importantly, future studies in the SAFEHEART cohort will be needed to validate whether one or more of the identified miRNAs and their target genes improve patient risk stratification when incorporated alongside the SAFEHEART risk equation.

### CRedit authorship contribution statement

**Teresa Padro:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Lina Badimon:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Pedro Mata:** Writing – original draft, Resources. **Marta Mauri:** Writing – original draft. **Juan F Sánchez Muñoz-Torrero:** Writing – original draft. **Rafael Escate:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization.

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### Declaration of Competing Interest

L.B. declares to have acted as SAB member of Sanofi, Ionnis and NovoNordisk; to have received speaker fees from Sanofi and NovoNordisk and to have founded the Spin-off Ivastatin Therapeutics S (all unrelated to this work). T.P. declares to be co-founder of the Spin-offs Ivastatin Therapeutics S (unrelated to this work). The remaining authors have nothing to disclose.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.csbj.2025.09.026](https://doi.org/10.1016/j.csbj.2025.09.026).

### Data Availability

The datasets and computer code produced in this study are available

in the following databases:•

Affymetrix microarray (GeneChip microRNA 4.0) dataset: miRNA profiling

GSE178126 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178126>)•

Affymetrix microarray (GeneChip microRNA 4.0) dataset: miRNA profiling

GSE265997 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE265997>)

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