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Original Research Article

Profiling of differentially expressed MicroRNAs in familial hypercholesterolemia via direct hybridization

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ABSTRACT

Background: Individuals with homozygous familial hypercholesterolemia (HoFH) have a severe clinical problem in their first decade of life, which is not usually present in heterozygous FH (HeFH) individuals. For this latter group of patients, FH diagnosis is mostly severely delayed with a significant increase in the risk of angina, myocardial infarction, peripheral artery disease, stroke, and cardiovascular and all-cause mortality.

Methods: This study used various bioinformatics tools to analyze microarray data and identify critical miRNAs and their target genes associated with FH and its severity. Differentially expressed serum miRNAs from direct hybridization microarray data in three groups of subjects: healthy, HeFH, and HoFH. The differential expressed miRNAs were determined according to a log of fold-change (LFC) <-0.5 or >0.5 and of $p < 0.05$. Then, we assessed their target genes *in silico*. Gene ontology (GO) enrichment was applied by Cytoscape. The protein-protein interaction and co-expression network were analyzed by the STRING and GeneMANIA plugins of Cytoscape, respectively.

Results: We identified increased expression of circulating hsa-miR-604, hsa-miR-652-5p, and hsa-miR-4451 as well as reduced expression of hsa-miR-3140-3p, hsa-miR-550a-5p, and hsa-miR-363-3p in both group of FH *vs.* healthy subjects. Higher levels of hsa-miR-1183, hsa-miR-1185-1-3p, hsa-miR-122-5p, hsa-miR-19a-3p, hsa-miR-345-3p, and hsa-miR-34c-5p were detected in HeFH in respect to HoFH when compared to healthy subjects. Most upregulated miRNAs mainly affected gene related to cardiac myofibrillogenesis, cholesterol synthesis, RNA editing for apolipoprotein B, and associated with LDL-cholesterol levels. In contrast, down-regulated miRNAs mainly affected gene related to plasma biomarker for coronary artery disease, lipids metabolism, cell adhesion and migration, genetic predictors of type 2 diabetes and cholesterol metabolism. The essential genes were primarily enriched in GO regarding biological regulation, intracellular nucleic acid binding, and the KEGG pathway of TGF-β signaling.

Conclusions: The case-control nature of this study precluded the possibility of assessing the predictive role of the identified differentially expressed miRNAs for cardiovascular events. Therefore, the signature of miRNAs reflecting the pathogenesis of both HeFH and HoFH.

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Abbreviation gene

QKI Quaking
PLAG1 Pleiomorphic Adenoma Gene 1 Protein
CELF2 CUGBP Elav-Like Family Member 2
CSNK1G3 Casein Kinase 1 Gamma 3
BRWD1 Bromodomain And WD Repeat Domain Containing 1
SH3TC2 SH3 Domain and Tetratricopeptide Repeats 2
SLC9A6 Solute Carrier Family 9 Member A6
NTRK2 Neurotrophic Receptor Tyrosine Kinase2
PTEN Phosphatase And Tensin Homolog
CPEB2 Cytoplasmic Polyadenylation Element Binding Protein 2
LPP LIM Domain Containing Preferred Translocation Partner In Lipoma
ONECUT2 One Cut Homeobox 2
HMGA2 High Mobility Group AT-Hook 2
TRIM2 Tripartite Motif Containing 2
KSR2 Kinase Suppressor of Ras 2
ASPH Aspartate Beta-Hydroxylase
ATP2B4 ATPase Plasma Membrane Ca^{2+} Transporting 4
LCOR Ligand Dependent Nuclear Receptor Corepressor
SNTB2 Syntrophin Beta 2
MEF2A Myocyte Enhancer Factor 2A
TNRC6B Trinucleotide Repeat Containing Adaptor 6B
TCF7L2 Transcription Factor 7 Like 2
ZNF704 Zinc Finger Protein 704
ATRX ATRX Chromatin Remodeler
FBXW7 F-Box and WD Repeat Domain Containing 7
ERBB4 Erb-B2 Receptor Tyrosine Kinase 4
IRGQ Immunity-Related GTPase Q
RSBN1 Round Spermatid Basic Protein 1
CREBRF CREB3 Regulatory Factor
LUC7L3 LUC7 Like 3 Pre-MRNA Splicing Factor
ZFX Zinc Finger Protein X-Linked
ARCN1 Archain 1
BCL11B BCL11 Transcription Factor B
SYNJ1 Synaptojanin 1
SOX4 SRY-Box Transcription Factor 4
PCSK9 Proprotein convertase subtilisin/kexin type 9
APOB Apolipoprotein B
LDLR Low-density lipoprotein receptor
TGFβ Transforming Growth Factor Beta
PI3K Phosphatidylinositol 4,5-Bisphosphate 3-Kinase
AKT AKT Serine/Threonine Kinase

1. Introduction

Familial hypercholesterolemia (FH) is known as a disease caused by a mutation in lipoprotein metabolism characterized by evaluated levels of low-density lipoprotein cholesterol (LDL-C) and a higher risk of coronary heart disease (CHD) [1]. Individuals with HoFH have a severe clinical problem in their first decade of life, which do not usually present in HeFH [2–6]. An LDL-C higher than 190 mg/dL and pathogenic variant genes have a 22-fold increased risk for coronary artery disease (CAD), while high LDL-C lacking pathogenic variants caused a 6-fold increased risk [7]. The molecular mechanisms of accelerated atherosclerosis in HeFH and HoFH have not been elucidated. Basic research in recent years has identified many molecules pertinent to the atherosclerotic process, which have improved our understanding of the underlying process of pathology, including microRNAs (miRNAs).

MiRNAs are non-coding RNAs, with approximately 22- nucleotides long, involved in gene expression regulation. Their roles in the molecular mechanisms and development of human dysmetabolic diseases have been shown in CHD pathophysiology [8–12]. Recent studies have shown that miRNAs have critical roles in regulating lipid metabolism and, therefore, are known as novel therapeutic approaches for hyperlipidemia [13]. Importantly, a change in the miRNA expression may alter its corresponding target gene expression, even cellular metabolism and homeostasis [14,15]. Previous studies have addressed unique alterations of gene expression and miRNAs in pediatric subjects with FH [16–19]. For example, the expression levels of hsa-miR-486-3p, hsa--miR-941, and BIRC5 in peripheral blood were found to be upregulated in HoFH compared to the healthy subjects [20]. Moreover, miR-24-3p and miR-130a-3p were reduced in circulating microvesicles of FH patients compared with controls [21]. miR-505-3p, which controls chemokine receptor up-regulation, was reported to be down-regulated in monocyte-derived macrophages of patients with FH [22]. It has also been found that miR-133a was significantly higher in FH patients suffering from a cardiovascular event within an 8-year follow-up period compared with FH patients not experiencing an event within the same time period of follow-up [23].

Thus, in this study, we performed a microarray analysis of miRNAs direct hybridization for the first time. We used various bioinformatic tools to identify critical and shared miRNAs and their target genes in three groups of subjects: HeFH patients, HoFH patients, and healthy subjects to have alternative biomarkers to assess a microRNA signature associated with the presence and severity of FH.

2. Materials and methods

2.1. Subjects and study design

Study populations: This case-control study comprised 16 patients with HoFH (case group) and 15 HeFH (control group) from family members of HoFH. This study was observational and approved by the IRB and Ethics Committee of the Mashhad University of Medical Sciences. Fifteen healthy individuals were also included as another control group. Selection criteria were defined previously [24]. According to the Dutch Lipid Clinic Network Criteria, FH scores were calculated for patients with suspected FH. After that, next-generation sequencing (NGS) and Sanger sequencing were used for the detection of mutations APOB, LDLR, and PCSK9 genes. In addition, pathogenic mutations, which were previously reported according to the ClinVar database, as well as novel mutations whose pathogenicity was found by the SIFT database and PolyPhen software, were defined. All participants fulfilled the written informed consent, and their medical history information and blood samples were collected; part of the serum samples was kept as such, and part was lyophilized and stored at -80 °C. Biochemical analyses were done using commercial kits (Pars Azmoon, Iran).

2.2. miRNA microarray

RNA/miRNA (100 ng) was applied for the n-counter flex of NanoString Technology. Firstly, the lyophilized serum was reconstituted in the same volume with nuclease-free water. Then, the total RNAs were extracted using miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany). A detailed method is reported in Supplementary data. Then, the Excel raw table data was exported for further bioinformatics analysis.

2.3. Microarray data analysis

The differential expressed miRNAs were determined according to a log fold-change (LFC) <-0.5 or >0.5 and $P < 0.05$. In addition, microarray data was used to compare miRNAs in groups of HeFH/healthy, HoFH/healthy, HeFH/HoFH, and HeFH and HoFH/healthy. Using

Table 1
Baseline features of patients.

Variables		FH (N = 31)		Healthy (N = 15)	*p*-Value
		HeFH (N = 15)	HoFH (N = 16)		
Sex (%)	Male	53.3	43.8	33.3	0.543
	Female	46.7	56.3	66.7	
Age (y)		30.2 ± 2.2[a]	14.1 ± 2.7	36.0 ± 1.6[a]	<0.001
TC (mmol/L)		6.8 ± 0.5[a]	16.1 ± 1.2	4.0 ± 0.2[a,b]	<0.001
TG(mmol/L)		1.6 ± 0.3	2.2 ± 0.4	1.1 ± 0.1[a]	<0.01
HDL-C (mmol/L)		1.6 ± 0.4	1.5 ± 0.1	1.3 ± 0.1	0.477
LDL-C (mmol/L)		4.7 ± 0.6[a]	10.9 ± 1.2	2.5 ± 0.2[a]	<0.001
FH score		15.0 ± 0.0	25.2 ± 0.6	–	<0.001
Number of patients with xanthoma symptoms (%)		0.0	100	–	<0.001
Number of patients with MI history (%)		0.0	56.3	–	
Mutation (%)	Previously reported	62.5	69.2	–	0.751
	Novel	37.5	30.8	–	
Mutation type (%)	Missense	46.2	60.0	–	<0.001
	Truncated	23.1	20.0	–	
	SNV	23.1	13.3	–	
	SNP	7.7	0.0	–	
	Missense, truncated	0.0	6.7	–	
Position of LDLR mutation (%)	Exon	57.1	84.6	–	0.290
	Intron	42.9	15.4	–	
Drugs consumption (%)	No drug	92.3	12.5	100	<0.001
	Only statin	7.7	18.8	0	
	Statin + ezetimibe	0	68.8	0	

Data are shown as mean ± SE; [a]: Significant in comparison with HoFH group; [b]: Significant in comparison with HeFH group.
HDL-C: High-density lipoprotein cholesterol; FH: Familial hypercholesterolemia; HeFH: Heterozygous familial hypercholesterolemia; HoFH: Homozygous familial hypercholesterolemia; LDL-C: Low-density lipoprotein cholesterol; mmol/L: Millimoles per liter; TC: Total cholesterol; TG: Triglyceride; y: Year. MI: Myocardial infarction. SNV: Single nucleotide variant; SNP: Single nucleotide polymorphism.

SRPLOT tools (http://www.bioinformatics.com.cn/), volcano plots were created to identify significantly differentially expressed microRNAs. In addition, heatmaps were created to display the microRNA expression profiles of the samples. The heatmap was made by the R software ("ggplot2" package). Co-differentially expressed miRNAs in heterozygous and homozygous FH groups were identified based on the LFC <-0.5 or >0.5 and $P < 0.05$. Venn plot was created to illustrate upregulated and downregulated miRNAs in HeFH and HoFH. Gene ontology (GO) as well as KEGG analysis of the predicted targets of the miRNAs were performed by DIANA miRPath tools (https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath).

2.4. Identification of target genes

miRDIP database (https://ophid.utoronto.ca/mirDIP/) was used to predict target genes of differently expressed microRNAs. These bioinformatic tools identified miRNA-gene targeting based on the results of 30 distinct databases.

2.5. Functional enrichment analysis of target genes

GO enrichment was performed by Cytoscape (BiNGO plugin) into Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) were created. Moreover, GO enrichment analysis parameters of BP, CC, and MF were obtained from http://geneontology.org/and three in a 1-bar plot created by SRPLOT tools.

2.6. Interaction networks of target genes

The GeneMANIA plugin in Cytoscape software performed the functional interaction networks. Moreover, the STRING tools were used to identify protein-protein interactions. Besides, targeted genes were clustered using STRING.

2.7. Statistical analysis

DESeq2 R package was used to perform data normalization based on miRNA raw counts. DESeq2 R package uses a statistical model to correct the variability in the microarray data and provides accurate results for identifying differentially expressed genes. Statistically significant differentially expressed microRNAs were defined as LFC <- 1 or >1 and $P < 0.05$.

3. RESULTS

3.1. Patients' characteristics

Groups differed in terms of age and lipid profile (Table 1). The HOFH group included significantly younger individuals (mean age 14.1 years) than the two other groups (30.2–36 years). Total cholesterol (TC) and LDL-C were the highest in HoFH patients than the other groups; also, TC levels showed significantly greater values in HeFH than in healthy subjects. In addition, triglyceride (TG) levels showed notably higher levels in HoFH than in healthy subjects. By contrast, HDL-C concentration showed no significant differences between the three studied groups. Furthermore, all subjects with HoFH had xanthomas; most (56.3%) experienced a myocardial infarction (MI). Therefore, the FH score was expected to be higher in the HoFH than in the HeFH group. All healthy (100%) and most HeFH (92.3%) individuals did not use any drugs, and just 7.7% of HeFH were on statin medication alone, while most HoFH patients (68.8%) were on both statin and ezetimibe therapy and only 18.8% of them were on statin medication.

3.2. miRNA microarray analysis

26 co-differentially expressed miRNAs that showed $|LFC| > 0.5$ and $P < 0.05$ (compared to the healthy group) in both heterozygous and homozygous samples were named key miRNAs. Moreover, it illustrated the overlapping between the two data sets using the Venn plot (Fig. 1).

16 miRNAs were upregulated in both HeFH vs. healthy and HoFH vs. healthy groups (Table 2).

10 miRNAs were downregulated in both HeFH vs. healthy and HoFH vs. healthy groups (Table 3).

Results showed high expression of hsa-miR-604, hsa-miR-652-5p, and hsa-miR-4451 as well as decreased expression of hsa-miR-3140-3p, hsa-miR-550a-5p, and hsa-miR-363-3p as highlighted in bold in Tables 2 and 3, respectively, based on the |LFC| compared with the healthy subjects. Volcano plots and heatmaps were created to compare HeFH *vs* healthy, HoFH *vs* healthy, HeFH *vs* HoFH, and total FH *vs* healthy (Fig. 2). Volcano plots were created to identify significantly differentially expressed microRNAs. Heatmaps were created to display the microRNA expression profiles of the samples. To distinguish HeFH form by HoFH hsa-miR-1183, hsa-miR-1185-1-3p, hsa-miR-122-5p, hsa-miR-19a-3p, hsa-miR-345-3p, and hsa-miR-34c-5p seems the best candidate since they are higher in HeFH then HoFH highlighted with underneath in Table 2.

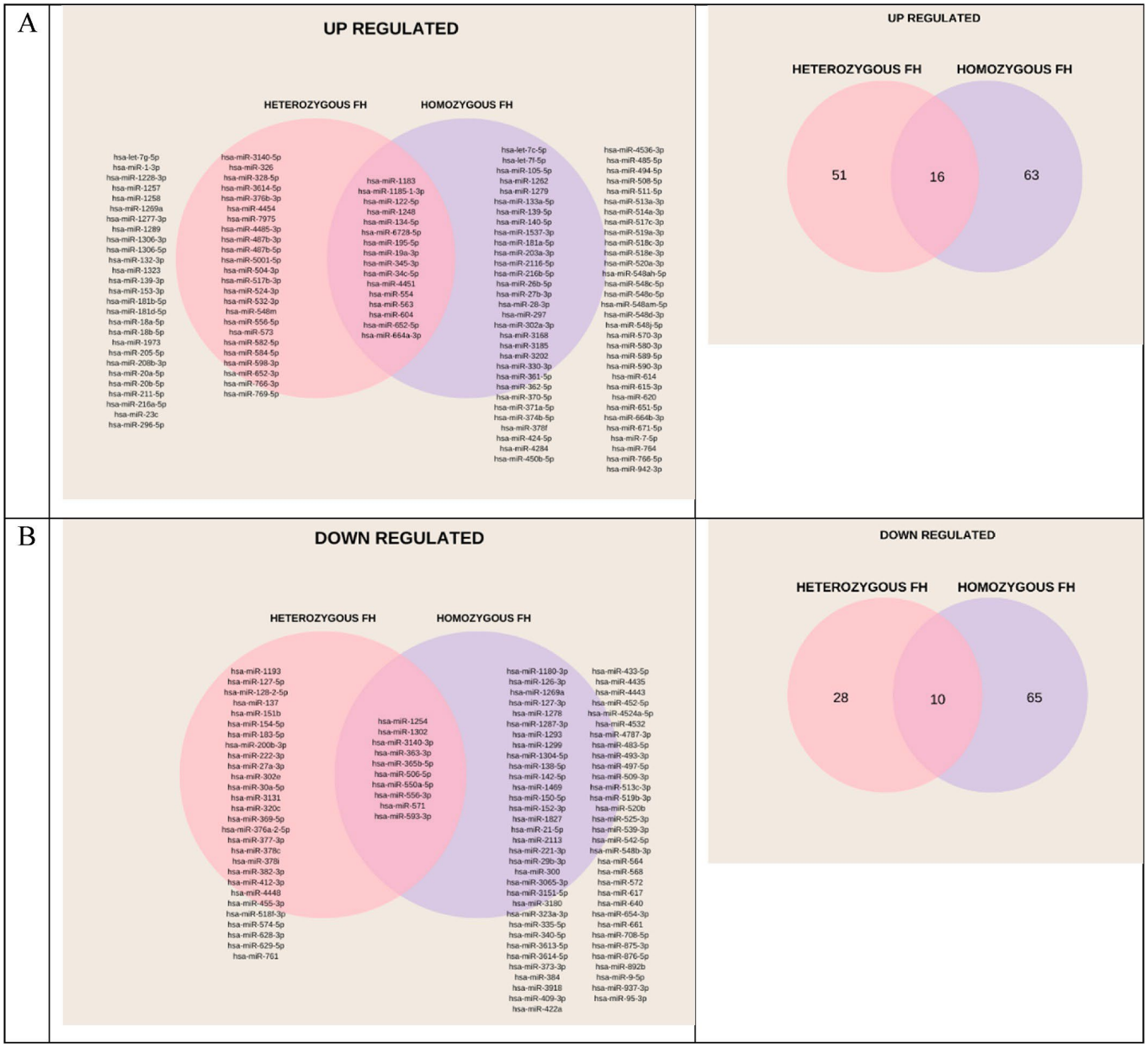

Fig. 1. Venn plot of key miRNAs in both HeFH and HoFH. (A) Venn plot shows the number of distinct and overlapping upregulated miRNAs in HeFH and HoFH. (B) Venn plot shows the number of distinct and overlapping downregulated miRNAs in HeFH and HoFH.

Table 2
List of 16 co-overexpressed miRNAs in HeFH and HoFH samples.

miRNA	LFC in HEFH	LFC in HOFH	mirbase accession No
hsa-miR-1183	0.943416	0.395929	MI0006276
hsa-miR-1185-1-3p	0.678072	0.192645	MI0003844
hsa-miR-122-5p	0.724366	0.389947	MI0000442
hsa-miR-1248	0.616671	0.616671	MI0006383
hsa-miR-134-5p	0.900464	0.719892	MI0000474
hsa-miR-6728-5p	0.900464	0.719892	MI0022573
hsa-miR-195-5p	0.526069	0.921997	MI0000489
hsa-miR-19a-3p	0.736966	0.473931	MI0000073
hsa-miR-345-3p	1	0.443607	MI0000825
hsa-miR-34c-5p	1.115477	0.378512	MI0000743
hsa-miR-4451	**0.902703**	**0.782409**	**MI0016797**
hsa-miR-554	0.736966	0.943416	MI0003559
hsa-miR-563	0.547488	0.440573	MI0003569
hsa-miR-604	**0.959358**	**1.04182**	**MI0003617**
hsa-miR-652-5p	**1.070389**	**0.691878**	**MI0003667**
hsa-miR-664a-3p	0.724366	0.341037	MI0006442

Table 3
List of 10 co-downregulated miRNAs in HeFH and HoFH samples.

miRNA	LFC in HEFH	LFC in HOFH	mirbase accession No
hsa-miR-1254	−0.70044	−0.80735	MIMAT0005905
hsa-miR-1302	−0.9027	−0.60572	MIMAT0005890
hsa-miR-3140-3p	**↓ 1**	**↓ 1.32193**	**MIMAT0015008**
hsa-miR-363-3p	**↓ 0.70627**	**↓ 1.14439**	**MIMAT0000707**
hsa-miR-365b-5p	−0.81943	−0.81943	MIMAT0022833
hsa-miR-506-5p	−0.65896	−0.50696	MIMAT0022701
hsa-miR-550a-5p	**↓ 1.19265**	**↓ 0.89308**	**MIMAT0004800**
hsa-miR-556-3p	−0.64636	−0.76184	MIMAT0004793
hsa-miR-571	−0.53051	−0.68806	MIMAT0003236
hsa-miR-593-3p	−0.64046	−0.40439	MIMAT0004802

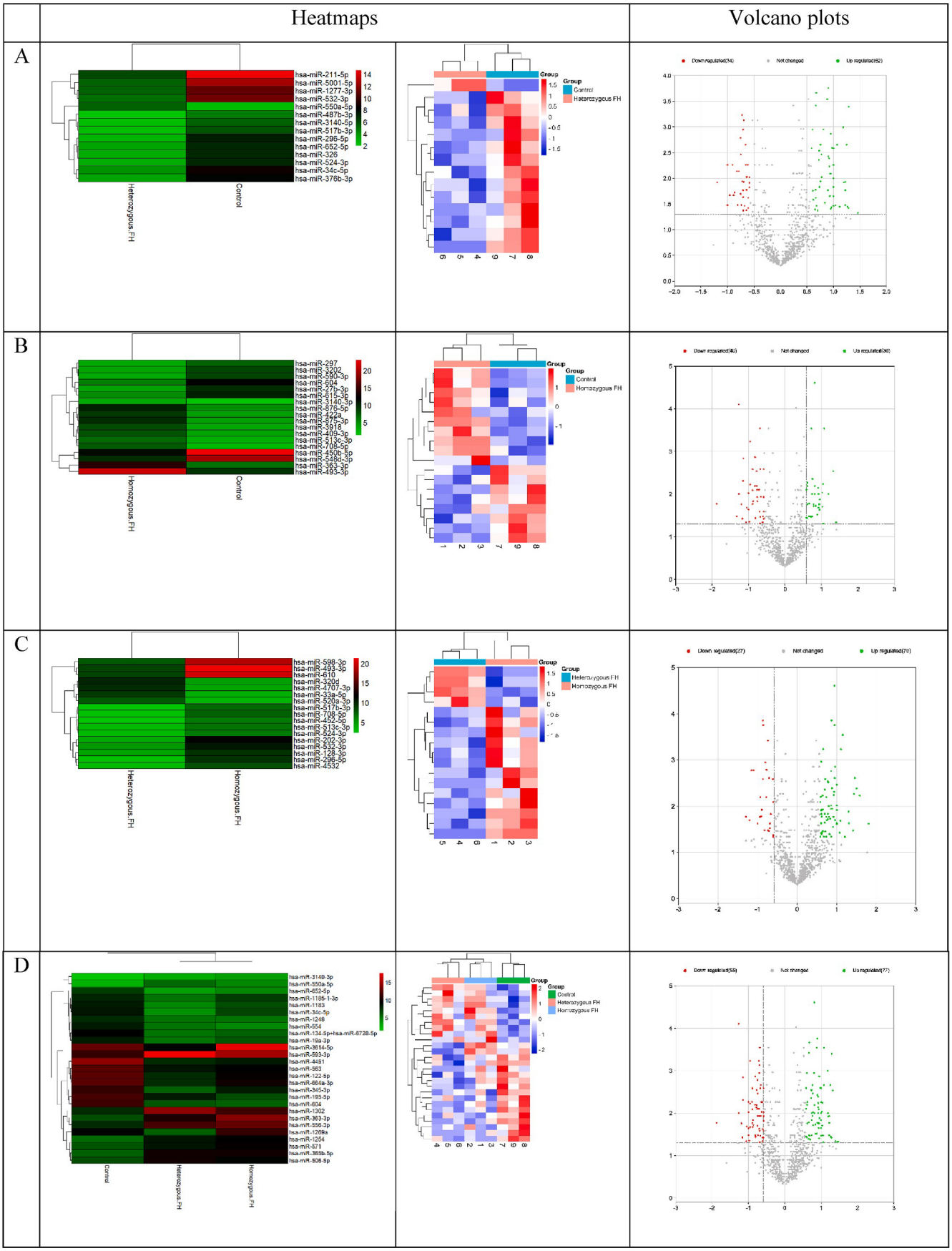

Fig. 2. Volcano plots and heat maps. (A) Heterozygous FH vs healthy. (B) Homozygous FH vs healthy. (C). Heterozygous FH vs Homozygous FH. (D). Heterozygous FH and Homozygous FH vs healthy.

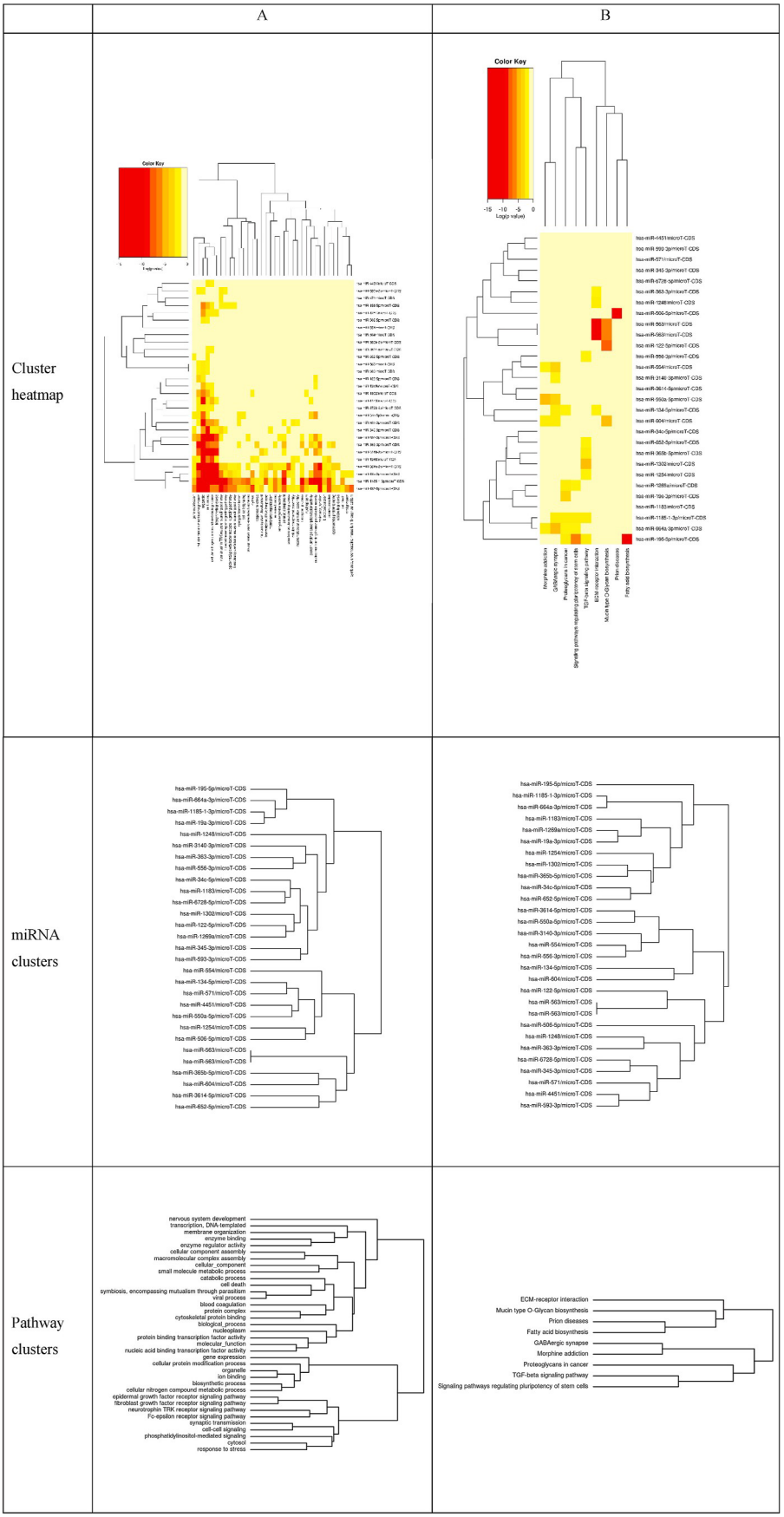

Fig. 3. Significance clusters heatmaps, miRNA clusters, and pathway clusters of key miRNAs in heterozygous FH and homozygous FH. (A) GO. (B) KEGG analysis.

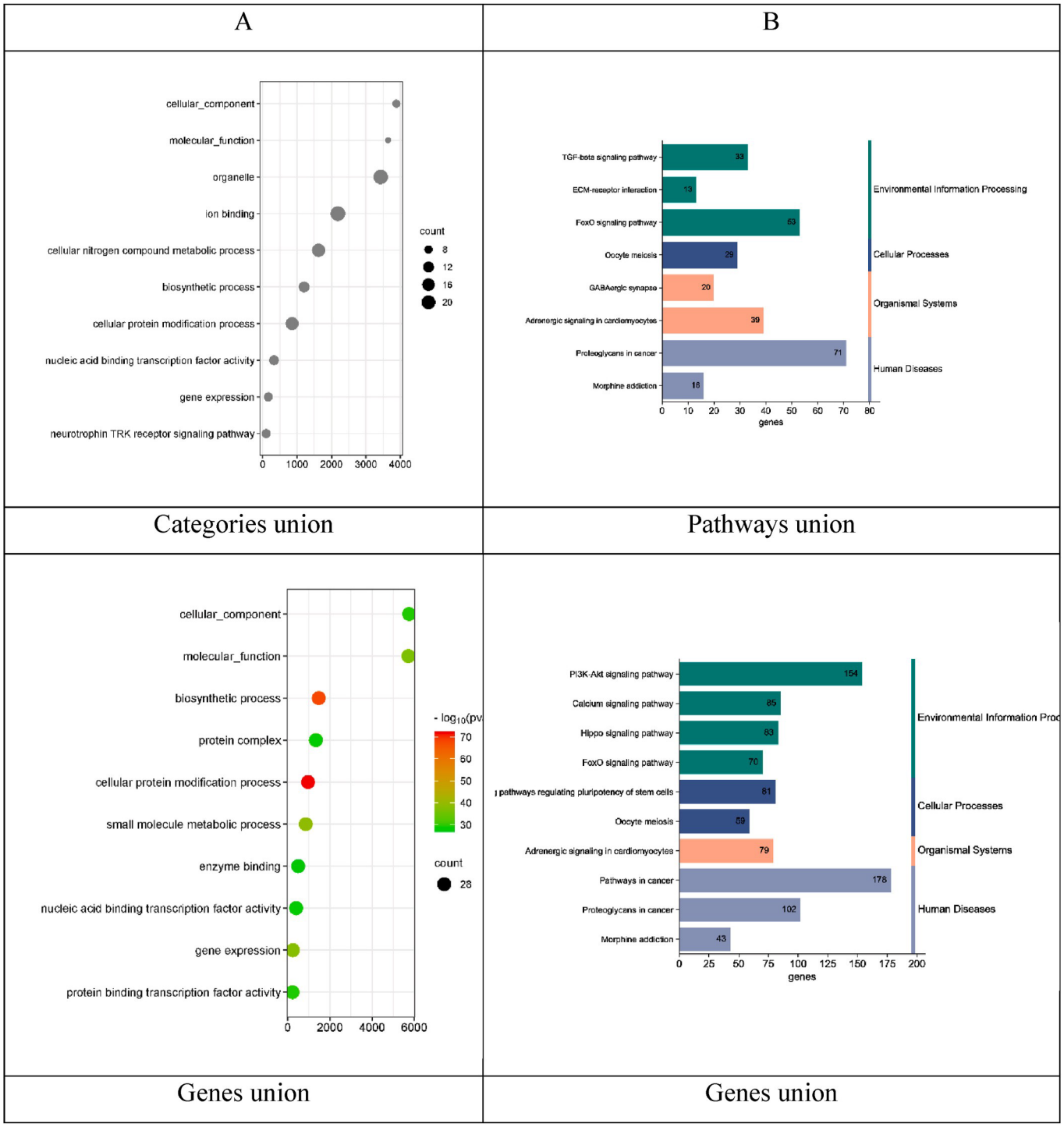

Fig. 4. GO and KEGG analysis of the key miRNAs in HeFH and HoFH (using the way of genes union and categories union). (A) GO enrichment bubble plot. (B) KEGG pathway enrichment category plot (using the way of genes union and pathways union).

Table 4
Predictive target genes of upregulated miRNAs. In bolt co-shared target gene.

Gene	Uniprot	Shared miRNAs				
		Very high	High	Medium	Low	Total
QKI	Q96PU8	8	4	4	0	16
PLAG1	Q6DJT9	6	5	3	2	16
CELF2	**O95319**	**6**	**2**	**8**	**0**	**16**
CSNK1G3	Q9Y6M4	6	2	3	5	16
BRWD1	Q9NSI6	5	5	6	0	16
SH3TC2	Q8TF17	5	4	7	0	16
SLC9A6	Q92581	5	4	4	3	16
NTRK2	Q16620	5	3	8	0	16
PTEN	P60484	5	3	7	1	16
CPEB2	Q7Z5Q1	5	2	3	6	16
LPP	Q93052	4	6	6	0	16
ONECUT2	**O95948**	**4**	**6**	**6**	**0**	**16**
HMGA2	P52926	3	8	5	0	16
TRIM2	Q9C040	3	8	5	0	16
KSR2	Q6VAB6	3	7	6	0	16
ASPH	Q12797	2	8	6	0	16
ATP2B4	P23634	2	8	6	0	16
LCOR	Q96JN0	2	8	6	0	16
SNTB2	Q13425	2	8	6	0	16

Table 5
Predictive target genes of down-regulated miRNAs. In bolt co-shared target gene.

Gene	Uniprot	Shared miRNAs				
		Very high	High	Medium	Low	Total
MEF2A	Q02078	4	0	3	3	10
TNRC6B	Q9UPQ9	3	5	1	1	10
ONECUT2	**O95948**	**3**	**3**	**3**	**1**	**10**
TCF7L2	Q9NQB0	3	3	3	1	10
ZNF704	Q6ZNC4	3	3	3	1	10
CELF2	**O95319**	**3**	**2**	**4**	**1**	**10**
ATRX	P46100	3	2	3	2	10
KSR2	Q6VAB6	3	1	5	1	10
FBXW7	Q969H0	3	1	2	4	10
ERBB4	Q15303	3	0	6	1	10
IRGQ	Q8WZA9	3	0	6	1	10
RSBN1	Q5VWQ0	3	0	5	2	10
CREBRF	Q8IUR6	3	0	4	3	10
LUC7L3	O95232	3	0	4	3	10
ZFX	P17010	3	0	4	3	10
ARCN1	P48444	3	0	3	4	10
BCL11B	Q9C0K0	3	0	3	4	10
SYNJ1	O43426	3	0	3	4	10
SOX4	Q06945	3	0	1	6	10

3.3. Gene ontology and KEGG analysis of miRNAs

GO and KEGG analysis of the key miRNAs in heterozygous and homozygous FH groups were performed DIANA mirpath tools. Significance clusters heatmaps, miRNA clusters, and pathway clusters for both of GO and KEGG analysis were illustrated in Fig. 3.

Moreover, in DIANA mirpath, GO analysis results were merged using the way of genes union and categories union. KEGG analysis results were merged using the way of genes union and pathways union. The best ten significant results of each analysis are shown in Fig. 4A and **B,** respectively.

3.4. Functional analysis of target genes

To explore a further analysis of significant miRNAs in heterozygous FH and homozygous FH, we performed miRNA-gene targeted analysis. microRNA-target predictions were done using miRDIP, collected across 30 different resources. As a result, 26 upregulated and downregulated miRNAs in the score class of high, medium, and low miRDIP were searched, producing 128 predictive target genes targeted by all 26 miRNAs. Tables 4 and 5 show the top 10 predictive target genes.

3.5. Gene oncology analysis of enrichment key genes

GO enrichment was done for targeted genes (**Fig. 5**). Genes were mainly enriched in BP, including biological regulation, regulation of the biological process, regulation of the cellular process, and regulation of the metabolic process (**Fig. 5A**); CC, including intracellular and nucleus (**Fig. 5B**); and MF, including nucleic acid binding and inositol or phosphatidylinositol phosphatase activity (**Fig. 5C**). A three-in-one bar plot GO enrichment analysis has also been shown in Fig. 5D.

3.6. Interaction network

Co-expression network of key genes performed with 55 nodes and 382 edges (Fig. 6).

The STRING online tool analyzed the PPI for top target genes of up and down-regulated miRNAs. Overall, 174 key genes were analyzed (Fig. 7).

174 nodes and 268 edges were resulted (PPI enrichment p-value $<$1.0e-16). Moreover, functional enrichment in this network was shown in Table 6.

Further PPI analysis was done by clustering the network into a 3-separate network (Fig. 8). Cluster 1 by 61 nodes and 27 edges (p-value: $1.47e^{-10}$), cluster 2 by 69 nodes and 137 edges (p-value $<1.0e^{-16}$), and cluster 3 by 44 nodes and 51 edges with p-value $<1.0e^{-16}$. Cluster 2 which has the best properties was selected as the main cluster. The top enriched terms of cluster 2 were collected inSupplementary Table 1.

4. Discussion

FH patients have a high heterogeneity in the clinical presentation of the disease. FH is still underdiagnosed and, therefore, undertreated, especially for children and young adults, there is an extensive ongoing discussion on the best moment to introduce the suitable less or more intensive statin and lipid-lowering combination therapy. Due to this fact, most children and young adults are over the LDL-C target with suitable clinical consequences [25,26]. There are several fences to diagnosing and treating FH since more than 2000 mutations of the LDLR gene have been identified so far [27]. Herein, we conduct a clinical study to assess a microRNA signature that could be associated with FH. It is worth reminding here that HeFH is asymptomatic in childhood; instead, HoFH has a severe clinical presentation in the first decade of life. Therefore, the diagnosis of the first group is more challenging. This latter condition is diagnosed clinically in people with a family history [28]. In 20–40% of the cases, the gene mutation causing FH remains unknown [29]. Therefore, some people with FH have mutations which are not found using genetic testing and therefore might be underdiagnosed. In the HeFH, this is of particular importance. In this view the scientific approach we used in performing the microarray led us to identify miRNAs which were differentially expressed in heterozygous and homozygous FH compared to healthy patients, 16 miRNAs were upregulated and 10 miRNAs were down-regulated in both groups. Here, we identified a miRNA signature reflecting the pathogenesis of both HoFH and HeFH from the patient's serum samples for the first time. Results showed elevated expression of hsa-miR-604, hsa-miR-652-5p, and hsa-miR-4451 and lower expression of hsa-miR-3140-3p, hsa--miR-550a-5p, and hsa-miR-363-3p. Furthermore, to stratify HeFH form by HoFH, hsa-miR-1183, hsa-miR-1185-1-3p, hsa-miR-122-5p, hsa--miR-19a-3p, hsa-miR-345-3p, and hsa-miR-34c-5p were recognized here as possible biomarker candidate since they are higher in HeFH then HoFH. Although of extreme interest also from a clinical point of view, having this possible signature to identify FH bypassing might not have tools for early diagnosis of HeFH in the future gene sequence. Of those miRNAs, one, the miR-122, was reported to be liver-enriched microRNA,

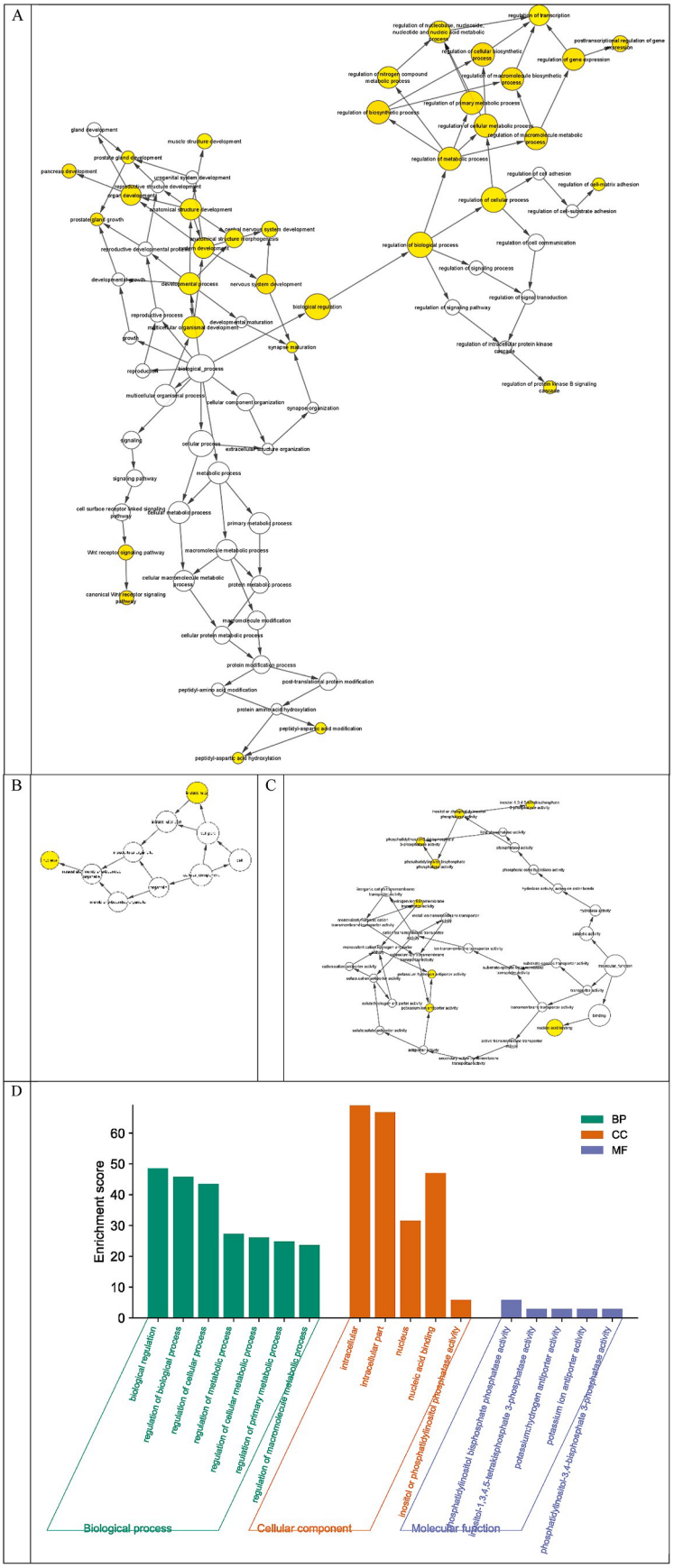

Fig. 5. GO enrichment of key genes. A: Biological Process of GO enrichment. B. Cellular components of GO enrichment. C. Molecular function of GO enrichment. D. Three-in-one bar plot GO enrichment analysis (BP, CC, and MF).

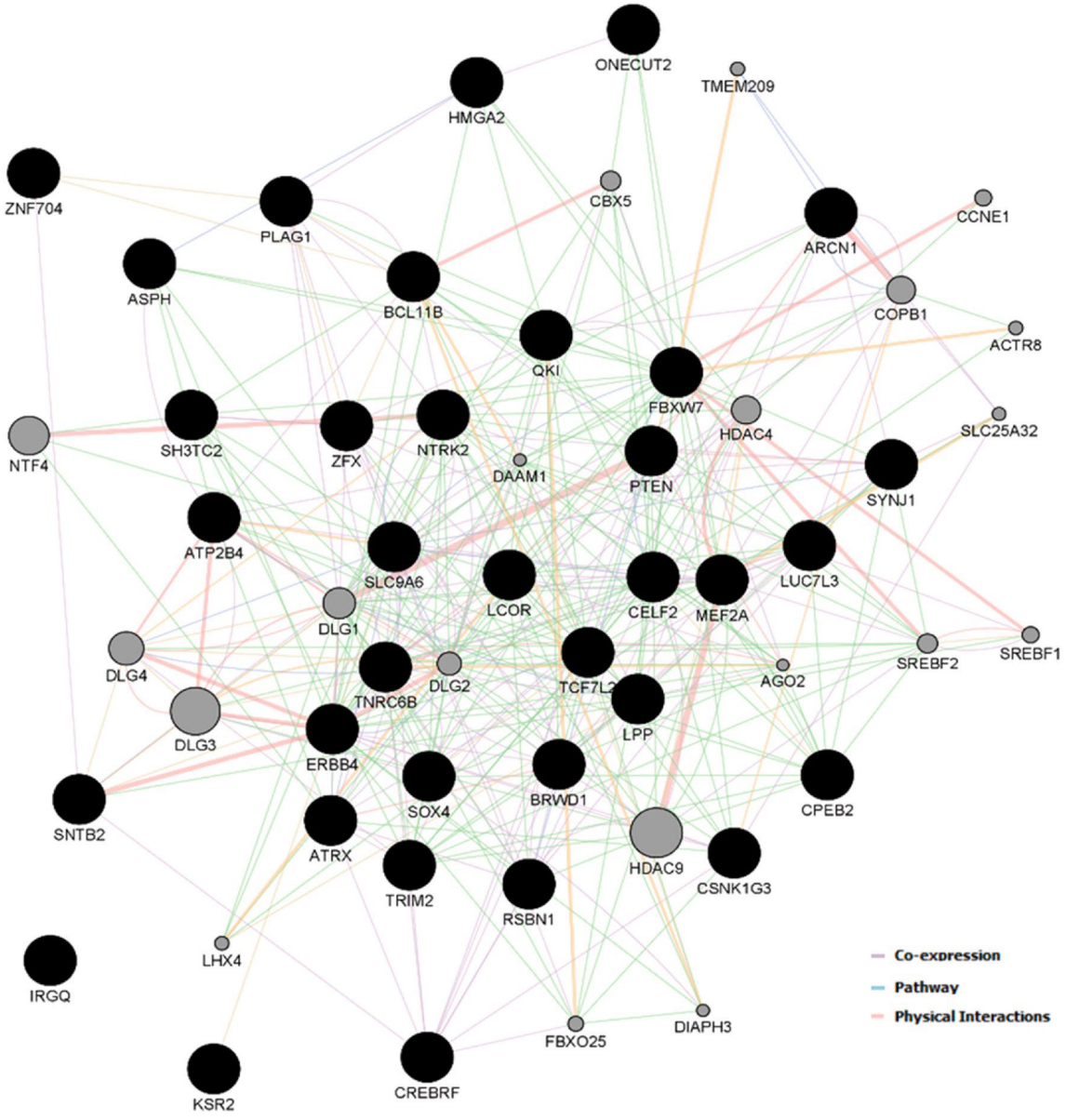

Fig. 6. Gene-gene interaction network. Black nodes show query genes, and gray nodes show additional related genes.

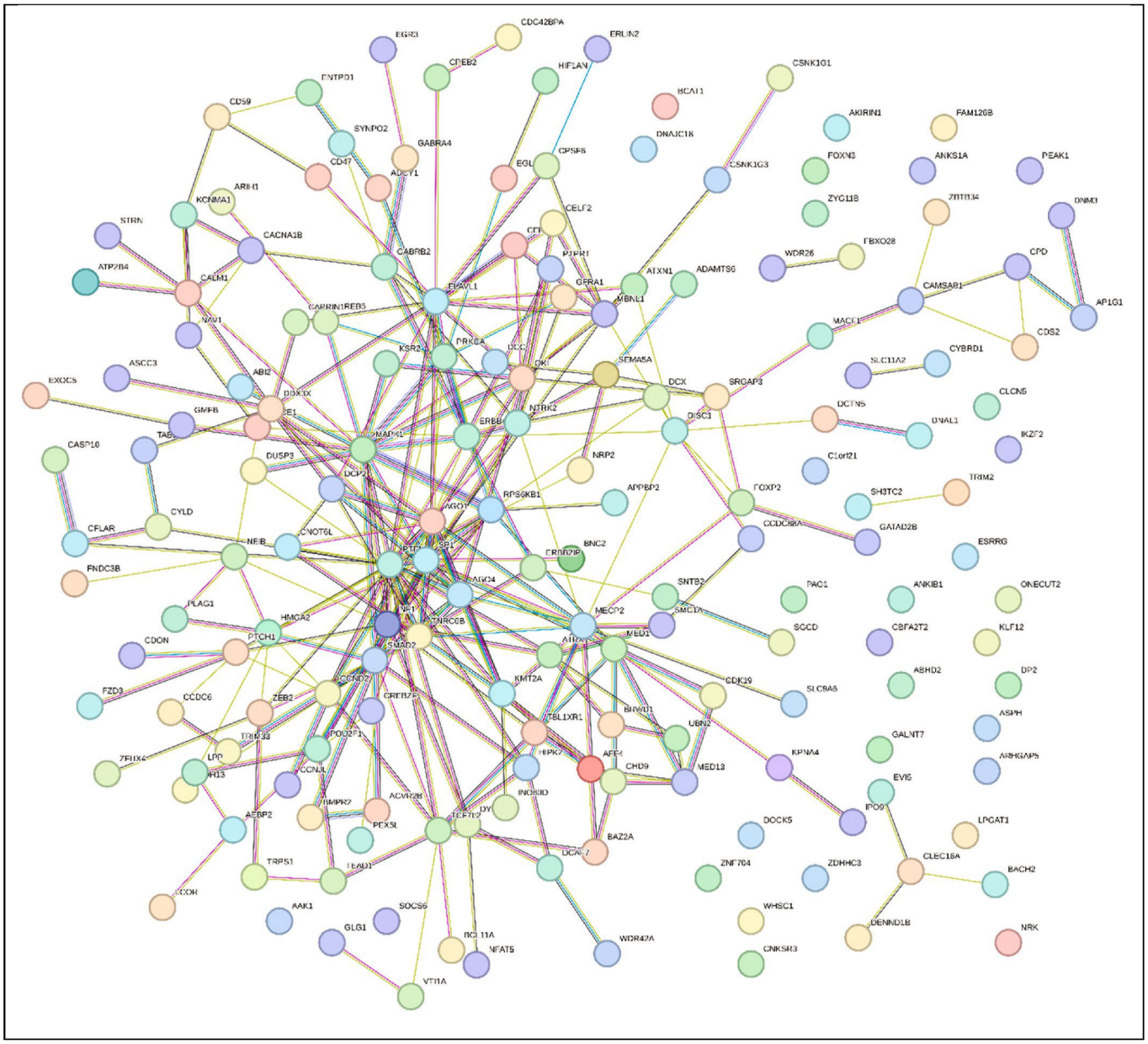

Fig. 7. PPI network. Circles: proteins encoded by the input genes. Lines: interactions between the proteins.

Table 6
Results of functional enrichment in PPI network.

Category	term ID	term description	strength	false discovery rate
GO Process	GO:0042264	Peptidyl-aspartic acid hydroxylation	2.05	0.0237
GO Process	GO:2001038	Regulation of cellular response to drug	1.87	0.0352
GO Process	GO:2001023	Regulation of response to drug	1.83	0.0033
GO Process	GO:0010501	RNA secondary structure unwinding	1.62	0.0077
GO Process	GO:0071679	Commissural neuron axon guidance	1.45	0.0163
GO Function	GO:0019904	Protein domain-specific binding	0.56	$6.16E^{-05}$
GO Function	GO:0004674	Protein serine/threonine kinase activity	0.56	0.0068
GO Function	GO:0004672	Protein kinase activity	0.55	0.00091
GO Function	GO:0001067	Regulatory region nucleic acid binding	0.52	$2.12E^{-05}$
GO Function	GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	0.51	0.0286
GO Component	GO:0016442	RISC complex	1.38	0.0187
GO Component	GO:0005901	Caveola	0.94	0.0058
GO Component	GO:0099060	Integral component of postsynaptic specialization membrane	0.87	0.0273
GO Component	GO:0000932	P-body	0.81	0.0411
GO Component	GO:0099055	Integral component of the postsynaptic membrane	0.75	0.0302
KEGG	hsa04340	Hedgehog signaling pathway	1.08	0.0314
KEGG	hsa04350	TGF-beta signaling pathway	0.87	0.0314
KEGG	hsa04360	Axon guidance	0.71	0.0314
KEGG	hsa05206	MicroRNAs in cancer	0.75	0.0314

and its presence in the blood circulation is due to early liver damage [30]. A pioneer work of Krützfeldt and colleagues showed that inhibiting liver-enriched miR-122 in mice impaired the expression of genes involved in cholesterol biosynthesis. They also showed that miR-122 inhibition in normal mice caused a reduction in plasma cholesterol through elevating hepatic fatty acid oxidation [31,32]. In our set of data, the hsa-miR-122-5p showed an LFC of 0.724366 and 0.389947 in HeFH and HoFH, respectively, highlighting that in FH disease, both type, gene mutation, and expression have an important role in the phenotype manifestation. Targets of up and downregulated miRNAs identified in our set of data highlight the following genes QKI, PLAG1, CSNK1G3, and CELF2, for upregulated and MEF2A, TNRC6B and ONECUT2 for the downregulated miRNAs were the most impacted targets. QKI is a splicing regulator of myofibrillogenesis in the cardiac and function of contractile, which activates Srebp2-mediated cholesterol biosynthesis [33,34]. CELF2 is an RNA-binding protein that encodes various functions at the posttranscriptional level, such as RNA editing for apolipoprotein B [35]. PLAG1 regulates genes involved in cholesterol synthesis [36]. PLAG1 is known to regulate plasma LDL and total cholesterol through upregulation of certain target genes [37]. CSNK1G3 was found in genome-wide association studies (GWAS) and Metabochip meta-analysis associated with LDL cholesterol regulation [38].

In our data, two genes, CELF2 and ONECTU2, recognized our attention particularly (in bolt in Tables 2 and 3). We noticed that they are co-shared amongst differently expressed miRNAs. CELF2 has pleiotropic effects on mRNA splicing, stability, and translation [18, 19]. Moreover, GWAS, CELF family members (CELF1 and 2) have been suggested to be genes conferring susceptibility to Alzheimer's Disease with which cholesterol biosynthetic pathway and LDL-C levels were seen to correlate. [20-22] Furthermore, ONECUT1 and ONECUT2 which are transcription factors, can control a set of genes which have roles in cell migration and adhesion [39]. From our data CELF2 and ONECUT2 gene results targeted by both miRNAs up and down-regulated, this data leads us to think that both genes are very important in FH patients since up and down-regulated miRNAs target them at the same moment. The levels of these two factors should stay in a homeostatic condition to sustain the FH disease and might be considered pharmacological targets in the future.

Moreover, ONECUT2 is a fundamental factor in controlling cell liver decision fate via activin/TGFβ signaling [40]. Pathway analysis of miRNAs target genes showed that these genes have roles in the TGFβ signaling pathway as well as they could affect hedgehog (Hh) signaling pathway [41]. It has been shown that secretion of Hh by modification of cholesterol and palmitate may occur by creating multimers or by association together with lipoproteins [42]. It is worth mentioning that inhibition of both TGF-β and Hh pathways in human hepatic stellate cells attenuates pro-fibrotic genes [43]. Bioinformatics data also highlighted that TGF-β s well as PI3K/Akt were the most impacted signaling pathways by these miRNAs. The PI3K-Akt as well as TGF-β signaling pathways in hyperlipidemia. Likewise, PI3K signaling pathway could be targeted by a some miRNAs, such as miR-147 and miR-223 [44]. This latter is transported also by high-density lipoprotein (HDL) in patients with HoFH [45]. In our set of microarray analyses, we did not recognize the difference of has-miR-223-3p in any of the three groups analyzed. We noticed that Vickers and colleagues purified HDL by ultracentrifugation and then tested the levels of miR-223-3p. TGF-β alteration signaling in humans causes vascular pathologies and cardiovascular disease [46]. It was found that TGF-β signaling pathway is involved in immune function, inflammation, differentiation, proliferation, and apoptosis *in vivo*. A crosstalk between TGF-β signaling and miRNAs has been previously demonstrated, and a role of mRNA in the modulation of TGF-β signaling by miRNAs has been reported. It may play in the pathogenesis of atherosclerosis [47,48]. The other genes found here affected by downregulated miRNAs were MEF2A, recently introduced as a potential plasma biomarker for CAD risk prediction, which directly upregulates PI3K gene expression in the PI3K/p-Akt pathway [49], and TNRC6B, which has indirect effects of on lipids metabolism since it was found impaired in type 2 diabetes [50] and have a role in pre-existing fatty liver disease [51].

The strength point of this research is its translational and interdisciplinary nature, which made it possible for the first time to find differentially expressed miRNAs in HeFH and HoFH patients vs. healthy subjects, as well as typical for HeFH and HoFH patients only. Moreover, the target genes for differentially expressed miRNAs were identified using a detailed bioinformatic approach. On the other hand, potential limitations were the lack of analyses on other non-coding RNAs, such as circular and long non-coding RNAs. Finally, the case-control nature of this study precluded the possibility of assessing the predictive role of the identified differentially expressed miRNAs for cardiovascular events.

5. Conclusions

Our study used various bioinformatics tools to analyze microarray data and identify critical miRNAs and their target genes. Here, we identified a miRNA signature reflecting the pathogenesis of both heterozygous and homozygous FH for the first time. The signature includes high expression of hsa-miR-604, hsa-miR-652-5p, and hsa-miR-4451 as well as reduced expression of hsa-miR-3140-3p, hsa-miR-550a-5p, and hsa-miR-363-3p. Furthermore, in a subset of analysis to distinguish HeFH form by HoFH the hsa-miR-1183, hsa-miR-1185-1-3p,hsa-miR-

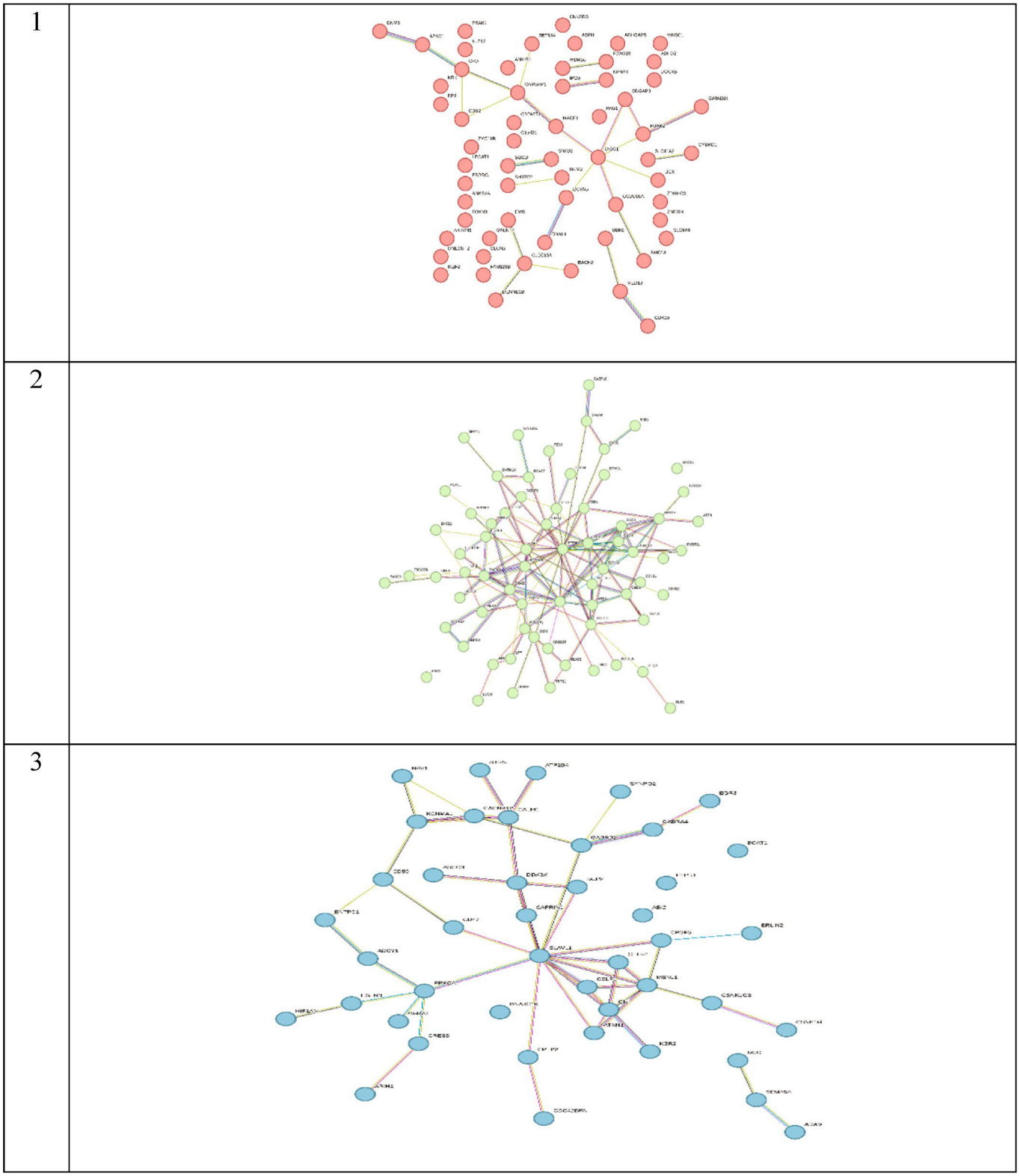

Fig. 8. Clustering the PPI network.

122-5p, hsa-miR-19a-3p, hsa-miR-345-3p, and hsa-miR-34c-5p seems the best biomarker candidates since they are higher in HeFH then HoFH. Overall, the upregulated miRNAs mainly affected QKI, PLAG1, CELF2, and CSNK1G3 genes, while down-regulated miRNAs mainly affected MEF2A, TNRC6B, and ONECUT2 genes. These genes are primarily involved in cholesterol/lipid metabolism or associated with cardiovascular diseases.

Moreover, the effects of identified miRNAs and their target genes in signaling pathways showed the critical roles of these miRNAs in the disease. These results provide insights into the underlying molecular mechanisms of FH that may contribute to disease development or progression toward atherosclerotic cardiovascular disease. It remains to be recognized if the identified differentially expressed miRNAs can serve as potential new therapeutic targets for FH and also supporting the FH diagnosis in case of lack of genetic confirmation of the disease, that it refers to the 20–40% of FH patients.

CRediT authorship contribution statement

Erika Cione: Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Maryam Mahjoubin-Tehran:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Data curation. **Tiziana Bacchetti:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Maciej Banach:** Writing – review & editing, Conceptualization. **Gianna Ferretti:** Writing – review & editing, Supervision, Funding acquisition, Data curation. **Amirhossein Sahebkar:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.02.017.

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