CHEST

Circulating microRNAs as Potential Biomarkers of Endothelial Dysfunction in Obese Children

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BACKGROUND: Cardiovascular disease (CVD) is a complex disease with multifactorial etiology. The presence of endothelial dysfunction constitutes an early risk factor for CVD in children. Circulating microRNAs (miRNAs) are small noncoding RNAs that regulate gene expression and represent a novel class of biomarkers and therapeutic targets; therefore, we examined whether the presence of endothelial dysfunction is associated with differential expression of plasma miRNAs in otherwise healthy children.

METHODS: A total of 70 children (aged 5-10 years) were recruited and classified into two groups (normal endothelial function [NEF] and endothelial dysfunction). Time to peak postocclusive reperfusion (Tmax) was considered as the indicator of either normal endothelial function (NEF; Tmax $<$ 45 s) or endothelial dysfunction (Tmax $\geq$ 45 s). Lipid profiles, high-sensitivity C-reactive protein, fasting glucose, and insulin were assayed using enzyme-linked immunosorbent assay. miRNAs isolated from plasma were assayed with a custom human CVD array, followed by quantitative polymerase chain reaction verification of candidates. In addition, bioinformatics approaches including combinatorial target prediction algorithms and gene ontology were applied.

RESULTS: Three miRNAs that have been previously linked to cardiomyopathy, hsa-miR-125a-5p, hsa-miR-342-3p, and hsa-miR-365b-3p, were identified as potential biomarkers of children with endothelial dysfunction. The miRNA predicted gene targets revealed 31 common targets among all three putative candidate biomarker miRNAs and encompass three biologic pathways, including transforming growth factor-β signaling, cytokine-cytokine receptor interactions, and activin receptor-like kinase in cardiac myocytes.

CONCLUSIONS: Plasma miRNAs may be useful as potential screening tools for the presence of endothelial dysfunction in children and may reveal endothelial dysfunction-relevant target genes.

CHEST 2016; 149(3):786-800

KEY WORDS: endothelium; microRNA; obesity

ABBREVIATIONS: CVD = cardiovascular disease; GO = gene ontology; HOMA = homeostasis model assessment; hsCRP = high-sensitivity C-reactive protein; miRNA = microRNA; NEF = normal endothelial function; qRT-PCR = quantitative real-time polymerase chain reaction; TGF-β = transforming growth factor-β; Tmax = time to peak postocclusive reperfusion

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FUNDING/SUPPORT: This work is supported by the National Institutes of Health [Grant HL-65270 to D. G. and L. K.-G.], the Herbert T. Abelson Endowed Chair in Pediatrics, and the American Heart Association [Grant 13SDG14780079 to R. B.].

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DOI: http://dx.doi.org/10.1378/chest.15-0799

Obesity is one of the world's greatest public health challenges in both developed and developing countries, affecting both adults and children. The presence of obesity has now been conclusively linked to heightened morbidity and mortality through increased risk for a multitude of chronic diseases, including type 2 diabetes mellitus, hypertension, dyslipidemia, and coronary artery disease.[1-5] The pathogenesis of obesity is multifactorial, incorporating both genetics and lifestyle factors. In children, obesity is associated with increased risk for multifaceted derangements in metabolic and cardiovascular function, including endothelial dysfunction.[6-9] Overweight children are more likely to prematurely develop endothelial dysfunction, hypertension, and type 2 diabetes mellitus, an array of conditions that would be expected in older obese adults,[4] a finding that has prompted the recommendation to screen for such morbidities at even earlier ages.[10]

Obesity in children is associated with an increased risk for the development of endothelial dysfunction prior to the onset of hypertension.[8] However, not every obese child will develop endothelial dysfunction, suggesting that both genetic and environmental factors may play a role. Conversely, a small subset of otherwise healthy children who are not obese may manifest abnormal endothelial function, and such functional phenotype may be determined by genetic variance in endothelial function-related genes.[11] Endothelial dysfunction, an early risk marker of cardiovascular disease, refers to a loss of normal homeostatic function in the blood vessels and is characterized by altered vasodilatory and vasoconstrictive functions and inflammatory activity.[12] Endothelial dysfunction is involved in the development of vascular complications related to dyslipidemia and cardiovascular disease (CVD) such as hypertension, coronary artery disease, and chronic heart failure.[13,14] Thus, extraordinary efforts have been directed to determine the molecular and pathologic characteristics of the diseased heart and vasculature and to develop novel diagnostic and therapeutic strategies.

One of the emerging and promising diagnostic tools is the use of circulating microRNAs (miRNAs). miRNAs are small noncoding RNAs (approximately 22 nucleotides) that posttranscriptionally regulate gene expression by blocking translation or inducing degradation of the targeted mRNA.[15] The presence of circulating miRNAs in plasma suggests that miRNAs may fulfill important biologic functions outside their corresponding cell sources in both physiologic and pathologic conditions and serve as potential biomarkers for disease states.[16] Circulating miRNAs are protected from RNase-dependent degradation by several mechanisms, including their inclusion in microvesicles, exosomes, and apoptotic bodies as well as through formation of protein-miRNA complexes that are resistant to degradation.[17] Of note, although the expression of miRNAs in plasma and serum are believed to reflect the extrusion of miRNAs from relevant remote tissues or organs or disease processes,[18] it is likely that peripheral blood miRNAs do not reflect miRNAs expressed in remote tissues. miRNAs are involved in the regulation of several key cellular processes, including cellular development, differentiation, proliferation, cell death, and metabolism.[19] miRNAs have been implicated in almost every cardiovascular disorder in which they have been examined, including heart failure, cardiac hypertrophy, remodeling after myocardial infarction, arrhythmias, atherosclerosis, atrial fibrillation, and peripheral artery disease.[20-24] Since miRNAs can be readily detected in various body fluids,[25,26] they have been advanced as useful biomarkers for the diagnosis and characterization of systemic diseases,[27] including type 2 diabetes mellitus,[28] hypertension,[29] obesity,[30] and cardiovascular disease.[31] Peripheral blood miRNAs, including miRNAs expressed by peripheral blood mononuclear cells, as well as extracellular/circulating miRNAs may, thus, provide an easy and rapid screening approach in clinical populations[32] and add to the accuracy of current CVD risk stratification criteria.

In this study, we hypothesized that circulating miRNAs may have predictive value for identification of children at increased risk for endothelial dysfunction. To examine this issue, plasma samples of children with and without endothelial dysfunction were examined using pathway-fused cardiovascular miRNA arrays, followed by bioinformatic assessments of functional information of the differentially expressed specific miRNAs and their related regulatory networks, as well as putative identification of their target genes as predicted by function and pathway enrichment analysis of at least three public datasets (TargetScan, PicTar, and miRanda).

Materials and Methods

Subjects

The study was approved by the University of Louisville Human Research Committee (Protocol 474.99), and informed consent was obtained from the legal caregiver of each participant. Consecutive healthy overweight and obese prepubertal children (aged 5-10 years) were recruited from the community to investigate endothelial function. Children were excluded if they had any chronic medical condition, were receiving medications, or had any genetic or craniofacial syndromes. All children who were hypertensive or using antihypertensive therapies were excluded. Also excluded from the study were children with a known episode of infection in the 8 weeks preceding the study or children with asthma or allergies receiving specific therapy (desensitization, leukotriene inhibitors, topical or systemic corticosteroids). Height and weight were obtained, systemic BP was measured,[33] and BMI z score was calculated (e-Appendix 1).

Endothelial Function

Endothelial function was assessed by using a modified hyperemic test after cuff-induced occlusion of the radial and ulnar arteries by placing the cuff over the wrist, as previously reported.[8,34-37] We defined endothelial dysfunction as a time to peak postocclusive reperfusion (Tmax) cutoff value of $\geq$ 45 s, whereas values $<$ 45 s were considered as normal endothelial function (NEF)[36] (see e-Appendix 1).

Biochemical Assays

Fasting blood samples were drawn from the subjects in the morning and immediately centrifuged at 2,000g for 20 min at 4°C, and aliquoted plasma samples were frozen at −80°C until assayed. Plasma high-sensitivity C-reactive protein (hsCRP), lipids, insulin, and glucose were measured (e-Appendix 1), and insulin resistance was assessed using the homeostasis model assessment (HOMA) equation (fasting insulin × fasting glucose/22.5).[38]

Circulating miRNA Isolation, Quality, and Integrity

Total RNA including miRNA was isolated from plasma using miRNeasy Mini Kit column-based system following the manufacturer's instructions (Qiagen). RNA quantity was evaluated by spectrophotometry using NanoDrop ND-1000 (Thermo Fisher Scientific Inc). The RNA quality and integrity were determined using the Eukaryote Total RNA Nano 6000 LabChip assay (Agilent Technologies) on the Agilent 2100 Bioanalyzer. The quality of miRNA was determined using Agilent Small RNA Kit according to the manufacturer's protocol. All the purified samples were stored at −80°C until further analyses (e-Appendix 1).

miRNA Polymerase Chain Reaction Array for CVD

Pathway-specific for human CVD miRNA arrays (84 miRNAs) were used in age-, sex-, ethnicity-, and BMI z score-matched children with either normal endothelial function (n = 8) or endothelial dysfunction (n = 8) (Qiagen) (Fig 1). Each of the arrays contains a specific set of selected miRNAs with CVD significance based on published studies. A set of 12 miRNA controls preset on this array (96-well plates) enables data analysis using the ΔΔCT method of relative quantification, assessment of reverse transcription performance, and assessment of polymerase chain reaction (PCR) performance using SYBR Green real-time PCR. For target verification purposes, quantitative real-time PCR (qRT-PCR) analyses were performed using ABI 7500 (Thermo Fisher Scientific Inc) using validated housekeeping genes[39,40] (e-Appendix 1).

Target Predictions

Gene targets for differentially expressed miRNAs were computationally predicted using established miRNA target-prediction programs: MicroInspector, miRanda, PicTar, RNA22, RNAhybrid, and TargetScan (Fig 1). To improve the reliability of the miRNA targets, only target genes predicted by at least three of the programs were selected for extraction. The predicted genes of individual miRNA were uploaded to the online DAVID (Database for Annotation, Visualization, and Integrated Discovery) program (http://david.abcc.ncifcrf.gov/) for their functional annotation and clustering analysis. The predominant biologic pathways for the selected miRNAs were identified.

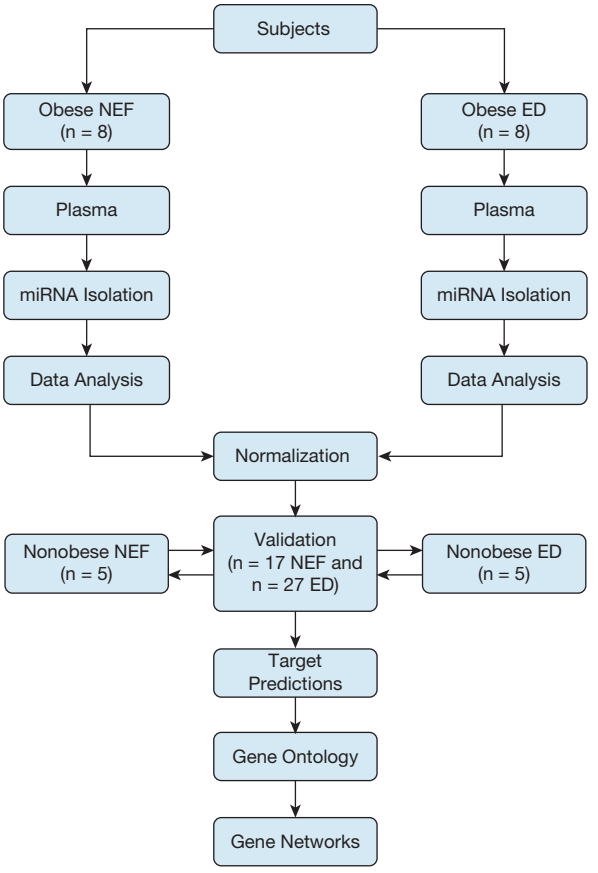

Figure 1 – *Schema illustrating subject recruitment and data analysis. The cohort was matched for age, sex, ethnicity, and BMI z score and differed only in their time to peak postocclusive reperfusion characteristics (ie, endothelial dysfunction or NEF). Plasma miRNAs were isolated from each subject and equal concentrations of each sample were converted into cDNA. Each individual sample was applied into Qiagen arrays (84 miRNAs). The data were normalized with housekeeping miRNA, and the averages of each miRNA were compared between NEF vs ED. Target predictions for each statistically significant miRNA were performed by at least four different software programs. The common target predictions genes as derived from Venn diagram were then used to build gene networks. The number of subjects tested in each step is indicated (eight subjects in ED and NEF groups served as the initial exploratory phase, 17 NEF and 27 ED subjects served as post hoc verification phase, and additional nonobese control subjects (n = 5 in endothelial dysfunction and NEF groups). ED = endothelial dysfunction; miRNA = microRNA; NEF = normal endothelial function.*

Gene Ontology and Functional Annotation

Analysis of gene ontology (GO) annotation was performed by applying DAVID 6.7 functional annotation tool. This DAVID software (http://david. abcc.ncifcrf.gov/) is able to identify the most relevant (overrepresented) biologic terms associated with a given gene list.[41] The DAVID functional annotation cluster tool groups genes based on their associated GO annotations, and the related terms are clustered into groups with enrichment scores calculated from their EASE Score, the modified Fisher exact *P* value.[42] Moreover, the web server hosts an updated version of the KEGG database providing a relevant search module based on KEGG pathway descriptions.[43]

Pathway Analysis Enrichment: The web-based computational tool DIANA-mirPath v2.1[44] was used to predict the target genes and altered pathways of the differentially expressed miRNAs. The software performs an enrichment analysis of multiple miRNA target genes by comparing each set of miRNA targets to all known KEGG pathways (Kyoto Encyclopedia of Genes and Genomes). The pathways exhibiting a false discovery rate adjusted *P* value of $< .05$ were considered significantly enriched between the compared classes.

Statistical Analysis

All data were expressed as mean ± SD. Analysis of variance tests or Student *t* tests were used for statistical analyses. We performed analysis of variance and/or paired *t* tests to study differences on quantitative variables between endothelial dysfunction and NEF groups. All analyses were conducted using SPSS software (version 21.0; IBM Corporation), and data are presented as mean ± SD. A *P* value $< .05$ was considered statistically significant for all analyses.

Results

Cohort Phenotype

The demographic and clinical parameters of the 60 children initially recruited to the study (25 in the NEF and 35 in the endothelial dysfunction groups) are summarized in Table 1, and illustrate their similar age, sex, ethnicity, and BMI *z* scores. The Tmax value for NEF children was 26.8 ± 11.4 s as compared with 62.4 ± 15.9 s for children with endothelial dysfunction (*P* value $< .0001$). However, there were no differences in either systolic or diastolic blood pressures between endothelial dysfunction and NEF groups.

The metabolic data for the two groups are shown in Table 1. Serum triglyceride levels were significantly higher in children with endothelial dysfunction (*P* value $\leq .04$), whereas high-density lipoprotein levels were significantly lower (*P* value $< .03$). Although fasting glucose levels were similar, plasma insulin concentrations were higher in children with endothelial dysfunction (*P* value $< .05$), and hsCRP levels also differed among the groups (*P* value $= 0.007$) (Table 1).

miRNA Profiling and qRT-PCR Validation

Microarray-based analyses revealed that three miRNAs were differentially expressed in children with endothelial dysfunction (Fig 1, Table 2). Out of 84 miRNAs listed on

TABLE 1] Demographic Characteristics and Metabolic Data in Children With and Without Endothelial Dysfunction

Variables	NEF (n = 25)	Endothelial Dysfunction (n = 35)	*P* Value
Age, y	7.59 ± 1.26	8.41 ± 1.20	.14
BMI *z*-score	1.69 ± 0.62	2.00 ± 0.68	.43
Sex, % male	70.4	63.8	.35
Ethnicity, %			
White	77.3	74.1	.21
African American	22.4	26.2	.26
SBP, mm Hg	107.55 ± 10.35	107.32 ± 10.68	.47
DBP, mm Hg	62.28 ± 8.15	63.56 ± 7.16	.32
Tmax, s	26.84 ± 11.35	62.42 ± 15.88	.0001
TG, mg/dL	67.30 ± 30.71	89.0.06 ± 53.64	.04
TC, mg/dL	160.34 ± 27.41	164.90 ± 26.98	.27
HDL-C, mg/dL	52.78 ± 7.44	49.01 ± 7.60	.03
LDL-C, mg/dL	94.04 ± 22.49	98.06 ± 23.87	.27
Glucose, mg/dL	76.23 ± 9.90	81.45 ± 13.43	.15
Insulin, mg/dL	5.88 ± 2.98	9.11 ± 6.53	.02
HOMA-IR	0.98 ± 0.79	1.66 ± 1.58	.04
hsCRP, mg/dL	0.43 ± 0.31	1.51 ± 1.18	.007

Data presented as mean ± SD unless otherwise noted. DBP = diastolic BP; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment-insulin resistance; hsCRP = high-sensitivity C-reactive protein; LDL-C = low-density lipoprotein cholesterol; NEF = normal endothelial function; SBP = systolic BP; TC = total cholesterol; TG = triglyceride; Tmax = time of maximum perfusion after occlusion release.

TABLE 2] List of Differentially Expressed miRNAs in Children With Endothelial Dysfunction and Validations of the Same miRNAs Using qRT-PCR Analysis

List of miRNAs	Reference Sequence	Chromosome Location	Transcript ID	Cardiovascular Arrays		Validation (qRT-PCR)	
				Fold Changes	*P* Value	Fold Changes	*P* Value
hsa-miR-365b-3p	NR_029856	17q11.2	ENSG00000199187	1.41 ± 0.14	.004	1.52 ± 0.23	.001
hsa-miR-125a-5p	NR_029693	19q13.41	ENST00000385273	−1.33 ± 0.11	.02	−1.27 ± 0.12	.01
hsa-miR-342-3p	NR_029888.1	14q32.2	ENST00000362212	−1.41 ± 0.08	.03	−1.22 ± 0.06	.02

miRNA = microRNA; qRT-PCR = quantitative real-time polymerase chain reaction.

the PCR array, 76 miRNAs were detected. We further confirmed these findings in 17 additional subjects with NEF and 27 subjects with endothelial dysfunction. Notably, qRT-PCR results on differentially expressed miRNAs (ie, hsa-miR-365b-3p, hsa-miR-125a-5p, and hsa-miR-342-3p) were consistent with the expression patterns observed in miRNA microarray profiling (Table 2). To ascertain that BMI *z*-score status was not biasing our findings and that the miRNA findings were specifically ascribable to endothelial dysfunction, we further compared additional nonobese children with otherwise normal endothelial function (n = 5) and additional nonobese children with endothelial dysfunction (n = 5). MiRNA expression patterns in these 10 nonobese children further confirmed the increased levels of hsa-miR-365b-3p in endothelial dysfunction and the reductions in circulating hsa-miR-125a-5p and hsa-miR-342-3p miRNAs in endothelial dysfunction. Thus, the number of subjects participating in this study were 60 obese children, either NEF or endothelial dysfunction, and 10 nonobese children (NEF or endothelial dysfunction), thereby totaling 70 subjects.

Prediction of Potential Targets of Differentially Expressed miRNAs

A Venn diagram in Figure 2 shows the intersections among the three target genes list, and the list of these 31 putative target genes is provided in Table 3. hsa-miR-125a-5p had the most gene target hit rates with 1,194, whereas hsa-miR-365-3p had 683 and hsa-miR-342-3p had 679 target genes, respectively (Fig 3). To evaluate the potential biologic roles of the three differentially expressed miRNAs, we assessed the GO of their potential gene targets using several established computational algorithms, and the annotated genes were classified according to the GO categories: cellular component (n = 3), biologic process (n = 50), and molecular function (n = 13) based on *P* value < .05. GO analyses further revealed that a broad range of biologic processes categories were enriched among the target genes list (Table 4), including metabolic process, anterior/posterior pattern formation, tube development, and respiratory tube development. The list of significant molecular functions associated with endothelial dysfunction is shown in Table 5, and the most common of these biologic processes were transmembrane receptor protein serine/threonine kinase activity and TGF-β receptor activity. Also, the lists of significant cellular components included cell surface, plasma membrane, and Srb-mediator complex (Table 6). Next, we found that three biologic pathways were associated with the gene target predictions, including transforming growth factor (TGF)-β signaling, cytokine-cytokine

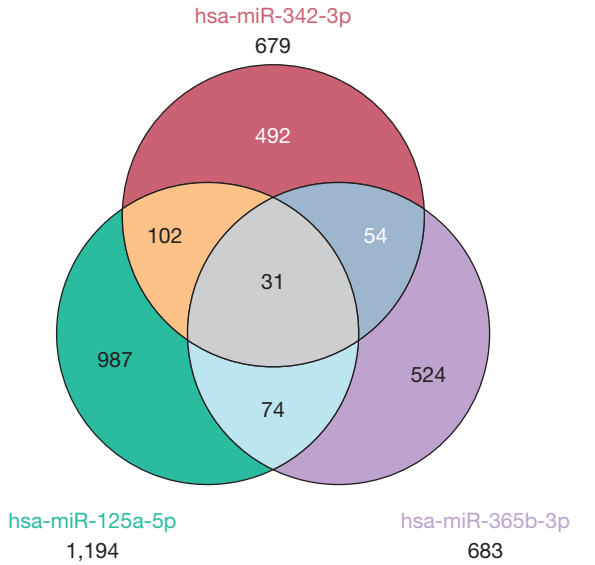

Figure 2 – *Venn diagram showing the intersections of the putative target gene predictions from the three differentially expressed microRNAs (miRNAs) in children with endothelial dysfunction. The final common list of target predictions genes among all three differentially expressed miRNAs were identified from the Venn diagram and used to define the putative common candidate target genes.*

TABLE 3] List of Predicted Target Genes Derived From Shared Venn Diagram of the Three Differentially Expressed miRNAs

Gene Name	Ensembl Gene	UniGene	RefseqRNA	Description	Chromosome	Start, bp	End, bp
ACVR2B	ENSG00000114739	Hs.174273	NM_001106	Activin A receptor, type IIB	3	38470794	38509637
APC	ENSG00000134982	Hs.158932	NM_000038	Adenomatosis polyposis coli	…	32236465	32246349
B4GALT1	ENSG00000071626	Hs.651277	NM_018959	UDP-Gal:betaGlcNAc β 1,4-galactosyltransferase, polypeptide 1	19	1358584	1386683
BMPR2	ENSG00000204217	Hs.471119	NM_001204	Bone morphogenetic protein receptor, type 2 (serine/threonine kinase)	2	202949916	203140719
C1orf21	ENSG00000116667	Hs.497159	NM_030806	Chromosome 1 open reading frame 21	1	182622773	182864778
CACNA1C	ENSG00000151067	Hs.118262	NM_000719.5	Calcium channel, voltage-dependent, L type, α 1C subunit	12	2032725	2677376
CBFB	ENSG00000067955	Hs.460988	NM_001755	Core-binding factor, β subunit	16	65620551	65692457
CREB5	ENSG00000146592	Hs.437075	NM_001011666.1	CAMP responsive element binding protein 5	7	28305465	28832036
DOCK3	ENSG00000129596	Hs.476284	NM_001801	Dedicator of cytokinesis 3	5	115168333	115180304
FBXO33	ENSG00000165355	Hs.324342	NM_203301	F-box protein 33	14	38936718	38971371
FRAS1	ENSG00000138759	Hs.369448	NM_025074.4	Fraser syndrome 1	4	79198120	79684447
GABRB2	ENSG00000121966	Hs.303527	NM_001008540	γ-aminobutyric acid (GABA) A receptor, β 2	5	160648014	160907708
GPR158	ENSG00000151025	Hs.499108	NM_020752	G protein-coupled receptor 158	10	25504296	25931164
GRB10	ENSG00000106070	Hs.164060	NM_001001549.1	Growth factor receptor-bound protein 10	7	50625260	50828652
HIATL1	ENSG00000148110	Hs.555996	NM_032558	Hippocampus abundant transcript-like 1	9	96176654	96263022
HIPK1	ENSG00000163349	Hs.532363	NM_152696	Homeodomain interacting protein kinase 1	1	114203267	114321949
KLHL24	ENSG00000114796	Hs.407709	NM_017644	Kelch-like 24 (Drosophila)	3	184836105	184884996
LMO3	ENSG00000048540	Hs.504908	NM_001001395	LIM domain only 3 (rhombotin-like 2)	12	16592574	16652291
MED13	ENSG00000108510	Hs.282678	NM_005121.2	Mediator complex subunit 13	17	57374749	57497425
MED14	ENSG00000180182	Hs.407604	NM_004229	Mediator complex subunit 14	2	28828118	28879300
MTF1	ENSG00000188786	Hs.471991	NM_005955	Metal-regulatory transcription factor 1	1	38047827	38097879
NFIB	ENSG00000147862	Hs.644095	NM_005596	Nuclear factor I/B	9	14071847	14388630
PCDH7	ENSG00000169851	Hs.479439	NM_002589	Protocadherin 7	4	30331135	30753569
PHF15	ENSG00000043143	Hs.483419	NM_015288	PHD finger protein 15	5	133889246	133946817
PLAG1	ENSG00000181690	Hs.14968	NM_002655	Pleomorphic adenoma gene 1	8	57236037	57286392
PURB	ENSG00000146676	Hs.349150	NM_033224	Purine-rich element binding protein B	7	44889059	44891485

(Continued)

TABLE 3] (Continued)

Gene Name	Ensembl Gene	UniGene	RefseqRNA	Description	Chromosome	Start, bp	End, bp
RICTOR	ENSG00000164327	Hs.407926	NM_152756	Rapamycin-insensitive companion of mTOR	5	38973779	39110260
SETD7	ENSG00000145391	Hs.480792	NM_030648	SET domain containing (lysine methyltransferase) 7	4	140646642	140697027
SLC44A1	ENSG00000070214	Hs.573495	NM_080546	Solute carrier family 44, member 1	9	10706724	10741273
SP7	ENSG00000170374	Hs.209402	NM_152860.1	Sp7 transcription factor	12	52008197	52009489
TGFBR1	ENSG00000128268	Hs.494622	NM_001098270.1	Transforming growth factor, β receptor I (activin A receptor type 2-like kinase, 53kDa)	22	38183271	38218143

receptor interactions, and activin receptor-like kinase in cardiac myocytes.

Gene Networks

Individual miRNAs are involved in a variety of biologic responses, and certain categories are also enriched in their own target genes. We therefore constructed gene networks for all target predicted genes (Fig 3, e-Figs 1A, 1B). We also determined gene networks for the putative predicted target genes for each of the three differentially expressed miRNAs (e-Figs 1-3, e-Tables 1-3).

Discussion

The present study demonstrates that endothelial dysfunction in otherwise healthy obese and nonobese children is associated with a distinctive circulating miRNA signature. Many diseases are characterized by an abnormal miRNA expression pattern, and identification of the miRNA signature for specific diseases should prove useful for early screening and diagnosis of asymptomatic diseases such as endothelial dysfunction. Here, we identified three unique miRNAs in the circulating plasma of children with endothelial dysfunction and further examined putative gene target prediction genes for those miRNAs through implementation of several previously validated and established algorithms. Venn diagrams of the intersections between predicted target genes showed that 31 genes are common gene targets among all three differentially expressed miRNAs linked to endothelial dysfunction and also revealed some of the putative biologic functions and signaling pathways that may be pathophysiologically relevant in the context of abnormal endothelial function in children.[45]

In the present study, we identified three miRNAs in plasma, namely hsa-miR-125a-5p, hsa-miR-342-3p, and hsa-miR-365b-3p, whose concentrations were significantly altered in the plasma of eight otherwise asymptomatic children with endothelial dysfunction when compared with eight matched children in whom normal endothelial function was present. Further confirmatory efforts revealed the specificity of these miRNAs, as the miRNA differential expression persisted in a post hoc cohort of 60 children (including the original 16 subjects), even when obese NEF children (n = 5) were compared with nonobese children with endothelial dysfunction (n = 5). It has been suggested that hsa-mir-125a-5p is involved in microarray expression profiling in human myocardial infarction and remodeling process during the progression of heart

TABLE 4] List of the Most Significant Gene Ontology Biologic Processes As Derived From Interrogation of Predicted mRNA Target Genes

GO ID	GO Term	Gene Count	Percentage	*P* Value	Genes
GO:0010604	Positive regulation of macromolecule metabolic process	10	32.26	.000026	*MTF1, TGFBR1, BMPR2, CREB5, MED14, MED13, RICTOR, CBFB, NFIB, APC*
GO:0009952	Anterior/posterior pattern formation	5	16.13	.00016	*ACVR2B, HIPK1, TGFBR1, BMPR2, APC*
GO:0006350	Transcription	13	41.94	.00034	*PLAG1, LMO3, BMPR2, MED14, CREB5, MED13, CBFB, PURB, HIPK1, MTF1, SETD7, SP7, NFIB*
GO:0003002	Regionalization	5	16.13	.00058	*ACVR2B, HIPK1, TGFBR1, BMPR2, APC*
GO:0045449	Regulation of transcription	14	45.16	.00063	*PLAG1, LMO3, TGFBR1, MED14, CREB5, MED13, CBFB, PURB, ACVR2B, HIPK1, MTF1, SETD7, SP7, NFIB*
GO:0045941	Positive regulation of transcription	7	22.58	.00071	*MTF1, TGFBR1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0010628	Positive regulation of gene expression	7	22.58	.00083	*MTF1, TGFBR1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0035295	Tube development	5	16.13	.00089	*B4GALT1, ACVR2B, TGFBR1, BMPR2, NFIB*
GO:0030324	Lung development	4	12.90	.00098	*ACVR2B, TGFBR1, BMPR2, NFIB*
GO:0030323	Respiratory tube development	4	12.90	.001067	*ACVR2B, TGFBR1, BMPR2, NFIB*
GO:0006366	Transcription from RNA polymerase II promoter	5	16.13	.00112	*BMPR2, CREB5, MED14, MED13, CBFB*
GO:0045935	Positive regulation of nucleic acid metabolic process	7	22.58	.001212	*MTF1, TGFBR1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0060541	Respiratory system development	4	12.90	.001259	*ACVR2B, TGFBR1, BMPR2, NFIB*
GO:0051173	Positive regulation of nitrogen compound metabolic process	7	22.58	.001427	*MTF1, TGFBR1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0010557	Positive regulation of macromolecule biosynthetic process	7	22.58	.001544	*MTF1, TGFBR1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0007389	Pattern specification process	5	16.13	.001821	*ACVR2B, HIPK1, TGFBR1, BMPR2, APC*
GO:0031328	Positive regulation of cellular biosynthetic process	7	22.58	.001957	*MTF1, TGFBR1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0009891	Positive regulation of biosynthetic process	7	22.58	.002106	*MTF1, TGFBR1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0045893	Positive regulation of transcription, DNA-dependent	6	19.35	.002265	*MTF1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0051254	Positive regulation of RNA metabolic process	6	19.35	.002349	*MTF1, CREB5, MED14, MED13, CBFB, NFIB*
GO:0006351	Transcription, DNA-dependent	5	16.13	.002522	*BMPR2, CREB5, MED14, MED13, CBFB*
GO:0032774	RNA biosynthetic process	5	16.13	.002649	*BMPR2, CREB5, MED14, MED13, CBFB*
GO:0045667	Regulation of osteoblast differentiation	3	9.68	.003294	*ACVR2B, BMPR2, APC*
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	5	16.13	.005928	*MTF1, MED14, MED13, CBFB, NFIB*

(Continued)

TABLE 4] *(Continued)*

GO ID	GO Term	Gene Count	Percentage	*P* Value	Genes
GO:0030278	Regulation of ossification	3	9.68	.010493	*ACVR2B, BMPR2, APC*
GO:0051247	Positive regulation of protein metabolic process	4	12.90	.012182	*TGFBR1, BMPR2, RICTOR, APC*
GO:0042325	Regulation of phosphorylation	5	16.13	.01301	*ACVR2B, TGFBR1, BMPR2, RICTOR, APC*
GO:0006357	Regulation of transcription from RNA polymerase II promoter	6	19.35	.013315	*MTF1, SP7, MED14, MED13, CBFB, NFIB*
GO:0001934	Positive regulation of protein amino acid phosphorylation	3	9.68	.013501	*TGFBR1, BMPR2, RICTOR*
GO:0051174	Regulation of phosphorus metabolic process	5	16.13	.014883	*ACVR2B, TGFBR1, BMPR2, RICTOR, APC*
GO:0042327	Positive regulation of phosphorylation	3	9.68	.015896	*TGFBR1, BMPR2, RICTOR*
GO:0010562	Positive regulation of phosphorus metabolic process	3	9.68	.016838	*TGFBR1, BMPR2, RICTOR*
GO:0051094	Positive regulation of developmental process	4	12.90	.017444	*B4GALT1, ACVR2B, BMPR2, APC*
GO:0007178	Transmembrane receptor protein serine/threonine kinase signaling pathway	3	9.68	.017803	*ACVR2B, TGFBR1, BMPR2*
GO:0042127	Regulation of cell proliferation	6	19.35	.018247	*B4GALT1, HIPK1, TGFBR1, BMPR2, NFIB, APC*
GO:0006355	Regulation of transcription, DNA-dependent	9	29.03	.019046	*MTF1, SETD7, CREB5, SP7, MED14, MED13, PURB, CBFB, NFIB*
GO:0001655	Urogenital system development	3	9.68	.020145	*ACVR2B, TGFBR1, APC*
GO:0051252	Regulation of RNA metabolic process	9	29.03	.02157	*MTF1, SETD7, CREB5, SP7, MED14, MED13, PURB, CBFB, NFIB*
GO:0007167	Enzyme linked receptor protein signaling pathway	4	12.90	.029914	*ACVR2B, GRB10, TGFBR1, BMPR2*
GO:0044087	Regulation of cellular component biogenesis	3	9.68	.032355	*TGFBR1, RICTOR, APC*
GO:0051896	Regulation of protein kinase B signaling cascade	2	6.45	.033413	*TGFBR1, RICTOR*
GO:0060491	Regulation of cell projection assembly	2	6.45	.035344	*TGFBR1, APC*
GO:0030501	Positive regulation of bone mineralization	2	6.45	.035344	*ACVR2B, BMPR2*
GO:0070169	Positive regulation of biomineral formation	2	6.45	.037272	*ACVR2B, BMPR2*
GO:0001932	Regulation of protein amino acid phosphorylation	3	9.68	.046311	*TGFBR1, BMPR2, RICTOR*
GO:0045778	Positive regulation of ossification	2	6.45	.046856	*ACVR2B, BMPR2*
GO:0032273	Positive regulation of protein polymerization	2	6.45	.048762	*RICTOR, APC*
GO:0045669	Positive regulation of osteoblast differentiation	2	6.45	.048762	*ACVR2B, BMPR2*
GO:0015850	Organic alcohol transport	2	6.45	.048762	*SLC44A1, HIATL1*

GO = gene ontology.

TABLE 5] List of the Most Significant Molecular Function Biologic Processes as Derived From Interrogation of Predicted mRNA Target Genes

GO ID	GO Term	Gene Count	Percentage	*P* Value	Genes
GO:0004675	Transmembrane receptor protein serine/threonine kinase activity	3	9.68	.00058	*ACVR2B, TGFBR1, BMPR2*
GO:0005024	Transforming growth factor β receptor activity	3	9.68	.00058	*ACVR2B, TGFBR1, BMPR2*
GO:0030145	Manganese ion binding	4	12.90	.00348327	*B4GALT1, ACVR2B, TGFBR1, BMPR2*
GO:0030528	Transcription regulator activity	9	29.03	.00754371	*PLAG1, MTF1, CREB5, SP7, MED14, MED13, PURB, CBFB, NFIB*
GO:0016563	Transcription activator activity	5	16.13	.0084461	*MTF1, MED14, MED13, CBFB, NFIB*
GO:0003713	Transcription coactivator activity	4	12.90	.00868169	*MTF1, MED14, MED13, CBFB*
GO:0008134	Transcription factor binding	5	16.13	.01805295	*MTF1, MED14, MED13, PURB, CBFB*
GO:0019838	Growth factor binding	3	9.68	.0185566	*ACVR2B, TGFBR1, BMPR2*
GO:0003712	Transcription cofactor activity	4	12.90	.03502498	*MTF1, MED14, MED13, CBFB*
GO:0003700	Transcription factor activity	6	19.35	.04146813	*PLAG1, MTF1, CREB5, PURB, CBFB, NFIB*
GO:0042809	Vitamin D receptor binding	2	6.45	.04509705	*MED14, MED13*
GO:0043167	Ion binding	14	45.16	.04984618	*PLAG1, B4GALT1, FRAS1, GABRB2, LMO3, TGFBR1, BMPR2, CREB5, PCDH7, ACVR2B, MTF1, PHF15, SP7, CACNA1C*

See Table 4 legend for expansion of abbreviation.

disease.[46] This mir-125a has been suggested to coordinate the suppression of ERBB2 and ERBB3 by enforced expression of miR-125a.[47] Plasma microRNAs can serve as biomarkers of therapeutic efficacy and disease progression in hypertension-induced heart failure and have included hsa-mir-342-3p.[48,49] For hsa-mir-365b, it has been reported to be involved in advanced heart failure and also following support with a left ventricular assist device.[50]

Increasing evidence indicates that miRNAs play critical roles in many key biologic processes, such as cell growth, tissue differentiation, embryonic development, cell proliferation, and apoptosis.[51] As such, dysregulation of miRNAs and their effects on downstream gene targets may result in disease states that include the cardiovascular system.[52,53] Recently, several studies have shown that plasma miRNA levels are significantly altered in specific CVDs and that some of these miRNAs

TABLE 6] List of the Most Significant Cellular Component Biologic Processes as Derived From Interrogation of Predicted mRNA Target Genes

GO ID	GO Term	Gene Count	Percentage	*P* Value	Genes
GO:0009986	Cell surface	4	12.90	.020994	*FRAS1, B4GALT1, ACVR2B, BMPR2*
GO:0044459	Plasma membrane part	9	29.03	.025195	*FRAS1, B4GALT1, ACVR2B, GABRB2, TGFBR1, BMPR2, PCDH7, CACNA1C, APC*
GO:0016592	Srb-mediator complex	2	6.45	.0487818	*MED14, MED13*

See Table 4 legend for expansion of abbreviation.

have the potential to serve as novel biomarkers for early diagnosis.[54] Since a single miRNA can target several genes, and multiple miRNAs may share common targets, miRNAs are particularly suited for regulating processes and pathways at the "network" level.[55] The current challenge is to accurately identify targets that are specifically regulated by miRNAs.

To further understand the function of the three differentially expressed plasma miRNAs in children with endothelial dysfunction, we proceeded to identify their putative targets using multiple bioinformatics tools. Although miRNAs traditionally influence the expression of their target genes in a negative manner via mRNA degradation and translational repression, there is also evidence supporting that they may also enhance the expression of genes.[56] Prediction of miRNA target genes is challenging in that most bioinformatics approaches, when used alone, usually yield an excessive number of genes. Thus, we applied a strict curation of such lists through combinatorial bioinformatics analyses. In this study, we found some potential gene targets involved in metabolic processing of macromolecules such as those included in the GO terms MED13 and MED14 (Tables 4-6). Mediators (MED) are multi-subunit complexes, which act as a bridge between DNA-bound transcription factors and RNA polymerase II, which joins transcription factors bound at the upstream regulatory elements, such as nuclear receptors and the transcription machinery at the promoter region.[57-60] A study demonstrated that MED13 functions in the heart to control metabolic homeostasis and energy expenditure,[58] whereas MED14 directly interacts with the N-terminus of PPARγ in a ligand-independent manner and is required for the transcription activity of peroxisome proliferator-activated receptor γ.[61] Activin A receptor, type IIB (ACVR2B) activins are dimeric growth and differentiation factors that belong to the TGF-β superfamily of structurally related signaling proteins. Activin A has been recognized as a multifunctional cytokine expressed in a wide range of tissues and cells with roles in regulation of wound repair,

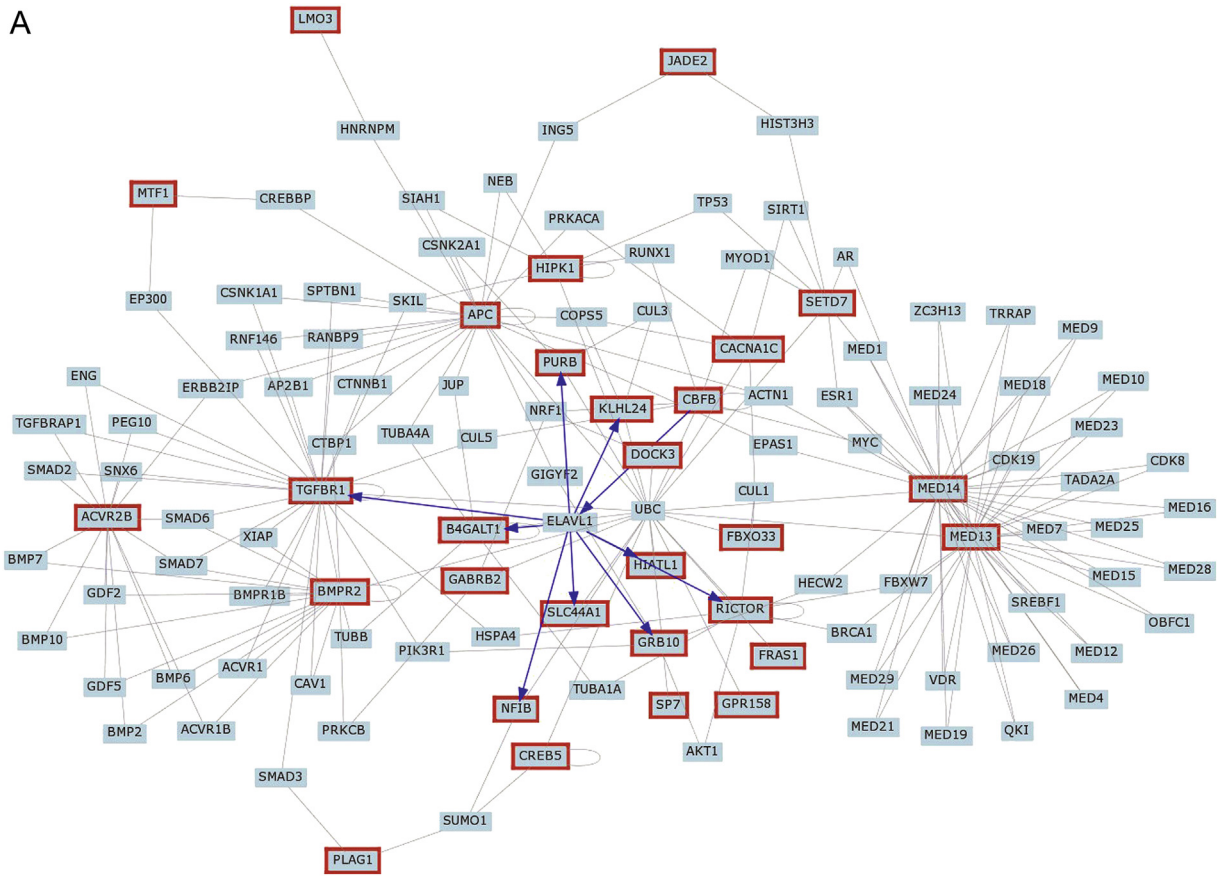

Figure 3 – *Gene network representation of interactions between common gene target predictions as identified from the Venn diagram in Figure 2. A, Interactions of the target genes with at least two list members of the query. B, Interactions of the target genes with all known interactions of gene products. Note that these densely connected nodes capture a significant proportion of the overall network connectivity.*

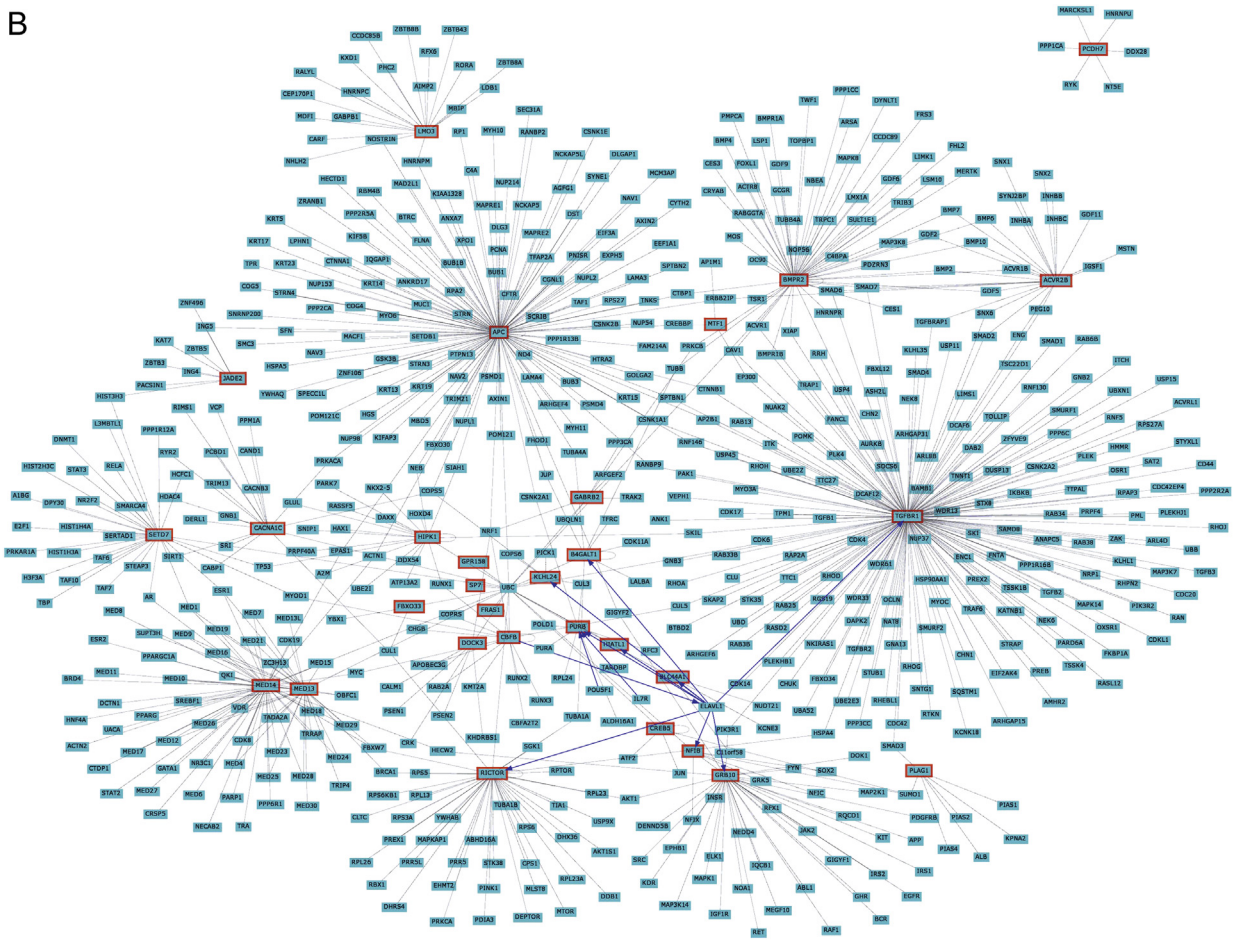

Figure 3 – *Continued*

cell differentiation, apoptosis, embryogenesis, and inflammation.[62] Activin A is induced by angiotensin II in smooth muscle cells and may be induced by inflammatory cytokines such as tumor necrosis factor-α and IL-1.[63] Thus, there is clearly biologic plausibility to the miRNA-RNA networks identified in children with endothelial dysfunction that could potentially be used in future multicenter studies aimed at validating the miRNA signature discovered herein.

Some of the strengths and limitations of the study should be mentioned. First, our recruitment approaches using a laser Doppler-based technique for assessment of endothelial function in children enabled inclusion of otherwise healthy children from the community. Furthermore, implementation of a previously validated 60-s occlusion duration rather than the more extensively used 5 min in adults prevents frequent motion-induced artifacts in children without detracting from the physiologic significance of the measurement.[36,64] In addition, all vascular assessments were conducted in the fasting state at the same time of the day to minimize potential confounders introduced by differences in meal content and timing relative to the testing and also to ensure that circadian variation in endothelial function would not play a role. Particular precautions were implemented to avoid hemolysis during plasma isolation, since it can affect circulating miRNA levels. However, we did not assess miR-15b and/or miR-16 as hemolysis indicators. In addition, exploratory in silico analyses (eg, Tarbase query) did not reveal any known relevant vascular function for two of the identified three miRNAs. Since these databases are continuously being updated, prospective re-evaluation of such miRNAs is justified. Finally, since the initial screening cohort was relatively small, the possibility exists that additional differentially expressed miRNAs may have not been identified, and as such additional biomarkers may be found in future larger studies.

Endothelial dysfunction induces impairment of regional blood flow in various organs and tissues. It is now evident from many studies that childhood obesity is correlated with adult excess weight status and the

development of risk factors for cardiovascular diseases in adulthood, including hypertension, type 2 diabetes mellitus, dyslipidemia, and metabolic syndrome.[65] Findings about obese children include not only the alteration characteristic of metabolic syndrome but also systemic low-grade inflammation and disorders indicative of endothelial dysfunction.[66-68] In this study we found that some of the metabolic parameters are significantly higher, such as triglyceride, insulin, and HOMA-insulin resistance, as well as hsCRP as shown in Table 1. The magnitude and characteristics of such metabolic and inflammatory parameters would ultimately underlie the probability of end-organ vascular injury with its attendant morbid consequences. However, the specificity of the three putative miRNAs as being indicative of endothelial dysfunction, rather than reflecting obesity-related or metabolic-related abnormalities, was confirmed by the differentially expressed miRNAs in five nonobese children with NEF and five nonobese children with endothelial dysfunction in whom there was no evidence of either dyslipidemia or insulin resistance. In the next a few years, we likely will see further breakthroughs in cardiovascular miRNA research, such as initial phase 1 and II projects using miRNA therapeutics in cardiovascular medicine that may pave the way for large-scale mechanism-orientated miRNA-based therapeutic trials in pediatric cardiovascular risk management approaches.

Conclusions

This study provides initial evidence that a subset of circulating miRNAs are deregulated in children with endothelial dysfunction. Biomarkers for early detection of children in general, and more particularly overweight and obese children, at risk for developing vascular abnormalities would greatly improve delineation of targeted interventions in this high-risk population. We further show that systems biology-based approaches provide new avenues for biologic interpretation of miRNA profiling and generation of experimentally testable hypotheses regarding collective regulatory functions of miRNAs in cardiovascular disease.

Acknowledgments

Author contributions: D. G. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, including and especially any adverse effects. A. K. contributed to performing miRNA experiments, performed data analyses, and drafted the initial version of the manuscript; L. K.-G. contributed to performing endothelial function studies, and performed data analyses; R. B. contributed to recruiting experimental subjects and assisted with endothelial function studies; A. A. K. contributed to performing bioinformatics analyses; D. G. contributed to conceptualizing the project, provided the funding for the project, critically reviewed data, and contributed to all phases of manuscript drafting; D. G., A. K., L. K.-G., R. B., and A. A. K. contributed to reviewing and approving the final version of the manuscript.

Financial/nonfinancial disclosures: None declared.

Role of sponsors: The sponsor had no role in the design of the study, the collection and analysis of the data, or the preparation of the manuscript.

Additional information: The e-Appendix, e-Figures, and e-Tables can be found in the Supplemental Materials section of the online article.

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