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ARTICLE

MicroRNA sequencing in patients with coronary artery disease – considerations for use as biomarker for thrombotic risk

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Abstract

MicroRNAs (miRNAs) are small RNAs integral in the regulation of gene expression. Analysis of circulating miRNA levels may identify patients with coronary artery disease (CAD) at risk for recurrent myocardial infarction (MI) after percutaneous coronary interventions (PCIs). Subjects with CAD were selected from the GENCATH cardiac catheterization biobank. Subjects with recurrent MI after PCI were compared with those without recurrent MI during follow-up in the initial ($n = 48$) and replication cohort ($n = 67$). Next generation MiRNA sequencing was performed on plasma samples and whole blood samples fixed with PAXGENE tubes upon collection. Overall, 164 miRNAs derived from whole blood were differentially expressed in the replication cohort between subjects with and without recurrent MI events ($p < 0.05$), with 69 remaining significant after false-discovery rate (FDR) correction. None of the miRNAs in plasma was significantly different by FDR among subjects with and without MI. Overall, correlation between direction of effects between plasma and whole blood assays was variable, and only two miRNAs were concordant and significant in both. Associations of miRNA with vascular disease, MI, and thrombosis were further explored. MiRNA profiling has potential as the future biomarker for disease prognosis and treatment response marker in secondary treatment of patients with CAD after PCI. Whole blood may be the preferred sample source as compared to plasma.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Micro RNAs (miRNAs) are promising biomarkers associated with various disease states, including coronary artery disease (CAD). Most studies evaluating miRNA as biomarkers in CAD have previously utilized miRNA extracted from plasma sources, mainly due to widely available sample sources. Few studies have specifically compared overlap between whole blood and plasma sources of miRNA in CAD, or relative contribution of cellular components to miRNA levels in whole blood.

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WHAT QUESTION DID THIS STUDY ADDRESS?

This study examined next-generation sequencing miRNA profiles in patients with established CAD comparing patients with recurrent myocardial infarction (MI) during follow-up versus those without recurrent events. We analyzed overlap of miRNA identified with differential expression in plasma versus whole blood.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Our study expands on prior work using miRNA to establish a high-risk biometric profile of patients with recurrent MI events. We identified a series of miRNAs in whole blood potentially associated with increased risk of recurrent MI after coronary intervention. No significant difference was found in samples derived from plasma. Several of these miRNAs have been previously linked to thrombosis, vascular inflammation, and coronary disease.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Knowledge about the significant impact of sample source on miRNA profiling should influence future investigations on use of miRNA in determining vascular thrombotic risk profiles, with larger variability and differential expression found in miRNA sequenced from whole blood sources. Specific miRNA associated with recurrent thrombotic events may be suitable for risk assessment or may be involved in gene regulation of potentially novel disease modifying pathways.

INTRODUCTION

One in five patients experiences a recurrent cardiac event within 5 years of their first cardiac event despite the success of drug therapy in reducing mortality and morbidity. This signifies a challenge to our current approaches to care. Recurrent ischemic events in patients with coronary artery disease (CAD) are associated with increased risk of sudden cardiac death, myocardial infarction (MI), stroke, and heart failure. Medical therapy for ischemic heart disease centers around attenuation of prothrombotic pathways implicated in pathogenesis of arterial thrombosis. Both pro-aggregatory changes in platelet function, as well as pro-coagulant environment, has been demonstrated in patients with CAD and metabolic syndrome at risk for recurrent ischemic events.[1,2]

Following an acute coronary syndrome, the American College of Cardiology (ACC)/American Heart Association (AHA) guideline recommends using dual antiplatelet therapy for 12 months after the index event, followed by aspirin monotherapy,[3,4] with extension of dual antiplatelet therapy beyond 12 months in some patients based on their individual risks.[3] While addition of low-dose FXa inhibitor to aspirin in patients with established cardiovascular (CV) disease has demonstrated reduction in risk of recurrent MI, the routine combination of anticoagulant and antiplatelet therapy is often limited by bleeding risk in net clinical benefit calculations. Interindividual variability in protein and gene expression related to coagulation and platelet function may influence a patient's response to antithrombotic medications, requiring adjustments or changes in drug therapy. This emphasizes the need to focus on antithrombotic therapy in a more individualized fashion, however, currently, there are few validated ex vivo biomarkers to diagnose and predict in vivo thrombosis.

Fibrin degradation product and D-dimer are coagulation biomarkers with characteristic elevation of levels when fibrinolysis occurs in acute diseases, however, their utility in future thrombotic risk prediction is limited.[5] Biomarkers associated with risk of specific clinical events in observational studies may be candidates for therapy modification and risk prediction in clinical practice. This approach may be utilized in the identification and treatment of patients at increased risk for recurrent ischemic cardiac events that may necessitate specific interventions tailored to coagulation or antiplatelet pathways.

A biomarker with growing utility is microRNA (miRNA). MiRNAs are small endogenous, noncoding RNAs that are ~20–24 nucleotides in length. MiRNAs bind to messenger RNA (mRNA) and post-transcriptionally repress the expression of target genes. They can be obtained through blood samples and analyzed within days, providing a feasible tool to quickly assess drug therapy.

In this study, we utilized the cardiac catheterization biobank GENCATH to examine circulating miRNAs from blood samples of patients. We sequenced miRNA in the whole blood and plasma samples of patients with angiographically confirmed CAD who underwent percutaneous

coronary intervention (PCI). Our primary aims of this study were to identify miRNAs with associations to CAD manifestations and prothrombotic phenotypes, and to replicate significant miRNAs from whole blood identified in our prior work.[6] We hypothesized that miRNAs with statistical significance could be identified in both whole blood and plasma samples. These miRNAs could have potential to serve as biomarkers to identify patients that require more aggressive antithrombotic therapies to mitigate their risk of subsequent cardiac events.

METHODS

Study design

The Krannert cardiac catheterization biobank (GENCATH) study is a 437-subject biobank at Indiana University that prospectively enrolled patients undergoing cardiac catheterization. Subjects were selected based on their history of CAD. The Indiana University Institutional Review Board approved the study protocol, and all subjects gave written informed consent prior to enrollment in GENCATH. Inclusion criteria for the GENCATH biobank were those undergoing coronary angiography or PCI at one of the participating institutions (Indiana University Health Methodist and Eskenazi Health) during the index presentation. Exclusion criteria for the biobank include patients under 18 years of age, and those who were unable to provide consent. Baseline variables, such as index coronary angiographic findings and index interventions, were recorded at the time of enrollment in GENCATH. Subjects were then prospectively followed for an average of 1.59 ± 1.1 years with a review of electronic medical records for the occurrence of clinical events. Coronary angiograms were reviewed to ascertain cases of stent thrombosis that occurred during the follow-up period. MI events were defined as per the universal definition of MI.[7]

Two cohorts of patients were selected from the GENCATH biobank (Figure 1). Cohort 1 was described and whole blood samples were sequenced in a previous paper (Kanuri et al.).[6] Plasma samples were not analyzed at that time, which was now completed as part of the current study in the same group of patients from cohort 1 in order to assess the potential source and contribution from cellular components to miRNA profiles. The patients from cohort 1 were divided into two groups: a stable CAD group ($n = 24$) without recurrent events, and a CAD group with recurrent MI events ($n = 17$; Figure 1).

In addition, miRNA sequencing of whole blood was performed in replication of cohort 1 in a similar second set of patients from the GENCATH biobank (named cohort 2; Figure 1). Cohort 2 consisted of 67 patients randomly selected from the GENCATH population with CAD who underwent PCI at the time of enrollment and who had whole blood samples available for sequencing. Cohort 2 subjects were divided into two groups, one group with stable CAD without recurring events during follow-up ($n = 51$) and the other group included those with recurrent MI events ($n = 16$; Figure 1).

A literature search of PubMed and Google Scholar was conducted to identify connections, associations, or causative relationships between the identified miRNA and cardiac manifestations. Studies were included in the discussion of our results if the researchers identified

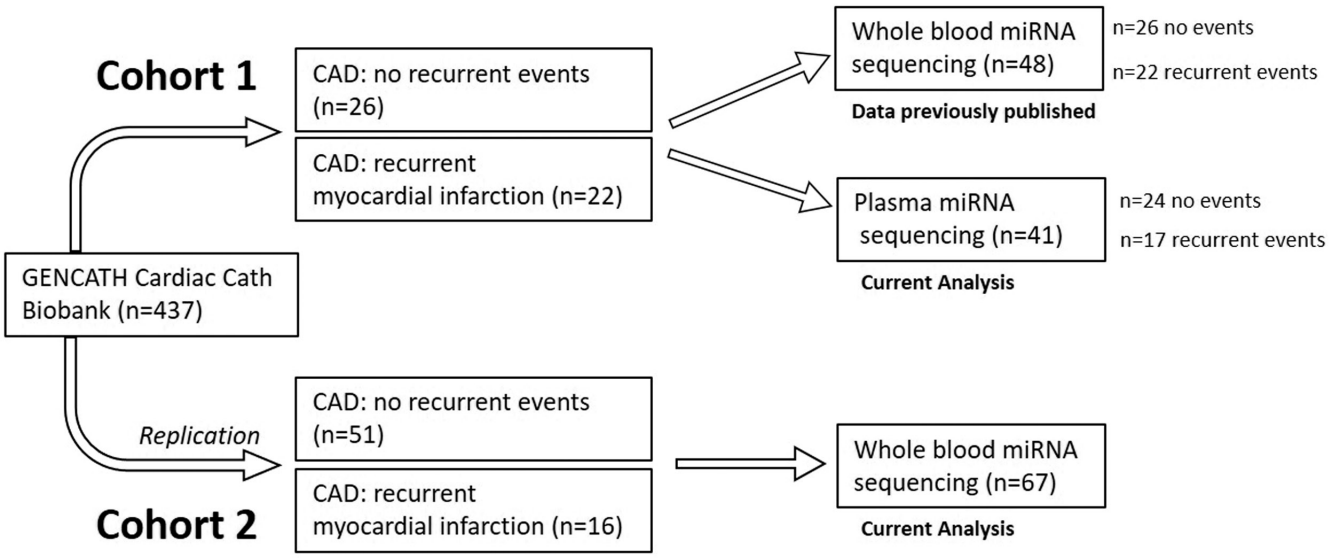

FIGURE 1 Study flowchart describing samples and cohorts used for analysis from GENCATH cardiac cath biobank. Results from cohort 1 whole blood miRNA analysis have been previously published[6] (Kanuri et al., Atherosclerosis 2019). CAD, coronary artery disease; miRNA, microRNA.

an association between the miRNA or its family and a cardiac-related manifestation. We created three categories based on phenotypes: myocardial disease (myocardial fibrosis, heart failure, and myocardial remodeling); and vascular disease (atherosclerosis, vascular inflammation, hypertension, and valvular heart disease); and thrombosis (arterial and venous thrombosis, pulmonary embolism, platelet aggregation, MI, and cerebral infarction).

Sample collection and RNA isolation

Whole blood analysis

Whole blood, sourced from either peripheral venipuncture or from arterial access sheaths, was collected during the index hospitalization period prior to cardiac catheterization and prior to administration of anticoagulants, such as heparin. None of the patients were receiving heparin at the time of blood sampling. Blood samples of patients who presented with ST-elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI) were sampled prior to hospital discharge. Samples were collected in PAXgene blood RNA vacutainer tubes and stored at −80°C (Qiagen). PAXgene blood RNA tubes were thawed on ice and incubated at room temperature overnight for processing. Total RNA was extracted, including miRNAs, using the PAXgene Blood miRNA kit (Qiagen; CAT #763134) according to the manufacturer's instructions for isolation of RNA from whole blood. Total RNA was then quantified using Qubit Fluorometers Quantification (Thermo Fisher Scientific). RNA quantity and the amount of miRNA was assessed using the Agilent Bioanalyzer (Agilent Technologies).

Plasma analysis

A citrated tube was used to collect whole blood from which plasma was isolated by centrifugation and frozen at –80°C. Prior to use, frozen plasma was thawed at room temperature. Cell-free total RNA, including miRNA, was isolated from 200 µl plasma using the Qiagen miRNeasy Serum/Plasma Kit per the manufacturer's protocol. Eluted miRNA was stored at −80°C until analysis.

miRNA sequencing and bioinformatics analysis

Whole blood and plasma sequencing libraries were prepared using the Qiagen QIAseq miRNA Library Kit, per the manufacturer's protocol. Whole blood miRNA was sequenced using an Illumina NovaSeq 6000 in single-end configuration with a read length of 75 bp. Plasma miRNA was sequenced on an Illumina NextSeq 500 in single-end configuration with a read length of 75 bp. All sequencing runs were required to have 75% or more reads with greater than or equal to Q30. After sequencing, read mapping and quantification were performed using Qiagen GeneGlobe Data Analysis Center. Details of the Qiagen GeneGlobe analysis pipeline can be found on the Qiagen website in the QIAseq miRNA Primary Quantification Supplementary Protocol documentation.[8] All samples analyzed met a threshold of having >2 million reads mapped to miRNAs. Differential expression analysis, comparing patients with one CAD event to those with recurrent CAD events, was done with edgeR version 3.28.1[9] using negative binomial generalized linear modeling and likelihood ratio tests according to the recommendations in the edgeR user's guide. Features (miRNAs or piwi interacting RNAs) with cpm ≥0.05 in at least the number of samples in the smallest group (in both experiments, this was 17 samples) were analyzed for differential expression. A p value of <0.05 was considered significant for miRNA, which were evaluated in replication between cohorts. Adjustment for de novo analysis with multiple comparisons was included with a false discovery rate (FDR) of 0.10.

RESULTS

Clinical characteristics and end points

The demographics and clinical variables of subjects enrolled in the GENCATH biobank cohort and those selected for miRNA sequencing are described in Table 1. Patients who experienced recurrence of MI during subsequent follow-up were categorized as subjects with recurrent ischemic events and compared with those who did not experience any subsequent events (no recurrent events; Table 2). There was no significant difference between the subjects regarding age, sex, weight, and most comorbidities (diabetes mellitus, hypertension, hyperlipidemia, history of coronary artery bypass grafting, history of PCI, and P2Y12 antiplatelet therapy). Subjects with recurrent MI events were less likely to have a history of MI and more likely to have congestive heart failure. The mean follow-up time was not significantly different between subjects with and without MI events (mean follow-up time for patients with MI vs. those without MI 1.68 ± 1 vs. 1.62 ± 1.1 years; $p = 0.73$).

Cohort 1 (plasma samples)

Differential expression analysis of the plasma samples identified 81 miRNAs that were statistically significantly

TABLE 1 GENCATH patient demographics

Clinical variables	GENCATH biobank ($n = 437$)	Selected CAD patients ($n = 108$)
Age, years	56.4 ± 10	57.2
Male	252 (57.7%)	67 (62%)
Weight, kg	94.8 ± 25.2	93.3
White	294 (67.3%)	77 (71.3%)
African American	126 (28.8%)	24 (22.2%)
Hypertension	364 (83.3%)	94 (87%)
Hyperlipidemia	318 (72.8%)	92 (88.5%)
Diabetes mellitus	199 (45.5%)	55 (50.9%)
History of coronary artery bypass grafting	40 (9.2%)	12 (11.1%)
History of PCI	189 (43.2%)	78 (72. 9%)
Congestive heart failure	92 (21.1%)	28 (26.2%)
Chronic obstructive pulmonary disease/asthma	123 (28.1%)	40 (37%)
Angiographic diagnosis of CAD	310 (70.9%)	108 (100%)
PCI during index presentation	181 (41.4%)	108 (100%)
Current presentation		
STEMI	61 (14%)	5 (4.6%)
NSTEMI	104 (23.8%)	38 (35.2%)
Unstable angina	111 (25.4%)	21 (19.4%)
Stable angina	55 (12.6%)	10 (9.3%)
Outcomes	**Mean follow-up 1.59 ± 1.1 years**	
Death	22 (5%)	5 (4.6%)
MI	44 (10.1%)	33 (30.6%)
Stent thrombosis	7 (1.6%)	4 (3.7%)
Major bleeding	13 (3%)	4 (3.7%)
Unplanned PCI	24 (5.5%)	12 (11.1%)

Demographics and baseline clinical variables of patients in the GENCATH cardiac biobank, and of those subjects included in the current analysis. Student *t*-test was used to compare continuous data and chi-square for categorical data.

Abbreviations: CAD, coronary artery disease; MI, myocardial infarction; NSTEMI, non-ST-elevation myocardial infarction; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction.

differentially expressed among subjects with recurrent versus no recurrent events ($p < 0.05$; Table S1). None were statistically significant after the FDR correction (≤0.1). The log 2-fold change (log2FC) effect size of the miRNA in the plasma samples ranged from −2.204 to 2.69. A list of all miRNAs with significant differential expressions identified in cohort 1 in plasma analysis is provided in Table S1.

Cohort 2 (whole blood samples)

The differential expression analysis of the whole blood samples identified 163 miRNAs that were statistically significantly different between subjects with no recurrent ischemic events and those with future recurrent MI events ($p < 0.05$), 69 of these remained significant after FDR correction (FDR ≤0.1; Table 3, Table S2). Log2FC effect sizes of the miRNAs that were significant after FDR correction ranged from −5.47 to 1.63. Most miRNAs with significant alteration among patients with recurrent MI had decreased expression levels as compared to those without recurrent MI. A list of all miRNAs with significant differential expressions identified in cohort 2 in whole blood analysis is provided in Table S2.

Comparison of cohorts and plasma versus whole blood samples

Five miRNAs were statistically significant ($p < 0.05$) in both the plasma samples from cohort 1 and the whole blood samples from cohort 2 (miR-106a-3p, miR-22-5p,

TABLE 2 Subjects with CAD and events during follow-up

Clinical variables	No recurrent events ($n = 75$)	Recurrent ischemic events ($n = 33$)	p value
Age, years	56.5	58.7	0.26
Male	48 (64%)	19 (57.6%)	0.53
Weight, kg	94.1	91.6	0.6
White	57 (76%)	20 (60.6%)	0.037
Black or African American	14 (18.7%)	10 (30.3%)	0.07
Smoking (current)	41 (54.67%)	16 (48.5%)	0.87
Family history of CAD	51 (81%)	19 (70%)	0.34
Diabetes mellitus	36 (48%)	19 (57.6%)	0.36
Hypertension	63 (84%)	31 (93.9%)	0.16
Hypercholesteremia	65 (87.8%)	27 (90%)	0.76
History of MI	49 (65.3%)	16 (48.5%)	0.038
Coronary artery bypass grafting	7 (9.3%)	5 (29.4%)	0.12
History of PCI	57 (77%)	21 (63.6%)	0.15
Congestive heart failure	15 (20%)	13 (39.4%)	0.038
P2Y12 inhibitor			
Clopidogrel	41 (54.7%)	13 (39.4%)	0.14
Prasugrel	15 (20%)	6 (18.2%)	0.83
Ticagrelor	4 (5.3%)	2 (6.1%)	0.88

Note: Chi-square for categorical and Student's *t*-test for continuous variables.

Comparison of baseline demographics, clinical variable and comorbidities of patients with recurrent myocardial infarction vs those without recurrent events. Student *t*-test was used to compare continuous data and chi-square for categorical data.

Abbreviations: CAD, coronary artery disease; MI, myocardial infarction; PCI, percutaneous coronary intervention.

miR-1304-3p, miR-4473, and miR-625-3p; Figure 2, Tables S1 and S2) using the same next-generation sequencing technique. Two of the whole blood miRNAs with FDR ≤0.1 showed significant concordant differential expression in plasma ($p < 0.05$, miR-1304-3p and miR-625-3p; Table 3). Radar plot for the log2FC of whole blood miRNA with FDR ≤0.1 demonstrates inconsistent direction of log2FC effects in plasma (Figure 3).

Only two miRNAs (miR-197-3p and miR-744-3p) were demonstrated as differentially expressed in both plasma and whole blood in the same patients from cohort 1 (Figure 2) using different miRNA sequencing technology (Kanuri et al.).[6]

Ten miRNAs were differentially expressed ($p < 0.05$) among subjects with recurrent MI events versus those without events in both cohorts 1 and 2 in whole blood sequencing: miR-1292-5p, miR-144-5p, miR-148a-5p, miR-181a-3p, miR-19a-3p, miR-301a-3p, miR-340-3p, miR-3605-3p, miR-4745-3p, and miR-589-5p (Figure 2).

Analysis of whole blood samples from cohort two yielded a significantly larger number of distinct catalogued miRNA as compared to whole blood samples analyzed from cohort 1 and published previously (cohort 2 whole blood: 1058 miRNAs vs. cohort 1 whole blood: 321 miRNAs [Kanuri et al.[6]]). Plasma samples from cohort 1 analyzed in this current study also showed significantly larger number of miRNAs (2122 miRNAs) using more recent sequencing technology as compared to whole blood analysis from the same patients in cohort 1 in a prior publication (Kanuri et al.[6]; Tables S1 and S2). Overall, there was a distinct separation between plasma samples and whole blood samples on principal component analysis cluster plot, suggestive of distinct differential miRNA expression based on sample source (Figure 4).

Associations with CV disease of differentially expressed miRNAs previously published in the literature are summarized (Table S3).

DISCUSSION

We previously sequenced circulating miRNAs from whole blood samples provided by patients in the GENCATH biobank to determine miRNA profiles associated with risk of recurrent MI after PCI.[6] Using whole blood miRNA sequencing, we detected miRNAs that were significantly differentially expressed in patients with CAD with no recurrent events versus in patients with CAD with recurrent MI events. Those findings raised the questions of whether the miRNAs we identified were from cell-free (plasma) or

TABLE 3 List of miRNAs in whole blood from cohort 2 with significant differential expression (FDR <0.1) among subjects with recurrent and those without recurrent ischemic events and expression of the same miRNAs in plasma in cohort 1

	Whole blood				Plasma			
miRNA	**Log2FC**	**FC**	***p* value**	**FDR**	**Log2FC**	**FC**	***p* value**	**FDR**
hsa-miR-6752-5p	−4.82	−28.28	7.76E-09	8.21E-06	−0.20	−1.15	0.52	1.00
hsa-miR-4690-5p	−5.42	−20.43	2.17E-07	7.64E-05	−0.01	−1.00	0.98	1.00
hsa-miR-541-5p	−4.35	−42.70	1.59E-07	7.64E-05	−0.14	−1.10	0.65	1.00
hsa-miR-6871-3p	−4.23	−18.72	2.99E-07	7.91E-05	0.00	1.00	0.99	1.00
hsa-miR-6862-5p	−3.27	−9.65	3.88E-07	0.000082	0.16	1.12	0.50	1.00
hsa-miR-6776-3p	−4.65	−25.08	8.65E-07	0.00015	0.20	1.15	0.68	1.00
hsa-miR-1304-3p	0.72	1.64	1.72E-06	0.0002	0.83	1.78	0.007	0.83
hsa-miR-4738-5p	−5.47	−44.46	1.95E-06	0.0002	−0.03	−1.02	0.95	1.00
hsa-miR-8055	−4.94	−30.76	1.11E-05	0.0011	0.17	1.12	0.54	1.00
hsa-miR-3189-3p	−4.69	−25.80	1.44E-05	0.0014	0.13	1.09	0.62	1.00
hsa-miR-1227-5p	−3.58	−11.98	1.69E-05	0.0015				
hsa-miR-6795-5p	−3.40	−10.53	2.02E-05	0.0016	0.06	1.04	0.83	1.00
hsa-let-7a-2-3p	−4.25	−18.97	2.21E-05	0.0017	−0.32	−1.25	0.13	1.00
hsa-miR-8064	−4.72	−26.30	2.37E-05	0.0017				
hsa-miR-6722-5p	−4.81	−28.04	2.66E-05	0.0018	−0.09	−1.07	0.80	1.00
hsa-miR-3909	−2.06	−4.16	3.24E-05	0.002	0.10	1.07	0.86	1.00
hsa-miR-3190-3p	−3.89	−14.81	4.36E-05	0.003	−0.04	−1.03	0.95	1.00
hsa-miR-4674	−4.08	−16.88	4.59E-05	0.003	−0.90	−1.87	0.20	1.00
hsa-miR-4800-3p	0.99	1.98	7.41E-05	0.004	−0.06	−1.04	0.84	1.00
hsa-miR-34a-5p	0.94	1.91	9.88E-05	0.005	0.25	1.19	0.45	1.00
hsa-miR-6769b-3p	−1.53	−2.89	0.0001	0.005	0.21	1.16	0.39	1.00
hsa-miR-6779-3p	−3.33	−10.03	0.0001	0.005	−0.08	−1.05	0.62	1.00
hsa-miR-144-3p	−2.02	−4.05	0.0001	0.006	−0.09	−1.06	0.89	1.00
hsa-miR-558	−4.85	−28.76	0.0002	0.007	0.40	1.32	0.58	1.00
hsa-miR-92a-1-5p	−3.34	−10.09	0.0002	0.007	0.31	1.24	0.13	1.00
hsa-miR-4473	−0.88	−1.84	0.0002	0.008	0.56	1.48	0.039	1.00
hsa-miR-4729	−3.13	−8.74	0.0003	0.01	0.00	1.00	0.99	1.00
hsa-miR-1299	1.64	3.11	0.0003	0.011	1.35	2.55	0.060	1.00
hsa-miR-6825-3p	−1.27	−2.41	0.0004	0.012	−0.21	−1.16	0.74	1.00
hsa-miR-3190-5p	−2.48	−5.57	0.0004	0.013	−0.20	−1.15	0.25	1.00
hsa-miR-6760-3p	−3.27	−9.63	0.0004	0.013	0.07	1.05	0.65	1.00
hsa-miR-23c	−0.94	−1.92	0.0005	0.014	0.31	1.24	0.15	1.00
hsa-miR-1225-5p	−4.95	−30.92	0.0005	0.015	−0.05	−1.03	0.91	1.00
hsa-miR-197-5p	−2.86	−7.25	0.0005	0.015	−0.10	−1.07	0.74	1.00
hsa-miR-596	−3.24	−9.43	0.0005	0.015	−0.22	−1.16	0.38	1.00
hsa-miR-6808-3p	−0.63	−1.54	0.0006	0.015				
hsa-miR-4632-5p	−2.99	−7.95	0.0008	0.02	−0.18	−1.13	0.39	1.00
hsa-miR-216b-3p	−2.66	−6.33	0.0008	0.021	0.30	1.24	0.20	1.00
hsa-miR-487b-5p	−2.76	−6.79	0.0009	0.022	−0.37	−1.29	0.11	1.00
hsa-miR-301a-3p	−1.33	−2.51	0.0011	0.026	0.06	1.04	0.89	1.00
hsa-miR-589-5p	0.39	1.31	0.0012	0.028	−0.20	−1.15	0.54	1.00
hsa-miR-625-3p	0.44	1.36	0.0012	0.028	0.80	1.74	0.015	1.00

TABLE 3 (Continued)

	Whole blood				Plasma			
miRNA	**Log2FC**	**FC**	***p* value**	**FDR**	**Log2FC**	**FC**	***p* value**	**FDR**
hsa-miR-6771-3p	−3.16	−8.91	0.0013	0.029	0.10	1.07	0.61	1.00
hsa-miR-6781-5p	−3.60	−12.08	0.0017	0.037	−0.13	−1.09	0.42	1.00
hsa-miR-1471	−3.91	−15.00	0.0021	0.045	0.05	1.04	0.94	1.00
hsa-miR-425-3p	0.41	1.33	0.0021	0.045	0.12	1.09	0.78	1.00
hsa-miR-4745-3p	−1.52	−2.86	0.0022	0.045	−0.16	−1.12	0.42	1.00
hsa-miR-6729-3p	−1.21	−2.32	0.0023	0.048	−0.01	−1.01	0.95	1.00
hsa-miR-4433a-3p	0.62	1.53	0.0027	0.054	0.01	1.01	0.98	1.00
hsa-miR-3124-5p	−0.81	−1.75	0.0028	0.054	−0.02	−1.01	0.89	1.00
hsa-miR-3621	−2.74	−6.69	0.0032	0.062	−0.16	−1.11	0.73	1.00
hsa-miR-4304	0.53	1.45	0.0034	0.063	0.05	1.04	0.72	1.00
hsa-miR-103a-2-5p	−0.60	−1.52	0.0040	0.073	−0.02	−1.01	0.99	1.00
hsa-miR-4999-3p	−3.51	−11.42	0.0041	0.073	−0.11	−1.08	0.54	1.00
hsa-miR-4784	0.59	1.51	0.0042	0.073	−0.20	−1.15	0.43	1.00
hsa-miR-4317	−1.70	−3.25	0.0044	0.075	−0.01	−1.00	0.98	1.00
hsa-miR-6720-3p	−3.01	−8.03	0.0044	0.075	−0.24	−1.18	0.48	1.00
hsa-miR-6764-5p	0.37	1.29	0.0047	0.077	−0.20	−1.15	0.26	1.00
hsa-miR-7106-3p	−1.06	−2.08	0.0048	0.078	0.15	1.11	0.49	1.00
hsa-miR-4687-5p	−1.42	−2.68	0.0050	0.081	0.02	1.01	0.91	1.00
hsa-miR-4739	−2.94	−7.69	0.0051	0.081	−0.31	−1.24	0.17	1.00
hsa-miR-6873-3p	0.35	1.28	0.0056	0.086	−0.02	−1.02	0.89	1.00
hsa-miR-744-5p	0.31	1.24	0.0057	0.087	−0.07	−1.05	0.85	1.00
hsa-miR-1539	0.48	1.39	0.0059	0.088	−0.08	−1.06	0.72	1.00
hsa-miR-660-3p	−0.53	−1.44	0.0064	0.094	0.46	1.38	0.16	1.00
hsa-miR-500a-3p	0.40	1.32	0.0068	0.098	0.00	−1.00	1.00	1.00
hsa-miR-3687	−2.68	−6.42	0.0069	0.099	0.00	1.00	1.00	1.00
hsa-let-7i-3p	−0.53	−1.44	0.0072	0.100	−0.31	−1.24	0.65	1.00
hsa-miR-148a-5p	−0.50	−1.41	0.0072	0.100	0.18	1.14	0.28	1.00

Abbreviations: FC, fold change; FDR, false discovery rate; Log2FC, log 2-fold change; miRNA, microRNA.

cellular sources (whole blood), and if we could replicate the same miRNA in whole blood samples of a second cohort of patients with CAD.

To address these questions, we sequenced whole blood and plasma samples of patients in our current study using a next-generation sequencing method. We identified several miRNAs differentially expressed in subjects with recurrent ischemic events versus those without events that have been previously associated with CAD manifestations and prothrombic states. Alterations in the strictly regulated balance of coagulation and fibrinolysis may be pivotal in the pathogenesis of recurrent arterial thrombotic events in patients with CAD and may be influenced by changes in miRNA expression.

The interpretation of previously published mechanistic pathways of interaction for specific miRNAs is often limited, because previous studies often used healthy controls for comparison, whereas, in our study, we compared two phenotypes of patients with established CAD, but variable clinical course. Therefore, mechanistic pathways not explored in usual disease models could be involved for many of the identified miRNAs. In addition, most research studies exploring miRNA expression in clinical cohorts of patients with CAD measured miRNAs isolated from plasma and used control groups without disease as comparison, thus limiting the ability to directly compare miRNA expression profiles derived from whole blood sources.

Role of identified miRNA in cardiovascular disease

Five miRNAs, miR-589-5p, miR-19a-3p, miR-144-5p, miR-181a-3p, and miR-148a-5p, have been previously

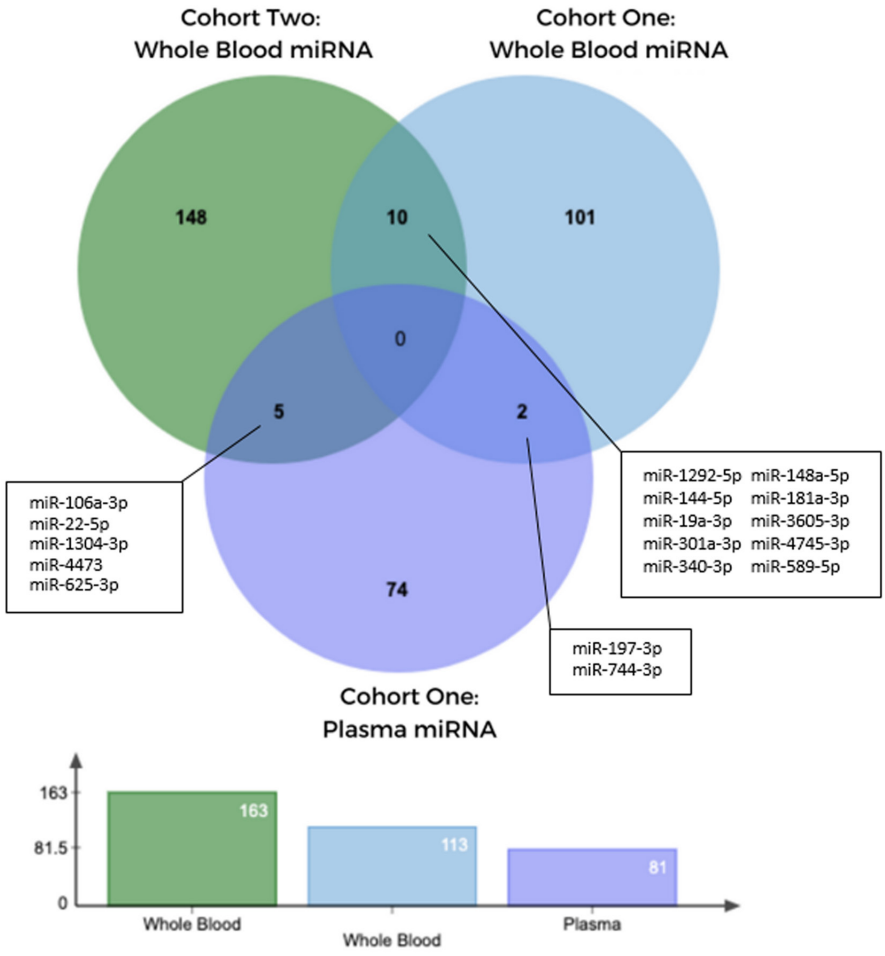

FIGURE 2 Top panel: Overlap of miRNA found significantly differentially expressed ($p < 0.05$) among subjects with established CAD with recurrent ischemic events versus those without events between cohort 1 (whole blood and plasma) and cohort 2 (whole blood). Bottom panel: Number of miRNAs with significant differential expression among subjects with established CAD with recurrent ischemic events versus those without events ($p < 0.05$). Green: Cohort 2- whole blood. Blue: Cohort 1 – whole blood. Purple: Cohort 1 – plasma. CAD, coronary artery disease; miRNA, microRNA.

associated with atherosclerosis as a manifestation of vascular inflammation, and were found differentially expressed in the whole blood samples of cohort 2. Notably, miR-589-5p is regulated by nuclear factor E2-related factor 2 (NRF2), a known mediator of cellular response to oxidized phospholipids. NRF2 represents a marker of lipid load and inflammatory microenvironment of the atherosclerotic plaque.[10] In our study, miR-589-5p was increased in patients with recurrent MI ($p = 0.028$) with a relatively large fold change (FC = 0.39), showing this miRNA is highly expressed in patients with recurrent MI. Another miRNA associated with inflammatory mediators is miR-148a-5p, which inhibits the proinflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β, and IL-6.[11] In our study, miR-148a-5p was lower in patients with recurrent MI events in whole blood ($p = 0.007$, FC −0.50), which would be consistent with higher inflammatory burden.

MiR-19a-3p and miR-144-5p have been previously associated with the macrophage response to vascular inflammation. A gene target of miR-19a-3p is HMG-Box Transcription Factor 1, which is a repressor of macrophage migration inhibiting factor, a cytokine that functions as a macrophage activator and inhibitor of migration. Antagonism of miR-19a-3p reduced atherosclerotic load in apoE-null mice. MiR-19a-3p has been also implicated in vascular disease through its targeting of PDE5A in angiotensin II-induced hypertrophy and cardiac remodeling.[12] In mouse models, expression of miR-19a-3p reduced progression of cardiac hypertrophy. In our study, miR-19a-3p was downregulated in patients with recurrent MI (FC −0.73), and thus could have been an indicator of more extensive cardiac hypertrophy and remodeling.

MiRNA mimic studies demonstrated that miR-144-5p suppressed oxidized low-density lipoprotein-induced upregulation of M1 macrophage markers, including IL-1β, TNF-α, prostaglandin-endoperoxide synthase 2 and nitric oxide synthase 2.[13] These markers are classically associated with vascular inflammation. In our study, miR-144-5p was downregulated in patients with recurrent MI (FC −0.91) consistent in direction with a higher inflammatory burden in those patients and therefore consistent with a protective effect of miR-144-5 on CV events.

Decreased expression of miR-181a has been observed in patients with CAD, however, the full function and role of this miRNA in atherogenesis is yet to be understood. Mechanistically, miR-181a-5p and its companion miRNA miR-181a-3p work cooperatively to prevent endothelial cell activation through blockade of NF-κB signaling

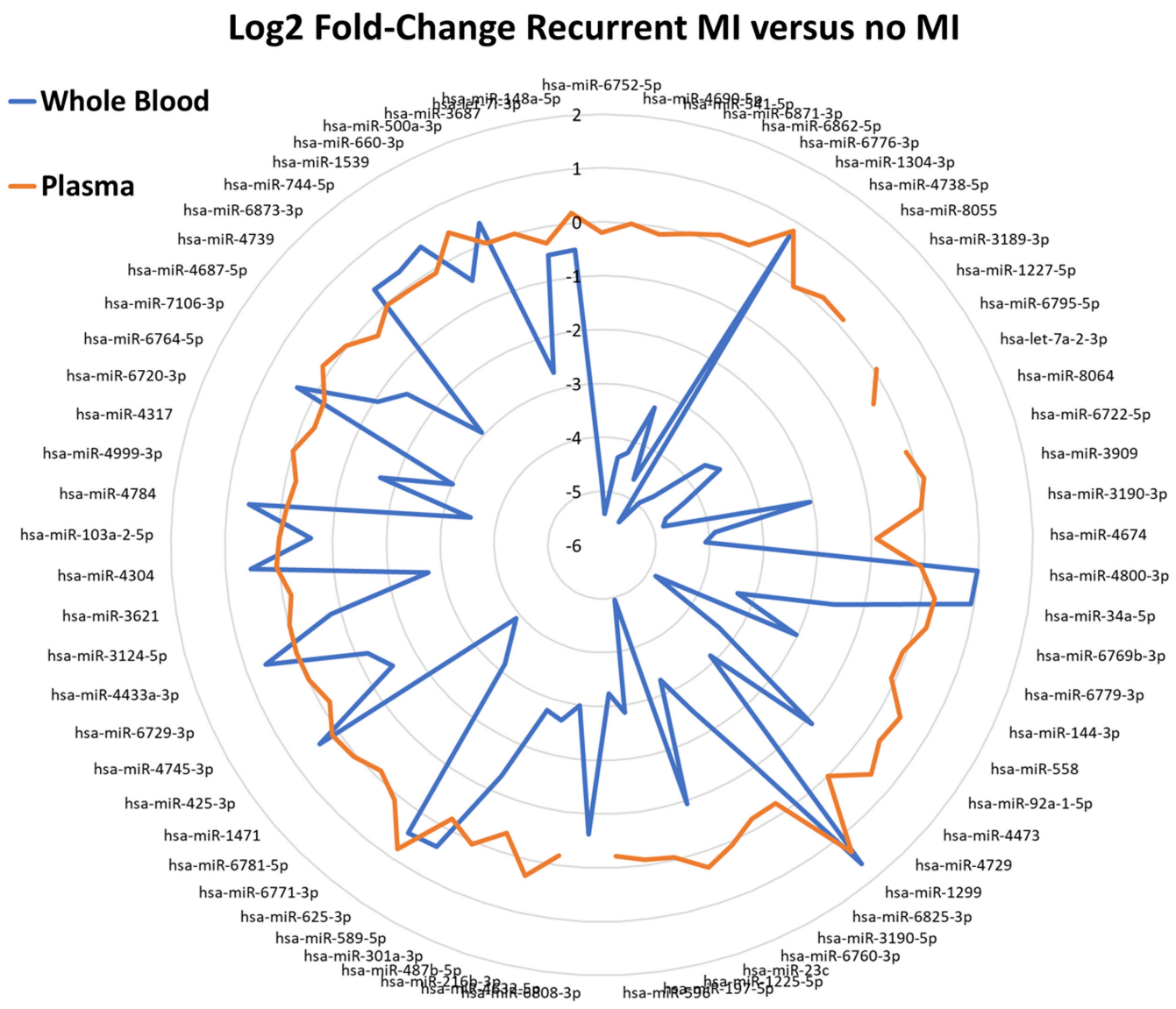

FIGURE 3 Radar plot demonstrating direction of log2FC of miRNA with significant differential expression between patients with CAD with recurrent MI vs those without recurrent events in whole blood (FDR <0.1) and respective expression in plasma. CAD, coronary artery disease; FDR, false discovery rate; log2FC, log 2-fold change; MI, myocardial infarction; miRNA, microRNA.

pathway. They target TAB2 kinase and NF-kappa-B essential modulator, respectively. NF-κB increases the production of inflammatory cytokines, chemokines, and adhesion molecules and regulates the cell proliferation, apoptosis, morphogenesis, and differentiation.[12] This leads to the conclusion that miR-181a-5p and miR-181a-3p are antiatherogenic miRNAs. However, in our study, miR-181a-3p was increased in patients with CAD with recurrent MI (FC 0.42).

Both miR-22-5p and miR-106a-3p have been associated with CV function and disease and were found to be significantly differentially expressed in plasma and whole blood in our study. Mir-22-5p is a cardiac-abundant miRNA, involved in stress-induced cardiac hypertrophy and remodeling.[14,15] Mir-22-5p was found to be decreased in patients with acute MI as compared to healthy controls in a prior study.[16] In our study, miR-22-5p was downregulated in patients with CAD with recurrent MI as compared to patients with CAD without recurrent events (FC −0.55), consistent in direction with an anti-thrombotic effect of miR-22-5p. Upregulation of MiR-106a-3p has been linked to repression of vascular smooth muscle cell apoptosis in mouse models.[17,18] In our study, decreased expression of miR-106a-3p was associated with risk of recurrent MI among patients with CAD (FC −0.57), indicating that reduced expression of miR-106a-3p may lead to promotion of vascular smooth muscle cell apoptosis and transition of CAD phenotype to plaque ulceration and thrombosis, as previously described.[19,20] MiR-625-3p has been found to be associated with cholesterol levels in the Young Finns

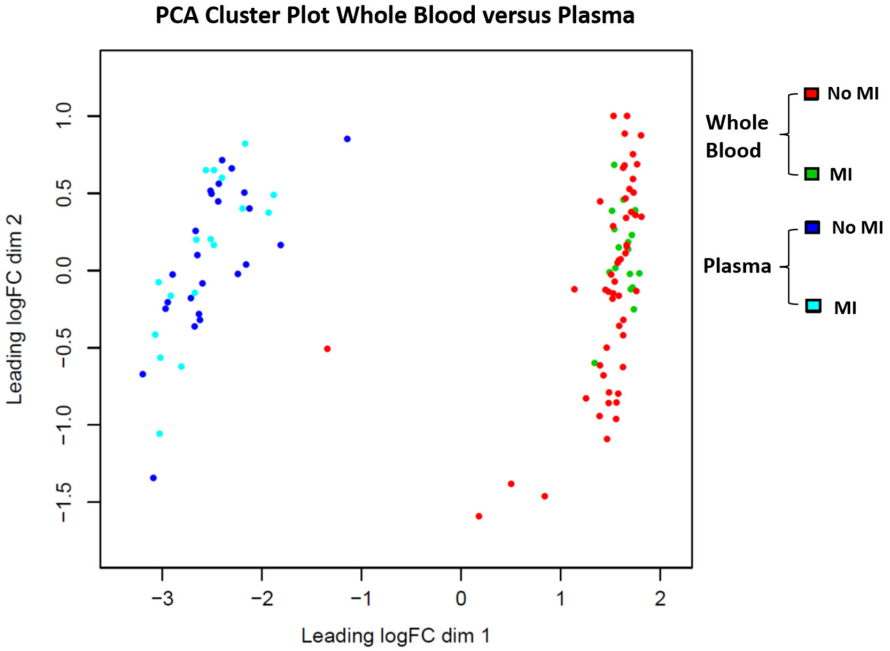

FIGURE 4 Principal Component Analysis (PCA) cluster plot showing expression of miRNA in plasma versus whole bloods in cohorts 1 and 2. MI, myocardial infarction; miRNA, microRNA.

study assessing miRNA associated with metabolic syndrome,[21] and it was also elevated among patients with recurrent MI (FC 0.44) in our study, possibly indicative of increased MI risk associated with hypercholesterolemia.

Role of identified miRNA in coagulation and thrombosis

In silico studies suggest that some miRNAs are associated with venous thromboembolism and markers of hypercoagulability.[22–24] Thrombosis is a complex process involving coagulation factors, fibrinogen, and platelets, and is tightly regulated in vivo to prevent pathologic bleeding as well as excess clot formation. MiRNA has been linked to alterations in endothelial function, platelet function, inflammation, coagulation, and smooth muscle proliferation, which are all linked to coronary thrombosis.[13,25,26] Three miRNAs differentially expressed in our study between patients with and without MI events were identified to have specific connections to components of the coagulation cascade or serve as regulators or modifiers of proteins along the cascade. They include miR-301a-3p, which has been associated with venous thromboembolism.[27,28] Studies of the miRNA-301a family demonstrate that they inhibit plasminogen activator inhibitor-1 (PAI-1), an endogenous inhibitor of fibrinolysis.[29] MiR-301a targets the 3′ UTR of PAI-1 mRNA for degeneration, resulting in downregulation of PAI-1 expression.[29] However, a connection between these miRNAs and arterial thrombosis has not been previously described. MiR-301a-3p was downregulated in patients with recurrent events in our study, which is in line with prior findings and may indicate inhibition of endogenous fibrinolysis in those patients.[25] MiR-144-5p is one of many miRNAs that influence the function of factor VIII (F8), and it was downregulated in patients with recurrent MI in our study consistent with the direction of such an effect.[27] The full impact of miR-144-5p on the F8 gene and its predictability for a hemophilia A phenotype has yet to be understood. MiR-19a-3p also influences factor VII (F7) and the F7 gene, although its full impact is also unknown.[27] MiR-19a-3p was downregulated in patients with recurrent MI in our study (FC −0.73), showing a consistent direction of effect suggestive of upregulation of F7 among patients with recurrent MI.

Although no prior publications have associated miR-1304-3p with distinct CV phenotypes, review of predicted gene targets demonstrate that mir-1304-3p has FXII, PAR-1, PAR-2, PAR-3, FX, thrombin, and FIX as potential targets associated, and has been found to be linked to cell fitness in a CRISPR-Cas9 library screen.[30] Similarly, miR-4473 has not been previously associated with specific CV disease phenotypes, however, it has been demonstrated to be implicated in modulation of IL-6 signaling pathway.[31] Predicted gene targets of miR-4473 include coagulation FXIIIA, which is the important final pathway of fibrin clot stabilization, which has been implicated in post PCI risk stratification.[1] MiR-4473 was downregulated among patients with recurrent MI (FC −0.88), suggesting a possible link to increased expression of FXIIIa.

Two miRNAs were differentially expressed in both plasma (current data) and whole blood (previously published data[6]) in cohort 1: miR-197-3p and miR-744-3p. MiR-197-3p has been associated with endothelial damage in Kawasaki disease and cardiometabolic risk.[32]

MiR-197-3p was also found to be associated with increased risk of recurrent venous thrombosis (VTE) in subjects after the first VTE diagnosis.[22]

MiR-744-3p has been previously associated with plaque rupture in ST elevation MI.[33]

Impact of sample sources

We discovered a surprising lack of overlap between miRNA identified as differentially expressed in plasma versus fixed whole blood samples using PAXGENE tubes, which incorporate miRNA from lysed cellular blood components, such as red cells, leukocytes, and platelets. As such, only five miRNAs differentially expressed in whole blood were also significant in plasma. Only a small number of miRNAs (=81) were differentially expressed between subjects with and without recurrent ischemic events in plasma samples, but none of those remained significant after FDR adjustment. This suggests that whole blood may be a preferred source for miRNA profiling to identify specific metabolic and prothrombotic phenotypes. Specific miRNAs of interest may derive preferentially from cellular contributions, likely leukocytes and platelets.

A limitation to our study is that confounding variables may have influenced our data in ways we could not control for, although groups were grossly balanced. In addition, the differential expression patterns of miRNAs in our study may have been influenced by the sample size. MiRNAs with a larger influence on CAD and coagulation may not be adequately represented in the differential expression analysis performed on a small sample. A larger sample population may allow detection of other miRNAs within whole blood samples with biomarker potential. Also, differences in samples related to modality of fixation, storage, and RNA extraction (Paxgene RNA whole blood tubes vs. citrate plasma) may have influenced the differential expression of miRNA. Further exploration of the specific genes at play and the effects of signal mediators is a necessary next step of exploration to understand the reliability of miRNA biomarker fingerprints.

CONCLUSION

Our current study expands on our prior work of miRNA profiling in patients with CAD to identify subsets at highest risk for recurrent thrombotic events. Our study demonstrates that a majority of significantly expressed miRNA in whole blood are likely from cellular blood components, such as leucocytes or platelets, and do not directly correlate with miRNA profiles derived from plasma samples.

Many of the miRNAs identified as differentially expressed in patients with future ischemic events have been previously linked to atherosclerosis, vascular inflammation, and thrombosis, and deserve further investigation.

AUTHOR CONTRIBUTIONS

C.O., J.I., E.S., T.C.S., and R.P.K wrote the manuscript. R.P.K., J.I., Y.L., and T.C.S. designed the research. R.P.K., J.I., E.S., and Y.L. performed the research. C.O., R.P.K., J.I., and E.S. analyzed the data. Y.L. contributed analytic tools.

CONFLICT OF INTEREST

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REFERENCES

1. Kreutz RP, Schmeisser G, Schaffter A, et al. Prediction of ischemic events after percutaneous coronary intervention: thrombelastography profiles and factor XIIIa activity. *TH Open*. 2018;2:e173-e181.
2. Kreutz RP, Schmeisser G, Maatman B, et al. Fibrin clot strength measured by thrombelastography and outcomes after percutaneous coronary intervention. *Thromb Haemost*. 2017;117:426-428.
3. Levine GN, Bates ER, Bittl JA, et al. 2016 ACC/AHA guideline focused update on duration of dual antiplatelet therapy in patients with coronary artery disease: a report of the American College of Cardiology/American Heart Association task force on clinical practice guidelines. *J Am Coll Cardiol*. 2016;68:1082-1115.
4. Berger JS. Oral antiplatelet therapy for secondary prevention of acute coronary syndrome. *Am J Cardiovasc Drugs*. 2018;18:457-472.
5. Aoki M, Hagiwara S, Oshima K. Clinical application of coagulation biomarkers. In: Begum G, ed. *Biomarker Indicator of Abnormal Physiological Process*. IntechOpen Book Series; 2018.
6. Kanuri SH, Ipe J, Kassab K, et al. Next generation MicroRNA sequencing to identify coronary artery disease patients at risk of recurrent myocardial infarction. *Atherosclerosis*. 2018;278:232-239.
7. Thygesen K, Alpert JS, Jaffe AS, et al. Fourth universal definition of myocardial infarction (2018). *J Am Coll Cardiol*. 2018;72:2231-2264.
8. Qiagen. *QIAseq miRNA Primary Quantification Supplementary Protocol Documentation*. Qiagen; 2022.
9. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26:139-140.
10. Linna-Kuosmanen S, Tomas Bosch V, Moreau PR, et al. NRF2 is a key regulator of endothelial microRNA expression under proatherogenic stimuli. *Cardiovasc Res*. 2021;117:1339-1357.

11. Li G, Tang X, Chen H, Sun W, Yuan F. miR-148a inhibits pro-inflammatory cytokines released by intervertebral disc cells by regulating the p38/MAPK pathway. *Exp Ther Med*. 2018;16:2665-2669.
12. Liu K, Hao Q, Wei J, Li GH, Wu Y, Zhao YF. MicroRNA-19a/b-3p protect the heart from hypertension-induced pathological cardiac hypertrophy through PDE5A. *J Hypertens*. 2018;36:1847-1857.
13. Shi X, Ma W, Li Y, et al. MiR-144-5p limits experimental abdominal aortic aneurysm formation by mitigating M1 macrophage-associated inflammation: suppression of TLR2 and OLR1. *J Mol Cell Cardiol*. 2020;143:1-14.
14. Huang ZP, Chen J, Seok HY, et al. MicroRNA-22 regulates cardiac hypertrophy and remodeling in response to stress. *Circ Res*. 2013;112:1234-1243.
15. Coffey S, Williams MJ, Phillips LV, Jones GT. Circulating microRNA profiling needs further refinement before clinical use in patients with aortic stenosis. *J Am Heart Assoc*. 2015;4:e002150.
16. Li H, Zhang P, Li F, et al. Plasma miR-22-5p, miR-132-5p, and miR-150-3p are associated with acute myocardial infarction. *Biomed Res Int*. 2019;2019:5012648.
17. Thum T, Catalucci D, Bauersachs J. MicroRNAs: novel regulators in cardiac development and disease. *Cardiovasc Res*. 2008;79:562-570.
18. Liu Y, Zhang WL, Gu JJ, et al. Exosome-mediated miR-106a-3p derived from ox-LDL exposed macrophages accelerated cell proliferation and repressed cell apoptosis of human vascular smooth muscle cells. *Eur Rev Med Pharmacol Sci*. 2020;24:7039-7050.
19. Clarke MC, Figg N, Maguire JJ, et al. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med*. 2006;12:1075-1080.
20. Clarke MC, Littlewood TD, Figg N, et al. Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res*. 2008;102:1529-1538.
21. Raitoharju E, Seppälä I, Oksala N, et al. Blood microRNA profile associates with the levels of serum lipids and metabolites associated with glucose metabolism and insulin resistance and pinpoints pathways underlying metabolic syndrome: the cardiovascular risk in young Finns study. *Mol Cell Endocrinol*. 2014;391:41-49.
22. Wang X, Sundquist K, Svensson PJ, et al. Association of recurrent venous thromboembolism and circulating microRNAs. *Clin Epigenetics*. 2019;11:28.
23. Starikova I, Jamaly S, Sorrentino A, et al. Differential expression of plasma miRNAs in patients with unprovoked venous thromboembolism and healthy control individuals. *Thromb Res*. 2015;136:566-572.
24. Hembrom AA, Srivastava S, Garg I, Kumar B. MicroRNAs in venous thrombo-embolism. *Clin Chim Acta*. 2020;504:66-72.
25. Shi J, Liu H, Wang H, Kong X. MicroRNA expression signature in degenerative aortic stenosis. *Biomed Res Int*. 2016;2016:4682172.
26. Shi J, Sun Y, Hua J. Functional genetic variation in the 3′-UTRNTRK2 is associated with risk of ischemic stroke. *Pharmgenomics Pers Med*. 2020;13:577-584.
27. Jankowska KI, Sauna ZE, Atreya CD. Role of microRNAs in hemophilia and thrombosis in humans. *Int J Mol Sci*. 2020;21:3598.
28. Rodriguez-Rius A, Lopez S, Martinez-Perez A, Souto JC, Soria JM. Identification of a plasma MicroRNA profile associated with venous thrombosis. *Arterioscler Thromb Vasc Biol*. 2020;40:1392-1399.
29. Patel N, Tahara SM, Malik P, Kalra VK. Involvement of miR-30c and miR-301a in immediate induction of plasminogen activator inhibitor-1 by placental growth factor in human pulmonary endothelial cells. *Biochem J*. 2011;434:473-482.
30. Kurata JS, Lin RJ. MicroRNA-focused CRISPR-Cas9 library screen reveals fitness-associated miRNAs. *RNA*. 2018;24:966-981.
31. Servais FA, Kirchmeyer M, Hamdorf M, et al. Modulation of the IL-6-signaling pathway in liver cells by miRNAs targeting gp130, JAK1, and/or STAT3. *Mol Ther Nucleic Acids*. 2019;16:419-433.
32. McManus DD, Rong J, Huan T, et al. Messenger RNA and MicroRNA transcriptomic signatures of cardiometabolic risk factors. *BMC Genomics*. 2017;18:139.
33. Li J, Tan Y, Sheng Z, et al. RNA-seq identifies circulating miRNAs as potential biomarkers for plaque rupture in patients with ST-segment elevation myocardial infarction. *Genomics*. 2021;113:1-10.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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