Supplemental Materials

Circulating MicroRNA Profiling in Non-ST Elevated Coronary Artery Syndrome Highlight Genomic Associations with Serial Platelet Reactivity Measurements

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Supplemental Materials and Methods

TRILOGY-ACS Cohort The primary outcome of the TRILOGY-ACS trial was a composite of cardiovascular events including cardiac death, MI and stroke at 36 months [70, 71]. Randomization occurred on average after 108 hours (IQR: 64, 155) of the initial cardiovascular event, specifically a NSTE-ACS event. Patients that were not able to be randomized to treatment within 72 hours of the primary event were first given a standard dose of open label clopidogrel prior to randomization [71]. Patients from the TRILOGY-ACS primary trial that were additionally enrolled in the secondary platelet function sub-study (N=2564) also provided whole blood samples at baseline, after 30 days and after six months of clopidogrel or prasugrel medication administration with simultaneous measurement of platelet reactivity (PR) at each time point [6]. PR was quantified at each individual trial site using a calibrated VerifyNow $P2Y_{12}$ Assay (Accumetrics Inc.). VerifyNow is a whole blood, adenosine diphosphate (ADP)/PGE_1 - based assay that measures platelet adhesion to fibrinogen-coated beads, with the secondary addition of PGE_1 increasing the test's specificity for $P2Y_{12}$ receptors [72-74].

Platelet reactivity profiling In the primary TRILOGY-ACS cohort, PR was measured at each participating site using the VerifyNow device $P2Y_{12}$ Assay (Accumetrics Inc), a whole blood, adenosine diphosphate (ADP) - based assay that measures platelet agglutination to fibrinogen-coated polystyrene beads. The addition of prostaglandin E_1 during the test reaction increases specificity of the test for $P2Y_{12}$ receptors. Test results are expressed as $P2Y_{12}$ reaction units (PRUs) and lower PRU readings reflected increased inhibition of the $P2Y_{12}$ receptor. PRU values were excluded if platelet adhesion measurements were performed within seven days of other anti-platelet medication administration (glycoprotein IIb/IIIa inhibitor therapy), if PRU values were determined at fewer than 10 minutes or greater than four hours after sample collection, if reported platelet inhibition was measured at greater than 100%, or if PRU values were greater > 500 [6].

In Singapore cohort A, the Vasodilator-Stimulated Phosphoprotein (VASP) flow cytometry assay (Diagnostica Stago, Asnières, France) was utilized to measure platelet sensitivity to $P2Y_{12}$ agonists and was completed within 48 hours of sample collection [40]. Citrated samples were incubated with prostaglandin E1 (PGE1) and 10 µmol/l adenosine diphosphate (ADP) for 10 min and set to plates with paraformaldehyde, and platelets were permeabilized with non-ionic detergent. VASP Analysis was performed on a FACS Canto II flow cytometer (Becton Dickinson, NJ, USA). The platelet population was identified via forward and side flow cytometry distribution and 5000 platelets were gated. We then calculated Platelet reactivity index (PRI) from median fluorescence intensity (MFI) after samples were inoculated with PGE1 or PGE1 and ADP according to the formula:

$$\mathbf{PRI = (MFI_{(PGE1)} - MFI_{(PGE1\ +\ ADP)}/MFI_{(PGE1)}) \times 100}$$

In both Singapore cohorts A and B, PR measurements were performed by whole blood impedance aggregometry on a Multiplate analyzer (Roche Diagnostics, Basel, Switzerland) without prostaglandin addition. Experimental technique consistency across study sites was ensured by conducting centralized training of those who conducted PR testing with only one trained person at each study site conducting the testing. VASP testing was performed on citrated whole blood within 48 h of collection. All Multiplate testing was performed on whole blood samples within 1 hour of collection during cardiac catheterization.

MiRNA sequencing (miRNA-seq) from whole blood. Total RNA was extracted from PaxGENE tubes using the PerfectPure RNA blood kit (5Prime, Gaithersburg, MD) with microRNA libraries prepared using TruSeq sample prep kits (Illumina). Prior to miRNA library pooling, a Bioanalyzer DNA1000 chip (Agilent) was used for library size validation and then computed using the KAPA Library Quantification kit. Library pools were comprised of 24 libraries. MicroRNA pooled-library sequencing was performed on an Illumina HiSeq2500 as

single-end 50bp sequence runs using rapid run flow cells. MiRNA clusters were also produced for single read flow cell. Raw miRNA sequence reads were then processed using c*utadapt* v1.5 to remove Illumina sequencing adapters and low quality 3' sequence ends and aligned to the human genome (GRCh38) using *bowtie* (*version 1.0.1*) [61, 62]. 1423 miRNAs were detected with ≥0.1 mapped reads/million aligned reads (rpm) in at least one sample. During alignment, reads required a minimum length of 18 NT and no more than one mismatch per read and < 6 alignments. Reads that mapped to > 10 locations were rejected. Aligned reads were then mapped to primary miRNA transcripts through miRBase (v. 21, 2813 miRNAs) using *bedtools* (v. 2.21.0) [64, 75]. A false enrichment of miR-486 was seen in the final mapped reads, however this is a known artifact of the Illumina library prep, which has previously shown enrichment of miR-486 in excess of 50x [65]. Therefore, reads mapping to miR-486 were removed. Overall, after applying a final cutoff of ≥ 1 rpm in 16 paired samples, 247 microRNAs were analyzed. From the 20 paired samples: one pair was removed for poor alignment, with an additional two pairs removed for having high levels of rRNA, and one was removed due to poor correspondence with its technical replicate.

Targeted miRNA profiling from plasma. MiRNA enriched total RNA was extracted from plasma samples using the Qiagen miRNAeasy Serum/Plasma kit. All samples had a miRNeasy Serum/Plasma Spike-in Control added. Following extraction, cDNA was acquired using 1.5µl of total RNA extracted from plasma in the Qiagen miScript II RT kit, 200 µL of water was added to the reaction volume after reverse-transcription reaction to dilute cDNA before storage at -20$_{o}$C. Custom miRNA arrays were designed using miRNA assays from Qiagen; specifically, the targeted array consisted of 46 miRNAs (Suppl. Table 1), chosen based on high concentration miRNAs associated with recurrent CVD event case-control status from the miRNA-seq analysis (N=35) as well as miRNAs determined to have association with potential CVD phenotypes from the literature (N=11) (Suppl. Table 2). Each array plate had 48 miRNA assays lyophilized into

the plate and repeated eight times. Three Qiagen control assays were added to the custom array, specifically: miRTC, our targeted array specific control miRNA (miR-30e-5p, miR-30d-5p, miR-23a-3p and SNORD61), cel-miR-39 miScript and the PPC assay. The Qiagen miScript SYBR Green PCR kit was used to run the PCR reaction for the array. Each PCR was performed on a Viia 7 Real-Time PCR system. For miRNA concentration normalization plasma samples were supplemented with a *C. elegans* miR-39 miRNA mimic. An automatic baseline was used with a Ct threshold of 0.02 and Ct values normalized to cel-miR-39* for Ct ≤ 35. Ct ≥ 35 were considered as being below the lower limit of quantification. Data below LLOQ for the remaining miRNAs was imputed using the minimum concentration values minus 10%. For the Singapore cohorts, the miRNeasy Kit (Qiagen) was used to isolate miRNAs from plasma samples. The nCounter Human miRNA Panel v2 (Nanostring) was then used to evaluate the concentration of ~800 miRNAs in these samples. MiRNA ligation and hybridization to fluorescent probes was performed at 65ºC for 18 hours, followed by probe purification and counting on the nCounter prep station and digital analyzer. Data from the nCounter analyzer contained individual fluorescent barcodes that mapped to individual miRNA species and allowed for an exact count of miRNAs present in the sample.

Supplemental Figures and Tables

Supplemental Table 1: 46 miRNA Species within the Targeted qRT-PCR Array Panel

Let-7a-5p	miR-1304-3p	miR-191-5p	miR-24-3p	miR-574-3p	
Let-7d-3p	miR-1307-5p	miR-192-5p	miR-25-3p/5p	miR-636	
Let-7g-5p	miR-15b-5p	miR-20a-5p	miR-29a-3p	miR-6087	

miR-1-3p*	miR-150-5p	miR-20b-5p	miR-29c-3p*	miR-92a-3p	
miR-126-3p	miR-151a-3p	miR-208a-3p*	miR-296-5p	miR-92b-3p	
miR-126-5p	miR-17-3p*	miR-21-5p	miR-30a-5p	miR-345-5p	
miR-133a-3p	miR-18a-5p	miR-22-3p	miR-484	miR-197-3p	
miR-133b*	miR-181b-5p	miR-222-3p	miR-4685-3p*	miR-324-5p	
miR-134-5p	miR-19b-3p	miR-223-3p	miR-4746-5p	miR-939-5p	

* 6 miRNA Species were excluded from the analysis following Internal Quality Control measures

Supplemental Table 2: Detailed reasoning for miRNA chosen for targeted qRT-PCR array.

MicroRNA	Involvement in Platelet/CVD [Reference]
Let-7a, Let-7d, Let-7g	• Part of Let-7 family, abundant in platelets
miR-1-3p	• Known platelet enriched miR • Highly cited in cardiac hypertrophy [76]
miR-126	• Athero-protective effects of endothelial apoptotic bodies • Known controller of angiogenesis [77]
miR-1304-3p	• Associated with lowering BNP and type I/II MI
miR-1307-5p	• miR-1307-5p; predictive of angiogenic response in medication [78] • Highly expressed in TRILOGY patients with high BNP.
miR-133a-3p	• Part of miR-1/133 cluster, many publications on role in cardiac hypertrophy and remodeling [79] • Considered a key biomarker in heart failure [80]

miR-133b	• Differentially expressed in sample patients on clopidogrel vs prasugrel at 30 days. • Potential biomarker in acute MI [81]
miR-134	• Reported to be circulating in Acute MI [82]
miR-150-5p	• Prognostic of ventricular remodeling and HF events [83]
miR-151a-3p	• Down regulated in experimental models of heart failure, increased susceptibility to post-MI arrhythmias [78]
miR-15b-5p	• MiR-15 family implicated in ventricular hypertrophy and modulation of infarct size [84]
miR-17-3p*	• Part of the miR-17/miR-92 supercluster [85] • Regulatory unit in ischemia/reperfusion injury [86] • Mir-17-5p but not miR-17-3p assessed in the Nanostring miRNA set
miR-181b-5p	• Down regulated in in response to transverse aortic constriction, implicated in cardiac hypertrophy via NF-κB [87]
miR-18a-5p	• miR-18a-5p; Part of the miR-17/miR-92 super cluster.
miR-191-5p	• Platelet enriched miRNA [49]
miR-192-5p	• A circulating p53-responsive microRNA predictive indicators of heart failure after acute MI [88]
miR-19b-3p	• Altered at baseline in miRNA-Seq (p=0.05).
miR-208a-3p	• Strongly highlighted as potential ACS biomarker [89]
miR-20a-5p	• Part of the miR-17/miR-92 super cluster.
miR-20b-5p	• Platelet enriched miR (anti-angiogenic). Responds differentially to antiplatelet therapy of different potency [90]
miR-21-5p	• Released from cardiac fibroblasts (pro-apoptotic) and induced in failing heart [91]
miR-22-3p	• Overexpression in cardiomyocyte causing hypertrophy [92]
miR-222-3p	• Inhibits endothelial cell migration, contributes to sex-dimorphic eNOS expression via ETS-1 [93]
miR-223-3p	• miR-223-3p; Platelet enriched miR, often cited as being key platelet-derived miR playing role in CV outcomes [15, 94]
miR-24-3p	• Platelet enriched miR, expression induces cardiac hypertrophy [95, 96]
miR-25-3p	• Highly expressed in miRNA-Seq dataset. • Inhibition improves contractility in heart failure [97]

miR-29a-3p	• Significant role of miR-29 in cardiac fibrosis following MI [98]
miR-29c-5p	• Critical role in cardiac fibrosis following MI [98] • Highly expressed in miR-Seq dataset
miR-30a-5p	• Highly expressed in miRNA-Seq dataset, particularly in those with low BNP/CRP.
miR-4685-3p*	• Highly expressed in miRNA-Seq dataset. • Not included in the Nanostring miRNA set
miR-4746-5p*	• Highly expressed in miRNA-Seq dataset, particularly in non-diabetic subjects. • Not included in the Nanostring miRNA set
miR-484	• Inhibitor of mitochondrial function and potential predictor of spontaneous MI [99]
miR-574-3p	• Highly expressed in miRNA-seq dataset
miR-6087*	• Highly expressed in miRNA-Seq dataset • Down regulates endothelial endoglin expression [100] • Not included in the Nanostring miRNA set
miR-636*	• Highly expressed in miRNA-Seq dataset • Early biomarker of MI in whole blood miRNA sequencing study [101] • Not included in the Nanostring miRNA set
miR-92a-3p	• Highly expressed in the miRNA-Seq dataset. • Member of the miR-17/miR-92 super-cluster [85]
miR-92b-3p	• Highly expressed in the miRNA-Seq dataset.

Supplemental Table 3A: Baseline characteristics of Singapore cohort A: A comparison between 24 patients with high on-treatment platelet reactivity (VASP PRI > 50%) and 24 patients with low on treatment platelet reactivity (VASP PRI < 50%).

	HPR	**LPR**	**P-value**
Age	57 (51–64)	53 (50–64.5)	0.705
Female	33.3	13.5	0.519
Ethnicity			0.132
Chinese	67.4	56.8	
Malay	20.4	32.4	

Indian	12.2	10.8	
Diabetes	34.7	48.7	0.426
BMI (kg/m2)	25 (23, 27)	26 (24, 28.5)	0.371
Serum creatinine (µmol/L)	75 (67.5, 85)	84.5 (70.5, 99.25)	0.07
Coronary artery disease†	91.8	86.4	0.945
Proton pump inhibitor	30.6	29.7	1
DHP calcium channel antagonist	26.9	32.5	0.893
Statin	66.7	78.4	0.461
Aspirin	100	100	1
Smoking	34.7	32.4	0.659

* Continuous variables are presented as median (25_{th} and 75_{th} percentile) and categorical variables are presented as percentages

Supplemental Table 3B: Baseline characteristics of Singapore cohort B: A comparison between 24 patients with high on-treatment platelet reactivity (ADP > 46 aggregation units) and 24 patients with low on treatment platelet reactivity (ADP < 46 aggregation units).

	HPR (N=24)	**LPR (N=24)**	**P-value**
Age (years)	56 (47, 60)	56 (50,63)	0.27
Men (%)	95	95	0.74
Race			0.08
Chinese (%)	50	85	
Malay (%)	14	5	
Indian (%)	36	10	
Body-Mass Index (kg/m2)	24.3 (23.4-27.1)	24.2 (23.4-26.8)	0.68
Hypertension (%)	67	63	0.08
Dyslipidemia (%)	68.2	52.4	0.36
Current Smoker (%)	40.9	33.3	0.70
Diabetes (%)	37	35	0.46
GRACE Score	102 (79-112)	86 (79-98)	0.11
NSTE-ACS (%)	82	79	0.16
ADP test result (AU*min)	926 (765-1191)	210 (185-226)	<0.01

Initial serum creatinine value (μmol/dL)	85 (67-101)	75 (68-84)	0.24
Baseline platelet count (×10³ cells per mm³)	254 (212-316)	210 (204-234)	<0.01

Continuous variables expressed as median (25_{th}, 75_{th} percentile) HPR = High on-ADP receptor antagonist platelet reactivity (ADP test ≥ 468 AU*min). LPR= Low on-ADP receptor antagonist platelet reactivity (ADP test < 468 AU*min)

Supplemental Table 4. MiRNAs significantly associated with platelet reactivity in the Singapore cohort in baseline plasma samples. Fold Changes represent Log_2 Fold Changes in miRNA concentration per 1 SD PRU unit. MiRNA species are listed from smallest to largest p-value in cohort A. MiRNA species significant in either cohort A or B were included. MiRNA effect sizes (Fold Changes) ranged from -0.279 to 0.630 with p-values from $1.6x10^{-13}$ to 0.18. **§** MiRNA species that were also significantly associated with PRU in the TRILOGY cohort.

MiRNA	Cohort A Log_2 Fold Change (P-value)	Cohort B Log_2 Fold Change (P-value)
Let-7a-5p	0.24 (0.033)	0.50 ($1.3x10^{-12}$)
Let-7b-5p	0.30 ($7.3x10^{-4}$)	0.35 ($3.1x10^{-7}$)
miR-106a-5p	0.21 (0.024)	0.24 ($4.5x10^{-4}$)
miR-126-3p**§**	0.33 (0.009)	0.45 ($3.2x10^{-11}$)
miR-142-3p	0.17 (0.091)	0.29 ($1.1x10^{-5}$)
miR-142-5p	-0.18 (0.07)	-0.1 (0.02)
miR-146a-5p	0.18 (0.07)	0.44 ($1.4x10^{-10}$)
miR-155-5p	-0.25 (0.01)	-0.44 ($7.2x10^{-8}$)
miR-15b-5p**§**	0.2 (0.06)	0.46 ($2.0x10^{-11}$)

miR-17-5p	0.21 (0.02)	0.24 ($4.5x10^{-4}$)
miR-181a-5p	0.34 ($2.4x10^{-3}$)	0.38 ($5.2x10^{-8}$)
miR-1912	-0.29 (0.01)	-0.1 (0.03)
miR-19b-3p	0.29 ($2.0x10^{-3}$)	0.21 ($2.1x10^{-3}$)
miR-20a-5p/miR-20b-5p	0.28 ($2.4x10^{-3}$)	0.25 ($2.6x10^{-4}$)
miR-223-3p	0.52 ($1.4x10^{-9}$)	0.27 ($3.8x10^{-5}$)
miR-23a-3p	0.38 ($9.1x10^{-5}$)	0.16 (0.013)
miR-361-3p	0.24 (0.074)	0.16 (0.045)
miR-4454	0.37 ($1.5x10^{-5}$)	0.32 ($8.5x10^{-7}$)
miR-451a	0.63 ($1.6x10^{-13}$)	0.16 (0.023)
miR-576-5p	-0.16 (0.16)	-0.1 (0.024)
miR-601	-0.13 (0.18)	-0.31 ($6x10^{-6}$)
miR-720	0.24 ($4.2x10^{-3}$)	0.28 ($5.4x10^{-5}$)
miR-92a-3p	0.34 ($3.4x10^{-4}$)	0.25 ($2.8x10^{-4}$)
miR-93-5p§	0.23 (0.01)	0.24 ($4.0x10^{-4}$)

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