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Combining Circulating MicroRNA and NT-proBNP to Detect and Categorize Heart Failure Subtypes

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ABSTRACT

BACKGROUND Clinicians need improved tools to better identify nonacute heart failure with preserved ejection fraction (HFpEF).

OBJECTIVES The purpose of this study was to derive and validate circulating microRNA signatures for nonacute heart failure (HF).

METHODS Discovery and validation cohorts (N = 1,710), comprised 903 HF and 807 non-HF patients from Singapore and New Zealand (NZ). MicroRNA biomarker panel discovery in a Singapore cohort (n = 546) was independently validated in a second Singapore cohort (Validation 1; n = 448) and a NZ cohort (Validation 2; n = 716).

RESULTS In discovery, an 8-microRNA panel identified HF with an area under the curve (AUC) 0.96, specificity 0.88, and accuracy 0.89. Corresponding metrics were 0.88, 0.66, and 0.77 in Validation 1, and 0.87, 0.58, and 0.74 in Validation 2. Combining microRNA panels with N-terminal pro-B-type natriuretic peptide (NT-proBNP) clearly improved specificity and accuracy from AUC 0.96, specificity 0.91, and accuracy 0.90 for NT-proBNP alone to corresponding metrics of 0.99, 0.99, and 0.93 in the discovery and 0.97, 0.96, and 0.93 in Validation 1. The 8-microRNA discovery panel distinguished HFpEF from HF with reduced ejection fraction with AUC 0.81, specificity 0.66, and accuracy 0.72. Corresponding metrics were 0.65, 0.41, and 0.56 in Validation 1 and 0.65, 0.41, and 0.62 in Validation 2. For phenotype categorization, combined markers achieved AUC 0.87, specificity 0.75, and accuracy 0.77 in the discovery with corresponding metrics of 0.74, 0.59, and 0.67 in Validation 1 and 0.72, 0.52, and 0.68 in Validation 2, as compared with NT-proBNP alone of AUC 0.71, specificity 0.46, and accuracy 0.62 in the discovery; with corresponding metrics of 0.72, 0.44, and 0.57 in Validation 1 and 0.69, 0.48, and 0.66 in Validation 2. Accordingly, false negative (FN) (81% Singapore and all NZ FN cases were HFpEF) as classified by a guideline-endorsed NT-proBNP ruleout threshold, were correctly reclassified by the 8-microRNA panel in the majority (72% and 88% of FN in Singapore and NZ, respectively) of cases.

CONCLUSIONS Multi-microRNA panels in combination with NT-proBNP are highly discriminatory and improved specificity and accuracy in identifying nonacute HF. These findings suggest potential utility in the identification of nonacute HF, where clinical assessment, imaging, and NT-proBNP may not be definitive, especially in HFpEF. (J Am Coll Cardiol 2019;73:1300–13) © 2019 by the American College of Cardiology Foundation.

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Heart failure (HF) imposes a high burden of morbidity, mortality, and health care costs worldwide. Current practice in the diagnosis of HF is based on clinical manifestations of a syndrome of symptoms and signs corroborated by cardiac imaging and plasma natriuretic peptide levels. The diagnostic challenge is greater in the nonacute setting when, in contrast to florid acute decompensated heart failure (ADHF), clinical bedside signs may be subtle or absent. HF can occur in the presence of preserved ejection fraction (HFpEF) or reduced ejection fraction (HFrEF), with the former accounting for a substantial proportion of clinical HF cases (1,2). It may be particularly challenging to diagnose compensated HFpEF, as neither cardiac imaging nor physical examination is sensitive in this situation.

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The plasma/serum B-type cardiac natriuretic peptide (BNP) and its cosecreted congener, N-terminal prohormone brain natriuretic peptide (NT-proBNP), are established HF biomarkers with proven diagnostic utility, especially in patients presenting to the emergency department with new-onset breathlessness (3). Although performing well in acute HF, plasma natriuretic peptide (NP) concentrations often fall below diagnostic cutpoint values in treated HF. For HFpEF patients, NT-proBNP levels are generally one-half of those observed in HFrEF, markedly reducing the marker's diagnostic performance in the incipient or treated (compensated) state (4). In the absence of better options, NT-proBNP at a threshold of 125 pg/ml is currently recommended as a rule-out test in the diagnosis of nonacute HF in current guidelines (3).

Circulating concentrations of microRNA (miRNA) vary in response to an array of acute and chronic disease states, and their stability in stored samples makes them plausible candidates as biomarkers in HF (5–9). There is no consensus on the choice of specific circulating serum/plasma miRNAs that might serve this function. The unmet need we target here is the difficult task, particularly in HFpEF, of diagnosing nonacute, incipient, or treated HF when the cardiac B-peptide levels are lower and more easily confounded and the signal to noise ratio for the marker is markedly diminished. We assess the diagnostic performance of circulating miRNAs (both alone and in combination with NT-proBNP) in patients with successfully treated HF (both recent and remote ADHF) entering the chronic phase of compensated HF.

ABBREVIATIONS AND ACRONYMS

ADHF = acute decompensated heart failure

HFpEF = heart failure with preserved ejection fraction

HFrEF = heart failure with reduced ejection fraction

LVEF = left ventricular ejection fraction

miRNA = microRNA

NT-proBNP = N-terminal prohormone brain natriuretic peptide

METHODS

STUDY DESIGN AND PARTICIPANTS. Our case-control study of 1,710 participants from Singapore and New Zealand (NZ) included 903 HF patients from the contemporaneous SHOP (Singapore Heart Failure Outcomes and Phenotype) and PEOPLE (NZ Prospective Evaluation of Outcome in Patients With Heart Failure With Preserved Left Ventricular Ejection

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Fraction) studies (PEOPLE and SHOP, Trial Registry ACTRN12610000374066) recruited and followed according to a common protocol (10,11). Controls included 807 non-HF cases from the SLAS (Singapore Longitudinal Aging Study) and the HVOL (NZ Healthy Volunteers for Heart Disease Research) study (Trial Registry ACTRN1260500448640). Discovery was performed on samples from 546 Singapore participants comprising 338 HF patients (180 HFrEF and 158 HFpEF) and 208 control subjects matched for ethnicity, sex, and age. The miRNA panels identified in the discovery phase were validated in 2 independent cohorts: Singapore Validation 1 (HF detection: 241 control subjects and 207 HF; HF subtype categorization: 116 HFrEF and 72 HFpEF) and NZ Validation 2 (HF detection: 358 control subjects and 358 HF; HF subtype categorization: 145 HFrEF and 179 HFpEF). The study design is shown in **Figure 1**. No participants overlapped between the 3 cohorts. All participants underwent Doppler echocardiography for assessment of cardiac structure and function. Left ventricular ejection fraction (LVEF) was assessed using the biplane method of disks as recommended by the American Society of Echocardiography guidelines. Patients with LVEF ≥50% were categorized as having HFpEF, whereas those with LVEF ≤40% were classified as having HFrEF. To ensure clear-cut phenotyping, 19 patients with HF from Validation 1 and 34 from Validation 2 with EF between 40% and 50% were excluded from the HF subtype categorization analysis. Patients were recruited either in hospital or in the outpatient clinic within 6 months of a documented episode of HF decompensation. Assessments and blood sampling were undertaken when patients were stable, symptomatically improved with resolution of bedside physical signs of HF and considered fit for discharge, or already ambulant and attending an outpatient clinic. This ensured assessment of marker performance specifically in the treated phase of HF. All participants provided informed consent. The study protocol was approved by the Singapore National Health Group Domain Specific Review Board (NHG DSRB Reference code: 2010/0114), the Institutional Review Board of the National University of Singapore (NUS-IRB Reference Code: 04-140), NZ Multi-region Ethics Committee (MEC/09/11/124) and Health and Disability Ethics Committees (CTY/01/05/062/AM11). For further details of blood plasma sample collection and NT-proBNP assay measurement, see the Online Appendix.

miRNA MEASUREMENT AND ANALYSIS. A total of 203 miRNA targets, selected according to pre-set criteria (see Online Methods on selection of miRNA candidates, and Online Table 1) were included in discovery analyses. The details of our method for isolating miRNA from plasma samples are also given in the Online Appendix.

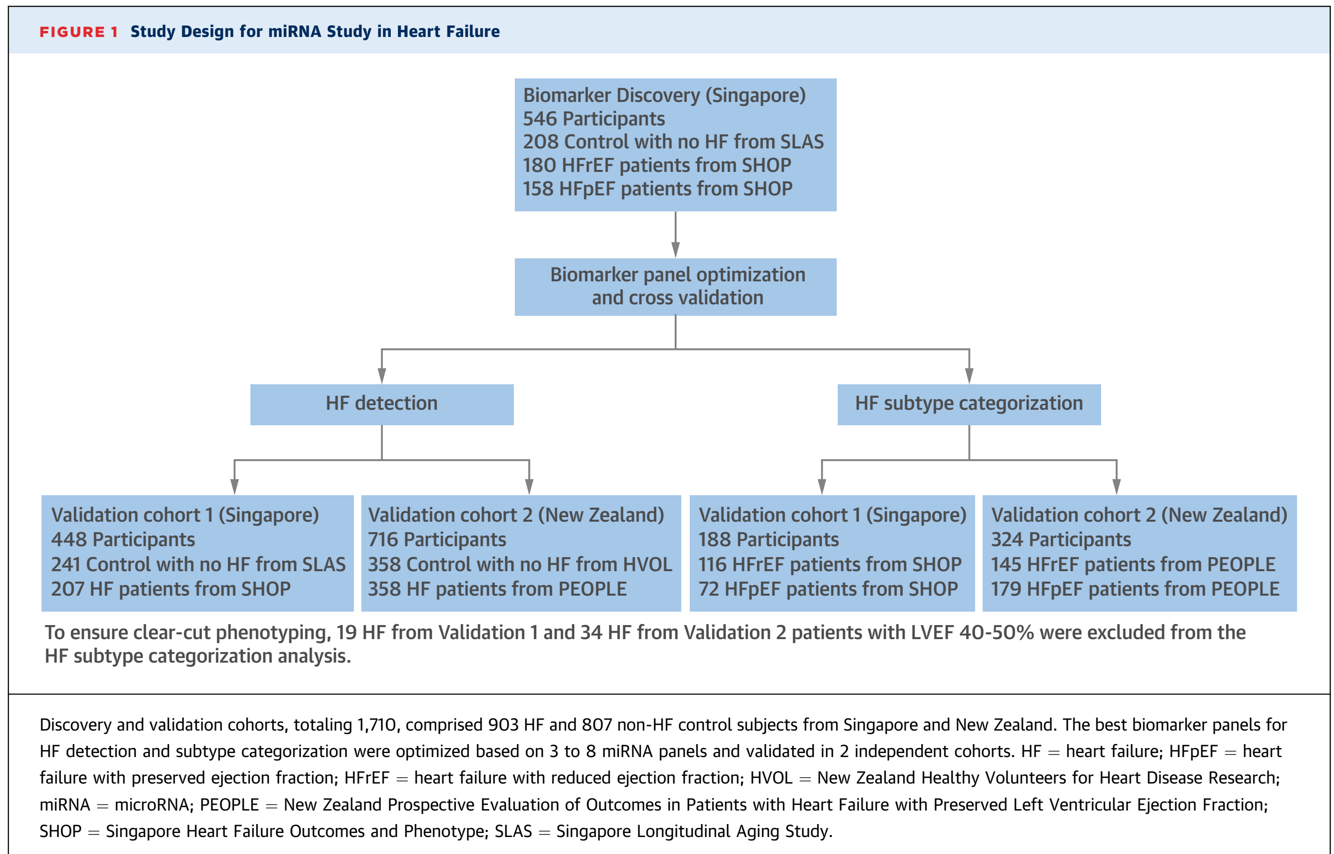
Extracted RNAs, with spike-in controls, were reverse transcribed and underwent multiplex augmentation by touch-down amplification to increase the amount of cDNA without changing the total miRNA levels. The augmented cDNA was diluted and quantified using a SYBR Green based single-plex qPCR assay (MiRXES, Singapore) on Applied Biosystems ViiA 7 384 Real-Time PCR System (Life Technologies, Singapore).

All spike-in controls were synthetic miRNA mimics (22 to 24 bases of small single-stranded RNAs) designed *in silico* for low sequence similarity to all known human miRNAs, thus minimizing cross-hybridization to the primers used in the assays. The spike-in controls serve to detect the presence of inhibitors and correct for technical variations during miRNA isolation, reverse transcription, augmentation, and qPCR. Synthetic miRNA standards of known concentration were diluted over at least 6 orders of magnitude, amplified, and generated a standard curve for copy number determination of samples for each miRNA assay. The standard curve was used to further correct for technical variation and to assess the efficiency of each miRNA assay in every PCR plate. This ensures the reliability of the assay. The miRNA assays were judiciously divided into a number of multiplex groups *in silico* to minimize nonspecific amplifications and primer-primer interactions (Online Figure 1).

The raw cycles to threshold values were processed, and the absolute copy numbers of the target miRNAs in each sample were determined by interpolation of standard curves derived from synthetic miRNA. MiRNAs at ≤500 copies/ml fall close to the detection limit of the single-plex qPCR assay (≤10 copies/well). Such low levels were considered undetectable and were excluded from analyses. Raw qPCR data was normalized in 2 steps: technical and global normalization. The technical variations introduced during RNA isolation and real time-quantitative polymerase chain reaction were normalized by the spike-in control. This was followed by performing global unsupervised analysis and principal component analysis (PCA) on data from all detected miRNAs with adjustment by global normalization to equalize the distribution of miRNA expression across samples. A flow chart of data processing was presented (Online Figure 2).

STATISTICAL ANALYSIS. Intergroup comparisons of miRNA levels were made using the Student's *t* test. All p values were corrected for false discovery rate (FDR) estimated using Bonferroni-type multiple

FIGURE 1 Study Design for miRNA Study in Heart Failure



Discovery and validation cohorts, totaling 1,710, comprised 903 HF and 807 non-HF control subjects from Singapore and New Zealand. The best biomarker panels for HF detection and subtype categorization were optimized based on 3 to 8 miRNA panels and validated in 2 independent cohorts. HF = heart failure; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; HVOL = New Zealand Healthy Volunteers for Heart Disease Research; miRNA = microRNA; PEOPLE = New Zealand Prospective Evaluation of Outcomes in Patients with Heart Failure with Preserved Left Ventricular Ejection Fraction; SHOP = Singapore Heart Failure Outcomes and Phenotype; SLAS = Singapore Longitudinal Aging Study.

comparison procedures. MiRNAs differing between groups with $p < 0.01$ were considered significant. For the discovery cohort, multi-miRNA panels for HF detection and HF subtype categorization were optimized by sequential forward floating selection (SFFS) (12) and support vector machine (SVM) (13) algorithms with repeated cross-validation in silico. The model yields a score based on a linear formula accounting for the weightage of each relevant miRNA representing its expression level. The SFFS algorithm determined the optimal combination of miRNAs to generate panels with the highest accuracy. The SVM method was used to train the prediction algorithms. In the prediction algorithm, the linear combination of the log2 transformed copy number/ml values for all miRNAs were used to calculate the prediction score where the higher the score the higher the likelihood for the subject to be positive (HF or HFpEF). The constant and coefficients for each of the miRNAs were listed.

$$\text{Score} = \sum_{i=1}^{n} \log2(\text{miRNA}_i) + \text{constant}$$

miRNA_i – copy number of ith miRNA per ml of plasma

The 3 to 8 miRNA biomarker panels for HF detection and HF subtype categorization were then validated in the validation cohorts. Receiver-operator characteristic curves characterizing the diagnostic performance of candidate miRNAs and NT-proBNP were calculated. To assess the incremental benefit of the miRNA panel with and without NT-proBNP, we calculated the continuous net reclassification improvement (NRI) and integrated discrimination improvement (IDI) to quantify improvement in diagnostic separation (14). The p values were estimated using the bootstrap method with $p < 0.01$ considered statistically significant. All statistical analyses were performed using MATLAB Toolbox (MathWorks, Natick, Massachusetts).

RESULTS

STUDY DESIGN AND CLINICAL CHARACTERISTICS. A total of 1,710 participants comprising Singapore and NZ participants participated in this study (**Figure 1**). Participant characteristics are summarized in **Table 1**. A Singapore cohort (n = 546) was used for discovery and 2 independent cohorts, 1 from Singapore

TABLE 1 Characteristics of the Healthy Subjects (Control) and Heart Failure Patients (Heart Failure With Reduced and Preserved Ejection Fraction)

	Discovery (Singapore)			Validation 1 (Singapore)			Validation 2 (New Zealand)		
	Control	HF	p Value	Control	HF	p Value	Control	HF	p Value
Population	208 (38.1)	338 (61.9)		241 (53.8)	207 (46.2)		358 (50.0)	358 (50.0)	
LVEF	64.0 ± 3.7	42.2 ± 18.7		64.4 ± 4.3	38.9 ± 16.0		63.3 ± 3.5	46.6 ± 17.0	
NT-proBNP, pg/ml	89 ± 140	4,249 ± 6,350		79 ± 77	2,653 ± 3,143		43 ± 47	3,413 ± 5,611	
Minimum	5	20.21		5	11.9		2	38	
Maximum	1,402	35,000		469	17,922		506	59,901	
Male	59.8	68.1	0.90	59.8	68.1	0.07	67.9	67.0	0.80
Age	59.7 ± 10.5	64.6 ± 12.0	<0.0001	60.4 ± 10.5	60.5 ± 12.7	0.90	73.2 ± 12.8	74.0 ± 11.8	0.40
Atrial fibrillation or flutter	1.0	25.1	<0.0001	1.7	23.4	<0.0001	56.7	63.7	0.06
Hypertension	33.7	76.3	<0.0001	35.9	70.9	<0.0001	56.7	63.7	0.06
Diabetes	9.62	58.9	<0.0001	10.9	54.9	<0.0001	20.4	22.3	0.50
	Discovery (Singapore)			Validation 1 (Singapore)			Validation 2 (New Zealand)		
	HFrEF	HFpEF	p Value	HFrEF	HFpEF	p Value	HFrEF	HFpEF	p Value
Population	180 (53.3)	158 (46.7)		116 (61.7)	72 (38.3)		145 (44.8)	179 (55.2)	
LVEF	25.9 ± 7.7	60.7 ± 5.9		26.4 ± 7.7	57.4 ± 6.1		28.5 ± 7.4	61.6 ± 6.5	
NT-proBNP	5,897 ± 7,630	2,327 ± 3,676		3,400 ± 3,517	1,598 ± 2,326		4,898 ± 7,887	2,557 ± 2,690	
Minimum	20.21	22.33		11.9	12.72		278	38	
Maximum	35,000	26,135		17,992	15,088		59,901	19,289	
Male	60.0	42.4	0.001	81.9	62.5	0.003	83.4	53.6	<0.0001
Age	60.8 ± 11.6	68.9 ± 11.0	<0.0001	57.1 ± 11.1	65.9 ± 12.9	1E-06	70.3 ± 14.0	76.6 ± 9.0	<0.0001
Atrial fibrillation or flutter	16.7	34.4	<0.0001	20.7	23.9	0.60	–	–	–
Hypertension	65.7	87.3	<0.0001	62.1	84.5	0.001	51.0	73.2	<0.0001
Diabetes	58.9	58.6	1.00	53.4	56.3	0.70	13.8	29.6	<0.001

Values are n (%), mean ± SD, or %, unless otherwise indicated. The percentage next to the variable name indicates the percentage of subjects with known value for the variable. For the comparisons of the variables between control and heart failure (Control vs. HF) and between HFpEF and HFrEF (HFrEF vs. HFpEF), Student's *t*-test was used for the comparisons of numerical variables and the chi-square test was used for the comparisons of categorical variables. To ensure clear-cut phenotyping, patients with LVEF of 40% to 50% (19 HF cases from Validation 1 and 34 HF cases from Validation 2) were excluded from the HF subtype categorization analysis.

HF = heart failure; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; LVEF = left ventricular ejection fraction; NT-proBNP = N-terminal prohormone brain natriuretic peptide.

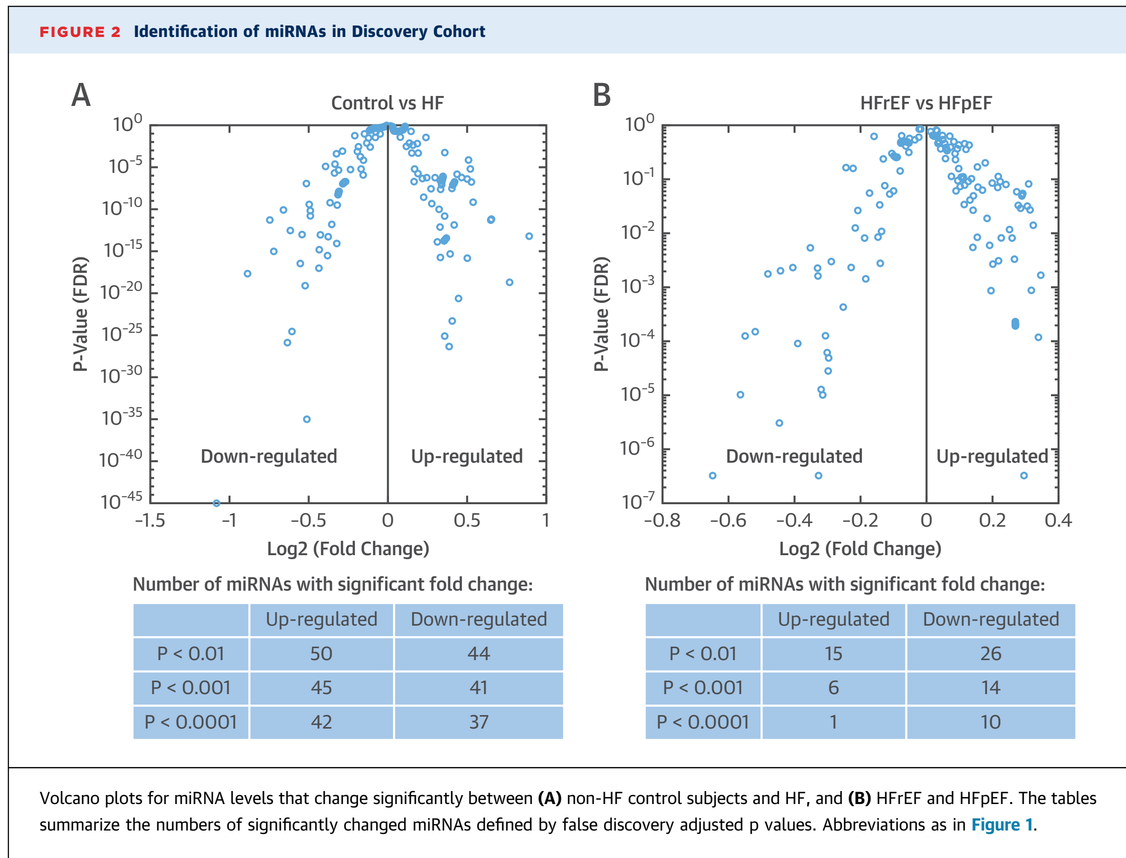
(Validation 1, n = 448) and 1 from NZ (Validation 2, n = 716), were used for validation. Overall, the HF patients in Singapore had a higher background prevalence of hypertension, atrial fibrillation (AF), and diabetes as compared with non-HF control subjects. Notably, the NZ participants were significantly older than the Singapore participants. As expected, HFrEF and HFpEF cases differed significantly with respect to sex, age, and hypertension in all 3 cohorts.

miRNA DISCOVERY COHORT. Of 203 miRNAs assayed, 132 miRNAs were detected (≥500 copies/ml) in >90% of samples. In the discovery cohort, univariate analysis demonstrated that 94 miRNAs differed significantly between HF patients and control subjects ($p < 0.01$ after FDR) (Online Table 2) (**Figure 2**). Of these, 82 miRNAs were not previously reported, and 12 were consistently identified in our study and also in the previous comparable reports. These included miR-125a-5p (6), miR-423-5p (8,15), miR-30a-5p (15), miR-21-5p (15), and miR-22-3p (15,16), which were up-regulated; and miR-30c-5p (5), miR-103a-3p (7), miR-30b-5p (7), miR-191-5p (15), miR-150-5p (7,17), miR-454-3p (18), and miR-500a-5p (18), which were down-regulated in HF compared with control subjects. The highest area under the curve (AUC) values achieved by single miRNAs (up- and down-regulated) for distinguishing HF (both subtypes combined) from control were hsa-let-7d-3p (Fold change = 1.33; $p < 0.001$ after FDR) (Online Figure 3A), with an AUC of 0.78 and hsa-miR-454-3p (Fold change = −2.12, FDR $p < 0.001$ after FDR) (Online Figure 3B), with an AUC of 0.85.

Our discovery cohort demonstrated that 41 miRNAs differed significantly between HFrEF and HFpEF ($p < 0.01$ after FDR) (Online Table 3) including miR-125a-5p and miR-550a-5p as previously reported in our earlier work (6). The highest AUC values attained by single miRNA (up- and down-regulated) for discriminating HFrEF from HFpEF were hsa-miR-223-5p, (Fold change = 1.23; $p < 0.001$ after FDR) (Online Figure 3C) and hsa-miR-185-5p (Fold change = −1.25; $p < 0.001$ after FDR) (Online Figure 3D) with AUCs of 0.68 and 0.69, respectively.

BIOMARKER PANELS FOR HF DETECTION AND SUBTYPE CATEGORIZATION. Within the discovery dataset, panels of miRNA with the highest AUCs for discrimination between groups were identified using SFFS and SVM. During the 2-fold cross validation in

FIGURE 2 **Identification of miRNAs in Discovery Cohort**



Number of miRNAs with significant fold change:

	Up-regulated	Down-regulated
P < 0.01	50	44
P < 0.001	45	41
P < 0.0001	42	37

Number of miRNAs with significant fold change:

	Up-regulated	Down-regulated
P < 0.01	15	26
P < 0.001	6	14
P < 0.0001	1	10

Volcano plots for miRNA levels that change significantly between **(A)** non-HF control subjects and HF, and **(B)** HFrEF and HFpEF. The tables summarize the numbers of significantly changed miRNAs defined by false discovery adjusted p values. Abbreviations as in **Figure 1**.

silico process, subsets of discovery cases were divided into 2 groups in >80 possible combinations. These paired groups were matched for HF subtype, sex, and ethnicity; one-half were used to derive the training set, and the remaining one-half of the samples were used as the testing set to verify the performance of the miRNA biomarker panels. The composition of the miRNA panels included a combination of miRNAs that were individually significant and nonsignificant for both HF detection and HF subtype categorization. For HF detection, the AUC values were similar between training sets and approached unity (AUC ~0.99) with increasing numbers of miRNAs in the panel. In the testing set, the AUC approached 0.94 in the 8-miRNA panel (Online Figure 4). Although the difference between 6 to 8 miRNA biomarker panels in the training set was statistically significant, the improvement was <0.01 in AUC. Thus, a biomarker panel with at least 8 miRNAs yielded an AUC of 0.94, comparable to NT-proBNP of AUC 0.96 in this discovery cohort. Next, we validated all the optimized 3 to 8 miRNA panels (Online Table 4) for HF detection in both validation cohorts (Online Figure 5A). The discovery panels of 8 selected miRNAs produced an AUC of 0.96 for discrimination between HF and controls (**Table 2**). In the Validation 1 cohort, we observed an AUC of 0.88, and in the Validation 2 cohort, the AUC was 0.87. A clustering heat map showing the 8-miRNA panel in validation cohorts is presented in Online Figure 6.

In discovery, miRNA panels of increasing size in the training set panel distinguished between HFrEF and HFpEF with an AUC of 0.87 (Online Figure 7). Subsequent to the 2-fold cross validation in silico process, the AUC approached 0.75 in the 6-miRNA panel in the testing set. There were no further improvements in AUCs in the testing sets once the miRNA-only biomarker panel contained >6 miRNAs. Thus, a biomarker panel with at least 6 miRNAs discriminated between HFrEF and HFpEF with an AUC of 0.75 compared with 0.71 for NT-proBNP in this discovery cohort. Next, we validated all the optimized 3 to 8 miRNA panels (Online Table 4) for HF subtype categorization in both validation cohorts (Online Figure 5B). For HF subtype categorization, the best performing diagnostic panel was a panel of 8 miRNAs demonstrating an AUC of 0.81 in discovery, with an AUC of 0.65 in Validation 1 and AUC of 0.65 in Validation 2 (**Table 2**). A clustering heat map showing the 8-miRNA panel in validation cohorts is presented in Online Figure 8.

TABLE 2 Performance of NT-proBNP, 8-miRNA Panel, and 8-miRNA Panel Coupled With NT-proBNP for HF Detection and Subtype Categorization

Biomarker	Performance	Discovery	Validation 1	Validation 2
HF detection, 90% sensitivity				
NT-proBNP	Sensitivity	90 (304/338)	90 (182/202)	90 (322/358)
	Specificity	91 (189/208)	90 (216/241)	100 (355/356)
	Accuracy	90 (493/546)	90 (398/443)	95 (677/714)
	PPV	94 (304/323)	88 (182/207)	100 (322/323)
	NPV	85 (189/223)	92 (216/236)	91 (355/391)
	AUC	0.96 (0.95–0.98)	0.95 (0.93–0.97)	0.996 (0.99–1.00)
8-miRNA panel	Sensitivity	90 (304/338)	90 (186/207)	90 (322/358)
	Specificity	88 (182/208)	66 (160/241)	58 (207/358)
	Accuracy	89 (486/546)	77 (346/448)	74 (529/716)
	PPV	92 (304/330)	70 (186/267)	68 (322/473)
	NPV	84 (182/216)	88 (160/181)	85 (207/243)
	AUC	0.96 (0.94–0.97)	0.88 (0.85–0.91)	0.87 (0.84–0.89)
8-miRNA panel + NT-proBNP	Sensitivity	90 (304/338)	90 (182/202)	90 (322/358)
	Specificity	99 (206/208)	96 (231/241)	99 (352/356)
	Accuracy	93 (510/546)	93 (413/443)	94 (674/714)
	PPV	99 (304/306)	94 (182/192)	99 (322/326)
	NPV	86 (206/240)	92 (231/251)	91 (352/388)
	AUC	0.99 (0.99–1.00)	0.97 (0.95–0.97)	0.994 (0.99–1.00)
HF subtype categorization, 80% sensitivity				
NT-proBNP	Sensitivity	80 (126/158)	79 (54/68)	80 (143/179)
	Specificity	46 (82/180)	44 (51/115)	48 (70/145)
	Accuracy	62 (208/338)	57 (105/183)	66 (213/324)
	PPV	56 (126/224)	46 (54/118)	66 (143/218)
	NPV	72 (82/114)	79 (51/65)	66 (70/106)
	AUC	0.71 (0.65–0.76)	0.72 (0.64–0.79)	0.69 (0.63–0.75)
8-miRNA panel	Sensitivity	80 (126/158)	81 (58/72)	80 (143/179)
	Specificity	66 (118/180)	41 (48/116)	41 (59/145)
	Accuracy	72 (244/338)	56 (106/188)	62 (202/324)
	PPV	67 (126/188)	46 (58/126)	62 (143/229)
	NPV	79 (118/150)	77 (48/62)	62 (59/95)
	AUC	0.81 (0.77–0.86)	0.65 (0.57–0.73)	0.65 (0.59–0.71)
8-miRNA panel + NT-proBNP	Sensitivity	80 (126/158)	79 (54/68)	80 (143/179)
	Specificity	75 (135/180)	59 (68/115)	52 (76/145)
	Accuracy	77 (261/338)	67 (122/183)	68 (219/324)
	PPV	74 (126/171)	54 (54/101)	68 (143/212)
	NPV	81 (135/167)	83 (68/82)	68 (76/112)
	AUC	0.87 (0.83–0.91)	0.74 (0.66–0.81)	0.72 (0.66–0.77)

The sensitivity, specificity, accuracy, positive (PPV) and negative (NPV) predictive values are presented as % (simple counts). The area under the curve (AUC) is presented as AUC value (95% confidence interval). The cutoff values for NT-proBNP for HF detection is 125 pg/ml, and the 8-miRNA panel score is 0. For HF detection, 7 samples without NT-proBNP read out were removed from the analysis, and for HF subtype classification, 5 cases without NT-proBNP read outs were removed from the analysis.

Abbreviations as in Table 1.

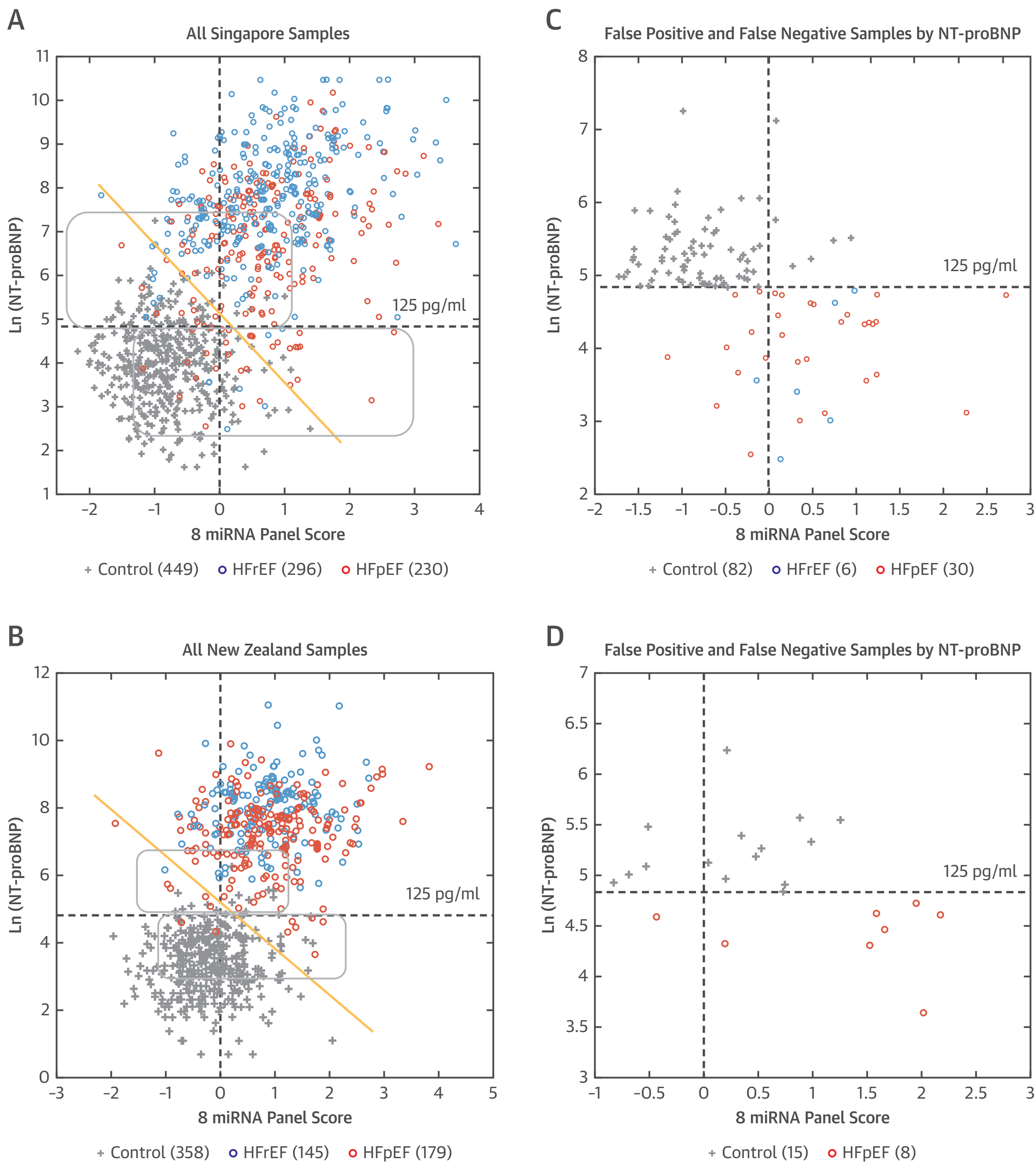
miRNA PANEL SCORE AND NT-proBNP ON HF DETECTION IN SINGAPORE AND NZ POPULATION. Scores from the 8-miRNA panel for HF were plotted against concurrent NT-proBNP levels. Applying the cut-off for NT-proBNP (125 pg/ml) recommended for ruling out nonacute HF (3), 82 (18%) and 15 (4%) of the healthy subjects in Singapore (combining both discovery and Validation 1) and NZ cohorts, respectively, were falsely classified as HF patients (false positive [FP], NT-proBNP >125 pg/ml), whereas 36 (7%) and 8 (2%) of HF patients in Singapore and NZ cohorts, respectively, had NT-proBNP levels lower than the cut-off (false negative [FN]) (Figures 3A and 3B). All of the FN results in the NZ cohort and most (83%) of the FN cases in Singapore were associated with HFpEF (n = 30). For the Singapore cohort, at least 76 (93%) of FP and 26 (72%) of FN cases were correctly reclassified by the 8-miRNA panel score, with 0 as the cut-off (Figure 3C). For the NZ cohort, 4 (27%) of FP and 7 (88%) of FN cases were correctly reclassified by the 8-miRNA panel score (Figure 3D).

COMBINING miRNAs WITH NT-proBNP FOR HF DETECTION AND SUBTYPE CATEGORIZATION. Next, we assessed the performance of our 8-miRNA panel coupled with NT-proBNP in validation cohorts. The receiver-operator characteristic curves were compared among the 3 candidate diagnostic biomarkers: 1) NT-proBNP alone; 2) the 8-miRNA panel; and 3) 8-miRNA panel combined with NT-proBNP as diagnostic panels. The combination of 8-miRNA score and NT-proBNP was optimized with logistic regression.

The best performing diagnostic panel for HF detection comprised the 8-miRNA panel combined with NT-proBNP, which achieved an AUC of 0.99, specificity of 0.99, and accuracy of 0.93 in the discovery cohort, with corresponding metrics of 0.97, 0.96, and 0.93 in Validation 1 (Table 2). This combination panel demonstrated improved specificity, accuracy, and diagnostic performance compared with NT-proBNP alone, with corresponding metrics of 0.96, 0.91, and 0.90 in the discovery and 0.95, 0.90, and 0.90 in Validation 1 (Figure 4A, Table 2). For Validation 2, our 8-miRNA panel coupled with NT-proBNP achieved near perfect diagnostic performance with an AUC 0.994, which was comparable to NT-proBNP (AUC of 0.996) (Table 2). Notably, any loss of specificity, accuracy, and/or positive predictive value observed when comparing the discovery dataset for miRNA panels alone to the Validation 1 cohort was completely restored or even improved beyond the initial derivation result when the panel was combined with NT-proBNP.

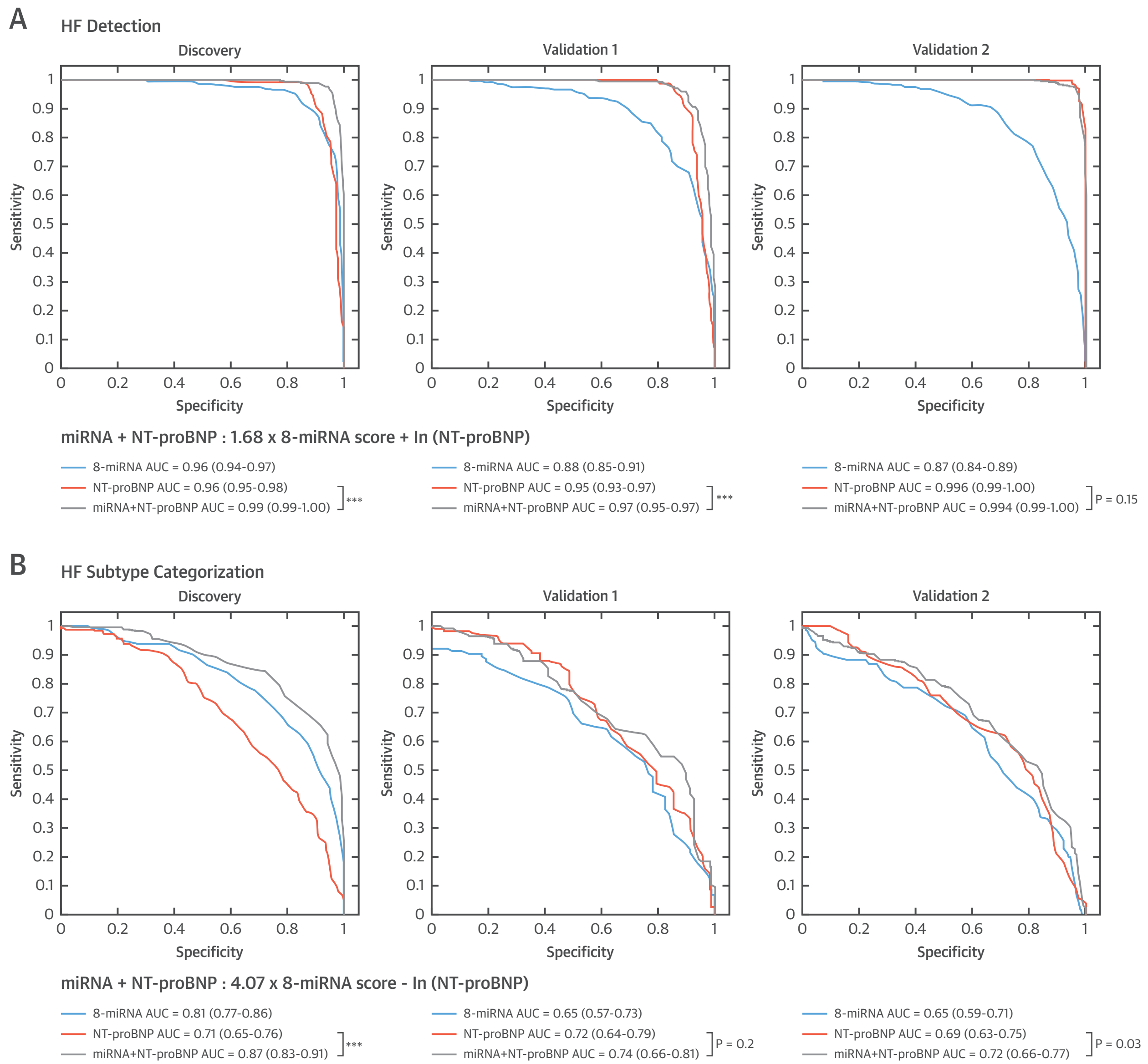
For HF subtype categorization, the best performing diagnostic panel comprised the 8-miRNA panel combined with NT-proBNP, achieving an AUC of 0.87, specificity of 0.75, and accuracy of 0.72 in the discovery cohort, with corresponding metrics of 0.74, 0.59, and 0.67 in Validation 1, and 0.75, 0.52, and 0.68 in Validation 2. This combined panel demonstrated

FIGURE 3 miRNA Panel Score and NT-proBNP on HF Detection in all Singapore and New Zealand Participants



The 2-dimensional plot of the ln (NT-proBNP) level **(y-axis)** and 8-miRNA panel score **(x-axis)** for **(A)** all Singapore subjects and **(B)** New Zealand subjects. The false positive and false negative subjects by NT-proBNP are **boxed**. The 2-dimensional plot for false positive and false negative subjects as classified by NT-proBNP using the 125-pg/ml threshold for **(C)** all Singapore subjects and **(D)** New Zealand subjects. The false positive and false negative subjects as reclassified by both NT-proBNP using the 125-pg/ml threshold and the 8-miRNA panel score (0) are indicated by the **dashed line**. The number of subjects plotted in the figures are shown in the legends. NT-proBNP = N-terminal prohormone brain natriuretic peptide; other abbreviations as in Figure 1.

FIGURE 4 Performance of NT-proBNP, miRNA Panels, and miRNA Panels Coupled With NT-proBNP



Receiver-operator characteristic curve plot for **(A)** HF detection and **(B)** HF subtype categorization in Discovery, Validation 1, and Validation 2. The combination of miRNA panel and NT-proBNP was optimized with logistic regression, and the formula is indicated in the figure. The comparison between the 8-miRNA panel, NT-proBNP, and 8-miRNA + NT-proBNP were evaluated with pairwise comparison. ***$p < 0.001$. AUC = area under the curve; other abbreviations as in Figures 1 and 3.

improved AUC, specificity, and accuracy compared with NT-proBNP with corresponding metric of 0.72, 0.44, and 0.57 in Validation 1, and 0.69, 0.48, 0.66 in Validation 2 (Figure 4B, Table 2).

For HF detection, the continuous NRI and IDI analysis using NT-proBNP as criterion standard and adding the 8-miRNA panel yielded continuous NRI of 1.52 ($p < 0.001$) and 1.18 ($p < 0.001$) in discovery and Validation 1, respectively, and an IDI of 0.03 ($p < 0.001$) in both discovery and Validation 1. The improvement of a combined marker as a new biomarker panel was significant compared with NT-proBNP alone, especially in discovery and Validation 1. For HF subtype categorization, adding the 8-miRNA panel yielded continuous NRI of 1.03 ($p < 0.001$), 0.67 ($p < 0.001$), and 0.48 ($p < 0.001$) in discovery,

TABLE 3 Reclassification for Adding miRNA Panel to NT-proBNP

	Model	AUC	p Value	IDI	p Value	NRI Events	p Value	NRI Nonevents	p Value	Continuous NRI	p Value
HF detection											
Discovery	NT-proBNP	0.96									
	NT-proBNP + 8-miRNA panel	0.99	<0.001	0.03	<0.001	0.82	<0.001	0.70	<0.001	1.52	<0.001
Validation 1	NT-proBNP	0.95									
	NT-proBNP + 8-miRNA panel	0.97	<0.001	0.03	<0.001	0.45	<0.001	0.73	<0.001	1.18	<0.001
Validation 2	NT-proBNP	0.996									
	NT-proBNP + 8-miRNA panel	0.994		−0.013	<0.001	0.88	<0.001	−0.12	0.03	0.77	<0.001
HF subtype categorization											
Discovery	NT-proBNP	0.71									
	NT-proBNP + 8-miRNA panel	0.87	<0.001	0.07	<0.001	0.53	<0.001	0.50	<0.001	1.03	<0.001
Validation 1	NT-proBNP	0.72									
	NT-proBNP + 8-miRNA panel	0.74	0.2	0.02	0.002	0.38	0.003	0.29	0.002	0.67	<0.001
Validation 2	NT-proBNP	0.69									
	NT-proBNP + 8-miRNA panel	0.72	0.03	0.02	<0.001	−0.05	0.51	0.53	<0.001	0.48	<0.001

The IDI is an absolute value. The NRI events and NRI nonevents values are presented as %. The overall continuous NRI has no units.
IDI = integrated discrimination improvement; NRI = net reclassification improvement; other abbreviations as in Table 1.

Validation 1, and Validation 2, respectively, and an IDI of 0.07 ($p < 0.001$), 0.02 ($p = 0.002$), and 0.02 ($p < 0.001$) in discovery, Validation 1, and Validation 2, respectively. The improved performance from combining markers was significant as compared with NT-proBNP alone in discovery, Validation 1, and Validation 2 (**Table 3**).

COMPARISON BETWEEN INPATIENT AND OUTPATIENT DATA. All treated HF patients were recruited either in hospital (inpatient) at the conclusion of an admission for ADHF or in the outpatient clinic within 6 months of a documented episode of ADHF requiring admission. Importantly, test performance of miRNAs with and without NT-proBNP was comparable in those with recent versus remote ADHF (Online Table 5, Online Figures 9 to 11). Notably, the ability to correctly reallocate HFpEF cases, which were initially erroneously designated as non-HF cases, was excellent in patients recruited in both inpatient (recent ADHF) and outpatient (remote ADHF) settings.

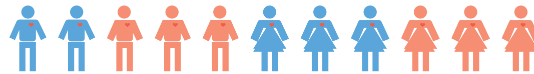
DISCUSSION

Our results established the strength of circulating miRNAs as diagnostic biomarkers in the discrimination of nonacute HF with performance comparable to NT-proBNP. We developed and validated two 8-miRNA panels as diagnostic tools for HF detection and subtype categorization. In the Validation 1, we demonstrated the discovery derived 8-miRNA panel alone generated an AUC of 0.88, and when coupled with NT-proBNP achieved an AUC of 0.97, for discrimination between HF and control subjects. In the Validation 2, the discovery derived 8-miRNA panel yielded an AUC of 0.87, and when coupled with NT-proBNP, the AUC was 0.994, which is comparable to the established NT-proBNP marker. NT-proBNP performed extremely well in the Validation 2 in distinguishing HF from control with an AUC of 0.996; therefore, it is challenging to improve on the performance of NT-proBNP. Combining our 8-miRNA panel with NT-proBNP clearly enhanced HF detection in the Validation 1 (Singapore) cohort. Importantly, at a given sensitivity, combining miRNA profiles with NT-proBNP markedly improved the specificity and accuracy of either used alone (**Table 2**). This was particularly notable for reducing FN designation of HFpEF cases with NT-proBNP values below 125 pg/ml. Notably, for the Singapore cohort, our 8-miRNA panel allowed correction of almost all FN and FP cases generated by application of the clinically endorsed diagnostic threshold for NT-proBNP (125 pg/ml) in the setting of nonacute HF (3,19). For NZ cohorts, our 8-miRNA panel corrected almost all FN cases generated by NT-proBNP cut-off threshold. Importantly, about 72% of Singapore and 88% of NZ FN cases that were correctly reallocated to HF by miRNAs were HFpEF. This fits well with the expected weaker performance of B-peptides in incipient, mild, or treated HFpEF.

Plasma NP concentrations reflect cardiac transmural distending pressures. In HFpEF plasma NP concentrations are on average one-half of that observed in HFrEF (4). This is in accord with the law of Laplace, with transmural distending forces directly related to intraventricular pressure and internal

CENTRAL ILLUSTRATION Combining miRNA Panel and NT-proBNP Improves Specificity and Accuracy for Heart Failure Detection and Subtype Categorization

Clinical Problem: Diagnosis of non-acute heart failure is often difficult, especially in patients with preserved ejection fraction (HFpEF)



Blue = No heart failure
Orange = Non-acute heart failure

Patient population: 1,710 participants with (903) and without (807) heart failure including both HFrEF and HFpEF cases from Singapore and New Zealand cohorts

Proposed heart failure panel: 8-miRNA panel + NT-proBNP
AUC 0.97-0.99; Specificity 0.96-0.99; Accuracy 0.93-0.94

No heart failure

Heart failure

Investigate for non-heart failure causes of presentation

Proposed heart failure subtype panel: 8-miRNA panel + NT-proBNP AUC 0.72-0.74; Specificity 0.52-0.59; Accuracy 0.66-0.67

HFrEF

HFpEF

Ensure imaging confirms reduced LVEF followed by full guideline-based Rx for HFrEF

Ensure imaging confirms preserved LVEF followed by symptomatic Rx plus ensure all contributory conditions (HTN, DM, CAD, dyslipidemia, renal dysfunction etc.) receive optimal Rx

Conclusion: Combining microRNA panel and NT-proBNP improves specificity and accuracy in identifying non-acute heart failure where clinical assessment and imaging may not be definitive, especially in HFpEF

Wong, L.L. et al. J Am Coll Cardiol. 2019;73(11):1300-13.

AUC = area under the curve; CAD = coronary artery disease; DM = diabetes mellitus; HF = heart failure; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; HTN = hypertension; LVEF = left ventricular ejection fraction; miRNA = microRNA; NT-proBNP = N-terminal prohormone brain natriuretic peptide; Rx = treatment.

diameter and inversely related to wall thickness. Hence, the nondilated, thick-walled HFpEF ventricle releases less NP at given intraventricular pressures than does the HFrEF ventricle. In ADHF, the levels of NT-proBNP in both HFrEF and HFpEF are so supranormal that the difference between phenotypes has only minor effects on the marker's diagnostic performance. However, between episodes of acute decompensation, plasma B-peptide levels may return to normal or near normal levels, especially in HFpEF (4). Even if not completely normal, plasma B-peptide levels often fall to values easily confounded by common factors including obesity, AF, age, and impaired renal function (20-22). Hence, in treated patients or in patients in the early stages of HF, the benchmark diagnostic marker loses diagnostic power especially in HFpEF. Our data indicate that the miRNA diagnostic panel augments the discriminative power of NT-proBNP in the nonacute setting, providing a markedly more accurate test panel for

this diagnosis. Therefore, it has the potential to be applied in community practice and in outpatient settings to improve early detection of incipient HF or partly treated HF, triggering timely diagnosis and/or clinical intervention. Its inclusion is likely to improve on the current recommendation for use of NT-proBNP 125 pg/ml as part of the diagnostic assessment for ambulant, nonacute HF, especially in the setting of HFpEF.

The strengths of our study include the relatively large number of HF patients and control subjects included for discovery of miRNA signatures. Validation was conducted in 2 sizable independent cohorts from Singapore and NZ, representative of Western and Asian populations. To date, reports on the biomarker performance of miRNAs in HF describe screening of miRNAs in relatively small samples conducted on various tissues ranging from (seldom accessible) cardiac biopsies (9,23) to readily stored serum/plasma samples (5,7,8). No prior published study has recruited >100 HF participants for high-throughput miRNA screening and discovery for diagnostic biomarker search (24); our sample size (n = 1,710) is large in comparison. Most studies employed a high-throughput array platform to screen a limited number of samples. This approach lacks sensitivity and reproducibility and has yielded small sets of targets for further validation. We have used stringently controlled methods for absolute qPCR-based quantification of miRNA copy number and we have used multistep spike-in controls to correct for any technical variation. With this approach, we have detected 82 dysregulated miRNAs not previously reported in HF compared with non-HF controls. The result obtained is highly reproducible and robust compared with the generally used calculation of relative miRNA expression (ΔCt by cel-miR spike-in only) considering there are no reference genes suitable for normalizing circulating miRNAs.

For discrimination between HFpEF and HFrEF, our 8-miRNA panel generated an AUC of 0.65 in distinguishing HFrEF from HFpEF for both Validation 1 and 2. Our 8-miRNA panel coupled with NT-proBNP achieved an AUC of 0.74 and 0.72 in Validation 1 and 2, respectively, showing significant improvement over NT-proBNP alone (AUC of 0.69 to 0.72). Thus far, 4 studies have investigated the potential of using miRNAs to aid distinction between HFrEF from HFpEF. Our earlier work (6) and the current study differ as we previously used whole-blood samples as a starting material in discovery of HF miRNAs signatures by microarray, followed by qPCR validation in independent plasma cohorts (30 participants in each group), resulting in 4 miRNAs distinguishing HFrEF and HFpEF (6). Of these, miR-125a-5p and miR-550a-5p were also dysregulated in the current study. Some miRNAs that are highly abundant in whole blood due to the presence of various blood cell types are absent in plasma samples. In this study, we improved our study design by using nonhemolyzed plasma samples as the starting material and employed an absolute quantification method to more accurately capture miRNA levels. Watson et al. (5) used the qPCR microfluidic card array approach in screening serum miRNAs (75 participants/group), showing 2 or more miRNAs in combination with BNP yielded an AUC >0.90 for diagnosis of undifferentiated HF and AUC >0.82 for differentiating HFrEF from HFpEF. Ellis et al. (7) reported 8 plasma miRNAs differentially expressed in HFrEF compared with HFpEF in the screening cohort of dyspnea cases, but this result could not be confirmed in a validation cohort. Among the 3 to 8 miRNAs used in our biomarker panels for both HF detection and subtype stratification, mir-24-3p has been reported to regulate apoptosis and vascularity in ischemic heart disease (25); mir-503-5p has been implicated in driving cardiomyocytes specification (26); miR-30a-5p has been shown to regulate autophagy during myocardial injury induced by Angiotensin II (27); and miR-106a-5p promotes hypertrophy through targeting mitofusin 2, a mitochondrial protein in regulating cardiac function (28).

NT-proBNP is the current benchmark HF biomarker. A comparison of the diagnostic performance of both BNP and NT-proBNP in a systematic review displayed an AUC range of 0.60 to 0.98 for BNP and 0.67 to 0.98 for NT-proBNP (29). In our present cohorts, NT-proBNP readily distinguished undifferentiated HF from non-HF cases with AUCs >0.9 in both Singapore and NZ. This may partly reflect the very clear phenotyping of our separately recruited cases and asymptomatic control subjects, who have been selected after extensive previous characterization rather than as part of assessment of a heterogeneous symptomatic group, such as those attending the emergency department with dyspnea (30,31). All control subjects were participants with no history of coronary artery disease or HF. Despite the excellent background performance of NT-proBNP, our miRNA panel augmented the diagnostic utility of NT-proBNP, especially in the Singapore cohort. The combination showed clear utility in correcting cases with FN designation as assessed by NT-proBNP alone, a particular problem in the setting of HFpEF.

STUDY LIMITATIONS. We selected a specific set of miRNAs for screening in the discovery cohort. We

cannot rule out the candidates that are not assayed in our current work as of potential utility. Our data is derived from comparison of very well-characterized HF and non-HF participants, and the results do require further corroboration in a "real-life" setting on an unbiased series of consecutive patients presenting in the nonacute setting with nonspecific symptoms and initially uncertain diagnosis. Furthermore, we have yet to assess the specific effect of comorbidities such as obesity, AF, age, and renal impairment on the test performance of our miRNA panel with NT-proBNP. In any case, we demonstrated overall our combined marker has better test performance, especially in identifying nonacute HF. Additional studies are warranted to underpin the association of identified miRNAs in understanding the mechanism of the disease. Future analyses will assess the relationship of patterns of miRNA derangement to prognosis, interrogate the possible significance of dysregulation of assorted biological pathways in the pathophysiology of HFrEF and HFpEF, and potentially point to new therapeutic targets.

CONCLUSIONS

Our findings highlight the potential applications of circulating miRNAs as diagnostic biomarkers in HF both alone and as adjuncts to the current benchmark biomarker, NT-proBNP (**Central Illustration**). Our miRNA panel improves upon the clinically endorsed HF biomarker, NT-proBNP, in detection of nonacute HF. miRNAs also aid in distinguishing HFpEF from HFrEF as evidenced through an improved AUC, especially when coupled with NT-proBNP. Notably, the use of miRNA panels correctly reallocates the vast majority of FN and FP cases resulting from application of the guideline-endorsed NT-proBNP threshold (125 pg/ml) currently used in ruling out nonacute HF. This advantage was particularly notable in the setting of HFpEF.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: As diagnostic biomarkers and adjuncts to NT-proBNP, panels of circulating microRNAs can identify nonacute HF and distinguish HFpEF from HFrEF.

TRANSLATIONAL OUTLOOK: Further studies are needed to assess the clinical utility of microRNA panels to improve HF detection and subtype categorization.

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KEY WORDS biomarker, diagnosis, heart failure, microRNA

APPENDIX For an expanded Methods section as well as supplemental figures and tables, please see the online version of this paper.