METHOD FOR DIAGNOSIS AND PROGNOSIS OF CHRONIC HEART FAILURE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore patent application No. 10201503644Q, filed 8 May 2015, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of molecular biology. In particular, the present invention relates to the use of biomarkers for the detection and diagnosis of heart failure.

BACKGROUND OF THE INVENTION

[0003] Cardiovascular disease including heart failure is a major health problem accounting for about 30% of human deaths worldwide [1]. Heart failure is also the leading cause of hospitalization in adults over the age of 65 years globally [2]. Adults at middle age have a 20% risk of developing heart failure in their life time. Despite treatment advances, morbidity and mortality (~50% at 5 years) for heart failure remain high and consume about 2% of health care budgets in many economies [3-6]. The prevalence of heart failure will increase due to the aging of the population, increasing prevalence of major risk factors such as diabetes, obesity and increased initial survival in acute myocardial infarction and severe hypertension.

[0004] Heart failure has been traditionally viewed as a failure of contractile function and left ventricular ejection fraction (LVEF) has been widely used to define systolic function, assess prognosis and select patients for therapeutic interventions. However, it is recognised that heart failure can occur in the presence of normal or near-normal EF: so-called "heart failure with preserved ejection fraction (HFPEF)" which accounts for a substantial proportion of clinical cases of heart failure [7-9]. Heart failure with severe dilation and/or markedly reduced EF: so- called "heart failure with reduced ejection fraction (HFREF)" is the best understood type of heart failure in terms of pathophysiology and treatment [10]. There are some epidemiological differences between patients with HFREF and those with HFPEF. The latter are generally older and more often women, are less likely to have coronary artery disease (CAD) and more likely to have underlying hypertension [7, 8, 11]. In addition, patients with HFPEF do not obtain similar clinical benefits from angiotensin converting enzyme inhibition or angiotensin receptor blockade as patients with HFREF [12, 13]. The symptoms of heart failure may develop suddenly- 'acute heart failure' leading to hospital admission, but they can also develop gradually.

[0005] Timely diagnosis, categorization of heart failure subtype - HFREF or HFPEF, and improved risk stratification are critical for the management and treatment of heart failure. Accordingly, there is a need to provide for methods of determining the risk of a subject in developing heart failure. There is also a need to provide for methods of categorizing heart failure subtypes.

SUMMARY OF THE INVENTION

[0006] In one aspect, there is provided a method of determining whether a subject suffers from heart failure or is at risk of developing heart failure. In some examples, the method includes the steps of a) measuring the level of at least one miRNA from a list of miRNAs "increased" or at least one from a list of miRNAs "reduced" as listed in Table 25, or Table 20, or Table 21, or Table 22, in a sample obtained from the subject. In some examples, the method further includes the step of b) determining whether the level of at least one miRNA from a list of miRNAs is different as compared to a control, wherein altered levels of the miRNA indicates that the subject has heart failure or is at a risk of developing heart failure.

[0007] In another aspect, there is provided a method of determining whether a subject suffers from a heart failure. In some examples, the heart failure is selected from the group consisting of a heart failure with reduced left ventricular ejection fraction (HFREF) and a heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, the method includes the steps of a) detecting the level of at least one miRNA as listed in Table 9 in a sample obtained from the subject. In some examples, the method further includes the step of determining whether the levels of the at least one miRNA indicates that the subject has, or is at a risk of, developing heart failure with reduced left ventricular ejection fraction (HFREF) or heart failure with preserved left ventricular ejection fraction (HFPEF).

[0008] In yet another aspect, there is provided a method for determining the risk of a heart failure patient having an altered risk of death. In some examples, the method includes the steps of a) detecting the levels of at least one miRNA as listed in Table 14 in a sample obtained from the subject. In some examples, the method also includes the step of b) measuring the levels of at least one miRNAs listed in Table 14. In some examples, the method also includes the step of c) determining whether the levels of at least one miRNAs listed in Table 14 is different as compared to the levels of the miRNAs of a control population, wherein altered levels of the miRNA indicates that the subject is likely to have an altered risk of death (altered observed (all-cause) survival rate) compared to the control population.

[0009] In yet another aspect, there is provided a method for determining the risk of a heart failure patient having an altered risk of disease progression to hospitalization or death. In some examples, the method comprises the step of a) detecting the levels of at least one miRNA as listed in Table 15 in a

sample obtained from the subject. In some examples, the method includes the step of b) measuring the levels of at least one miRNAs listed in Table 15. In some examples, the method further includes the step of c) determining whether the levels of at least one miRNAs listed in Table 15 is different as compared to the levels of the miRNAs of a control population, wherein altered levels of the miRNA indicates that the subject is likely to have an altered risk of disease progression to hospitalization or death (altered event free survival rate) compared to the control population.

[0010] In yet another aspect, there is provided a method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure. In some examples, the method includes the step of: (a) detecting the presence of miRNA in a sample obtained from the subject. In some examples, the method further includes the step of (b) measuring the levels of at least three miRNAs listed in Table 16 or Table 23 in the sample. In some examples, the method further includes the step of (c) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure.

[0011] In yet another aspect, there is provided a method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure. In some examples, the method includes the steps of: (a) detecting the presence of miRNA in a sample obtained from the subject. In some examples, the method also includes the step of (b) measuring the levels of at least three miRNAs listed in Table 17 in the sample. In some examples, the method also includes the step of (c) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure.

[0012] In yet another aspect, there is provided a method of determining the likelihood of a subject to be suffering from a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, the method includes the step of: (a) detecting the presence of miRNA in a sample obtained from the subject. In some examples, the method includes the step of (b) measuring the levels of at least three miRNA listed in Table 18 in the sample. In some examples, the method includes (c) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

[0013] In yet another aspect, there is provided a method of determining the likelihood of a subject having a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, the method includes the step of: (a) detecting the presence of miRNA in a sample obtained from the subject. In some examples, the method includes the step of (b) measuring the levels of at least three miRNAs listed in Table 19 or Table 24 in the sample. In some examples, the method also includes the step of (c) using a score based

on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0015] **Figure 1** shows a schematic diagram showing a summary of the number of miRNAs identified from studies described herein.

[0016] **Figure 2** shows histogram and skewness diagrams of N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and natural logarithm of the N-terminal prohormone of brain natriuretic peptide level (ln_NT-proBNP). Distribution of NT-proBNP level (A-C) and ln_NT-proBNP level (the natural logarithm of NT-proBNP, D-F) for the control subjects (A, D), heart failure with reduced left ventricular ejection fraction subjects (HFREF) (B, E) and heart failure with preserved left ventricular ejection fraction subjects (HFPEF) (C, F). The skewness of each graph was calculated and is displayed. Figure 2 shows that the N-terminal prohormone of brain natriuretic peptide (NT-proBNP) in all groups was positively skewed. In contrast, the natural logarithm of the N-terminal prohormone of brain natriuretic peptide (ln_NT-proBNP) level has less skewness. Therefore, the natural logarithm of the N-terminal prohormone of brain natriuretic peptide level was used for all analysis involving NT-proBNP.

[0017] **Figure 3** shows the results of the analysis of the performance of natural logarithm of the N-terminal prohormone of brain natriuretic peptide (ln_NT-proBNP) as a biomarker for heart failure. In particular, (A) shows a boxplot representation of ln_NT-proBNP (the natural logarithm of NT-proBNP) levels. Each boxplot presents the 25th, 50th, and 75th percentiles in the distribution. (B-D) show the receiver operating characteristic curves of ln_NT-proBNP for the of control vs heart failure (HFREF and HFPEF, B), HFREF vs heart HFPEF (C), control vs HFREF (D) and control vs HFPEF (E). AUC: area under the receiver operating characteristic curve, C: control (healthy), HF: heart failure, HFREF: heart failure with reduced left ventricular ejection fraction subjects, HFPEF: heart failure with preserved left ventricular ejection fraction subjects. Figure 3A shows the loss of NT-proBNP test performance is more pronounced in HFPEF. Figure 3B-D shows the natural logarithm of the N-terminal prohormone of brain natriuretic peptide (ln_NT-proBNP) performed better in detecting HFREF than HFPEF.

[0018] **Figure 4** shows an exemplary workflow of a high-throughput miRNA RT-qPCR measurement. The steps shown in Figure 4 includes isolation, multiplex groups, multiplex RT, augmentation, single-plex PCR and synthetic miRNA standard curve. Details of each steps are as

follows: Isolation refers to the step of isolating and purifying the miRNA from plasma samples; Spike-in miRNA refers to the non-natural synthetic miRNAs mimics (small single-stranded RNA with length range from 22-24 bases) that were added into the samples to monitor the efficiencies at each step including isolation, reverse transcription, augmentation and qPCR; Multiplex Design refers to the miRNA assays that were deliberately divided into a number of multiplex groups (45-65 miRNA per group) *in silico* to minimize non-specific amplifications and primer-primer interaction during the RT and augmentation processes; Multiplex reverse transcription refers to the various pools of reverse transcription primers that were combined and added to different multiplex groups to generate cDNA; Augmentation refers to a pool of PCR primers were combined and added to the each cDNA pool generated from a certain multiplex group and the optimized touch down PCR was carried out to enhance the amount of all cDNAs in the group simultaneously; Single-plex qPCR refers to the augmented cDNA pools that were distributed in to various wells in the 384 well plates and single-plex qPCR reactions were then carried out; and Synthetic miRNA standard curve refers to Synthetic miRNA stand curves that were measured together with the samples for the interpolation of absolute copy numbers in all the measurements.

[0019] **Figure 5** shows bar graph results of principal component analysis. Principal component analysis was performed for all 137 reliably detected mature miRNA (Table 4) based on the log2 scale expression levels (copy/mL). (A): the eigenvalues for the topped 15 principal components. (B), the classification efficiencies (AUC) of the topped 15 principal components on separating control (C) and heart failure (HF). (C), the classification efficiencies (AUC) of the topped 15 principal components on separating HFREF (heart failure with reduced ejection fraction) and HFPEF (heart failure with preserved ejection fraction). AUC: area under the receiver operating characteristic curve. Figure 5 shows a multivariate assay may be required to capture the information in multiple dimensions for the classification of HFREF and HFPEF.

[0020] **Figure 6** shows a scatter plot of the top (AUC) principal components in heart failure subjects as compared to control. In particular, the top (AUC) principal components used for discrimination between control (C, black cycle) and heart failure (HF, white triangle) subjects are shown in A. The top (AUC) two principal components for distinguishing HFREF (heart failure with reduced ejection fraction, black cycle) from HFPEF (heart failure with preserved ejection fraction, white triangle) subjects are shown in B. AUC: area under the receiver operating characteristic curve. PC: principal component number based on Figure 10. Variation: the percentage of the variations represented by the principal components calculated by eigenvalues. Figure 6 shows it is possible to separate the control, HFREF and HFPEF subjects based on their miRNA profiles.

[0021] **Figure 7** shows Venn diagrams showing the overlap of biomarkers that could be used for the detection of heart failure. The comparisons between control (healthy) and various groups of heart

failure patients (HF, HFREF and HFPEF) were carried out by univariate analysis (t-test) and multivariate analysis (logistic regression) incorporating age and AF (Atrial Fibrillation or Flutter), hypertension and diabetes. For the three comparisons: C vs HF (HFREF and HFPEF), C vs HFREF and C vs HFPEF, the numbers and overlaps of miRNAs with p-values (after false discovery rate correction) lower than 0.01 for in univariate analysis (A) and multivariate analysis (B) are shown. HF: heart failure, HFPEF: heart failure with preserved ejection fraction, HFREF: heart failure with reduced ejection fraction, C: control (healthy). Figure 7 shows many of the miRNAs were found to differ between control and only one of the two heart failure subtypes, thus demonstrate genuine differences between the two subtypes in terms of miRNA expression.

[0022] **Figure 8** shows boxplot and receiver operative characteristics curves of the top up-regulated and down-regulated miRNAs between healthy control and heart failure patients. The boxplot and receiver operating characteristic (ROC) curves of top (based on AUC) up-regulated (A: ROC curve, C: boxplot) and down-regulated (B: ROC curve, D: boxplot) miRNAs in all heart failure patients compared to the control (healthy) subjects. The expression levels (copy/ml) of miRNAs were presented in log2 scale. The boxplot presented the 25th, 50th, and 75th percentiles in the distribution of the expression levels. C: control (healthy), HF: heart failure. AUC: area under the receiver operating characteristic curve. Figure 8 shows combination of multiple miRNAs may enhance the performance of heart failure diagnosis.

[0023] **Figure 9** shows Venn diagrams showing the overlap of biomarkers for the detection of heart failure and the categorization of heart failure subtypes. Comparisons between HFREF and HFPEF were carried out by univariate analysis (t-test) and multivariate analysis (logistic regression) incorporating age, gender, BMI (Body Mass Index) and AF (Atrial Fibrillation or Flutter), hypertension (p-value, ln_BNP). The miRNAs with p-values (after false discovery rate correction) lower than 0.01 in univariate analysis (A) and multivariate analysis (B) were compared to the miRNAs for the detection of heart failure (either C vs HF or C vs HFREF or C vs HFPEF, Figure 5). HF: heart failure, HFPEF: heart failure with preserved ejection fraction, HFREF: heart failure with reduced ejection fraction, C: control (healthy) subject.

[0024] **Figure 10** shows boxplots and receiver operating characteristics (ROC) curve of top up-regulated and down-regulated miRNAs in HFPEF patients compared to that of HFREF patients. The boxplot and receiver operating characteristic (ROC) curves of topped (based on AUC) up-regulated (A: ROC curve, C: boxplot) and down-regulated (B: ROC curve, D: boxplot) miRNAs in HFPEF patients compared to that of HFREF patients. The expression levels (copy/ml) of miRNAs were presented in log2 scale. The boxplot presented the 25th, 50th, and 75th percentiles in the distribution of the expression levels. HFPEF: heart failure with preserved ejection fraction, HFREF: heart failure with reduced ejection fraction, AUC: area under the receiver operating characteristic curve. Figure 10

shows that combining the multiple miRNAs in a multivariate index assay may provide more diagnostic power for subtype categorization.

[0025] **Figure 11** shows line graphs of the overlapped miRNAs for the detection of heart failure and for the categorization of heart failure subtypes. The 38 overlapped miRNAs between control, heart failure (HFREF or HFPEF) and HFREF, HFPEF (Figure 7, A) were separated into 7 groups based on the changes. The two groups were defined as equal if the p-value (t-test) of the miRNA after false discovery test was higher than 0.01. The expression levels were based on the log2 scale and were standardized to zero mean for each miRNA. HFPEF: heart failure with preserved ejection fraction, HFREF: heart failure with reduced ejection fraction, C: control (healthy). Figure 11 shows that unlike the LVEF and NT-proBNP, HFPEF had more distinct miRNA profiles than the HFREF subtype compared to the healthy control. Figure 11 demonstrates miRNA could complement NT-proBNP to provide better discrimination of HFPEF.

[0026] **Figure 12** shows the scatter plot of the correlation analysis between all reliably detected miRNAs. Based on the log2 scale expression levels (copy/mL), Pearson's linear correlation coefficients were calculated between all 137 reliable detected miRNA targets (Table 4). Each dot represents a pair of miRNAs where the correlation coefficient is higher than 0.5 (A, positively correlated) or below -0.5 (B, negatively correlated). The differentially expressed miRNAs for C vs HF and HFREF vs HFPEF are indicated as black in the horizontal dimension. HF: heart failure, HFPEF: heart failure with preserved ejection fraction, HFREF: heart failure with reduced ejection fraction, C: control (healthy). Figure 12 demonstrates that many pairs of miRNAs were regulated similarly among all subjects.

[0027] **Figure 13** shows bar graph representing the pharmacotherapy for HFREF and HFPEF. The numbers of cases for various anti-HF drug treatments are summarized for the 327 subjects included in the prognosis analysis, divided into HFREF and HFPEF subtypes. The Chi-square test was applied to compare the two subtypes for each treatment. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001. Figure 13 is a summary of treatments according to the current clinical practice and was included among clinical variables for the analysis of prognostic markers.

[0028] **Figure 14** shows the survival analyses of subjects. In particular, (A) shows the Kaplan-Meier plots of clinical variables significantly predictive of observed survival (Table 14) based on univariate analysis (p-values < 0.05). For the categorical variables, the positive group (black) and negative groups (gray) were compared. For normally distributed variables, subjects with supra-median (black) and infra-median (gray) values were compared. The log-rank test was performed to test the between the two groups for each variable and the p-values were shown above each plot. (B) shows a bar graph representing the percentage of observed survival (OS) at 750 days after treatment.

[0029] **Figure 15** shows the survival analysis for event free survival. In particular, (A) shows Kaplan-Meier plots of clinical variables significantly predictive of for event free survival (Table 14) based on univariate analysis (p-values < 0.05). For the categorical variables, the positive group (black) and negative groups (gray) were compared. For normally distributed variables, subjects with supra-median (black) and infra-median (gray) values were compared. The log-rank test was performed to test the between the two groups for each variable and the p-values were shown above each plot. (B), shows bar graph representing the percentage of event free survival (EFS) at 750 days after treatment.

[0030] **Figure 16** shows Venn diagrams of the comparison between biomarkers for observed survival (OS) and event free survival (EFS). In particular, (A) shows the comparison between the miRNAs significantly prognostic for OS identified by univariate analysis and multivariate analysis with CoxPH model. (B) shows the comparison between the significant miRNAs for the prognosis of OS and for the prognosis of EFS. The miRNAs were either identified by univariate analysis or multivariate analysis with CoxPH model. Figure 16 demonstrates differing mechanisms for death and recurrent decompensated heart failure.

[0031] **Figure 17** shows Venn diagrams of the comparison between biomarkers for observed survival (OS) and event free survival (EFS). In particular, (A) shows the comparison between the miRNAs significantly prognostic by CoxPH model (either for OS or for EFS) and for detection of HF (either subtype). All the miRNAs were either identified by univariate analysis or multivariate analysis. (B) shows the comparison between the significant miRNAs for the prognosis identify by CoxPH model (either for OS or for EPS) and for categorization of two HF subtypes. All the miRNAs were either identified by univariate analysis or multivariate analysis. Figure 17 shows a large portion of the prognostic markers were not found in the other two lists indicating that a separate set of miRNA may be used or combined to form an assay for the prognosis.

[0032] **Figure 18** shows the analysis of miRNA with maximum and minimum hazard ratio for observed survival (OS). In (A), miRNA with the maximum hazard ratio (hsa-miR-503) and minimum hazard ratio (hsa-miR-150-5p) for observed survival (OS) were used to construct the univariate CoxPH model or the multivariate CoxPH model including six additional clinical variables: gender, hypertension, BMI, ln_NT-proBNP, BetaBlockers and Warfarin for observed survival (OS). All the level of normal variables including BMI, ln_NT-proBNP and the miRNA expression level (log2 scale) were scaled to have one standard deviation. Based on the value of the explanation score according to the on CoxPH model, the top 50% of the subjects (black) and the bottom 50% of the subjects (gray) were compared. The log-rank test was performed to test the between the two groups and the p-values were shown (B), the observed survival (OS)at 750 days after treatment.

[0033] **Figure 19** shows the analysis of miRNA with maximum and minimum hazard ratio for EFS. In (A), the miRNA with the maximum hazard ratio (hsa-miR-331-5p) and minimum hazard ratio

(hsa-miR-191-5p) for EFS were used to construct the univariate CoxPH model or the multivariate CoxPH model including 2 additional clinical variables: diabetes condition and ln_NT-proBNP for EFS. All the level of normal variables including diabetes condition ln_NT-proBNP and the miRNA expression level (log2 scale) were scaled to have one standard deviation. Based on the value of the explanation score according to the on CoxPH model, the top 50% of the subjects (black) and the bottom 50% of the subjects (gray) were compared. The log-rank test was performed to test the between the two groups and the p-values were shown (B), the EFS at 750 days after treatment.

[0034] **Figure 20** shows the representative results that generates multivariate biomarker panels for heart failure detection. In (A), the boxplots show the diagnostic power (AUC) of multivariate biomarker panels (number of miRNAs = 3-10) in the discovery and validation phases for heart failure detection during the two fold cross validation *in silico*. The boxplot presents the 25th, 50th, and 75th percentiles in the AUC for the classification of healthy and heart failure patients. The quantitative representation of the result for the discovery set (black) and validation set (gray) are shown in (B). The error bar represents the standard deviation of the AUC. In order to test the significance of the AUC improvement in the validation set when more miRNAs were included in the panel, the right-tailed t-test was carried to compare all the adjacent gray bars. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001.

[0035] **Figure 21** shows the comparison between multivariate miRNA score and NT-proBNP on HF detection using 2 dimensional plot. (A) shows 2 dimensional plot of the NT-proBNP level (y-axis) and one of the six-miRNA panel score (x-axis) for all subjects. The threshold for NT-proBNP (125) is indicated by the dashed line. The false positive and false negative subjects by NT-proBNP were boxed. (B) shows 2 dimensional plot of the NT-proBNP level (y-axis) and the six-miRNA panel score (x-axis) for false positive and false negative subjects as classified by NT-proBNP using the 125pg/ml threshold. The threshold miRNA score (0) is indicated by the dashed line. Control subjects are indicated by crosses; HFREF subjects by filled circles and HFPEF subjects by empty triangles. Figure 21 validated the hypothesis that miRNA biomarkers carry different information from that of N-terminal prohormone of brain natriuretic peptide (NT-proBNP).

[0036] **Figure 22** shows the analysis of multivariate biomarker panels for heart failure detection combining miRNAs with NT-proBNP. (A) show a series of boxplots of the diagnostic power (AUC) of multivariate biomarker panels (ln_NT-proBNP plus 2-8 miRNAs) in the discovery and validation phases for HF detection during the two fold cross validation *in silico*. The boxplot presented the 25th, 50th, and 75th percentiles in the AUC for the classification of healthy and HF patients. (B) shows the quantitative representation the result for discovery set (black) and validation set (gray) as well as the ln-NT-proBNP itself (the first column). The error bar represented the standard deviation of the AUC. In order to test the significance of the AUC improvement in the validation set when more miRNAs

were included in the panel, the right-tailed t-test was carried to compare all the adjacent gray bars. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. Thus, Figure 22 shows significantly improved classification efficiency when miRNA is combined with N-terminal prohormone of brain natriuretic peptide (NT-proBNP).

[0037] **Figure 23** shows Venn diagram of the overlap of miRNAs selected for multivariate HF detection panels with or without the addition of N-terminal prohormone of brain natriuretic peptide (NT-proBNP). Comparison between biomarkers selected for HF detection using miRNA along (Table 16) or using miRNA together with NT-proBNP (Table 17) during the multivariate biomarker search process. The significant miRNAs (A) and insignificant miRNAs (B) were compared separately. Figure 23 shows when using NT-proBNP, a different list of miRNAs may be used.

[0038] **Figure 24** shows the representative results that generates multi-miRNA panels for heart failure subtype stratification with and without the addition of NT-proBNP. (A) shows multivariate miRNA biomarker panel search (3-10 miRNAs) for heart failure subtype categorization The AUC result for discovery set (black bars) and validation set (gray bars) are shown. (B) shows multivariate miRNA and NT-proBNP biomarker panel search (ln_NT-proBNP plus 2-8 miRNAs) for heart failure subtype categorization. The AUC result for discovery set (black bars) and validation set (gray bars) as well as the ln_NT-proBNP itself (the first column) are shown. The error bar represents the standard deviation of the AUC. The right-tailed t-test was carried to compare all the adjacent gray bars. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. Figure 24 shows even clearer classifications may be achieved when both miRNA and NT-proBNP are used.

BRIEF DESCRIPTION OF TABLES

[0039] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying tables, in which:

[0040] **Table 1** is a summary of reported serum/ plasma miRNA biomarkers for heart failure. The studies that measured the cell-free serum/plasma miRNAs or the whole blood were included in the table. Only miRNAs validated with qPCR are shown. Up-regulated: miRNAs that had a higher level in HF patients than in the control (healthy) subject. Down-regulated: miRNAs that had a lower level in HF patients than in the control (healthy) subject. The numbers in "Study design" indicated the number of samples used in the study. PBMC: Peripheral blood mononuclear cells, AMI: acute myocardial infarction, HF: heart failure, HF: heart failure, HFPEF: heart failure with preserved left ventricular ejection fraction, HFREF: heart failure with reduced left ventricular ejection fraction, BNP: brain natriuretic peptide, C: control (healthy subjects).

[0041] **Table 2** is a table listing the clinical information of the subjects included in the study. The clinical information of the 546 subjects included in the study. All the plasma samples were stored at -

80 °C prior to use. N.A.: not available, C: control (healthy subjects), PEF: heart failure with preserved left ventricular ejection fraction, REF: heart failure with reduced left ventricular ejection fraction

[0042] **Table 3** is a table listing the characteristics of the healthy subjects and heart failure patients. The Ejection Fraction (left ventricular ejection fraction), ln_NT-proBNP, Age, Body Mass Index are shown as arithmetic mean ± standard deviation and the NT-proBNP is shown as geometric mean. The percentage next to the variable name indicates the percentage of subjects with known value for the variable. HF: heart failure, HFPEF: heart failure with preserved ejection fraction, HFREF: heart failure with reduced ejection fraction, C: control (healthy) subject. For the comparisons of the variables between control and heart failure (C vs HF) and between HFPEF and HFREF (HFREF vs HFPEF), t-test was used for normal variables and chi-squared test were used for categorical variables.

[0043] **Table 4** is a table listing the sequences of 137 reliably detected mature miRNA. The 137 mature miRNA were reliably detected in the plasma samples. The definition of "reliably detected" was that at least 90% of the plasma samples had a concentration higher than 500 copies per ml. The miRNAs were named according to the miRBase V18 release.

[0044] **Table 5** is a table listing miRNAs that are differentially expressed between control and all heart failure subjects. Comparisons between control (healthy) and all heart failure subjects (both HFREF and HFPEF) were carried out by univariate analyses (p-value, t-test) and multivariate analyses with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension, diabetes (p-value, Logistic regression). The enhancements by miRNAs to the diagnostic performance of ln_NT-proBNP for heart failure were tested with logistic regression with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, ln_BNP). All the p-values were adjusted for false discovery rate correction using Bonferroni method. Only those miRNAs had p-values lower than 0.01 for both the "p-value, t-test" test and "p-value, Logistic regression" test were shown. Fold change: the miRNA expression level in heart failure subjects divided by that in the control subjects.

[0045] **Table 6** is a table listing miRNAs that are differentially expressed between control and HFREF subjects. Comparisons between control (healthy) and HFREF subjects (heart failure with reduced left ventricular ejection fraction) were carried out by univariate analyses (p-value, t-test) and multivariate analyses with adjustment for age, AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, Logistic regression). The enhancements by miRNAs of the discrimination of HFREF by ln_NT-proBNP were tested by logistic regression with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, ln_BNP). All p-values were adjusted for false discovery rate correction using the Bonferroni method. Only those miRNAs with p-values < 0.01 for both the "p-value, t-test" test and "p-value, Logistic regression" test were shown. Fold change: the miRNA expression level in HFREF subjects divided by that in the control subjects.

[0046] **Table 7** is a table listing miRNAs that are differentially expressed between control and HFPEF subjects. Comparisons between control (healthy) and HFPEF subjects (heart failure with preserved left ventricular ejection fraction) were carried out by univariate analyses (p-value, t-test) and multivariate analyses with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, Logistic regression). The enhancements by miRNAs of the discrimination by ln_NT-proBNP of HFPEF diagnosis were tested with logistic regression with adjustment for age, AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, ln_BNP). All the p-values were adjusted for false discovery rate correction using the Bonferroni method. Only those miRNAs with p-values < 0.01 for both the "p-value, t-test" test and "p-value, Logistic regression" test were shown. Fold change: the miRNA expression level in HFPEF subjects divided by that in the control subjects.

[0047] **Table 8** is a table listing the comparison between the current study and previously published reports. The miRNAs not listed in Table 4 (expression levels ≥500 copies/ml) were indicated as N.A. (not available) which may not be included in the study or were below detection limit. Up: the miRNA had a higher expression level in heart failure patients compared to that of control (healthy) subjects. Down: the miRNA had a lower expression level in heart failure patients compared to that of control (healthy) subjects. Those miRNAs with p-values after false discovery rate correction lower than 0.01 were indicated as No Change. For hsa-miR-210, there were contradictions for the direction of changes in various literature reports (indicated Up & Down).

[0048] **Table 9** is a table listing miRNAs that are differentially expressed between HFREF and HFPEF subjects. Comparisons between HFREF (heart failure with reduced left ventricular ejection fraction) and HFPEF subjects (heart failure with preserved left ventricular ejection fraction) were carried out by univariate analyses (p-value, t-test) and multivariate analyses with adjustment for age, gender, BMI (Body Mass Index) and AF (Atrial Fibrillation or Flutter)and hypertension (p-value, Logistic regression). The enhancements by miRNAs to the ability of ln_NT-proBNP to discriminate between HFREF and HFPEF categorization were tested with logistic regression with adjustment for age, gender, BMI (Body Mass Index),AF (Atrial Fibrillation or Flutter)and hypertension (p-value, ln_BNP). All the p-values were adjusted for false discovery rate correction using the Bonferroni method. Only those miRNAs with p-values < 0.01 for the "p-value, t-test" test were shown. Fold change: the miRNA expression level in HFPEF subjects divided by that in the HFREF subjects.

[0049] **Table 10** is a table listing the clinical information of the subjects included in the prognosis study. The clinical information of the 327 subjects included in the prognosis study. All subjects were followed-up for two years after recruitment to the SHOP cohort study. 49 patients passed away during follow up.

[0050] **Table 11** is a table listing the treatments of subjects included in the prognosis study. Drug treatment of the 327 subjects included in the prognosis study; Name of the medicine, Me1: ACE

Inhibitors, Me2: Angiotensin 2 Receptor Blockers, Me3: Loop/thiazide Diuretics, Me4: Beta Blockers, Me5: Aspirin or Plavix, Me6: Statins, Me7: Digoxin, Me8: Warfarin, Me9: Nitrates Calcium, Me10: Channel Blockers, Me11: Spironolactone, Me12: Fibrate, Me13: Antidiabetic, Me14: Hydralazine, Me15: Iron supplements.

[0051] **Table 12** is a table listing the analysis of clinical variables for observed survival. The clinical parameters included in analyses on observed survival using Cox proportional hazard model included drug treatments and other variables. The level of age, BMI, LVEF and ln_NT-proBNP were scaled to have one standard deviation. In the multivariate analysis, all variables were included. The cells for those variables with p-value less than 0.05 are indicated in gray. ln(HR): natural logarithm of hazard ratio (a positive value indicated a higher chance of death with the higher value of the variable), SE: standard error.

[0052] **Table 13** is a table listing the analysis of clinical variables for Event free survival. The clinical parameters for analysis of Event free survival used Cox proportional hazards models with the level of age, BMI, LVEF and ln_NT-proBNP scaled to have one standard deviation. Drug treatments were also included. In the multivariate analysis, all variables were included. The cells for those variables with p-value < 0.05 were indicated gray. ln(HR): natural logarithm of hazard ratio (a positive value indicated a higher chance of death with the higher value of the variable), SE: standard error.

[0053] **Table 14** is a table listing miRNAs that are significantly predictive of observed survival. Each of the miRNAs was analyzed for association with observed survival using Cox proportional hazard model with univariate and multivariate analyses which included additional clinical variables: gender, hypertension, BMI, ln_NT-proBNP, BetaBlockers and Warfarin. All the normally distributed variables including ln_NT-proBNP, BMI and miRNA expression level (log2 scale) were scaled to have one standard deviation. Those p-values < 0.05 are indicated as gray cells. ln(HR): natural logarithm of hazard ratio (a positive value indicated a higher chance of death with the higher value of the variable), SE: standard error.

[0054] **Table 15** is a table listing miRNAs significantly predictive of event free survival. Each of the miRNA was analyzed for associations with event free survival using Cox proportional hazard model with univariate and multivariate analyses which included additional clinical variables: diabetes and ln_NT-proBNP. All the normally distributed variables including ln_NT-proBNP and miRNA expression level (log2 scale) were scaled to have one standard deviation. Those p-values < 0.05 are indicated as gray cells. ln(HR): natural logarithm of hazard ratio (a positive value indicated a higher chance of death with the higher value of the variable), SE: standard error.

[0055] **Table 16** is a table listing miRNAs identified in multivariate panel search process for heart failure detection. The miRNAs selected for the assembly of biomarker panels with 6, 7, 8, 9, and 10

miRNAs for heart failure detection are listed. Prevalence was defined by the counts of the miRNA in all panels divided by the total number of panels. The panels with the top 10% and bottom 10% AUC were excluded to avoid counting of falsely discovered biomarkers due to fitting of inaccurate data from subpopulations generated by the randomization process in cross-validation analysis. Only the miRNAs used in more than 2% of the panels were listed. The changes of the miRNAs in various subtypes of heart failure were defined based on Table 5-7.

[0056] **Table 17** is a table listing the miRNAs that are identified in multivariate panel search process for heart failure (HF) detection in conjunction with NT-proBNP. The miRNAs selected for the assembly of biomarker panels with ln_NT-proBNP and 3, 4, 5, 6, 7 and 8 miRNAs for heart failure detection are listed. Prevalence was defined by the counts of the miRNA in all panels divided by the total number of panels. The panels with the top 10% and bottom 10% AUC were excluded to avoid counting of falsely discovered biomarkers due to fitting of inaccurate data from subpopulations generated by the randomization process in cross-validation analysis. Only the miRNAs used in more than 2% of the panels were listed. The significances of the miRNAs additional to ln_NT-proBNP in discriminating various subtypes of heart failure were determined based on the logistic regression using the selected miRNA and ln_NT-proBNP as predictive variables where the p-values for the significant miRNAs after FDR correction were < 0.01.

[0057] **Table 18** is a table listing the miRNAs that are identified in multivariate panel search process for HF subtype categorization. The miRNAs selected for the assembly of biomarker panels with 6, 7, 8, 9, and 10 miRNAs for heart failure (HF) subtype categorization are listed. Prevalence was defined by the counts of the miRNA in all panels divided by the total number of panels. The panels with the top 10% and bottom 10% AUC were excluded to avoid counting of falsely discovered biomarkers due to fitting of inaccurate data from subpopulations generated by the randomization process in cross-validation analysis. Only the miRNAs used in more than 2% of the panels were listed. The changes of the miRNAs in between the HFREF and HFPEF subtypes were defined based on Table 9.

[0058] **Table 19** is a table listing the miRNAs identified in multivariate panel search process for HF subtype categorization in conjunction with NT-proBNP. The miRNAs selected for the assembly of biomarker panels with ln_NT-proBNP and 5, 6, 7 and 8 miRNAs for HF subtype categorization are listed. Prevalence was defined by the counts of the miRNA in all panels divided by the total number of panels. The panels with the top 10% and bottom 10% AUC were excluded to avoid counting of falsely discovered biomarkers due to fitting of inaccurate data from subpopulations generated by the randomization process in cross-validation analysis. Only the miRNAs used in more than 2% of the panels were listed. The significances of the miRNAs additional to ln_NT-proBNP were determined

based on the logistic regression using the selected miRNA and ln_NT-proBNP as predictive variables where the p-values for the significant miRNAs after FDR correction were < 0.01.

[0059] **Table 20** is a table listing miRNAs identified for heart failure (HF) detection. Comparisons between control (healthy) and all heart failure subjects (both HFREF and HFPEF) were carried out by univariate analyses (p-value, t-test) and multivariate analyses with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension, diabetes (p-value, Logistic regression). The enhancements by miRNAs to the diagnostic performance of ln_NT-proBNP for heart failure were tested with logistic regression with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, ln_BNP). All the p-values were adjusted for false discovery rate correction using Bonferroni method. Only those miRNAs had p-values lower than 0.01 for both the "p-value, t-test" test and "p-value, Logistic regression" test were shown. Fold change: the miRNA expression level in HF subjects divided by that in the control subjects. Table 20 corresponds to Table 5 with the exception that the miRNAs listed in Table 20 are not part of the miRNAs known in the art (i.e. as listed in Table 1 and Table 8).

[0060] **Table 21** is a table listing the miRNAs identified for HFREF detection. Comparisons between control (healthy) and HFREF subjects (heart failure with reduced left ventricular ejection fraction) were carried out by univariate analyses (p-value, t-test) and multivariate analyses with adjustment for age, AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, Logistic regression). The enhancements by miRNAs of the discrimination of HFREF by ln_NT-proBNP were tested by logistic regression with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, ln_BNP). All p-values were adjusted for false discovery rate correction using the Bonferroni method. Only those miRNAs with p-values < 0.01 for both the "p-value, t-test" test and "p-value, Logistic regression" test were shown. Fold change: the miRNA expression level in HFREF subjects divided by that in the control subjects. Table 21 corresponds to Table 6 with the exception that the miRNAs listed in Table 21 are not part of the miRNAs known in the art (i.e. as listed in Table 1 and Table 8).

[0061] **Table 22** is a table listing the miRNAs identified for HFPEF detection. Comparisons between control (healthy) and HFPEF subjects (heart failure with preserved left ventricular ejection fraction) were carried out by univariate analyses (p-value, t-test) and multivariate analyses with adjustment for age and AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, Logistic regression). The enhancements by miRNAs of the discrimination by ln_NT-proBNP of HFPEF diagnosis were tested with logistic regression with adjustment for age, AF (Atrial Fibrillation or Flutter), hypertension and diabetes (p-value, ln_BNP). All the p-values were adjusted for false discovery rate correction using the Bonferroni method. Only those miRNAs with p-values < 0.01 for both the "p-value, t-test" test and "p-value, Logistic regression" test were shown. Fold change: the

miRNA expression level in HFPEF subjects divided by that in the control subjects. Table 22 corresponds to Table 7 with the exception that the miRNAs listed in Table 22 are not part of the miRNAs known in the art (i.e. as listed in Table 1 and Table 8).

[0062] **Table 23** is a table listing frequently selected miRNAs for heart failure detection in multivariate panel search process. The miRNAs selected for the assembly of biomarker panels with 6, 7, 8, 9, and 10 miRNAs for heart failure detection are listed. Prevalence was defined by the counts of the miRNA in all panels divided by the total number of panels. The panels with the top 10% and bottom 10% AUC were excluded to avoid counting of falsely discovered biomarkers due to fitting of inaccurate data from subpopulations generated by the randomization process in cross-validation analysis. Only the miRNAs used in more than 2% of the panels were listed. The changes of the miRNAs in various subtypes of heart failure HF were defined based on Table 20-22. Table 23 corresponds to Table 16 with the exception that the miRNAs listed in Table 23 are not part of the miRNAs known in the art (i.e. as listed in Table 1 and Table 8).

[0063] **Table 24** is a table listing frequently selected miRNAs for HF detection in multivariate panel search process in conjunction with NT-proBNP. The miRNAs selected for the assembly of biomarker panels with ln_NT-proBNP and 3, 4, 5, 6, 7 and 8 miRNAs for HF detection are listed. Prevalence was defined by the counts of the miRNA in all panels divided by the total number of panels. The panels with the top 10% and bottom 10% AUC were excluded to avoid counting of falsely discovered biomarkers due to fitting of inaccurate data from subpopulations generated by the randomization process in cross-validation analysis. Only the miRNAs used in more than 2% of the panels were listed. The significances of the miRNAs additional to ln_NT-proBNP in discriminating various subtypes of HF were determined based on the logistic regression using the selected miRNA and ln_NT-proBNP as predictive variables where the p-values for the significant miRNAs after FDR correction were < 0.01. Table 24 corresponds to Table 17 with the exception that the miRNAs listed in Table 24 are not part of the miRNAs known in the art (i.e. as listed in Table 1 and Table 8).

[0064] **Table 25** is a table listing microRNAs that may be used specifically for heart failure detection. To the best of the inventors' knowledge, these miRNAs are only associated with heart failure. miRNAs listed in Table 25 are not part of miRNAs known in the art (i.e. as listed in Table 1 and Table 8).

[0065] **Table 26** is a table listing exemplary biomarker panels for heart failure detection. Based on the biomarkers provided, an example of the formula, cutoffs and performance of the panel are provided in the table.

[0066] **Table 27** is a table listing exemplary biomarker panels for heart failure subtype detection. Based on the biomarkers provided, an example of the formula, cutoffs and performance of the panel are provided in the table.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0067] Timely diagnosis, accurate categorization of heart failure subtype, including, but not limited to heart failure with reduced left ventricular ejection fraction (HFREF), heart failure with preserved left ventricular ejection fraction (HFPEF), and the like, and improved risk stratification are important for the management and treatment of heart failure. An attractive approach is the use of circulating biomarkers [14]. The established circulating biomarkers in heart failure are the cardiac natriuretic peptides, B type natriuretic peptide (BNP) and its co-secreted congener, N-terminal prohormone brain natriuretic peptide (NT-proBNP). Both have proven diagnostic utility in acute heart failure and are independently related to prognosis at all stages of heart failure leading to their inclusion in all major international guidelines for the diagnosis and management of heart failure [14, 15]. However, confounders including age, renal function, obesity and atrial fibrillation do impair their diagnostic performance [16, 17]. In asymptomatic left ventricular dysfunction, early symptomatic heart failure and treated heart failure, the discriminating power of B peptides is markedly diminished with half of all stable HFREF cases exhibiting BNP below 100 pg/ml and 20% with NT-proBNP below values employed to rule out heart failure in the acutely symptomatic state [18]. This loss of test performance is even more pronounced in the cases of HFPEF [19]. B peptides reflect cardiac ventricular transmural distending pressures and myocyte stretch which (being dependent on chamber diameter as well as intra-ventricular pressures and wall thickness) is far less elevated in HFPEF with normal or reduced ventricular lumen volume and thickened ventricular walls, compared with HFREF with typically dilated ventricles and eccentric remodeling [20]. Therefore there is an unmet need for biomarkers that complement or replace B type peptides in screening for heart failure in its early or partly treated state and in monitoring status in the chronic phase of heart failure. This is particularly true for HFPEF with B peptides level lower than HFREF and often normal [21]. Currently, the categorization of heart failure subtype is dependent on imaging and imaging interpretation by a cardiologist. There is no biomarker based test available for this purpose. Therefore, a minimally invasive method to improve the diagnosis of heart failure as well as categorization into HF subtype is desirable.

[0068] MicroRNAs (miRNAs) are small non-coding RNAs that play central roles in the regulation of gene expression dysregulation of microRNAs is implicated in the pathogenesis of various diseases [22-26]. Since their discovery in 1993 [27], miRNAs have been estimated to regulate more than 60% of all human genes [28], with many miRNAs identified as key players in critical cellular functions such as proliferation [29] and apoptosis [30]. The discovery of miRNAs in human serum and plasma has raised the possibility of using circulating miRNA as biomarkers for diagnosis, prognosis, and treatment decisions for many diseases [31-35]. An integrated multidimensional method

for the diagnosis of HF using miRNA or miRNA in conjunction with BNP/NT-proBNP may improve the diagnosis. Combining genomic marker(s), such as miRNAs, and protein marker(s), such as BNP/NT-proBNP may strengthen diagnostic power in HF compared to sole use of BNP/NT-proBNP. Recently, various attempts had been made to identify circulating cell-free miRNA biomarkers in serum or plasma to distinguish HF patients from healthy subjects [36-47] (Table 1).

[0069] **Table 1. Summary of reported serum/ plasma miRNA biomarkers for heart failure**

Publication	**Study design**	**Up-regulated**	**Down-regulated**	**Discovery Study design**	**Validation Study design**
Vogel et al [1]	Predict HFREF	miR-200b*, miR-622, miR-1228*	-	whole blood, 53 HFREF/39 C, microarray	serum, 14 REF/8 C, qPCR
Endo et al [2]	Outcome as change of BNP in 3 weeks	-	miR-210	Start with miR-210 only	Plasma, 39 NYHA II heart failure, qPCR
Zhang et al [3]	Predict the development of HF after AMI	-	miR-1	Start with miR-1 only	Plasma, 49 AMI patients with various EF, qPCR
Fukushima et al [4]	Predicts HF	-	miR-126	Start with three miRNAs	Plasma, 10 HF/ 17 C
Corsten et al [5]	Predicts acute HF	miR-499	-	Start with six miRNAs	Plasma, 33 HF/34 C
Matsumoto et al [6]	Predict the development of HF after AMI	miR-192, miR-194, miR-34a,	-	Serum, 7 HF/ 7 C, Taqman, qPCR array	Serum, 21 HF/ 65 C, qPCR of 14 miRNAs (additional 2 not based on discovery)
Goren et al [7]	Predict HFREF	miR-423-5p, miR-320a, miR-22, miR-92b, miR-17*, miR-532-3p, miR-92a, miR-30a, miR-21, miR-29c, miR-101	miR-199b-5p, miR-33a, miR-27b, miR-331-3p, miR-744, miR-28-5p, miR-574-3p, miR-223, miR-142-3p, miR-27a, miR-191, miR-335, miR-24, miR-151-5p	Serum, pooled samples, 2 HF/ 2 C, qPCR 370 miRNAs	Serum 30 HF/30 C, qPCR 186 miRNAs
Xiao et al [8]	Predict chronic HF	miR-142-3p miR-29b	miR-107, miR-139, miR-142-5p, miR-107, miR-125b,	PBMC 15 HC/9 C, qPCR 159 miRNAs	PBMC 34 HC/19 C, qPCR 12 miRNAs

			miR-497,		
Tijsen et al [9]	Predict acute HF	miR-423-5p, miR-18b*, miR-129-5p, miR-1254, miR-675, miR-622	-	Plasma, HF 12/C 12, microarray	Plasma, HF 30/C 39, qPCR 16 miRNAs
Zhao et al [10]	Predict chronic HF	miR-210, miR-30a	-	Serum, pooled samples HF 1/C 1, qPCR 27 miRNAs	Serum, HF 22/C 18, qPCR 9 miRNAs
Goren et al [11]	Predict chronic HF - PEF	-	-	-	Serum, HF 41/C 35, qPCR: miR-150
Ellis et al [12]	Predict chronic HF – HFPEF/REF	miR-185,	miR-103, miR-142-3p, miR-30b, miR-342-3p, miR-150, miR-199a-3p, miR-23a, miR-27b, miR-324-5p	Plasma, HF 32/C 29, qPCR array	Plasma, HF 44/C 106, qPCR, 17 miRNAs

[0070] These studies reported a set of miRNAs differentially regulated in heart failure subjects. However, there is a lack of concordance between these published works. Among 67 reported miRNAs, only three were found up-regulated in more than one report. In particular, hsa-miR-210 was reported as up-regulated in HF in one report and down-regulated in another (Table 1). The lack of agreement between studies could be due to a number of reasons including the use of small sample sizes or the variability in the sample selection such as stage of disease and, importantly, the controls used [32, 48]. Pre-analytical process including experimental design and workflow are critical in biomarkers identification and validation. Most studies to date have used a high-throughput array platform to screen a limited number of samples. This approach lacks sensitivity and reproducibility. It has yielded a small set of targets (less than 10 miRNAs) identified for further validation. Most of the studies have yet to be corroborated in larger patient groups. Another approach which has been adopted widely is based on screening of reported candidate miRNAs using quantitative real time polymerase chain reaction (qPCR). Evaluation of different technologies based on array versus qPCR platforms showed there are substantial differences in the performance of these platforms for miRNAs measurement. This could contribute to the observed inconsistency between studies [49]. Thus far, there is no consensus on the specific circulating serum/plasma miRNAs that might be used as heart failure biomarkers. None of the miRNA profiles previously reported was useful for categorization into

heart failure subtypes. Hence, there is a need to build a robust pre-designated technology platform for heart failure biomarkers discovery and validation, and to ensure the reproducibility of the results.

[0071] In this disclosure, panels of circulating miRNAs were identified as potential heart failure biomarkers. These multivariate index assays are defined by Food and Drug Agency (FDA) guidelines, quoted as below: "combines the values of multiple variables using an interpretation function to yield a single, patient-specific result (e.g., a "classification," "score," "index," etc.), that is intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment or prevention of disease, and provides a result whose derivation is non-transparent and cannot be independently derived or verified by the end user." Thus, highly reliable qPCR-based quantitative data following the MIQE (Minimum Information for publication of Quantitative Real-Time PCR Experiments) guidelines is a pre-requisite and the use of the state-of-the art mathematical and biostatistics tools are essential to determine the inter-relationship of these multiple variables simultaneously.

[0072] There are a variety of miRNA measurement methods including hybridization-based (microarray, northern blotting, bioluminescent), sequencing-based and qPCR-based [50]. Due to the small size of miRNA's (about 22 nucleotides), the most robust technology that provides precise, reproducible and accurate quantitative result with the greatest dynamic range is the qPCR-based platform [51]; currently, it is a gold standard commonly used to validate the results from other technologies, such as sequencing and microarray data. A variation of this method is digital PCR [52], an emerging technology based on similar principles but yet to gain widespread acceptance and use.

[0073] In this study, 203 miRNAs were profiled by qPCR in the plasma of 338 chronic heart failure patients (180 HFREF and 158 HFPEF) and 208 non-heart failure subjects (control group). This is a larger cohort of miRNA screening in heart failure than any reported in the literature to date. A summary of the number of miRNAs identified for various proposed approaches used in this study is depicted in Figure 1.

[0074] The inventors of the present disclosure have established a well-designed workflow with multi-layered technical and sample controls. This is to ensure the reliability of the assay and minimize the possible cross-over of contaminants and technical noise. For heart failure diagnosis biomarkers discovery, 203 miRNAs were screened and the inventors detected 137 miRNAs expressed across all the plasma samples. Of which, 75 miRNAs were identified to be significantly altered between heart failure (HFREF and/or HFPEF) and controls. A list of 52 miRNAs was able to distinguish HFREF from controls and 68 were found to be significantly differentially expressed between HFPEF and controls. Accordingly, the present inventors found a group of miRNAs that were able to distinguish HFREF from HFPEF. The present inventors have also found a group of miRNAs that are dysregulated in heart failure compared to controls.

[0075] Thus, in one aspect, there is provided a method of determining whether a subject suffers from heart failure or is at risk of developing heart failure. In some examples, the method comprises the steps of a) measuring the level of at least one miRNA from a list of miRNAs "increased" (above control) or at least one from a list of miRNAs "reduced" (below control) as listed in Table 25, or Table 20, or Table 21, or Table 22, in a sample obtained from the subject. In some examples, the method further comprises b) determining whether the level of miRNA is different as compared to a control, wherein altered levels of the miRNA indicates that the subject has heart failure or is at a risk of developing heart failure.

[0076] **Table 20. miRNAs for heart failure detection**

Increased (n=33)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-let-7d-3p	8.9E-23	4.1E-09	3.8E-05	1.32	0.78
hsa-miR-197-3p	8.9E-23	2.7E-08	7.9E-05	1.27	0.77
hsa-miR-24-3p	2.8E-22	5.5E-10	6.7E-05	1.30	0.76
hsa-miR-221-3p	5.4E-19	4.9E-09	6.2E-05	1.35	0.73
hsa-miR-503	1.1E-17	1.2E-07	9.7E-04	1.69	0.73
hsa-miR-130b-3p	1.2E-14	3.9E-07	1.3E-03	1.27	0.72
hsa-miR-23b-3p	1.1E-13	2.6E-06	8.9E-04	1.31	0.71
hsa-miR-21-3p	2.4E-14	8.0E-06	>0.01	1.25	0.70
hsa-miR-223-5p	4.4E-13	1.6E-06	9.2E-04	1.23	0.70
hsa-miR-34b-3p	9.5E-14	4.6E-04	>0.01	1.84	0.69
hsa-miR-148a-3p	2.0E-12	2.3E-06	1.8E-03	1.28	0.68
hsa-miR-23a-5p	6.2E-11	4.3E-04	>0.01	1.25	0.67
hsa-miR-335-5p	1.3E-10	2.5E-06	2.3E-04	1.33	0.67
hsa-miR-124-5p	3.8E-09	9.8E-04	>0.01	1.54	0.66
hsa-miR-382-5p	6.0E-10	1.7E-05	7.6E-03	1.56	0.66
hsa-miR-134	6.4E-10	2.9E-05	6.7E-03	1.57	0.66
hsa-let-7e-3p	7.6E-07	1.1E-03	>0.01	1.33	0.65
hsa-miR-598	4.9E-08	4.8E-05	>0.01	1.20	0.65
hsa-miR-627	2.8E-08	5.5E-04	>0.01	1.31	0.65
hsa-miR-199a-3p	1.3E-05	4.1E-03	>0.01	1.27	0.64
hsa-miR-27b-3p	1.6E-06	8.7E-04	3.8E-04	1.20	0.64
hsa-miR-146b-5p	6.3E-07	8.7E-04	3.4E-04	1.25	0.64
hsa-miR-146a-5p	3.1E-06	4.3E-03	9.7E-04	1.25	0.64
hsa-miR-331-5p	2.7E-07	2.7E-03	>0.01	1.13	0.64
hsa-miR-654-3p	7.4E-08	2.0E-03	>0.01	1.44	0.63
hsa-miR-375	1.1E-05	7.9E-03	>0.01	1.43	0.63
hsa-miR-132-3p	9.8E-07	7.4E-04	>0.01	1.12	0.63
hsa-miR-27a-3p	2.0E-05	2.4E-03	4.9E-03	1.16	0.63
hsa-miR-128	5.9E-06	8.6E-04	>0.01	1.11	0.63

hsa-miR-299-3p	2.9E-06	3.3E-03	>0.01	1.43	0.62
hsa-miR-424-5p	4.0E-07	1.3E-03	>0.01	1.25	0.62
hsa-miR-154-5p	5.9E-06	1.0E-03	>0.01	1.41	0.62
hsa-miR-377-3p	1.3E-05	3.9E-03	>0.01	1.37	0.60
Reduced n=(34)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, BNP**	**Fold change**	**AUC**
hsa-miR-454-3p	3.3E-43	3.0E-14	5.6E-06	0.47	0.85
hsa-miR-30c-5p	8.9E-23	1.9E-10	3.2E-04	0.65	0.75
hsa-miR-17-5p	2.4E-19	1.4E-06	3.4E-04	0.73	0.74
hsa-miR-196b-5p	2.2E-15	7.8E-06	2.0E-04	0.79	0.73
hsa-miR-500a-5p	5.4E-19	1.1E-07	3.8E-04	0.68	0.73
hsa-miR-106a-5p	1.1E-16	1.3E-06	4.7E-05	0.76	0.72
hsa-miR-20a-5p	2.6E-17	1.4E-06	7.9E-05	0.74	0.72
hsa-miR-451a	5.4E-19	9.8E-08	7.9E-05	0.54	0.72
hsa-miR-29b-3p	1.5E-16	4.6E-08	6.7E-05	0.76	0.71
hsa-miR-374b-5p	2.4E-16	1.1E-07	1.8E-03	0.69	0.71
hsa-miR-20b-5p	1.5E-16	2.3E-06	8.1E-05	0.60	0.71
hsa-miR-501-5p	2.2E-14	3.3E-06	1.2E-04	0.71	0.70
hsa-miR-18b-5p	4.4E-13	3.9E-05	4.7E-05	0.78	0.69
hsa-miR-23c	3.1E-12	1.2E-06	>0.01	0.68	0.69
hsa-miR-551b-3p	3.0E-12	3.2E-05	>0.01	0.65	0.69
hsa-miR-26a-5p	4.7E-13	3.9E-05	>0.01	0.74	0.69
hsa-miR-183-5p	1.8E-12	2.8E-05	3.8E-04	0.59	0.68
hsa-miR-16-5p	4.2E-12	1.9E-05	8.4E-04	0.71	0.68
hsa-miR-532-5p	1.2E-11	8.0E-06	4.9E-04	0.77	0.67
hsa-miR-363-3p	3.9E-11	1.7E-04	2.7E-03	0.70	0.67
hsa-miR-374c-5p	4.5E-10	3.7E-04	>0.01	0.71	0.67
hsa-let-7b-5p	3.5E-11	3.8E-04	>0.01	0.80	0.66
hsa-miR-15a-5p	3.8E-09	9.8E-04	4.7E-03	0.82	0.66
hsa-miR-144-3p	3.9E-11	9.4E-05	3.8E-04	0.63	0.66
hsa-miR-93-5p	1.3E-09	3.8E-04	1.3E-03	0.82	0.66
hsa-miR-181b-5p	3.1E-09	1.2E-07	>0.01	0.80	0.66
hsa-miR-19b-3p	2.3E-09	3.4E-05	8.3E-05	0.80	0.65
hsa-miR-4732-3p	2.4E-08	4.7E-04	3.5E-03	0.70	0.64
hsa-miR-484	5.9E-07	9.9E-03	>0.01	0.89	0.64
hsa-miR-25-3p	3.3E-07	4.4E-03	8.8E-03	0.79	0.63
hsa-miR-192-5p	8.9E-06	9.9E-04	>0.01	0.76	0.63
hsa-miR-205-5p	3.2E-05	2.0E-03	>0.01	0.75	0.62
hsa-miR-19a-3p	2.2E-06	1.1E-03	6.9E-04	0.84	0.61
hsa-miR-32-5p	7.5E-06	8.3E-03	>0.01	0.88	0.61

[0077] **Table 21. miRNAs identified for HFREF detection**

Increased (n=21)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-let-7d-3p	5.0E-15	1.2E-06	>0.01	1.26	0.75
hsa-miR-24-3p	5.9E-15	5.1E-07	>0.01	1.27	0.74
hsa-miR-503	5.0E-15	1.9E-06	>0.01	1.74	0.74
hsa-miR-197-3p	6.6E-14	6.7E-05	>0.01	1.22	0.73
hsa-miR-130b-3p	1.6E-10	1.1E-05	>0.01	1.23	0.71
hsa-miR-221-3p	4.4E-12	1.2E-06	>0.01	1.31	0.71
hsa-miR-34b-3p	2.4E-12	7.2E-04	>0.01	1.90	0.70
hsa-miR-21-3p	1.1E-09	3.6E-04	>0.01	1.22	0.69
hsa-miR-132-3p	7.4E-09	6.8E-04	>0.01	1.16	0.68
hsa-miR-331-5p	2.7E-09	2.2E-03	>0.01	1.18	0.68
hsa-miR-124-5p	3.3E-09	5.6E-04	>0.01	1.62	0.67
hsa-miR-148a-3p	1.2E-08	2.6E-04	>0.01	1.26	0.66
hsa-miR-23b-3p	3.2E-07	2.0E-04	>0.01	1.22	0.66
hsa-miR-375	1.0E-06	3.8E-03	>0.01	1.55	0.65
hsa-miR-134	2.4E-06	4.2E-04	>0.01	1.48	0.64
hsa-miR-627	1.4E-05	2.2E-03	>0.01	1.28	0.64
hsa-miR-382-5p	6.2E-06	6.4E-04	>0.01	1.45	0.63
hsa-miR-598	6.1E-05	2.6E-04	>0.01	1.16	0.63
hsa-miR-23a-5p	1.4E-05	3.3E-03	>0.01	1.17	0.63
hsa-miR-223-5p	3.7E-04	9.3E-03	>0.01	1.12	0.62
hsa-miR-335-5p	1.6E-04	2.6E-04	>0.01	1.19	0.61
Reduced (n=23)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-miR-454-3p	2.9E-30	2.0E-11	>0.01	0.48	0.83
hsa-miR-30c-5p	3.7E-19	1.1E-08	>0.01	0.64	0.76
hsa-miR-374b-5p	1.1E-15	5.7E-07	>0.01	0.66	0.73
hsa-miR-23c	1.3E-13	2.7E-07	>0.01	0.63	0.72
hsa-miR-551b-3p	6.9E-11	1.5E-04	>0.01	0.63	0.70
hsa-miR-17-5p	1.5E-11	2.6E-04	>0.01	0.80	0.70
hsa-miR-26a-5p	6.9E-11	6.7E-05	>0.01	0.73	0.70
hsa-miR-181b-5p	1.9E-11	1.4E-06	>0.01	0.73	0.70
hsa-miR-500a-5p	2.1E-10	6.7E-05	>0.01	0.73	0.69
hsa-miR-196b-5p	1.0E-07	1.7E-03	>0.01	0.84	0.68
hsa-miR-374c-5p	1.4E-08	6.3E-04	>0.01	0.70	0.68
hsa-miR-451a	2.1E-10	1.1E-04	>0.01	0.62	0.68
hsa-miR-29b-3p	2.8E-09	8.8E-05	>0.01	0.80	0.67
hsa-miR-20a-5p	1.7E-08	7.8E-04	>0.01	0.81	0.67
hsa-miR-106a-5p	3.0E-07	4.7E-03	>0.01	0.85	0.65
hsa-miR-181a-2-3p	1.1E-04	5.9E-03	>0.01	0.84	0.65

hsa-miR-501-5p	4.3E-06	4.3E-03	>0.01	0.80	0.64
hsa-miR-20b-5p	2.0E-06	4.0E-03	>0.01	0.75	0.63
hsa-miR-183-5p	5.8E-06	1.4E-03	>0.01	0.69	0.63
hsa-miR-16-5p	1.6E-05	3.8E-03	>0.01	0.79	0.63
hsa-miR-125a-5p	7.9E-05	6.4E-04	>0.01	0.81	0.62
hsa-miR-205-5p	3.3E-04	5.9E-03	>0.01	0.76	0.61
hsa-miR-532-5p	2.3E-04	9.3E-03	>0.01	0.85	0.61

[0078] **Table 22. miRNAs identified for HFPEF detection**

Increased (n=30)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-let-7d-3p	4.40E-22	5.90E-07	4.10E-05	1.39	0.81
hsa-miR-197-3p	4.10E-24	2.10E-07	6.40E-05	1.34	0.81
hsa-miR-223-5p	6.80E-21	2.10E-07	7.80E-05	1.37	0.79
hsa-miR-24-3p	5.80E-19	2.10E-07	4.10E-05	1.33	0.78
hsa-miR-221-3p	5.90E-17	5.90E-07	4.10E-05	1.4	0.76
hsa-miR-23b-3p	1.60E-16	1.40E-05	2.60E-04	1.41	0.76
hsa-miR-130b-3p	2.20E-14	9.00E-05	9.10E-04	1.32	0.74
hsa-miR-335-5p	2.40E-14	5.30E-06	4.10E-05	1.5	0.73
hsa-miR-21-3p	8.50E-13	8.20E-05	3.60E-03	1.29	0.72
hsa-miR-23a-5p	1.90E-12	1.40E-03	3.60E-03	1.34	0.72
hsa-miR-503	1.40E-11	3.80E-05	5.80E-04	1.64	0.72
hsa-miR-148a-3p	4.40E-11	1.10E-04	2.20E-03	1.3	0.7
hsa-miR-146a-5p	3.10E-08	2.50E-04	1.50E-04	1.37	0.69
hsa-miR-199a-3p	2.70E-07	8.40E-04	2.20E-03	1.38	0.69
hsa-let-7e-3p	4.00E-08	1.30E-03	8.50E-03	1.43	0.68
hsa-miR-134	3.90E-09	2.90E-04	1.60E-03	1.67	0.68
hsa-miR-382-5p	1.00E-09	1.00E-04	1.10E-03	1.69	0.68
hsa-miR-128	2.60E-07	2.90E-04	2.50E-03	1.15	0.67
hsa-miR-146b-5p	9.90E-08	1.60E-04	6.40E-05	1.33	0.67
hsa-miR-27a-3p	1.60E-06	3.40E-04	5.80E-04	1.21	0.67
hsa-miR-27b-3p	5.10E-07	6.70E-04	1.50E-04	1.26	0.67
hsa-miR-598	3.10E-08	1.20E-03	>0.01	1.25	0.67
hsa-miR-101-3p	2.70E-08	5.10E-04	6.70E-03	0.74	0.67
hsa-miR-551b-3p	3.30E-08	8.90E-03	>0.01	0.67	0.67
hsa-miR-627	4.20E-07	8.70E-03	>0.01	1.36	0.66
hsa-miR-185-5p	1.70E-09	1.80E-03	4.60E-03	0.8	0.66
hsa-miR-299-3p	2.30E-07	2.30E-03	>0.01	1.59	0.65
hsa-miR-425-3p	1.30E-04	9.30E-04	1.60E-03	1.15	0.65
hsa-miR-154-5p	1.10E-06	9.10E-04	3.60E-03	1.55	0.64
hsa-miR-377-3p	2.40E-06	8.30E-03	>0.01	1.52	0.64
Reduced (n=33)					

Name	p-value, t-test	p-value, Logistic regression	p-value, ln_BNP	Fold change	AUC
hsa-miR-454-3p	8.9E-36	5.9E-07	4.1E-05	0.45	0.87
hsa-miR-106a-5p	3.4E-22	5.9E-07	4.1E-05	0.68	0.80
hsa-miR-17-5p	4.0E-21	2.5E-05	2.9E-04	0.67	0.80
hsa-miR-20b-5p	4.1E-24	5.0E-07	4.1E-05	0.47	0.79
hsa-miR-20a-5p	7.0E-21	2.1E-06	6.4E-05	0.66	0.79
hsa-miR-196b-5p	5.6E-18	2.8E-05	1.8E-04	0.75	0.78
hsa-miR-451a	3.3E-21	5.9E-07	7.0E-05	0.46	0.78
hsa-miR-18b-5p	3.0E-17	8.2E-05	9.2E-05	0.69	0.77
hsa-miR-500a-5p	2.1E-19	7.0E-06	1.6E-04	0.62	0.77
hsa-miR-29b-3p	1.0E-18	1.3E-06	6.2E-05	0.72	0.76
hsa-miR-501-5p	5.2E-19	2.4E-06	4.5E-05	0.62	0.76
hsa-miR-532-5p	1.3E-16	1.2E-06	7.0E-05	0.69	0.75
hsa-let-7b-5p	1.1E-16	3.8E-05	9.0E-04	0.71	0.74
hsa-miR-30c-5p	1.3E-15	1.0E-04	2.1E-03	0.67	0.74
hsa-miR-183-5p	2.9E-15	3.8E-05	3.8E-04	0.50	0.74
hsa-miR-144-3p	1.9E-16	3.6E-06	1.5E-04	0.51	0.73
hsa-miR-93-5p	4.6E-15	2.5E-05	5.0E-04	0.74	0.73
hsa-miR-16-5p	6.5E-15	4.1E-06	2.6E-04	0.62	0.73
hsa-miR-363-3p	1.5E-13	4.5E-05	1.3E-03	0.62	0.73
hsa-miR-25-3p	3.3E-12	8.2E-05	2.5E-03	0.68	0.71
hsa-miR-4732-3p	1.1E-13	1.1E-05	9.1E-04	0.57	0.71
hsa-miR-192-5p	2.2E-09	4.1E-04	>0.01	0.65	0.70
hsa-miR-19b-3p	1.5E-11	1.6E-05	1.0E-04	0.74	0.70
hsa-miR-15a-5p	3.3E-08	3.0E-03	3.6E-03	0.80	0.69
hsa-miR-486-5p	2.2E-10	3.4E-04	>0.01	0.64	0.69
hsa-miR-374b-5p	3.4E-10	5.1E-03	>0.01	0.72	0.68
hsa-miR-484	4.0E-08	9.9E-03	>0.01	0.86	0.67
hsa-miR-194-5p	1.0E-06	4.6E-03	>0.01	0.71	0.67
hsa-miR-101-3p	2.7E-08	5.1E-04	6.7E-03	0.74	0.67
hsa-miR-551b-3p	3.3E-08	8.9E-03	>0.01	0.67	0.67
hsa-miR-185-5p	1.7E-09	1.8E-03	4.6E-03	0.80	0.66
hsa-miR-19a-3p	3.3E-08	1.9E-04	9.1E-04	0.79	0.66
hsa-miR-550a-5p	3.5E-05	3.0E-03	5.7E-03	0.81	0.62

[0079] **Table 25. Specific novel microRNAs for Heart failure detection**

Increased (n=6), AUC> 0.7					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-let-7d-3p	8.90E-23	4.10E-09	3.80E-05	1.32	0.78
hsa-miR-197-3p	8.90E-23	2.70E-08	7.90E-05	1.27	0.77

hsa-miR-221-3p	5.40E-19	4.90E-09	6.20E-05	1.35	0.73
hsa-miR-503	1.10E-17	1.20E-07	9.70E-04	1.69	0.73
hsa-miR-130b-3p	1.20E-14	3.90E-07	1.30E-03	1.27	0.72
hsa-miR-23b-3p	1.10E-13	2.60E-06	8.90E-04	1.31	0.71

Reduced n=(6), AUC >0.7					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-miR-30c-5p	8.90E-23	1.90E-10	3.20E-04	0.65	0.75
hsa-miR-17-5p	2.40E-19	1.40E-06	3.40E-04	0.73	0.74
hsa-miR-196b-5p	2.20E-15	7.80E-06	2.00E-04	0.79	0.73
hsa-miR-106a-5p	1.10E-16	1.30E-06	4.70E-05	0.76	0.72
hsa-miR-20a-5p	2.60E-17	1.40E-06	7.90E-05	0.74	0.72
hsa-miR-451a	5.40E-19	9.80E-08	7.90E-05	0.54	0.72

[0080] As used herein throughout the disclosure, the term "miRNA" refers to microRNA, small non-coding RNA molecules, and are found in plants, animals and some viruses. miRNA are known to have functions in RNA silencing and post-transcriptional regulation of gene expression. These highly conserved RNAs regulate the expression of genes by binding to the 3'-untranslated regions (3'-UTR) of specific mRNAs. For example, each miRNA is thought to regulate multiple genes, and since hundreds of miRNA genes are predicted to be present in higher eukaryotes. miRNA may be at least 10 nucleotides and of not more than 35 nucleotides covalently linked together. In some examples, the miRNA may be molecules of 10 to 33 nucleotides, or of 15 to 30 nucleotides in length, or 17 to 27 nucleotides, or 18 to 26 nucleotides in length. In some examples, the miRNA may be molecules of 10, or 11, or 12, or 13, or 14, or 15, or 16, or 17, or 18, or 19, or 20, or 21, or 22, or 23, or 24, or 25, or 26, or 27, or 28, or 29, or 30, or 31, or 32, or 33, or 34, or 35 nucleotides in length, not including optionally labels and/or elongated sequences (e.g. biotin stretches). The miRNAs regulate gene expression and are encoded by genes from whose DNA they are transcribed but miRNAs are not translated into protein (i.e. miRNAs are non- coding RNAs). As used herein throughout the disclosure, the miRNA measured may be at least 90%, 95%, 97.5%, 98%, or 99% sequence identity to the miRNAs as listed in any one of the tables provided in the present disclosure. Thus, in some examples, the measure miRNA has at least 90%, 95%, 97.5%, 98%, or 99% sequence identity to the miRNAs as listed in any one of, Table 9, Table 14, Table 15, Table 16, Table 17, Table 18, Table 19,

Table 20, Table 21, Table 22, Table 23, Table 24 or Table 25. As used herein, the term “sequence identity” "sequence identity" refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences are "identical" at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by methods known to the person skilled in the art.

[0081] As used herein throughout the disclosure, the term “heart failure”, or “HF”, refers to a complex clinical syndrome in which the pumping function of the heart becomes insufficient (ventricular dysfunction) to meet the needs of the vital system and tissues of the body. The severity of heart failure may range from non-severe (mild), which manifest in the subject having no limitation of physical activity, to increasing severity, which manifest in the subject unable to carry on any physical activity without discomfort. Heart failure is a progressive and chronic disease, worsening over time. In extreme cases, heart failure may lead to the need for a heart transplant. In some examples, the subject may be determined to be at risk of developing heart failure if the subject may have further heart failure, such as deterioration into recurrent acute decompensated heart failure or death among those with known chronic heart failure.

[0082] As used herein throughout the disclosure, the terms “subject” and “patient” are to be used interchangeably to refer to individual or mammal suspected to be affected by heart failure. The patient may be predicted (or determined, or diagnosed) to be affected by heart failure, i.e. diseased, or may be predicted to be not affected by heart failure, i.e. healthy. The subject may also be determined to be affected by a specific form of heart failure. In some examples, the heart failure patient may be a subject who has had primary diagnosis of heart failure and/or being treated 3-5 days when symptomatically improved, with resolution of bedside physical signs of heart failure and considered fit to discharge. Thus, the subject may further be determined to develop heart failure or a specific form of heart failure. It should be noted that a subject that is determined as being healthy, i.e. not suffering from heart failure or from a specific form of heart failure, may possibly suffer from another disease not tested/known. As used herein, the subject of the present disclosure may be any mammal, including both a human and another mammal, e.g. an animal such as a dog, cat, rabbit, mouse, rat, or monkey. In some examples, the subject may be human. Therefore, the miRNA from a subject may be a human miRNA or a miRNA from another mammal, e.g. an animal miRNA such as a mouse,

monkey or rat miRNA, or the miRNAs comprised in a set may be human miRNAs or miRNAs from another mammal, e.g. animal miRNAs such as mouse, monkey or rat miRNAs. As illustrated by Table 2 in the Experimental Section, the subject of the present disclosure may be of Asian descent or ethnicity. In some examples, the subject may include, but is not limited to any Asian ethnicity, including, Chinese, Indian, Malay, and the like.

[0083] On the other hand, the term "control" or "control subject", as used in the context of the present invention, may refer to (a sample obtained from) subject known to be affected with heart failure (positive control, e.g .good prognosis, poor prognosis), i.e. diseased, and/or a subject with heart failure subtype HFPEF, and/or heart failure subtype HFREF, and/or a subject known to be not affected with heart failure (negative control), i.e. healthy. It may also refer to (a sample obtained from) a subject known to be effected by another disease/condition. It should be noted that a control subject that is known to be healthy, i.e. not suffering from heart failure, may possibly suffer from another disease not tested/known. Thus, in some examples, the control may be a non-heart failure subject (or sometimes referred to as a normal subject). The control subject may be any mammal, including both a human and another mammal, e.g. an animal such as a rabbit, mouse, rat, or monkey. In some examples, the control is human. In some examples, the control may be (samples obtained from) an individual subject or a cohort of subjects.

[0084] It would be appreciated by the person skilled in the art that the methods as described herein are not to be used to replace the physician's role in diagnosing the condition in a subject. As would be appreciated, clinical diagnosis of heart failure in a subject would require the physician's analysis of other symptoms and/or other information that may be available to the physicians. The methods as described herein are meant to provide support or additional information for the physicians to make the final diagnosis of the patient/subject.

[0085] As used herein throughout the disclosure, the term "sample" refers to a bodily fluid or extracellular fluid. In some examples, the bodily fluid may include, but is not limited to, cellular and non-cellular components of amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid, colostrum, interstitial fluid, peritoneal fluids, pleural fluid, saliva, seminal fluid, urine, tears, whole blood, including plasma, red blood cells, white blood cells, serum, and the like. In some examples, the bodily fluid may be blood, serum plasma, and/or plasma.

[0086] In some examples, an increase in the level of miRNAs as listed as "increased" in Table 20 or Table 25, as compared to the control, indicates the subject to have heart failure or is at a risk of developing heart failure.

[0087] In some examples, a reduction in the level of miRNAs as listed as "reduced" in Table 20 or Table 25 as compared to the control, indicates the subject to have heart failure or is at a risk of developing heart failure.

[0088] As used herein the term "miRNA level" or “level of miRNA” as used in the context of the present disclosure, represents the determination of the miRNA expression level (or miRNA expression profile) or a measure that correlates with the miRNA expression level in a sample. The miRNA expression level may be generated by any convenient means known in the art, such as, but are not limited to, nucleic acid hybridization (e.g. to a microarray), nucleic acid amplification (PCR, RT-PCR, qRT-PCR, high-throughput RT- PCR), ELISA for quantitation, next generation sequencing (e.g. ABI SOLID, Illumina Genome Analyzer, Roche/454 GS FLX), flow cytometry (e.g. LUMINEX) and the like, that allow the analysis of miRNA expression levels and comparison between samples of a subject (e.g. potentially diseased) and a control subject (e.g. reference sample(s)). The sample material measured by the aforementioned means may be a raw or treated sample or total RNA, labeled total RNA, amplified total RNA, cDNA, labeled cDNA, amplified cDNA, miRNA, labeled miRNA, amplified miRNA or any derivatives that may be generated from the aforementioned RNA/DNA species. By determining the miRNA expression level, each miRNA is represented by a numerical value. The higher the value of an individual miRNA, the higher is the (expression) level of said miRNA, or the lower the value of an individual miRNA, the lower is the (expression) level of said miRNA. When a higher value of an individual miRNA is detected over and beyond the control, the miRNA expression is referred to as “increased” or “upregulated”. On the other hand, when a lower value of an individual miRNA is detected that is below the control, the miRNA expression is then referred to as “decreased” or “downregulated”.

[0089] The "miRNA (expression) level", as used herein, represents the expression level/expression profile/expression data of a single miRNA or a collection of expression levels of at least two miRNAs, or least 3, or least 4, or least 5, or least 6, or least 7, or least 8, or least 9, or least 10, or least 11, or least 12, or least 13, or least 14, or least 15, or least 16, or least 17, or least 18, or least 19, or least 20, or least 21, or least 22, or least 23, or least 24, or least 25, or least 26, or least 27, or least 28, or least 29, or least 30, or least 31, or least 32, or least 33, or least 34, or least 35, or more, or up to all known miRNAs.

[0090] In some examples, the method of determining whether a subject suffers or is at risk of suffering heart failure may include measuring the change in levels of at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 11, at least two to at least 20, at least 10 to at least 50, at least 40 to at least 66, or all miRNA as listed in Table 20. In some examples, the method of determining whether a subject suffers or is at risk of suffering heart failure may include measuring the change in levels of at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 11, or all miRNA as listed in Table 25.

[0091] In some examples, in the method as described herein, an increase in the level of miRNAs as listed as "increased" in Table 21, as compared to the control, may indicate the subject to have heart failure with reduced left ventricular ejection fraction (HFREF) or may be at a risk of developing heart failure with reduced left ventricular ejection fraction (HFREF). In some examples, in the method as described herein, a reduction in the level of miRNAs as listed as "reduced" in Table 21 as compared to the control, may indicate the subject to have heart failure with reduced left ventricular ejection fraction (HFREF) or may be at a risk of developing heart failure with reduced left ventricular ejection fraction (HFREF). In some examples, the method of determining whether a subject suffers or is at risk of suffering HFREF may include measuring the change in levels of at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least 10, or at least 11, or at least two to at least 20, or at least 10 to at least 43, or at least 15 to at least 43, or at least 30 to at least 43, or at least 40, or all miRNA as listed in Table 21.

[0092] As used herein, the term "heart failure with reduced left ventricular ejection fraction (HFREF)" or "heart failure with preserved left ventricular ejection fraction (HFPEF)" refers to the same term as commonly used in the art. For example, the term HFREF may also be referred to as systolic heart failure. In HFREF, the heart muscle does not contract effectively and less oxygen-rich blood is pumped out to the body. In contrast, the term "heart failure with preserved left ventricular ejection fraction (HFPEF)" refers to a diastolic heart failure. In HFPEF, the heart muscle contracts normally but the ventricles do not relax as they should during ventricular filling or when the ventricles relax).

[0093] In some examples, in the method as described herein, an increase in the level of miRNAs as listed as "increased" in Table 22, as compared to the control, may indicate the subject to have heart failure with preserved left ventricular ejection fraction (HFPEF) or may be at a risk of developing heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, in the method as described herein, a reduction in the level of miRNAs as listed as "reduced" in Table 22 as compared to the control, may indicate the subject to have heart failure with preserved left ventricular ejection fraction (HFPEF) or may be at a risk of developing heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, the method of determining whether a subject may suffer or may be at risk of suffering HFPEF may include measuring the change in levels of at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least 10, or at least 11, or at least two to at least 20, or at least 10 to at least 50, or at least 20 to at least 55, or at least 30 to at least 60, or at least 35 to at least 60, or at least 40 to at least 60, or at least 40 to at least 62, or all miRNA as listed in Table 22.

[0094] In another aspect, there is provided a method of determining whether a subject suffers from a heart failure selected from the group consisting of a heart failure with reduced left ventricular

ejection fraction (HFREF) and a heart failure with preserved left ventricular ejection fraction (HFPEF), the method comprising the steps of a) detecting (or measuring) the levels of at least one miRNA as listed in Table 9 in a sample obtained from the subject and b) determining whether it is different as compared to a control, wherein altered levels of the miRNA may indicate that the subject has, or may be at a risk of, developing heart failure with reduced left ventricular ejection fraction (HFREF) or heart failure with preserved left ventricular ejection fraction (HFPEF).

[0095] **Table 9. miRNAs differentially expressed between HFREF and HFPEF subjects**

Up-regulated (n=10)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-miR-223-5p	3.5E-07	2.4E-04	7.3E-05	1.22	0.68
hsa-miR-335-5p	3.4E-04	2.9E-03	1.9E-03	1.26	0.63
hsa-miR-452-5p	2.4E-03	>0.17	>0.04	1.26	0.62
hsa-miR-23b-3p	7.1E-03	>0.12	5.3E-03	1.16	0.62
hsa-miR-181b-5p	1.2E-03	>0.01	>0.01	1.20	0.62
hsa-miR-146a-5p	8.5E-03	>0.16	>0.03	1.20	0.62
hsa-miR-181a-2-3p	3.8E-04	2.9E-03	9.2E-03	1.20	0.61
hsa-miR-199b-5p	1.3E-03	3.3E-03	1.9E-03	1.24	0.61
hsa-miR-126-5p	5.7E-03	>0.02	>0.01	1.14	0.61
hsa-miR-23a-5p	6.4E-03	>0.15	>0.02	1.14	0.60
Down-regulated (n=30)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-miR-185-5p	1.9E-08	5.2E-05	4.2E-05	0.79	0.69
hsa-miR-20b-5p	3.2E-07	1.0E-03	1.5E-04	0.63	0.68
hsa-miR-550a-5p	3.5E-07	2.7E-04	1.1E-03	0.73	0.68
hsa-miR-106a-5p	9.6E-07	2.9E-03	4.5E-04	0.80	0.67
hsa-miR-486-5p	2.7E-06	9.5E-04	3.3E-04	0.67	0.66
hsa-let-7b-5p	6.3E-06	2.9E-03	3.3E-04	0.81	0.66
hsa-miR-93-5p	2.0E-06	1.0E-03	3.3E-04	0.81	0.65
hsa-miR-20a-5p	2.3E-05	9.3E-03	7.8E-04	0.81	0.65
hsa-miR-25-3p	2.4E-05	2.9E-03	8.7E-04	0.76	0.65
hsa-miR-18b-5p	9.9E-06	9.4E-03	1.9E-03	0.80	0.65
hsa-miR-532-5p	3.7E-05	4.0E-03	1.5E-03	0.80	0.65
hsa-miR-501-5p	4.7E-05	2.1E-03	7.5E-04	0.77	0.64
hsa-miR-4732-3p	8.4E-05	2.9E-03	1.5E-03	0.69	0.64
hsa-miR-144-3p	1.1E-04	2.9E-03	7.5E-04	0.68	0.63
hsa-miR-192-5p	2.3E-03	>0.08	>0.04	0.75	0.63
hsa-miR-17-5p	2.7E-04	>0.21	5.7E-03	0.83	0.62
hsa-miR-363-3p	1.2E-03	>0.06	>0.02	0.79	0.62
hsa-miR-103a-3p	1.2E-03	>0.07	>0.03	0.87	0.62
hsa-miR-16-5p	9.8E-04	>0.22	3.2E-03	0.79	0.62

hsa-miR-194-5p	5.3E-03	>0.18	>0.05	0.78	0.62
hsa-miR-183-5p	1.4E-03	>0.14	>0.01	0.71	0.62
hsa-miR-451a	1.7E-03	>0.13	3.2E-03	0.73	0.61
hsa-miR-19b-3p	1.7E-03	>0.04	7.0E-03	0.85	0.61
hsa-miR-30a-5p	1.8E-03	>0.20	>0.07	0.81	0.60
hsa-miR-106b-3p	6.4E-03	>0.03	>0.01	0.90	0.60
hsa-miR-19a-3p	7.3E-03	>0.09	>0.05	0.87	0.60
hsa-let-7i-5p	1.2E-03	>0.11	>0.07	0.90	0.59
hsa-miR-196b-5p	7.7E-03	>0.19	>0.06	0.90	0.59
hsa-miR-500a-5p	8.5E-03	>0.10	>0.06	0.86	0.58
hsa-miR-122-5p	8.7E-03	>0.05	>0.01	0.68	0.58

[0096] In some examples, in the method as described herein, an increase in the level of miRNAs as listed as "increased" in Table 9, as compared to the control, may indicate the subject has heart failure with reduced left ventricular ejection fraction (HFREF) or heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, in the method as described herein, a reduction in the level of miRNAs as listed as "reduced" in Table 9 as compared to the control, may indicate the subject has developing heart failure with reduced left ventricular ejection fraction (HFREF) or heart failure with preserved left ventricular ejection fraction (HFPEF).

[0097] In some examples of the method for determining whether a subject suffers from a heart failure selected from the group consisting of a heart failure with reduced left ventricular ejection fraction (HFREF) and a heart failure with preserved left ventricular ejection fraction (HFPEF), the method may comprise measuring the change in levels of at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least 10, or at least 11, or at least two to at least 20, or at least 10 to at least 39, or all miRNA as listed in Table 9.

[0098] In the examples of the method for determining whether a subject suffers from a heart failure selected from the group consisting of a heart failure with reduced left ventricular ejection fraction (HFREF) and a heart failure with preserved left ventricular ejection fraction (HFPEF), the control may be a subject that has either a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, the control may be a patient with a heart failure with reduced left ventricular ejection fraction (HFREF), differential expression of miRNAs as listed in Table 9 indicates the subject to have a heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, when the control is a patient with a heart failure with preserved left ventricular ejection fraction (HFPEF), differential expression of miRNAs as listed in Table 9 indicates the subject to have a heart failure with reduced left ventricular ejection fraction (HFREF).

[0099] In addition, the inventors of the present disclosure also examined the use of these miRNAs as prognostic markers. That is, the methods of the present disclosure may be used to predict possible

risk of events of death or hospitalization in the future or prospects of progress determined by diagnosing a disease. Prognosis in patients with heart failure means predicting the possibility of observed survival (survival free of death) or event free survival (survival free of hospitalization or death). As used herein, the term "observed survival", or "all-cause survival", or "all-cause mortalilty", or "all-cause of death", refers to observed survival rate of subjects in view of any causes of death. This term is in contrast to the term "event free survival (EFS)", which refers to the absence of the recurrent hospital admission for heart failure (i.e. the length of time after heart failure treatment during which recurrent admission for decompensated heart failure is avoided) and any causes of death.

[00100] The inventors of the present disclosure found that there were a number of miRNAs that were found to be good predictors for either the observed (all-cause) survival (OS) (i.e. observed survival rate due to all causes of death) or event free survival (EFS) combination of recurrent admission for heart failure (i.e. the length of time after heart failure treatment during which recurrent admission for decompensated heart failure is avoided) and all causes of death in chronic heart failure patients. Thus, the present disclosure may also be used in a method of predicting the prognosis of a subject. Thus, in another aspect of the present disclosure, there is provided a method for determining the risk of a heart failure patient having an altered risk of death (or decreased observed (all-cause) survival rate). In some examples, the method may comprise the steps of a) detecting the levels of at least one miRNA as listed in Table 14 in a sample obtained from the subject; and/or measuring the levels of at least one miRNAs listed in Table 14; and b) determining whether the levels of at least one miRNAs listed in Table 14 is different as compared to the levels of the miRNAs of a control population, wherein altered levels of the miRNA indicates that the subject is likely to have an altered risk of death (altered observed (all-cause) survival rate) compared to the control population.

[00101] **Table 14. miRNAs that may be used in predicting observed survival**

HR > 1 OR ln(HR) > 0 (n = 26)								
	Univariate analysis				**Multivariate analysis**			
	HR	ln(HR)	SE of ln(HR)	p-value	HR	ln(HR)	SE of ln(HR)	p-value
hsa-miR-503	1.90	0.64	0.17	1.40E-04	1.79	0.58	0.19	2.80E-03
hsa-miR-186-5p	1.72	0.54	0.17	1.70E-03	1.54	0.43	0.17	9.50E-03
hsa-miR-21-3p	1.65	0.5	0.15	1.00E-03	1.48	0.39	0.17	2.70E-02
hsa-miR-337-3p	1.60	0.47	0.15	1.80E-03	1.60	0.47	0.16	3.10E-03
hsa-miR-424-5p	1.57	0.45	0.14	1.50E-03	1.38	0.32	0.16	4.20E-02
hsa-miR-127-3p	1.57	0.45	0.16	5.10E-03	1.51	0.41	0.17	1.40E-02
hsa-miR-369-3p	1.57	0.45	0.16	6.50E-03	1.52	0.42	0.17	1.60E-02
hsa-miR-487b	1.52	0.42	0.15	5.80E-03	1.72	0.54	0.17	1.80E-03
hsa-miR-485-3p	1.52	0.42	0.16	6.90E-03	1.57	0.45	0.17	6.90E-03

hsa-miR-379-5p	1.52	0.42	0.16	9.00E-03	1.62	0.48	0.17	4.40E-03
hsa-miR-299-3p	1.51	0.41	0.15	6.90E-03	1.39	0.33	0.16	3.30E-02
hsa-miR-377-3p	1.49	0.4	0.15	7.80E-03	1.51	0.41	0.15	6.90E-03
hsa-miR-495	1.48	0.39	0.16	1.30E-02	1.62	0.48	0.17	5.10E-03
hsa-miR-654-3p	1.48	0.39	0.15	8.10E-03	1.46	0.38	0.15	1.00E-02
hsa-miR-493-5p	1.46	0.38	0.16	1.50E-02	1.46	0.38	0.16	1.70E-02
hsa-miR-382-5p	1.45	0.37	0.14	9.30E-03	1.35	0.3	0.14	3.60E-02
hsa-miR-23a-3p	1.45	0.37	0.17	3.40E-02	1.48	0.39	0.18	3.00E-02
hsa-miR-154-5p	1.43	0.36	0.14	1.10E-02	1.34	0.29	0.15	4.40E-02
hsa-miR-134	1.43	0.36	0.14	1.00E-02	1.30	0.26	0.14	6.70E-02
hsa-miR-136-5p	1.40	0.34	0.15	1.80E-02	1.48	0.39	0.16	1.40E-02
hsa-miR-128	1.39	0.33	0.15	2.70E-02	1.39	0.33	0.16	3.50E-02
hsa-miR-200c-3p	1.38	0.32	0.15	3.00E-02	1.38	0.32	0.16	4.40E-02
hsa-miR-1226-3p	1.38	0.32	0.16	5.50E-02	1.55	0.44	0.18	1.70E-02
hsa-miR-24-3p	1.35	0.3	0.15	4.50E-02	1.23	0.21	0.15	1.60E-01
hsa-miR-29c-5p	1.30	0.26	0.15	7.90E-02	1.39	0.33	0.17	4.80E-02
hsa-miR-374b-5p	1.19	0.17	0.15	2.60E-01	1.42	0.35	0.17	4.00E-02
HR < 1 OR ln(HR) < 0 (n = 14)								
	Univariate analysis				**Multivariate analysis**			
	HR	ln(HR)	SE of ln(HR)	p-value	HR	ln(HR)	SE of ln(HR)	p-value
hsa-miR-150-5p	0.52	-0.66	0.13	1.30E-07	0.59	-0.52	0.14	3.20E-04
hsa-miR-192-5p	0.64	-0.44	0.15	3.80E-03	0.76	-0.28	0.18	1.10E-01
hsa-miR-122-5p	0.68	-0.39	0.15	9.90E-03	0.76	-0.28	0.17	9.60E-02
hsa-miR-500a-5p	0.70	-0.36	0.15	1.70E-02	0.79	-0.23	0.16	1.50E-01
hsa-miR-181a-2-3p	0.70	-0.35	0.11	9.70E-04	0.67	-0.4	0.14	3.50E-03
hsa-miR-194-5p	0.70	-0.35	0.15	2.10E-02	0.79	-0.24	0.17	1.50E-01
hsa-miR-92a-3p	0.70	-0.35	0.16	2.60E-02	0.70	-0.36	0.16	2.20E-02
hsa-miR-660-5p	0.71	-0.34	0.15	2.50E-02	0.73	-0.32	0.16	3.80E-02
hsa-miR-486-5p	0.72	-0.33	0.14	2.20E-02	0.74	-0.3	0.15	4.30E-02
hsa-miR-375	0.72	-0.33	0.14	1.80E-02	0.77	-0.26	0.14	6.40E-02
hsa-miR-101-3p	0.73	-0.32	0.15	3.30E-02	0.81	-0.21	0.16	1.90E-01
hsa-miR-30c-5p	0.73	-0.31	0.15	3.50E-02	0.84	-0.18	0.16	2.40E-01
hsa-miR-20b-5p	0.74	-0.3	0.13	2.00E-02	0.84	-0.17	0.15	2.50E-01
hsa-miR-10b-5p	0.74	-0.3	0.14	3.30E-02	0.80	-0.22	0.15	1.30E-01

[00102] As used herein, the term "hazard ratio" refers to a term commonly known in the art to relate to a rate, or an estimate of the potential for "death" or "hospital admission" per unit time at a particular instant, given that the subject has "survived" until that instant (of "death" or "hospital admission". It is used to measure the magnitude of difference between two survival curves. Hazard

ratio (HR) > 1 indicates the higher risk of having short survival time and Hazard ratio (HR) < 1 indicates the higher risk of having longer survival time. As known in the art, the hazard ratio may be calculated by Cox proportional hazards (CoxPH) model.

[00103] In some examples, in the method as described herein, an increase in the level of miRNA as listed as "hazard ratio > 1" in Table 14, as compared to the control, may indicate the subject has an increased risk of death (decreased observed (all-cause) survival rate). In some examples, in the method as described herein, a reduction in the level of miRNA as listed as "hazard ratio > 1" in Table 14, as compared to the control, may indicate the subject has a decreased risk of death (increased observed (all-cause) survival rate).

[00104] In some examples, in the method as described herein, an increase in the level of miRNA as listed as "hazard ratio < 1" in Table 14, as compared to the control, may indicate the subject has a decreased risk of death (increased observed (all-cause) survival rate). In some examples, in the method as described herein, a reduction in the level of miRNA as listed as "hazard ratio < 1" in Table 14, as compared to the control, may indicate the subject has an increased risk of death (decreased observed (all-cause) survival rate).

[00105] In another aspect, there is provided a method for determining the risk of a heart failure patient having an altered risk of disease progression to hospitalization or death (decreased event free survival rate). In some examples, the method comprises the steps of a) detecting the levels of at least one miRNA as listed in Table 15 in a sample obtained from the subject; and/or measuring the levels of at least one miRNAs listed in Table 15; and b) determining whether the levels of at least one miRNAs listed in Table 15 is different as compared to the levels of the miRNAs of a control population, wherein altered levels of the miRNA indicates that the subject is likely to have an altered risk of disease progression to hospitalization or death (altered event free survival rate)compared to the control population.

[00106] **Table 15. miRNAs predictive of event free survival**

HR >1 OR ln(HR) > 0 (n = 4)								
	Univariate analysis				**Multivariate analysis**			
	HR	ln(HR)	SE of ln(HR)	p-value	HR	ln(HR)	SE of ln(HR)	p-value
hsa-miR-331-5p	1.27	0.24	0.08	0.0025	1.12	0.11	0.08	0.15
hsa-miR-21-3p	1.25	0.22	0.08	0.01	1.11	0.1	0.09	0.27
hsa-miR-497-5p	1.20	0.18	0.08	0.033	1.07	0.07	0.08	0.39
hsa-miR-22-3p	1.25	0.22	0.06	0.00048	1.06	0.06	0.07	0.34
HR< 1 OR ln(HR) < 0 (n = 9)								
	Univariate analysis				**Multivariate analysis**			
	HR	ln(HR)	SE of ln(HR)	p-value	HR	ln(HR)	SE of ln(HR)	p-value

hsa-miR-30e-3p	0.80	-0.22	0.08	0.007	0.88	-0.13	0.09	0.14
hsa-miR-191-5p	0.81	-0.21	0.08	0.013	0.90	-0.1	0.09	0.27
hsa-miR-30b-5p	0.82	-0.2	0.08	0.018	0.97	-0.03	0.09	0.7
hsa-miR-454-3p	0.83	-0.19	0.08	0.018	0.94	-0.06	0.09	0.47
hsa-miR-150-5p	0.83	-0.19	0.08	0.017	0.89	-0.12	0.09	0.17
hsa-miR-17-5p	0.83	-0.19	0.07	0.0054	0.88	-0.13	0.08	0.09
hsa-miR-103a-3p	0.83	-0.19	0.08	0.017	0.91	-0.09	0.08	0.29
hsa-miR-374b-5p	0.84	-0.17	0.08	0.048	0.98	-0.02	0.09	0.8
hsa-miR-551b-3p	0.85	-0.16	0.08	0.036	0.96	-0.04	0.08	0.62

[00107] In some examples, in the method as described herein, an increase in the level of miRNA as listed as "hazard ratio > 1" in Table 15, as compared to the control, may indicate the subject has an increased risk of disease progression to hospitalization or death (decreased event free survival rate). In some examples, in the method as described herein, a reduction in the level of miRNA as listed as "hazard ratio > 1" in Table 15, as compared to the control, may indicate the subject has a decreased risk of disease progression to hospitalization or death (increased event free survival rate).

[00108] In some examples, in the method as described herein, an increase in the level of miRNA as listed as "hazard ratio < 1" in Table 15, as compared to the control, may indicate the subject has a decreased risk of disease progression to hospitalization or death (increased event free survival rate). In some examples, in the method as described herein, a reduction in the level of miRNA as listed as "hazard ratio < 1" in Table 15, as compared to the control, may indicate the subject has an increased risk of disease progression to hospitalization or death (decreased event free survival rate).

[00109] In some examples, in the method as described herein, the control may be control population or cohort of heart failure subjects. In some examples, the control population may be a population or cohort of heart failure patients where the microRNA expression levels and risk of death or disease progression for the population can be determined. In some examples, the expression level of microRNAs for the control population may be the mean or median expression level for all subjects (including the patient in question) in the population. In some examples, if 10% of the patients in the control population died within 5 years, the risk of death within 5 years is 10% for the control population. In some examples, the control population include the heart failure patient whose risk of death or disease progression is to be determined with the microRNA expression levels.

[00110] In some examples, in the method as described herein, the heart failure patient may be a subject who has had primary diagnosis of heart failure and/or being treated 3-5 days when symptomatically improved, with resolution of bedside physical signs of heart failure and considered fit to discharge. In some examples, the patient may be a stable compensated heart failure patient,

which have yet to have further deterioration into recurrent acute decompensated heart failure that require re-hospitalization or death.

[00111] In yet another aspect, there is provided a method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure, comprising the steps of: (a) detecting the presence of miRNA in a sample obtained from the subject; and/or measuring the levels of at least three miRNAs listed in Table 16 or Table 23 in the sample; and (b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure. In some examples, the method may further comprise measuring the levels of at least one miRNA as listed in "insignificant group" in Table 16, or Table 23 and wherein the at least one miRNA is hsa-miR-10b-5p.

[00112] As used herein throughout the disclosure and with reference to all methods as described herein, the term "score" refers to an integer or number, that can be determined mathematically, for example by using computational models a known in the art, which can include but are not limited to, SMV, as an example, and that is calculated using any one of a multitude of mathematical equations and/or algorithms known in the art for the purpose of statistical classification. Such a score is used to enumerate one outcome on a spectrum of possible outcomes. The relevance and statistical significance of such a score depends on the size and the quality of the underlying data set used to establish the results spectrum. For example, a blind sample may be input into an algorithm, which in turn calculates a score based on the information provided by the analysis of the blind sample. This results in the generation of a score for said blind sample. Based on this score, a decision can be made, for example, how likely the patient, from which the blind sample was obtained, has heart failure or not. The ends of the spectrum may be defined logically based on the data provided, or arbitrarily according to the requirement of the experimenter. In both cases the spectrum needs to be defined before a blind sample is tested. As a result, the score generated by such a blind sample, for example the number "45" may indicate that the corresponding patient has heart failure, based on a spectrum defined as a scale from 1 to 50, with "1" being defined as being heart failure-free and "50" being defined as having heart failure. Therefore, the term "score", refers to a mathematical score, which can be calculated using any one of a multitude of mathematical equations and/or algorithms known in the art for the purpose of statistical classification. Examples of such mathematical equations and/or algorithms can be, but are not limited to, a (statistical) classification algorithm selected from the group consisting of support vector machine algorithm, logistic regression algorithm, multinomial logistic regression algorithm, Fisher's linear discriminant algorithm, quadratic classifier algorithm, perceptron algorithm, k-nearest neighbours algorithm, artificial neural network algorithm, random forests algorithm, decision tree algorithm, naive Bayes algorithm, adaptive Bayes network algorithm, and ensemble learning method combining multiple learning algorithms. In another example, the

classification algorithm is pre-trained using the expression level of the control. In some examples, the classification algorithm compares the expression level of the subject with that of the control and returns a mathematical score that identifies the likelihood of the subject to belong to either one of the control groups. In some examples, the classification algorithm may compare the expression level of the subject with that of the control and returns a mathematical score that identifies the likelihood of the subject to belong to either one of the control groups. Examples of algorithms that may be used in the present disclosure are provided below.

[00113] **Table 16. miRNAs for multivariate detection of heart failure**

Significant miRNAs				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-551b-3p	59.7%	Yes	Yes	Yes
hsa-miR-24-3p	57.3%	Yes	Yes	Yes
hsa-miR-576-5p	39.7%	Yes	No	Yes
hsa-miR-375	39.7%	Yes	Yes	Yes
hsa-miR-451a	37.5%	Yes	Yes	Yes
hsa-miR-503	37.0%	Yes	Yes	Yes
hsa-miR-374b-5p	25.5%	Yes	Yes	Yes
hsa-miR-423-5p	24.9%	Yes	Yes	Yes
hsa-miR-181b-5p	24.2%	Yes	Yes	Yes
hsa-miR-454-3p	24.2%	Yes	Yes	Yes
hsa-miR-484	23.0%	Yes	Yes	Yes
hsa-miR-191-5p	20.2%	Yes	Yes	Yes
hsa-miR-1280	14.8%	Yes	Yes	Yes
hsa-miR-205-5p	12.3%	Yes	Yes	Yes
hsa-miR-424-5p	11.9%	Yes	Yes	Yes
hsa-miR-106a-5p	11.8%	Yes	Yes	Yes
hsa-miR-532-5p	11.1%	Yes	Yes	Yes
hsa-miR-197-3p	10.9%	Yes	Yes	Yes
hsa-miR-598	10.9%	Yes	Yes	Yes
hsa-miR-34b-3p	10.6%	Yes	Yes	Yes
hsa-miR-103a-3p	9.9%	Yes	Yes	Yes
hsa-miR-30b-5p	9.7%	Yes	Yes	Yes
hsa-miR-199a-3p	9.7%	Yes	No	Yes
hsa-let-7b-3p	9.6%	Yes	Yes	Yes
hsa-miR-374c-5p	6.1%	Yes	Yes	Yes
hsa-miR-148a-3p	5.7%	Yes	Yes	Yes
hsa-miR-23c	5.2%	Yes	Yes	Yes
hsa-miR-132-3p	5.0%	Yes	Yes	No
hsa-miR-200b-3p	4.5%	No	Yes	No
hsa-miR-21-5p	4.5%	Yes	Yes	Yes

hsa-miR-130b-3p	4.2%	Yes	Yes	Yes
hsa-miR-221-3p	4.0%	Yes	Yes	Yes
hsa-miR-223-5p	3.9%	Yes	Yes	Yes
hsa-miR-627	3.7%	Yes	Yes	Yes
hsa-miR-550a-5p	3.4%	No	No	Yes
hsa-miR-382-5p	3.4%	Yes	Yes	Yes
hsa-miR-19b-3p	3.2%	Yes	Yes	Yes
hsa-miR-20a-5p	3.2%	Yes	Yes	Yes
hsa-miR-23b-3p	3.0%	Yes	Yes	Yes
hsa-miR-30a-5p	2.7%	Yes	Yes	No
hsa-miR-363-3p	2.4%	Yes	Yes	Yes
hsa-miR-30c-5p	2.4%	Yes	Yes	Yes
Insignificant miRNAs				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-10b-5p	35.0%	No	No	No
hsa-miR-29c-3p	13.9%	No	No	No
hsa-miR-660-5p	12.1%	No	No	No
hsa-miR-133a	7.9%	No	No	No
hsa-miR-379-5p	5.2%	No	No	No
hsa-miR-10a-5p	4.7%	No	No	No
hsa-miR-92a-3p	4.0%	No	No	No
hsa-miR-222-3p	3.7%	No	No	No
hsa-miR-200c-3p	3.5%	No	No	No

[00114] **Table 23. miRNAs for heart failure detection**

Significant miRNAs (n=35)				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-551b-3p	59.7%	Yes	Yes	Yes
hsa-miR-24-3p	57.3%	Yes	Yes	Yes
hsa-miR-576-5p	39.7%	Yes	No	Yes
hsa-miR-375	39.7%	Yes	Yes	Yes
hsa-miR-451a	37.5%	Yes	Yes	Yes
hsa-miR-503	37.0%	Yes	Yes	Yes
hsa-miR-374b-5p	25.5%	Yes	Yes	Yes
hsa-miR-181b-5p	24.2%	Yes	Yes	Yes
hsa-miR-454-3p	24.2%	Yes	Yes	Yes
hsa-miR-484	23.0%	Yes	Yes	Yes
hsa-miR-205-5p	12.3%	Yes	Yes	Yes
hsa-miR-424-5p	11.9%	Yes	Yes	Yes
hsa-miR-106a-5p	11.8%	Yes	Yes	Yes
hsa-miR-532-5p	11.1%	Yes	Yes	Yes

hsa-miR-197-3p	10.9%	Yes	Yes	Yes
hsa-miR-598	10.9%	Yes	Yes	Yes
hsa-miR-34b-3p	10.6%	Yes	Yes	Yes
hsa-miR-199a-3p	9.7%	Yes	No	Yes
hsa-let-7b-3p	9.6%	Yes	Yes	Yes
hsa-miR-374c-5p	6.1%	Yes	Yes	Yes
hsa-miR-148a-3p	5.7%	Yes	Yes	Yes
hsa-miR-23c	5.2%	Yes	Yes	Yes
hsa-miR-132-3p	5.0%	Yes	Yes	No
hsa-miR-200b-3p	4.5%	No	Yes	No
hsa-miR-130b-3p	4.2%	Yes	Yes	Yes
hsa-miR-221-3p	4.0%	Yes	Yes	Yes
hsa-miR-223-5p	3.9%	Yes	Yes	Yes
hsa-miR-627	3.7%	Yes	Yes	Yes
hsa-miR-550a-5p	3.4%	No	No	Yes
hsa-miR-382-5p	3.4%	Yes	Yes	Yes
hsa-miR-19b-3p	3.2%	Yes	Yes	Yes
hsa-miR-20a-5p	3.2%	Yes	Yes	Yes
hsa-miR-23b-3p	3.0%	Yes	Yes	Yes
hsa-miR-363-3p	2.4%	Yes	Yes	Yes
hsa-miR-30c-5p	2.4%	Yes	Yes	Yes
Insignificant miRNAs (n=7)				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-10b-5p	35.0%	No	No	No
hsa-miR-660-5p	12.1%	No	No	No
hsa-miR-133a	7.9%	No	No	No
hsa-miR-379-5p	5.2%	No	No	No
hsa-miR-10a-5p	4.7%	No	No	No
hsa-miR-222-3p	3.7%	No	No	No
hsa-miR-200c-3p	3.5%	No	No	No

[00115] In some examples, the method as disclosed herein measures the change in levels of: at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least 10, or at least 11, or at least two to at least 20, or at least 10 to at least 45, or at least 40 to at least 50, or all miRNA as listed in Table 16. In some examples, the method as disclosed herein measures at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least 10, or at least 11, or at least two to at least 20, or at least 10 to at least 41, or all miRNA as listed in Table 23.

[00116] In some examples, the method as disclosed herein measures the level of at least one (or multiple) miRNAs (in a subject's plasma sample). The measurement of at least one miRNAs may be

combined to generate a score for the prediction of heart failure or the classification of HFREF and HFPEF subtypes. In some examples, the formula to generate the score may be Formula 1, which formula is as follows:

Formula 1 -

$$prediction\ score = B + \sum_{i=1}^{n} K_i \times \log_2 copy_miRNA_i,$$

where $\log_2 copy_miRNA_i$ is log transformed copy numbers (copy/ml of plasma) of individual miRNAs'; K_i is the coefficients used to weight multiple miRNA targets; and B is a constant value to adjust the scale of the prediction score.

[00117] Formula 1 here demonstrated the use of a linear model for the prediction of heart failure or classification of HFREF and HFPEF subtypes. The prediction score (unique for each subject) is the number to make the predictive or diagnostic decisions.

[00118] In the Experimental Section of the present disclosure, the diagnostic utility of the identified miRNAs underwent further statistical evaluation. Multivariate miRNA biomarker panels (HF panel, HFREF and HFPEF panels) were then formulated by sequence forward floating search (SFFS) [53] and support vector machine (SVM) [54] with repeated cross-validation *in silico*. The inventors of the present disclosure found some of the miRNAs in the biomarker panels consistently produced AUC values (Areas Under the Curve) of ≥0.92 for HF detection (Figure 20, B) and AUC ≥0.75 for subtype categorization (Figure 24, A) in the receiver operating characteristic (ROC) plot. The miRNA panels, when used in combination with NT-proBNP, exhibited marked improved discriminative power and better classification accuracies for both purposes (Figure 22, B and 24, B).

[00119] Thus, in another aspect, there is provided a method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure, comprising the steps of: (a) detecting the presence of miRNA in a sample obtained from the subject; and/or measuring the levels of at least two miRNAs listed in Table 17 in the sample; and (b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure.

[00120] **Table 17. miRNAs to be used in conjunction with NP-proBNP in detection of heart failure**

Significant miRNAs (additional to ln_NT-proBNP)				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-454-3p	42.0%	Yes	No	Yes
hsa-miR-451a	38.6%	Yes	No	Yes
hsa-miR-503	36.7%	Yes	No	Yes
hsa-miR-1280	30.9%	Yes	No	Yes
hsa-miR-103a-3p	22.1%	Yes	No	Yes

hsa-miR-106a-5p	19.2%	Yes	No	Yes
hsa-miR-375	16.2%	Yes	No	Yes
hsa-miR-148a-3p	13.8%	Yes	No	Yes
hsa-miR-24-3p	12.9%	Yes	No	Yes
hsa-miR-17-5p	11.6%	Yes	No	Yes
hsa-miR-25-3p	11.0%	Yes	No	Yes
hsa-miR-30b-5p	10.9%	Yes	No	Yes
hsa-miR-196b-5p	9.8%	Yes	No	Yes
hsa-miR-34b-3p	7.3%	Yes	No	Yes
hsa-miR-363-3p	6.8%	Yes	No	Yes
hsa-miR-374b-5p	6.7%	Yes	No	No
hsa-miR-193a-5p	6.6%	Yes	No	No
hsa-miR-197-3p	5.1%	Yes	No	Yes
hsa-miR-101-3p	4.8%	Yes	No	Yes
hsa-miR-532-5p	4.7%	Yes	No	Yes
hsa-miR-30c-5p	4.3%	Yes	No	Yes
hsa-miR-16-5p	4.3%	Yes	No	Yes
hsa-miR-144-3p	4.2%	Yes	No	Yes
hsa-miR-183-5p	4.2%	Yes	No	Yes
hsa-miR-20b-5p	4.1%	Yes	No	Yes
hsa-miR-501-5p	4.0%	Yes	No	Yes
hsa-miR-423-5p	3.9%	Yes	No	No
hsa-miR-130b-3p	3.9%	Yes	No	Yes
hsa-miR-20a-5p	3.6%	Yes	No	Yes
hsa-miR-29b-3p	3.2%	Yes	No	Yes
hsa-let-7b-5p	3.1%	Yes	No	Yes
hsa-miR-500a-5p	2.6%	Yes	No	Yes
hsa-miR-19b-3p	2.3%	Yes	No	Yes
hsa-miR-4732-3p	2.2%	Yes	No	Yes
hsa-let-7d-3p	2.2%	Yes	No	Yes
hsa-miR-15a-5p	2.0%	Yes	No	Yes
Insignificant miRNAs (additional to ln_NT-proBNP)				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-576-5p	25.4%	No	No	No
hsa-miR-124-5p	15.9%	No	No	No
hsa-miR-192-5p	9.6%	No	No	No
hsa-miR-551b-3p	8.1%	No	No	No
hsa-miR-150-5p	7.6%	No	No	No
hsa-miR-191-5p	6.6%	No	No	No
hsa-miR-10b-5p	6.5%	No	No	No
hsa-miR-181a-2-3p	4.2%	No	No	No
hsa-miR-181b-5p	2.8%	No	No	No

hsa-miR-26a-5p	2.8%	No	No	No
hsa-miR-205-5p	2.6%	No	No	No
hsa-miR-92a-3p	2.4%	No	No	No
hsa-miR-424-5p	2.3%	No	No	No

[00121] In some examples, the methods as described herein may further comprise the step of determining the level of Brain Natriuretic Peptide (BNP) and/or N-terminal prohormone of brain natriuretic peptide (NT-proBNP). In some examples, both NT-proBNP and BNP are good markers of prognosis and diagnosis of heart failure, such as chronic heart failure.

[00122] In some examples, the method as described herein may measure the altered levels of at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least 10, or at least 11, or at least two to at least 20, or at least 10 to at least 45, or at least 40 to at least 48, or all miRNA as listed in Table 17.

[00123] In some examples, in the methods as disclosed herein, where BNP and/or NT-proBNP are used together with miRNA, Formula 2 can be used instead. In Formula 2, the level of BNP/NT-proBNP in the plasma sample is included into the linear model. In one example, Formula 2 is as follows:

[00124] **Formula 2 -**

$$prediction\ score = B + BNP + \sum_{i=1}^{n} K_i \times \log_2 copy_miRNA_i,$$

wherein, $\log_2 copy_miRNA_i$ is log transformed copy numbers (copy/ml of plasma) of individual miRNAs'; K_i is the coefficients used to weight multiple miRNA targets; B is a constant value to adjust the scale of the prediction score; BNP is a measure positively or negatively correlated with the level of BNP and/or NT-proBNP in the sample.

[00125] Additionally, for the prediction of heart failure, the prediction score (which would be unique for each subject) is the number that indicates the likelihood of a subject having heart failure. In some examples, the outcome of the methods as described herein (i.e. prediction of likelihood or diagnosis) may be found in Formula 3. If the value is higher than a pre-set cutoff value, the subject will be diagnosed or predicted to have heart failure. If the value is lower than a pre-set cutoff value, the subject will be diagnosed or predicted to be without heart failure. Formula 3 is as follows:

Formula 3 -

[00126] $$outcome = \begin{cases} have\ heart\ failure,\ if\ prediction\ score > cutoff \\ no\ heart\ failure,\ if\ prediciton\ score < cutoff \end{cases}$$

[00127] In some examples, for the classification of HFREF and HFPEF subtypes, the prediction score (which is unique for each subject) may be the number that indicates the likelihood of a heart

failure subject having HFPEF subtype of heart failure. In some examples, the outcome of the diagnosis may be found in Formula 4. If the value is higher than a pre-set cutoff value, the heart failure subject will be diagnosed as (or predicted to) having HFPEF subtype of heart failure. If the value is lower than a pre-set cutoff value, the heart failure subject will be diagnosed as (or predicted to) have HFREF subtype of heart failure by this test.

Formula 4 -

$$outcome = \begin{cases} have\ HFPEF\ subtype\ of\ heart\ failure, & if\ prediction\ score > cutoff \\ have\ HFREF\ subtype\ of\ heart\ failure, & if\ prediciton\ score < cutoff \end{cases}$$

[00128] In another aspect, there is provided a method of determining the likelihood of a subject to be suffering from a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF). In some examples, the method comprises the steps of: (a) detecting the presence of miRNA in a sample obtained from the subject; and/or measuring the levels of at least three miRNA listed in Table 18 in the sample; and (b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

[00129] **Table 18. miRNA for determining heart failure subtype categorization**

Significant miRNAs	
Name	**prevalence in biomarker panels**
hsa-miR-30a-5p	94.6%
hsa-miR-181a-2-3p	83.7%
hsa-miR-486-5p	64.6%
hsa-miR-199b-5p	55.6%
hsa-miR-451a	39.3%
hsa-miR-144-3p	37.7%
hsa-miR-20b-5p	24.2%
hsa-miR-223-5p	21.6%
hsa-miR-20a-5p	16.3%
hsa-miR-106a-5p	10.1%
hsa-miR-93-5p	4.9%
hsa-miR-18b-5p	4.4%
hsa-miR-103a-3p	4.1%
hsa-miR-500a-5p	4.1%
hsa-let-7i-5p	3.5%
hsa-miR-196b-5p	3.5%
hsa-miR-335-5p	3.4%
hsa-miR-183-5p	3.3%

hsa-miR-146a-5p	2.6%
hsa-miR-25-3p	2.4%
hsa-miR-17-5p	2.4%
hsa-miR-185-5p	2.3%
Insignificant miRNAs	
Name	**prevalence in biomarker panels**
hsa-miR-191-5p	42.3%
hsa-miR-1275	32.8%
hsa-miR-124-5p	31.7%
hsa-miR-532-3p	28.0%
hsa-miR-23a-3p	22.0%
hsa-miR-484	15.1%
hsa-miR-125a-5p	11.3%
hsa-miR-10b-5p	11.0%
hsa-miR-101-3p	8.4%
hsa-miR-423-5p	7.6%
hsa-miR-660-5p	7.3%
hsa-miR-374b-5p	7.1%
hsa-miR-193a-5p	6.4%
hsa-miR-92a-3p	5.2%
hsa-miR-15a-5p	4.9%
hsa-miR-200c-3p	4.1%
hsa-miR-497-5p	3.8%
hsa-miR-425-3p	2.9%
hsa-miR-32-5p	2.7%
hsa-miR-139-5p	2.7%
hsa-miR-503	2.6%
hsa-miR-221-3p	2.3%
hsa-miR-345-5p	1.9%
hsa-miR-551b-3p	1.8%

[00130] In some examples, the method as described herein may measure the altered levels of at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 11, at least two to at least 20, at least 10 to at least 30, at least 40 to at least 45 or all miRNA as listed in Table 18.

[00131] In some examples, the score in the method as disclosed herein may be calculated by the formulas provided herein. In some examples, the formula may be at least one of the formula, including, but is not limited to, Formula 1, and/or Formula 2. The outcome of the methods as disclosed herein may be determined by the formula, such as, but not limited to, Formula 3, Formula 4, and the like.

[00132] In yet another aspect, there is provided a method of determining the likelihood of a subject having a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF), comprising the steps of: (a) detecting the presence of miRNA in a sample obtained from the subject; and/or measuring the levels of at least two miRNAs listed in Table 19 or Table 24 in the sample; and (b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

[00133] **Table 19. miRNAs for use in conjunction of NT-proBNP when categorizing heart failure subtype**

Significant miRNAs (additional to ln_NT-proBNP)	
Name	**prevalence in biomarker panels**
hsa-miR-199b-5p	91.5%
hsa-miR-30a-5p	66.7%
hsa-miR-486-5p	49.3%
hsa-miR-181a-2-3p	35.5%
hsa-miR-20b-5p	31.4%
hsa-miR-122-5p	10.8%
hsa-miR-223-5p	10.0%
hsa-miR-144-3p	9.8%
hsa-miR-106a-5p	8.9%
hsa-miR-20a-5p	6.5%
hsa-miR-451a	5.3%
hsa-miR-25-3p	3.9%
hsa-miR-103a-3p	3.6%
hsa-miR-335-5p	2.3%
Insignificant miRNAs (additional to ln_NT-proBNP)	
Name	**prevalence in biomarker panels**
hsa-miR-191-5p	74.9%
hsa-miR-186-5p	29.1%
hsa-miR-1275	18.1%
hsa-miR-484	16.6%
hsa-miR-532-3p	13.9%
hsa-miR-132-3p	11.0%
hsa-miR-124-5p	10.6%
hsa-miR-15a-5p	8.8%
hsa-miR-425-3p	8.7%
hsa-miR-374b-5p	7.4%

hsa-miR-23a-3p	6.4%
hsa-miR-92a-3p	5.8%
hsa-miR-150-5p	4.5%
hsa-miR-26a-5p	2.7%
hsa-miR-598	2.4%
hsa-miR-660-5p	2.3%
hsa-miR-454-3p	2.0%

[00134] **Table 24. miRNAs for use in conjunction of NT-proBNP when categorizing heart failure subtype**

Significant miRNAs (additional to ln_NT-proBNP) (n=32)				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-454-3p	42.0%	Yes	No	Yes
hsa-miR-451a	38.6%	Yes	No	Yes
hsa-miR-503	36.7%	Yes	No	Yes
hsa-miR-106a-5p	19.2%	Yes	No	Yes
hsa-miR-375	16.2%	Yes	No	Yes
hsa-miR-148a-3p	13.8%	Yes	No	Yes
hsa-miR-24-3p	12.9%	Yes	No	Yes
hsa-miR-17-5p	11.6%	Yes	No	Yes
hsa-miR-25-3p	11.0%	Yes	No	Yes
hsa-miR-196b-5p	9.8%	Yes	No	Yes
hsa-miR-34b-3p	7.3%	Yes	No	Yes
hsa-miR-363-3p	6.8%	Yes	No	Yes
hsa-miR-374b-5p	6.7%	Yes	No	No
hsa-miR-193a-5p	6.6%	Yes	No	No
hsa-miR-197-3p	5.1%	Yes	No	Yes
hsa-miR-101-3p	4.8%	Yes	No	Yes
hsa-miR-532-5p	4.7%	Yes	No	Yes
hsa-miR-30c-5p	4.3%	Yes	No	Yes
hsa-miR-16-5p	4.3%	Yes	No	Yes
hsa-miR-144-3p	4.2%	Yes	No	Yes
hsa-miR-183-5p	4.2%	Yes	No	Yes
hsa-miR-20b-5p	4.1%	Yes	No	Yes
hsa-miR-501-5p	4.0%	Yes	No	Yes
hsa-miR-130b-3p	3.9%	Yes	No	Yes
hsa-miR-20a-5p	3.6%	Yes	No	Yes
hsa-miR-29b-3p	3.2%	Yes	No	Yes
hsa-let-7b-5p	3.1%	Yes	No	Yes
hsa-miR-500a-5p	2.6%	Yes	No	Yes
hsa-miR-19b-3p	2.3%	Yes	No	Yes

hsa-miR-4732-3p	2.2%	Yes	No	Yes
hsa-let-7d-3p	2.2%	Yes	No	Yes
hsa-miR-15a-5p	2.0%	Yes	No	Yes
Insignificant miRNAs (additional to ln_NT-proBNP) (n=10)				
Name	**prevalence in biomarker panels**	**Significant for all HF**	**Significant for HFREF**	**Significant for HFPEF**
hsa-miR-576-5p	25.4%	No	No	No
hsa-miR-124-5p	15.9%	No	No	No
hsa-miR-192-5p	9.6%	No	No	No
hsa-miR-551b-3p	8.1%	No	No	No
hsa-miR-10b-5p	6.5%	No	No	No
hsa-miR-181a-2-3p	4.2%	No	No	No
hsa-miR-181b-5p	2.8%	No	No	No
hsa-miR-26a-5p	2.8%	No	No	No
hsa-miR-205-5p	2.6%	No	No	No
hsa-miR-424-5p	2.3%	No	No	No

[00135] As illustrated in the Experimental Section and Figure, for example Figures 22 and 24, when a method present disclosure is used with an additional step of determining NT-proBNP, the method provides a surprisingly accurate prediction. Thus, in some examples, the method may further comprise the step of determining the level of Brain Natriuretic Peptide (BNP) and/or N-terminal prohormone of brain natriuretic peptide (NT-proBNP).

[00136] In some examples, the method as described herein may measure the altered levels of at least three, or at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 11, at least two to at least 20, at least 10 to at least 30, or all miRNA as listed in Table 19 or at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 11, at least two to at least 20, at least 10 to at least 41, or all miRNA as listed in Table 24.

[00137] In some examples, the levels of at least one of the miRNAs measured in step (b), when compared to a control, is not altered in the subject. In such examples, the miRNA which levels when compared to a control is not altered in the subject is the miRNAs listed as "insignificant" in the respective tables.

[00138] In some examples, the score in the method as disclosed herein may be calculated by Formula 2.

[00139] As would be understood by the person skilled in the art, the classification algorithm, as used herein in any of the methods described in the disclosure, may be pre-trained using the expression level of the control. In examples where the classification algorithm is to be pre-trained using pre-existing clinical data, the control may be at least one selected from the group consisting of a heart failure free control (normal) and a heart failure patient. The control may include a cohort of subject(s)

having heart failure and/or not having heart failure (i.e. heart failure free). Thus, in some examples of the method as disclosed herein, the control may include, but not limited to, a heart failure free control, and a heart failure patient, a HFPEF subtype heart failure patient, a HFREF subtype heart failure patient, and the like.

[00140] The present disclosure discusses the differential comparison of expression levels of miRNA in the establishment of a panel of miRNAs, based on which a determination of whether a subject is at risk of developing heart failure, or a determination whether a subject suffers from heart failure can be made. As disclosed therein, the methods as disclosed herein require the differential comparison of miRNA expression levels, usually from different groups. In one example, the comparison is made between two groups. These comparison groups can be defined as being, but are not limited to, heart failure, heart failure-free (normal). Within the heart failure groups, further subgroups, for example but not limited to, HFREF and HFPEF, can be found. Differential comparisons can also be made between these groups described herein. Thus, in some examples, the expression level of the miRNAs can be expressed as, but not limited to, concentration, log(concentration), threshold cycle/quantification cycle (Ct/Cq) number, two to the power of threshold cycle/quantification cycle (Ct/Cq) number and the like.

[00141] In any of the methods as described herein, the methods may further include, but is not limited to, the steps of obtaining a sample from the subject at different time points, monitoring the course of the heart failure, staging the heart failure, measuring the miRNA level and/or NT-proBNP level in the (sample obtained from) subject, and the like.

[00142] In some examples, based on the current cohort as described in the Experimental Section below, biomarker panels including multiple miRNAs or biomarker panels including multiple miRNAs and BNP/NT-proBNP may be developed. The prediction score calculation may be optimized by methods known in the art, for example with a linear SVM model. In some examples, the biomarker panels consisting various number of miRNAs targets may be optimized by SFFS and SVM, where the AUC was optimized for the prediction of heart failure (Table 26) or classifications of heart failure subtypes (Table 27). Exemplary formulas, cutoffs and the performance of the panel are provided in Tables.

[00143] **Table 26**. Exemplary biomarker panels for HF detection.

Biomarker	**Combination of biomarker panel**	**cutoff value**	**AUC (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Accuracy (95% CI)**
3 miRNAs Panel	-0.58*miR-454-3p - 0.57*miR-551b-3p +1.31*miR-24-3p -11.47	0	0.93 (0.91 - 0.95)	85.2% (81.9% - 88.0%)	87.0% (83.8% - 89.7%)	85.9% (82.6% - 88.7%)
4 miRNAs Panel	-0.54*miR-454-3p - 0.62*miR-551b-3p +1.39*miR-24-3p -	0	0.94 (0.93 - 0.96)	87.3% (84.1% - 89.9%)	88.0% (84.9% - 90.5%)	87.5% (84.4% - 90.2%)

	0.54*miR-10b-5p -7.13					
5 miRNAs Panel	-0.95*miR-451a +0.38*miR-503 +1.12*miR-576-5p -1.17*miR-30b-5p +0.73*let-7b-3p +21.47	0	0.95 (0.93 - 0.97)	87.3% (84.1% - 89.9%)	90.4% (87.5% - 92.7%)	88.5% (85.4% - 91.0%)
6 miRNAs Panel	-0.84*miR-451a -0.46*miR-551b-3p +0.34*miR-503 +1.11*miR-576-5p +1.02*miR-24-3p -1.02*miR-374b-5p +6.61	0	0.96 (0.94 - 0.98)	87.6% (84.4% - 90.2%)	92.3% (89.7% - 94.4%)	89.4% (86.4% - 91.8%)
7 miRNAs Panel	-0.73*miR-451a -0.54*miR-551b-3p +0.30*miR-503 +1.06*miR-576-5p -1.16*miR-374b-5p +0.85*miR-24-3p +0.37*miR-199a-3p +4.60	0	0.97 (0.95 - 0.98)	87.3% (84.1% - 89.9%)	93.8% (91.3% - 95.6%)	89.7% (86.8% - 92.1%)
8 miRNAs Panel	-0.84*miR-451a -0.45*miR-551b-3p +0.35*miR-503 +1.13*miR-576-5p +0.30*miR-375 +0.94*miR-24-3p -0.28*miR-205-5p -0.98*miR-374b-5p +6.52	0	0.97 (0.96 - 0.98)	91.4% (88.7% - 93.6%)	94.2% (91.8% - 96.0%)	92.5% (89.9% - 94.5%)
2 miRNAs panel + NTproBNP	-0.88*miR-103a-3p +0.88*miR-24-3p +0.43*log2(BNP) -3.48	0	0.98 (0.98 - 0.99)	92.9% (90.3% - 94.9%)	95.2% (93.0% - 96.8%)	93.8% (91.3% - 95.6%)
3 miRNAs panel + NTproBNP	-1.09*miR-103a-3p +0.73*miR-24-3p +0.44*miR-148a-3p +0.45*log2(BNP) -2.87	0	0.99 (0.98 - 0.99)	94.4% (92.0% - 96.1%)	95.2% (93.0% - 96.8%)	94.7% (92.4% - 96.4%)
4 miRNAs panel + NTproBNP	+0.93*miR-24-3p -0.98*miR-103a-3p +0.50*miR-148a-3p -0.42*miR-181b-5p +0.42*log2(BNP) -5.06	0	0.99 (0.98 - 1.00)	93.8% (91.3% - 95.6%)	96.2% (94.1% - 97.6%)	94.7% (92.4% - 96.4%)
5 miRNAs panel + NTproBNP	+0.27*miR-503 +0.84*miR-576-5p -0.92*miR-451a -0.99*miR-30b-5p +0.69*miR-148a-3p +0.46*log2(BNP) +14.70	0	0.99 (0.99 - 1.00)	95.0% (92.7% - 96.6%)	97.6% (95.8% - 98.7%)	96.0% (93.9% - 97.4%)
6 miRNAs panel + NTproBNP	-0.87*miR-451a +1.30*miR-576-5p -1.13*miR-30b-5p +0.81*miR-148a-3p -0.57*miR-15a-5p +0.47*miR-99b-5p	0	0.99 (0.99 - 1.00)	96.2% (94.1% - 97.6%)	96.6% (94.7% - 98.0%)	96.3% (94.3% - 97.7%)

	+0.48*log2(BNP) +16.75					
7 miRNAs panel + NTproBNP	-0.99*miR-451a +0.28*miR-503 +1.16*miR-576-5p +0.83*miR-148a-3p +0.35*miR-181a-2-3p - 1.20*miR-30b-5p - 0.60*miR-15a-5p +0.53*log2(BNP) +22.38	0	1.00 (0.99 - 1.00)	95.9% (93.7% - 97.3%)	96.6% (94.7% - 98.0%)	96.2% (94.1% - 97.6%)

[00144] As used herein in Table 26, the symbol "*" refers to "×" or multiplication symbol; "-" refers to negative value; "+" refers to addition; and "log2(BNP)" refers to the log 2 value of BNP expression. The second column of Table 26 also illustrates exemplary formulas for calculating the score as used in the method used herein. In the formula, the measuring unit for microRNA is copy/ml plasma and for NT-proBNP is pg/ml plasma. As would be apparent to the person skilled in the art, the coefficients and cutoffs in the formulas would have to be adjusted in accordance with different detection system used for the measurement and/or different units used to represent the microRNA expression level and BNP level/type. The adjustment of the formula would not be beyond the skill of the average person skilled in the art.

[00145] Thus, in another aspect, there is provided a method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure, comprising the steps of (a) detecting the presence of miRNAs of a selected panel as listed in Table 26 in a sample obtained from the subject; or measuring the levels of miRNAs as listed in the selected panel of Table 26 in the sample; and (b) assigning a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure. In one example, the score is calculated based on the formula as listed in Table 26. In one example, when a two miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 26 as "2 miRNAs Panel". In some examples, when a three miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 26 as "3 miRNAs Panel". In some examples, when a four miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 26 as "4 miRNAs Panel". In some examples, when a five miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 26 as "5 miRNAs Panel". In some examples, when a six miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 26 as "6 miRNAs Panel". In some examples, when a seven miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 26 as "7 miRNAs Panel". In some examples, when a eight miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 26 as "8 miRNAs Panel". In some examples, the methods may be performed with an additional step of detecting and measuring the level of NTproBNP in the sample thereof.

[00146] In some examples, for the prediction of heart failure, the prediction score (which would be unique for each subject) is the number that indicates the likelihood of a subject having heart failure. In some examples, the outcome of the methods as described herein (i.e. prediction of likelihood or diagnosis) may be found in Formula 3. If the value is higher than a pre-set cutoff value, the subject will be diagnosed or predicted to have heart failure. If the value is lower than a pre-set cutoff value, the subject will be diagnosed or predicted to be without heart failure. Formula 3 is as follows:

Formula 3 -

$$outcome = \begin{cases} have\ heart\ failure, & if\ prediction\ score > cutoff \\ no\ heart\ failure, & if\ prediciton\ score < cutoff \end{cases}$$

[00147] **Table 27.** Exemplary biomarker panels for heart failure subtype classification.

Biomarker	**Combination of biomarker panel**	**cutoff value**	**AUC (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Accuracy (95% CI)**
3 miRNAs Panel	-0.31*miR-486-5p -0.35*miR-30a-5p +0.37*miR-181a-2-3p +8.14	0	0.77 (0.72 - 0.82)	69.6% (64.4% - 74.4%)	70.6% (65.3% - 75.3%)	70.1% (64.9% - 74.9%)
4 miRNAs Panel	-0.29*miR-30a-5p +0.43*miR-181a-2-3p -0.24*miR-1275 -0.27*miR-20b-5p +8.80	0	0.79 (0.74 - 0.84)	78.5% (73.6% - 82.7%)	71.7% (66.5% - 76.4%)	74.9% (69.8% - 79.3%)
5 miRNAs Panel	-0.51*miR-20b-5p -0.23*miR-1275 +0.24*miR-451a +0.43*miR-181a-2-3p -0.27*miR-30a-5p +6.51	0	0.80 (0.75 - 0.85)	78.5% (73.6% - 82.7%)	72.2% (67.1% - 76.9%)	75.1% (70.1% - 79.6%)
6 miRNAs Panel	-0.50*miR-486-5p -0.35*miR-30a-5p +0.34*miR-199b-5p +0.42*miR-181a-2-3p -0.46*miR-191-5p +0.39*miR-484 +9.98	0	0.82 (0.77 - 0.86)	74.1% (69.0% - 78.6%)	73.3% (68.2% - 77.9%)	73.7% (68.6% - 78.2%)
2 miRNAs panel + NTproBNP	-0.39*miR-20b-5p +0.34*miR-199b-5p -0.19*log2(BNP) +5.47	0	0.83 (0.78 - 0.87)	74.1% (69.0% - 78.6%)	76.1% (71.1% - 80.5%)	75.1% (70.1% - 79.6%)
3 miRNAs panel + NTproBNP	-0.34*miR-20b-5p +0.41*miR-199b-5p -0.27*miR-30a-5p -0.19*log2(BNP) +8.02	0	0.84 (0.80 - 0.89)	71.5% (66.3% - 76.2%)	78.9% (74.1% - 83.1%)	75.4% (70.4% - 79.9%)
4 miRNAs panel + NTproBNP	-0.47*miR-20b-5p +0.47*miR-199b-5p -0.52*miR-191-5p +0.50*miR-186-5p -0.24*log2(BNP) +7.35	0	0.87 (0.83 - 0.91)	77.2% (72.3% - 81.5%)	78.9% (74.1% - 83.1%)	78.1% (73.2% - 82.3%)
5 miRNAs panel +	-0.44*miR-20b-5p +0.49*miR-199b-5p -	0	0.88 (0.85 -	79.7% (75.0% -	77.2% (72.3% -	78.4% (73.6% -

NTproBNP	0.50*miR-191-5p +0.56*miR-186-5p - 0.29*miR-30a-5p - 0.24*log2(BNP) +9.79		0.92)	83.8%)	81.5%)	82.6%)
6 miRNAs panel + NTproBNP	-0.43*miR-20b-5p - 0.18*miR-1275 +0.47*miR-199b-5p - 0.49*miR-191-5p +0.54*miR-186-5p - 0.27*miR-30a-5p - 0.24*log2(BNP) +11.85	0	0.89 (0.85 - 0.92)	80.4% (75.7% - 84.4%)	76.7% (71.7% - 81.0%)	78.4% (73.6% - 82.6%)

[00148] As used herein in Table 27, the symbol "*" refers to "×" or multiplication symbol; "-" refers to negative value; "+" refers to addition; and "log2(BNP)" refers to the log 2 value of BNP expression. The second column of Table 27 also illustrates exemplary formulas for calculating the score as used in the method used herein. In the formula, the measuring unit for microRNA is copy/ml plasma and for NT-proBNP is pg/ml plasma. As would be apparent to the person skilled in the art, the coefficients and cutoffs in the formulas would have to be adjusted in accordance with different detection system used for the measurement and/or different units used to represent the microRNA expression level and BNP level/type. The adjustment of the formula would not be beyond the skill of the average person skilled in the art.

[00149] In yet another aspect, there is provided a method of determining the likelihood of a subject to be suffering from a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF), comprising the steps of (a) detecting the presence of miRNAs of a selected panel as listed in Table 27 in a sample obtained from the subject; or measuring the levels of miRNAs as listed in the selected panel of Table 27 in the sample; and (b) assigning a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF). In one example, the score is calculated based on the formula as listed in Table 27. In one example, when a two miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 27 as "2 miRNAs Panel". In some examples, when a three miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 27 as "3 miRNAs Panel". In some examples, when a four miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 27 as "4 miRNAs Panel". In some examples, when a five miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 27 as "5 miRNAs Panel". In some examples, when a six miRNAs biomarker panel is required, the method may detect and measure the level of miRNAs listed in Table 27 as "6 miRNAs Panel". In some examples, the methods may be performed with an additional step of detecting and measuring the level of NTproBNP in the sample thereof.

[00150] In some examples, for the classification of HFREF and HFPEF subtypes, the prediction score (which is unique for each subject) may be the number that indicates the likelihood of a heart failure subject having HFPEF subtype of heart failure. In some examples, the outcome of the diagnosis may be found in Formula 4. If the value is higher than a pre-set cutoff value, the heart failure subject will be diagnosed as (or predicted to) having HFPEF subtype of heart failure. If the value is lower than a pre-set cutoff value, the heart failure subject will be diagnosed as (or predicted to) have HFREF subtype of heart failure by this test.

Formula 4 -

$$\text{outcome} = \begin{cases} \text{have HFPEF subtype of heart failure,} & \text{if prediction score} > \text{cutoff} \\ \text{have HFREF subtype of heart failure,} & \text{if prediciton score} < \text{cutoff} \end{cases}$$

[00151] In one example, the methods as described herein may be implemented into a device capable to (or adapted to) perform all of (or part of) the steps described in the present disclosure. Thus, in one example, the present disclosure provides for a device adapted to (or capable of) adapting to perform the methods as described herein.

[00152] In another aspect, there is provided a kit for use (or adapted to be used, or when used) in any of the methods as described herein. In one example, the kit may comprise reagents for determining the expression of the at least one gene listed in Table 9, or at least one gene listed in Table 14, or at least one gene listed in Table 15, or at least two genes listed in Table 16, or at least two genes listed in Table 17, or at least two genes listed in Table 18, or at least two genes listed in Table 19, or at least one genes listed in Table 20; or at least one gene listed in Table 21; or at least one gene listed in Table 22; or at least one gene listed in Table 23; or at least one gene listed in Table 24; or at least one gene listed in Table 25.

[00153] In some examples, the reagents may comprise a probe, primer or primer set adapted to or capable of ascertaining the expression of at least one gene listed in Table 9, or ate last one gene listed in Table 14, or at least one gene listed in Table 15, or at least two genes listed in Table 16, or at least two genes listed in Table 17, or at least two genes listed in Table 18, or at least two genes listed in Table 19, or at least one genes listed in Table 20; or at least one gene listed in Table 21; or at least one gene listed in Table 22; or at least one gene listed in Table 23; or at least one gene listed in Table 24; or at least one gene listed in Table 25.

[00154] In some examples, the kit may further comprise a reagent for determining the level of Brain Natriuretic Peptide (BNP) and/or N-terminal prohormone of brain natriuretic peptide (NT-proBNP).

[00155] In some examples, the methods as described herein may further comprise a step of treating the subject predicted to (or diagnosed as) having heart failure or heart failure subtype to at least one

therapeutic agent for treating heart failure (or heart failure subtype). In some examples, the method may further comprise therapies known for alleviating and/or reducing the symptoms of heart failure. In some examples, the method as described herein may further comprise the administration of agents including, but not limited to, classes of drugs that are proven to improve prognosis in heart failure (for example, ACEI's/ARB's, angiotensin receptor blockers, Loop/thiazide diuretics, beta blockers, mineralocorticoid antagonists, aspirin or Plavix, statins, digoxin, warfarin, nitrates, calcium channel blockers, spironolactone, fibrate, antidiabetic, hydralazine, iron supplements, anticoagulant, antiplatelet and the likes).

[00156] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00157] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00158] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

[00159] **METHOD**

[00160] **Pre-analytics (sample collection and miRNA extraction)**: Plasma samples were stored frozen at -80°C prior to use. Total RNA from 200 µl of each plasma sample was isolated using the well-established TRI Reagent (Sigma-Aldrich®) following the manufacturer's protocol. Plasma contains minute amounts of RNA. To reduce the loss of RNA and monitor extraction efficiency,

rationally designed isolation enhancers (MS2) and spike-in control RNAs (MiRXES™) were added to the specimen prior to isolation.

[00161] **RT-qPCR:** The isolated total RNAs and synthetic RNA standards were converted to cDNA in optimized multiplex reverse transcription reactions with a second set of spike-in control RNAs to detect the presence of inhibitors and monitor the RT-qPCR efficiency. The Improm II (Promega®) reverse transcriptase was used to perform the reverse transcription following manufacturer's instruction. The synthesized cDNA is then subjected to a multiplex augmentation step and quantified using a Sybr Green based single-plex qPCR assays (MIQE compliant) (MiRXES™). Applied Biosystems® ViiA 7 384 Real-Time PCR System or Bio-rad® CFX384 Touch Real-Time PCR Detection System was used for qPCR reactions. The overview and details of miRNA RT-qPCR measurement workflow was summarized in Figure 2.

[00162] **Data processing:** The raw Cycles to Threshold (Ct) values were processed and the absolute copy numbers of the target miRNAs in each sample were determined by intrapolation of the synthetic miRNA standard curves. The technical variations introduced during RNA isolation and the processes of RT-qPCR were normalized by the spike-in control RNAs. For the analysis of single miRNA, the biological variations were further normalized by a set of validated endogenous reference miRNAs stably expressed across all control and disease samples.

[00163] **RESULTS**

[00164] **I. Characteristics of study participants**

[00165] A well-designed clinical study (case-control study) was carried out to ensure the accurate identification of biomarkers for chronic heart failure (HF). A total number of 338 chronic heart failure patients (180 HFREF and 158 HFPEF) from the Singapore population were used in this study and comparisons were made with 208 non-heart failure subjects matched for race, gender and age, serving as the control group. Patients with heart failure were recruited from the Singapore Heart Failure Outcomes and Phenotypes (SHOP) study [55]. Patients were included if they presented with a primary diagnosis of acute decompensated heart failure (ADHF) or attended clinics for management of heart failure within 6 months of a known episode of ADHF . Controls without overt coronary artery disease or history of heart failure were recruited through the ongoing epidemiological Singapore Longitudinal Ageing Study (SLAS) [56]. All patients and controls underwent detailed clinical examination including comprehensive Doppler echocardiography for confirmation of the presence (or absence) of clinical heart failure. LVEF was assessed using the biplane method of disks as recommended by the American Society of Echocardiography (ASE) guidelines. Patients with validated heart failure and LVEF $\geq$50% were categorized as HFPEF, whereas those with LVEF $\leq$40% were classified as HFREF. Patients with EF between 40% and 50% were excluded. Assessments including blood plasma samples were deliberately undertaken when patients had received treatment (typically for 3 -5 days), were

symptomatically improved with resolution of bedside physical signs of heart failure and were considered fit for discharge. This ensured assessment of marker performance in the treated or "chronic" phase of heart failure. Clinical characteristics and demographic information are given in Table 2. All plasma samples were stored at -80 °C prior to use.

[00166] **Table 2. Clinical information of the subjects included in the study**

Sample Type	Gender	Race	Atrial Fibrillation or Flutter	Hypert-ension	Diabetes	Age	Body Mass Index
C	Female	Indian	No	Yes	No	70	26.67
C	Male	Chinese	No	Yes	No	66	30.86
C	Male	Indian	No	No	No	61	25.36
C	Female	Indian	No	Yes	Yes	64	26.58
C	Male	Chinese	No	Yes	No	69	24.45
C	Male	Chinese	No	No	No	68	16.73
C	Female	Chinese	No	No	No	61	28.69
C	Male	Chinese	No	No	No	70	25.43
C	Female	Chinese	No	No	No	56	25.63
C	Male	Chinese	No	No	No	60	22.15
C	Male	Chinese	No	Yes	No	64	24.13
C	Male	Chinese	No	Yes	No	78	25.76
C	Male	Chinese	No	No	No	51	22.16
C	Male	Chinese	No	No	No	62	72.8
C	Female	Chinese	No	Yes	No	66	22.39
C	Female	Chinese	No	No	No	63	22.97
C	Female	Chinese	No	No	No	71	24.85
C	Female	Chinese	No	Yes	No	64	24.78
C	Female	Chinese	No	Yes	Yes	52	32.7
C	Female	Chinese	No	Yes	No	71	17.5
C	Female	Chinese	No	No	No	70	21.62
C	Male	Chinese	No	Yes	No	62	30.4
C	Female	Indian	No	No	Yes	65	27.82
C	Female	Malay	No	No	No	56	22.54
C	Male	Malay	No	No	No	74	26.26
C	Female	Malay	No	Yes	No	56	33.25
C	Female	Malay	No	Yes	No	79	25.22
C	Female	Chinese	No	No	No	71	31.6
C	Female	Malay	No	Yes	No	71	32.37
C	Male	Indian	No	No	No	68	25.51
C	Male	Indian	No	No	No	54	29.12
C	Female	Malay	No	No	No	64	28.22
C	Female	Malay	No	Yes	Yes	66	25.26
C	Male	Malay	No	Yes	No	58	32.44

C	Male	Chinese	No	No	No	74	27.02
C	Female	Chinese	No	Yes	No	71	28.68
C	Male	Malay	No	Yes	No	53	30.91
C	Male	Malay	No	No	No	60	22.38
C	Female	Indian	No	No	No	58	35.81
C	Male	Malay	No	No	No	69	20.96
C	Male	Chinese	No	No	No	75	19.69
C	Female	Chinese	No	Yes	Yes	75	31.03
C	Male	Malay	No	Yes	No	66	25.15
C	Male	Indian	No	No	No	40	23.44
C	Male	Malay	No	Yes	No	63	26.61
C	Female	Chinese	No	Yes	No	65	25.51
C	Male	Chinese	No	Yes	No	68	22.61
C	Female	Chinese	No	No	No	46	19.35
C	Female	Chinese	No	Yes	No	53	23.19
C	Male	Chinese	No	Yes	Yes	69	20.83
C	Male	Chinese	No	No	No	50	26.53
C	Female	Chinese	No	No	No	64	22.38
C	Female	Chinese	No	Yes	No	47	23.65
C	Male	Chinese	No	No	No	54	26.33
C	Male	Chinese	No	Yes	No	65	25.38
C	Male	Chinese	No	Yes	No	67	23.95
C	Female	Chinese	No	No	No	48	20.49
C	Female	Chinese	No	No	Yes	62	19.08
C	Male	Chinese	No	Yes	No	65	21.6
C	Female	Malay	No	Yes	No	73	25.7
C	Female	Chinese	No	No	No	67	25.8
C	Male	Malay	No	No	No	57	30.42
C	Male	Chinese	No	Yes	No	79	24.87
C	Male	Chinese	No	Yes	No	64	23.73
C	Female	Chinese	No	No	No	62	21.7
C	Female	Chinese	No	No	No	51	26.31
C	Male	Indian	No	Yes	Yes	70	27.07
C	Female	Malay	No	No	No	71	23.28
C	Male	Chinese	Yes	Yes	No	81	19.47
C	Female	Chinese	No	No	No	58	23.26
C	Female	Chinese	No	No	No	48	21.16
C	Male	Malay	No	No	Yes	55	28.77
C	Female	Chinese	No	No	No	51	28.06
C	Male	Chinese	No	Yes	No	70	32.32
C	Male	Malay	No	No	No	46	21.22
C	Female	Chinese	No	Yes	No	61	24.99
C	Male	Indian	No	Yes	No	56	30.2

C	Female	Indian	No	No	Yes	62	25.85
C	Male	Chinese	No	Yes	No	59	26.43
C	Male	Indian	No	No	Yes	68	32.01
C	Female	Chinese	No	No	No	55	26.85
C	Female	Chinese	No	No	Yes	63	18.72
C	Male	Chinese	No	No	No	59	27.49
C	Female	Chinese	No	No	No	45	21.1
C	Female	Chinese	No	Yes	No	73	24.24
C	Male	Indian	No	No	Yes	51	28.86
C	Female	Chinese	No	Yes	No	72	23.77
C	Female	Chinese	No	No	No	73	17.63
C	Male	Chinese	No	No	No	64	23.02
C	Female	Chinese	No	No	No	59	21.63
C	Male	Chinese	No	Yes	No	67	31.2
C	Female	Chinese	No	No	No	49	21.87
C	Male	Malay	No	Yes	No	48	28.36
C	Male	Chinese	No	No	No	39	26.23
C	Female	Chinese	No	No	No	61	27.82
C	Male	Malay	No	No	No	51	21.63
C	Male	Chinese	No	Yes	No	61	23.73
C	Male	Malay	No	No	No	50	29.07
C	Male	Chinese	No	No	No	59	26.45
C	Male	Chinese	No	No	No	36	23.53
C	Female	Chinese	No	No	No	57	24.8
C	Male	Indian	No	Yes	No	49	22.86
C	Female	Malay	No	Yes	No	61	25.48
C	Male	Chinese	No	Yes	No	54	23.47
C	Male	Chinese	No	Yes	No	57	25.96
C	Female	Indian	No	No	No	62	26.56
C	Female	Chinese	No	No	No	59	22.65
C	Female	Chinese	Yes	No	No	68	21.59
C	Male	Chinese	No	Yes	Yes	59	31.04
C	Male	Chinese	No	Yes	No	73	18.89
C	Female	Chinese	No	No	No	38	17.47
C	Male	Chinese	No	Yes	No	77	26.26
C	Female	Malay	No	No	Yes	59	30.18
C	Male	Chinese	No	No	No	80	24.38
C	Female	Chinese	No	Yes	No	74	23.46
C	Female	Chinese	No	No	No	70	23.82
C	Male	Chinese	No	Yes	No	38	31.88
C	Male	Malay	No	No	No	52	32.23
C	Male	Malay	No	No	No	53	24.81
C	Male	Chinese	No	Yes	No	48	33.51

C	Female	Chinese	No	No	No	55	25.13
C	Female	Chinese	No	No	No	47	22.89
C	Female	Chinese	No	No	No	75	26.08
C	Female	Chinese	No	No	No	52	25.94
C	Male	Chinese	No	No	No	59	23.95
C	Male	Chinese	No	No	No	48	26.83
C	Female	Chinese	No	No	No	67	22.02
C	Female	Chinese	No	No	No	59	21.3
C	Female	Chinese	No	No	No	63	21.64
C	Male	Malay	No	No	No	39	19.96
C	Female	Chinese	No	No	No	54	21.36
C	Female	Malay	No	No	No	50	29.9
C	Male	Malay	No	No	No	48	30.93
C	Female	Indian	No	No	No	42	24.12
C	Female	Chinese	No	No	No	47	25
C	Male	Chinese	No	No	No	63	26.4
C	Male	Chinese	No	No	No	61	28.3
C	Male	Chinese	No	Yes	Yes	77	22.32
C	Female	Indian	No	Yes	No	63	35.96
C	Female	Indian	No	Yes	Yes	49	25.89
C	Female	Malay	No	No	No	63	22.15
C	Male	Chinese	No	No	No	49	21.42
C	Male	Chinese	No	No	No	62	21.38
C	Female	Chinese	No	Yes	No	49	26.05
C	Female	Malay	No	No	No	41	21.18
C	Male	Indian	No	Yes	No	53	28.8
C	Male	Chinese	No	No	No	52	18.28
C	Female	Chinese	No	No	Yes	69	20.04
C	Female	Chinese	No	No	No	43	26.29
C	Male	Indian	No	No	No	54	24.73
C	Male	Malay	No	Yes	No	77	23.26
C	Male	Malay	No	No	No	51	20.76
C	Female	Chinese	No	No	No	52	25.82
C	Female	Chinese	No	No	No	72	20.58
C	Male	Chinese	No	No	No	39	24.64
C	Female	Indian	No	No	No	61	27.6
C	Female	Chinese	No	No	No	59	27.38
C	Male	Chinese	No	No	No	71	23.41
C	Female	Indian	No	No	No	59	27.41
C	Female	Chinese	No	No	No	60	16.65
C	Female	Chinese	No	Yes	No	72	31.23
C	Male	Malay	No	Yes	No	66	27.22
C	Male	Chinese	No	No	No	41	26.42

C	Male	Malay	No	No	No	52	22.77
C	Female	Indian	No	No	No	54	25.19
C	Female	Chinese	No	No	No	36	20.28
C	Male	Chinese	No	No	No	59	28.64
C	Female	Chinese	No	Yes	Yes	83	18.37
C	Male	Malay	No	No	No	51	18.38
C	Male	Chinese	No	No	No	37	28.57
C	Female	Chinese	No	No	No	71	19.05
C	Male	Chinese	No	No	No	63	21.93
C	Female	Chinese	No	No	No	63	19.84
C	Male	Malay	No	No	No	59	30.93
C	Male	Chinese	No	No	No	64	24.61
C	Male	Chinese	No	Yes	No	72	23.58
C	Female	Indian	No	No	No	48	27.67
C	Male	Chinese	No	No	No	73	18.17
C	Female	Chinese	No	No	No	64	17.89
C	Male	Chinese	No	No	No	72	26.52
C	Female	Chinese	No	No	No	57	18.94
C	Female	Chinese	No	No	No	62	25.63
C	Female	Malay	No	Yes	Yes	48	27.1
C	Female	Malay	No	No	No	39	35.91
C	Female	Malay	No	Yes	No	56	26.86
C	Female	Malay	No	No	No	60	32.97
C	Male	Chinese	No	Yes	No	76	27.94
C	Male	Chinese	No	No	No	43	26.68
C	Female	Malay	No	No	No	53	30.99
C	Male	Chinese	No	No	No	55	24.5
C	Male	Malay	No	No	No	40	22.91
C	Female	Chinese	No	Yes	No	74	25.78
C	Male	Chinese	No	Yes	No	77	18.68
C	Male	Chinese	No	No	No	63	30.15
C	Female	Chinese	No	No	No	55	22.18
C	Male	Chinese	No	No	No	75	20.37
C	Male	Indian	No	Yes	No	51	32.14
C	Female	Chinese	No	No	No	59	29.05
C	Female	Chinese	No	Yes	No	59	19.96
C	Male	Chinese	No	No	No	55	21.74
C	Male	Chinese	No	No	No	54	23.02
C	Female	Chinese	No	No	No	73	24.09
C	Male	Indian	No	No	No	48	25.28
C	Male	Chinese	No	No	No	67	27.1
C	Male	Malay	No	No	No	44	28.9
C	Male	Chinese	No	No	No	59	22.05

C	Female	Chinese	No	No	No	49	35.67
C	Male	Chinese	No	No	No	56	23.51
PEF	Female	Malay	No	Yes	No	80	26.44
PEF	Female	Chinese	Yes	Yes	No	72	23.33
PEF	Female	Chinese	Yes	Yes	No	71	26.67
PEF	Male	Malay	No	Yes	Yes	62	30.22
PEF	Male	Chinese	No	Yes	Yes	67	24.5
PEF	Male	Chinese	No	Yes	No	69	27.35
PEF	Male	Malay	No	Yes	No	75	26.1
PEF	Female	Malay	Yes	No	No	76	27.61
PEF	Female	Chinese	Yes	Yes	Yes	72	23.52
PEF	Male	Malay	No	No	Yes	64	32.31
PEF	Female	Malay	No	Yes	No	56	34.37
PEF	Female	Indian	No	Yes	Yes	61	26
PEF	Female	Chinese	No	Yes	Yes	52	32.89
PEF	Female	Malay	No	Yes	Yes	56	40.22
PEF	Male	Malay	No	Yes	Yes	70	24
PEF	Male	Indian	No	No	No	40	24.77
PEF	Female	Chinese	No	Yes	No	71	24
PEF	Male	Chinese	No	Yes	Yes	72	25.77
PEF	Female	Malay	No	Yes	Yes	64	25.33
PEF	Female	Chinese	Yes	No	No	71	31.25
PEF	Female	Chinese	Yes	Yes	Yes	56	29.89
PEF	Male	Chinese	No	Yes	Yes	70	26.56
PEF	Male	Indian	Yes	Yes	Yes	55	28.41
PEF	Male	Malay	No	Yes	Yes	60	33.2
PEF	Female	Indian	No	Yes	Yes	56	39.17
PEF	Male	Malay	Yes	No	Yes	52	32.23
PEF	Female	Malay	No	Yes	Yes	67	29.76
PEF	Female	Indian	No	Yes	Yes	65	18.29
PEF	Male	Malay	No	Yes	Yes	53	30.75
PEF	Male	Malay	No	Yes	Yes	60	21.16
PEF	Female	Chinese	No	Yes	Yes	78	23.23
PEF	Female	Indian	No	Yes	Yes	73	28.05
PEF	Female	Chinese	No	Yes	Yes	55	24.88
PEF	Female	Malay	No	Yes	Yes	52	27.24
PEF	Female	Chinese	Yes	Yes	No	74	20.81
PEF	Female	Chinese	Yes	No	No	48	33.22
PEF	Male	Indian	No	Yes	Yes	53	43.12
PEF	Female	Malay	No	Yes	Yes	60	27.79
PEF	Female	Chinese	Yes	Yes	Yes	67	22.37
PEF	Male	Chinese	No	Yes	Yes	60	37.04
PEF	Female	Chinese	No	Yes	Yes	66	47.3

PEF	Female	Chinese	Yes	Yes	No	78	29.9
PEF	Female	Chinese	No	Yes	Yes	76	23.59
PEF	Female	Malay	Yes	Yes	Yes	62	N.A.
PEF	Female	Chinese	Yes	Yes	No	91	21.52
PEF	Female	Chinese	No	No	No	83	23.5
PEF	Female	Chinese	No	Yes	No	91	20.98
PEF	Male	Chinese	No	Yes	No	59	24.57
PEF	Male	Chinese	No	Yes	Yes	63	27.37
PEF	Female	Chinese	Yes	Yes	No	77	25
PEF	Female	Chinese	No	Yes	Yes	68	29.41
PEF	Male	Chinese	Yes	No	No	60	23.14
PEF	Male	Chinese	Yes	Yes	Yes	51	27.4
PEF	Female	Chinese	No	Yes	Yes	68	25.87
PEF	Female	Chinese	No	Yes	Yes	73	27.89
PEF	Female	Chinese	No	Yes	Yes	89	20.45
PEF	Female	Chinese	Yes	Yes	Yes	87	28.31
PEF	Female	Indian	No	Yes	No	69	30
PEF	Female	Chinese	Yes	Yes	No	74	25.54
PEF	Female	Chinese	No	Yes	No	65	39.26
PEF	Female	Malay	Yes	Yes	Yes	86	32.09
PEF	Female	Chinese	Yes	Yes	No	86	26.37
PEF	Female	Malay	Yes	Yes	Yes	64	39.58
PEF	Female	Chinese	No	Yes	Yes	84	22.59
PEF	Female	Chinese	Yes	Yes	Yes	83	24.14
PEF	Female	Indian	Yes	Yes	No	64	38.75
PEF	Female	Chinese	No	Yes	Yes	83	19.31
PEF	Male	Chinese	No	Yes	Yes	66	23.66
PEF	Female	Chinese	Yes	Yes	No	73	20.03
PEF	Female	Chinese	No	Yes	No	81	24.03
PEF	Female	Chinese	Yes	Yes	Yes	52	27.64
PEF	Male	Malay	No	Yes	Yes	82	N.A.
PEF	Female	Chinese	No	Yes	No	52	40.27
PEF	Male	Indian	No	N.A.	Yes	54	30.59
PEF	Male	Malay	No	Yes	Yes	50	31.89
PEF	Male	Chinese	No	Yes	Yes	62	25.15
PEF	Female	Chinese	No	Yes	Yes	74	28.48
PEF	Male	Chinese	No	Yes	No	78	26.67
PEF	Female	Chinese	No	Yes	Yes	82	24.97
PEF	Female	Chinese	Yes	Yes	No	63	42.8
PEF	Male	Chinese	No	Yes	No	71	23.59
PEF	Female	Malay	No	Yes	No	48	25.27
PEF	Female	Chinese	No	Yes	Yes	79	29.22
PEF	Female	Chinese	Yes	No	No	59	27.55

PEF	Male	Chinese	No	Yes	Yes	57	25.8
PEF	Male	Malay	No	Yes	Yes	83	N.A.
PEF	Female	Malay	No	Yes	Yes	68	38.93
PEF	Male	Chinese	No	Yes	No	57	23.48
PEF	Male	Chinese	No	Yes	Yes	62	20.69
PEF	Male	Indian	Yes	Yes	Yes	71	28.21
PEF	Male	Chinese	Yes	No	No	36	21.6
PEF	Male	Chinese	No	Yes	No	70	26.61
PEF	Male	Chinese	Yes	Yes	Yes	76	24.59
PEF	Male	Malay	No	Yes	Yes	52	28.94
PEF	Male	Malay	No	Yes	Yes	55	26.41
PEF	Female	Malay	No	Yes	Yes	77	N.A.
PEF	Male	Chinese	No	Yes	No	61	25.31
PEF	Female	Chinese	No	No	No	78	26.9
PEF	Male	Malay	No	No	No	74	27.48
PEF	Female	Chinese	Yes	Yes	No	77	27.62
PEF	Female	Malay	No	Yes	No	83	17.86
PEF	Male	Chinese	No	Yes	Yes	63	28.12
PEF	Male	Chinese	No	No	No	81	26.12
PEF	Female	Indian	No	Yes	Yes	69	30.25
PEF	Male	Chinese	No	Yes	No	84	21.91
PEF	Female	Chinese	No	Yes	Yes	82	19.56
PEF	Female	Malay	No	Yes	Yes	77	32.58
PEF	Female	Chinese	Yes	Yes	N.A.	79	22.37
PEF	Male	Indian	No	Yes	Yes	85	20.55
PEF	Female	Chinese	Yes	Yes	No	74	34.27
PEF	Female	Malay	No	Yes	No	62	20.82
PEF	Male	Chinese	No	Yes	Yes	66	26.93
PEF	Female	Indian	No	Yes	Yes	68	26.56
PEF	Male	Chinese	Yes	Yes	Yes	77	24.87
PEF	Male	Malay	Yes	Yes	No	72	24.52
PEF	Female	Chinese	Yes	Yes	No	72	20.4
PEF	Female	Chinese	N.A.	Yes	Yes	84	27.78
PEF	Male	Chinese	No	Yes	No	65	31.63
PEF	Male	Chinese	Yes	No	No	66	31.51
PEF	Female	Malay	No	Yes	Yes	58	30.5
PEF	Male	Chinese	No	Yes	Yes	62	35.25
PEF	Female	Chinese	Yes	Yes	No	88	23.71
PEF	Female	Chinese	No	Yes	Yes	80	24.26
PEF	Female	Chinese	No	Yes	No	75	26.64
PEF	Male	Chinese	Yes	No	Yes	68	0.01
PEF	Male	Malay	No	Yes	Yes	65	29.38
PEF	Female	Chinese	No	No	Yes	63	21.76

PEF	Female	Chinese	Yes	Yes	Yes	69	31.61
PEF	Female	Indian	No	Yes	No	79	28.8
PEF	Female	Chinese	No	No	No	75	23.83
PEF	Female	Chinese	No	Yes	No	73	22.22
PEF	Male	Chinese	Yes	Yes	No	83	N.A.
PEF	Male	Chinese	No	Yes	Yes	76	25.22
PEF	Female	Chinese	No	Yes	No	68	23.5
PEF	Female	Chinese	Yes	Yes	Yes	61	25.38
PEF	Male	Malay	No	Yes	Yes	64	25.7
PEF	Female	Chinese	No	Yes	No	77	26.43
PEF	Male	Chinese	Yes	Yes	No	81	21.08
PEF	Female	Chinese	Yes	Yes	Yes	78	25.63
PEF	Female	Chinese	No	Yes	Yes	67	38.67
PEF	Female	Chinese	No	No	No	87	38.27
PEF	Female	Malay	No	Yes	No	81	32.19
PEF	Male	Chinese	No	Yes	No	75	22.22
PEF	Male	Chinese	Yes	Yes	No	52	28.7
PEF	Male	Chinese	No	Yes	Yes	75	28.98
PEF	Male	Chinese	No	Yes	No	78	22.09
PEF	Male	Chinese	Yes	Yes	Yes	72	32.57
PEF	Male	Chinese	No	Yes	Yes	47	34.01
PEF	Female	Malay	Yes	Yes	No	72	35.25
PEF	Male	Chinese	Yes	Yes	No	81	22.41
PEF	Male	Chinese	No	Yes	Yes	68	25.08
PEF	Female	Chinese	Yes	No	No	76	30.5
PEF	Male	Chinese	Yes	No	Yes	64	25.09
PEF	Male	Chinese	No	Yes	Yes	53	28.72
PEF	Female	Chinese	Yes	Yes	Yes	78	35.18
PEF	Male	Chinese	Yes	Yes	Yes	65	32.42
PEF	Male	Chinese	No	Yes	Yes	57	23.15
PEF	Female	Chinese	Yes	Yes	Yes	78	20.27
REF	Male	Chinese	Yes	Yes	Yes	71	21.38
REF	Male	Indian	No	No	No	38	22.65
REF	Female	Chinese	No	Yes	Yes	65	23.07
REF	Male	Indian	Yes	Yes	Yes	62	20.72
REF	Male	Chinese	No	No	Yes	77	22.63
REF	Female	Indian	No	No	Yes	68	28.74
REF	Male	Chinese	No	Yes	No	68	26.17
REF	Male	Chinese	Yes	Yes	Yes	62	23.26
REF	Male	Chinese	Yes	No	No	59	20.24
REF	Female	Chinese	No	Yes	No	72	31.3
REF	Male	Malay	Yes	No	Yes	60	23.58
REF	Male	Chinese	No	Yes	No	63	20.02

REF	Male	Chinese	No	No	No	64	19.81
REF	Female	Chinese	No	Yes	Yes	63	22
REF	Male	Indian	No	Yes	Yes	63	25.03
REF	Female	Chinese	No	Yes	No	72	22.21
REF	Male	Malay	No	Yes	No	51	21.84
REF	Male	Chinese	No	Yes	Yes	71	21.91
REF	Male	Malay	No	Yes	No	71	18.2
REF	Male	Malay	No	Yes	Yes	76	30.16
REF	Male	Malay	No	No	No	62	21.15
REF	Male	Indian	No	No	Yes	55	20.76
REF	Female	Chinese	Yes	Yes	Yes	66	20.08
REF	Male	Chinese	Yes	Yes	No	69	18.47
REF	Female	Malay	No	Yes	Yes	67	23.47
REF	Female	Malay	No	Yes	Yes	56	25.45
REF	Female	Chinese	No	Yes	Yes	55	19.53
REF	Female	Chinese	No	No	No	76	22.52
REF	Male	Malay	No	No	Yes	64	24.22
REF	Male	Malay	No	Yes	Yes	67	24.52
REF	Female	Malay	No	Yes	Yes	57	29.96
REF	Male	Malay	Yes	Yes	No	60	26.76
REF	Female	Chinese	Yes	No	No	56	23.29
REF	Female	Malay	No	Yes	Yes	72	24.89
REF	Female	Chinese	No	No	Yes	65	22.72
REF	Male	Chinese	No	Yes	No	70	16.8
REF	Female	Indian	No	Yes	No	63	19.07
REF	Male	Chinese	No	Yes	Yes	75	23.25
REF	Female	Chinese	No	Yes	Yes	73	26.29
REF	Female	Chinese	Yes	Yes	Yes	72	17.1
REF	Female	Indian	No	Yes	Yes	74	33.19
REF	Female	Chinese	Yes	Yes	Yes	69	27.44
REF	Female	Indian	No	No	Yes	60	17.78
REF	Female	Malay	No	Yes	Yes	78	18.3
REF	Male	Malay	No	Yes	No	68	21.5
REF	Female	Malay	No	Yes	No	47	19.33
REF	Male	Chinese	No	Yes	Yes	53	36.57
REF	Male	Malay	No	Yes	Yes	62	24.24
REF	Female	Chinese	Yes	Yes	No	70	21.33
REF	Male	Indian	No	Yes	Yes	50	22.56
REF	Male	Chinese	No	Yes	No	60	23.57
REF	Male	Chinese	Yes	No	No	59	35.67
REF	Female	Chinese	No	Yes	No	59	23.78
REF	Male	Chinese	No	No	No	40	22.46
REF	Male	Chinese	No	Yes	No	70	15.66

REF	Female	Chinese	No	Yes	Yes	58	20.17
REF	Female	Indian	No	No	Yes	41	29.87
REF	Female	Chinese	Yes	Yes	Yes	62	22.27
REF	Female	Chinese	No	Yes	Yes	79	24.7
REF	Female	Chinese	No	No	Yes	57	23.11
REF	Male	Malay	No	No	Yes	67	23.05
REF	Male	Chinese	Yes	No	Yes	62	22.99
REF	Female	Chinese	No	Yes	Yes	61	31.96
REF	Male	Malay	No	Yes	Yes	56	25.89
REF	Male	Malay	Yes	Yes	Yes	81	17.26
REF	Female	Malay	No	Yes	Yes	64	22.06
REF	Male	Indian	No	No	No	54	27.02
REF	Female	Chinese	No	No	No	57	16.14
REF	Female	Indian	No	Yes	Yes	68	29.85
REF	Male	Malay	No	Yes	Yes	74	25.88
REF	Female	Chinese	No	Yes	Yes	77	21.03
REF	Female	Malay	Yes	Yes	Yes	46	23.98
REF	Male	Chinese	No	No	No	53	22.56
REF	Male	Chinese	No	Yes	No	49	32.32
REF	Male	Malay	No	Yes	Yes	63	25.73
REF	Male	Chinese	No	Yes	Yes	71	25.25
REF	Female	Indian	No	No	Yes	45	20.13
REF	Female	Chinese	No	No	No	59	24.67
REF	Female	Malay	No	Yes	Yes	50	25.69
REF	Male	Chinese	No	Yes	No	46	24.22
REF	Male	Chinese	No	Yes	No	54	28.21
REF	Male	Chinese	No	Yes	Yes	64	25.61
REF	Female	Chinese	No	No	No	81	18.63
REF	Male	Chinese	No	No	No	31	26.54
REF	Male	Malay	No	Yes	Yes	48	28.67
REF	Female	Chinese	Yes	Yes	No	61	28.71
REF	Male	Chinese	No	No	Yes	69	22.27
REF	Female	Chinese	No	No	No	64	20.88
REF	Male	Chinese	No	Yes	No	57	17.44
REF	Male	Chinese	No	Yes	Yes	66	23.15
REF	Female	Chinese	No	Yes	Yes	62	24.22
REF	Male	Chinese	No	Yes	Yes	84	18.81
REF	Female	Chinese	No	No	No	34	19.83
REF	Male	Malay	No	No	Yes	38	28.86
REF	Female	Malay	No	Yes	Yes	61	34.7
REF	Female	Chinese	Yes	No	No	76	17.5
REF	Male	Chinese	No	Yes	No	84	24.61
REF	Male	Chinese	No	Yes	No	39	33.91

REF	Female	Chinese	No	No	Yes	45	24.17
REF	Male	Chinese	No	No	Yes	63	24.5
REF	Female	Indian	No	No	Yes	72	24.85
REF	Male	Chinese	Yes	No	No	60	30.27
REF	Female	Chinese	No	Yes	Yes	73	29.06
REF	Female	Malay	No	No	No	37	35.84
REF	Male	Chinese	No	No	Yes	69	23.25
REF	Female	Indian	No	Yes	Yes	45	29.91
REF	Male	Indian	No	Yes	No	71	20.65
REF	Male	Malay	No	No	Yes	52	25.21
REF	Male	Chinese	No	No	No	55	31.6
REF	Female	Malay	No	Yes	No	50	29.77
REF	Female	Malay	Yes	Yes	No	81	N.A.
REF	Male	Malay	No	Yes	Yes	73	26.01
REF	Male	Malay	No	No	Yes	64	28.91
REF	Male	Chinese	No	No	No	66	17.49
REF	Female	Chinese	No	No	No	49	20.48
REF	Male	Indian	No	Yes	Yes	54	20.61
REF	Male	Chinese	No	Yes	No	73	27.34
REF	Female	Chinese	No	Yes	No	80	24.13
REF	Female	Indian	No	Yes	No	46	43.28
REF	Male	Chinese	No	No	Yes	61	22.14
REF	Female	Chinese	No	Yes	Yes	43	30.1
REF	Male	Malay	No	No	No	50	20.76
REF	Male	Chinese	No	Yes	Yes	54	24.13
REF	Female	Malay	No	Yes	Yes	74	17.12
REF	Female	Chinese	No	Yes	Yes	55	23.07
REF	Female	Chinese	No	No	No	49	33.45
REF	Male	Chinese	No	Yes	No	63	21.23
REF	Male	Chinese	No	Yes	Yes	64	28.35
REF	Female	Malay	No	Yes	Yes	58	24.43
REF	Male	Chinese	No	No	Yes	53	17.07
REF	Female	Indian	No	Yes	No	79	N.A.
REF	Male	Chinese	Yes	Yes	Yes	56	24.68
REF	Male	Chinese	No	Yes	Yes	78	24.44
REF	Male	Malay	No	Yes	Yes	60	28.34
REF	Male	Indian	Yes	No	No	83	16.81
REF	Female	Chinese	No	Yes	Yes	71	N.A.
REF	Female	Chinese	No	No	Yes	56	30.04
REF	Male	Chinese	No	Yes	Yes	54	26.3
REF	Female	Chinese	Yes	No	Yes	57	30.49
REF	Male	Chinese	No	Yes	No	40	25.82
REF	Male	Chinese	Yes	Yes	Yes	59	25.1

REF	Female	Chinese	No	Yes	Yes	63	23.11
REF	Male	Chinese	No	Yes	No	50	26.33
REF	Female	Indian	No	No	No	50	29.24
REF	Female	Malay	No	Yes	Yes	47	26.62
REF	Male	Chinese	No	No	No	38	26.2
REF	Male	Chinese	No	No	No	42	28.74
REF	Male	Malay	No	Yes	No	47	23.94
REF	Male	Indian	No	N.A.	Yes	57	27.28
REF	Female	Malay	No	N.A.	No	35	28.86
REF	Male	Chinese	No	No	Yes	43	28.26
REF	Male	Malay	No	Yes	No	47	27.66
REF	Female	Indian	No	No	Yes	45	27.77
REF	Female	Malay	No	Yes	Yes	40	27.47
REF	Male	Chinese	No	Yes	No	81	32.38
REF	Male	Chinese	No	N.A.	Yes	81	18.09
REF	Male	Chinese	No	No	No	61	21.11
REF	Male	Indian	No	Yes	Yes	82	23.92
REF	Male	Chinese	No	Yes	Yes	62	18.55
REF	Male	Chinese	No	Yes	Yes	53	23.87
REF	Male	Chinese	No	No	No	61	24.62
REF	Male	Chinese	No	Yes	Yes	58	20.76
REF	Male	Malay	No	Yes	Yes	57	23.44
REF	Male	Chinese	No	No	No	45	45.74
REF	Male	Indian	No	Yes	Yes	56	23.44
REF	Male	Chinese	No	Yes	Yes	63	24.53
REF	Male	Indian	No	Yes	Yes	53	30.33
REF	Female	Chinese	No	Yes	No	61	29.83
REF	Male	Chinese	Yes	Yes	Yes	64	29.07
REF	Male	Chinese	No	N.A.	No	76	22.49
REF	Female	Chinese	No	Yes	Yes	71	25.44
REF	Male	Chinese	No	No	Yes	50	26
REF	Male	Chinese	Yes	Yes	Yes	73	N.A.
REF	Male	Chinese	No	No	Yes	55	28.63
REF	Male	Malay	Yes	No	Yes	65	22.68
REF	Male	Chinese	No	Yes	No	65	25.15
REF	Male	Chinese	Yes	N.A.	No	57	19.36
REF	Male	Chinese	Yes	Yes	No	76	31.09
REF	Male	Indian	No	Yes	Yes	62	20.65
REF	Male	Chinese	No	Yes	No	60	19.23

[00167] Plasma NT-proBNP was measured in all samples by electro-chemiluminescence immunoassay (Elecsys proBNP II assay) on an automated Cobas e411 analyzer according to the

manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). A preliminary examination of the distributions in Control, HFREF and HFPEF groups (Figure 2, A-C) showed that the NT-proBNP levels in all groups were positively skewed (skewness/skewing > 2). Since the statistical methods to be applied require an un-skewed distribution (Student's t distribution or logistic distribution), the natural logarithm was calculated for NT-proBNP to generate a new variable: ln_NT-proBNP for which skewness was close to zero (Figure 2, D-F). The ln_NT-proBNP was used for all analyses involving NT-proBNP.

[00168] The characteristics of subject groups are summarized in Table 3.

[00169] **Table 3. characteristics of the healthy subjects and heart failure subjects**

Variables:	**C (n=208)**	**HF (n=338)**	**p-value (C v.s. HF)**	**HFREF (n=180)**	**HFPEF (n=158)**	**p-value (HFREF v.s. HFPEF)**
Left ventricular ejection fraction (100%)	64.0 ± 3.7	42.2 ± 18.7	-	25.9 ± 7.7	60.7 ± 5.9	-
ln_NT-proBNP (100%)	4.02 ± 0.92	7.44 ± 1.5	-	7.95 ± 1.32	6.86 ± 1.49	<0.0001
NT-proBNP	55.8	1704.6		2834.2	955.1	
Gender (male) (100%)	51.0%	51.8%	0.85	60.0%	42.4%	0.0012
Age (100%)	59.7 ± 10.5	64.6 ±12.0	<0.0001	60.8 ± 11.6	68.9 ± 11.0	<0.0001
Race (100%)			0.66			0.27
Chinese	66.8%	63.6%		60.6%	67.1%	
Malay	20.7%	24.0%		24.4%	23.4%	
Indian	12.5%	12.4%		15.0%	9.5%	
Body Mass Index (98.4%)	25.4 ± 5.2	26.0 ± 5.5	0.22	24.7 ± 4.9	27.4 ± 5.9	<0.0001
Arial Fibrillation or Flutter (99.8%)	0.96%	24.9%	<0.0001	16.7%	34.4%	0.00017
Hypertension (99.0%)	33.7%	75.9%	<0.0001	65.7%	87.3%	<0.0001
Diabetes (99.8%)	9.6%	58.8%	<0.0001	58.9%	58.6%	0.96

[00170] Besides demographic variables including age, race and gender, clinical variables critical for HF were recorded including LVEF, ln_NT-proBNP, Body Mass Index (BMI), Atrial Fibrillation or Flutter (AF), hypertension and diabetes. HFPEF patients had similar mean LVEF (60.7 ± 5.9) as healthy control subjects (64.0 ± 3.7) whilst, as expected and as per patient selection and allocation, HFREF patients clearly had lower LVEF (25.9 ± 7.7). 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 between control and HF (C vs HF, Table 3) and between HFPEF and HFREF (HFREF vs HFPEF,

Table 3). In general, HF patients were older, with higher prevalence of hypertension, AF and diabetes compared to controls. HFREF and HFPEF patients differed with respect to distributions of gender, age, BMI, hypertension and AF. All these differently distributed variables were taken into account in the discovery of miRNA biomarkers for HF detection or for HF subtype categorization by multivariate logistic regression.

[00171] Ln_NT-proBNP was lower in HFPEF than HFREF with some results falling below the ESC- promoted NT-proBNP cut-off (<125 pg/ml) for diagnosis of HF in the non-acute setting [57]. The loss of NT-proBNP test performance is pronounced in HFPEF (Figure 3, A). The performance of ln_NT-proBNP as a biomarker for the diagnosis of HF was examined by the ROC analysis. In this study, ln_NT-proBNP had 0.962 AUC (area under the ROC curve) for the diagnosis of HF overall. It performed better for detecting HFREF (AUC=0.985) than HFPEF (AUC=0.935) (Figure 3, B-D). ln_NT-proBNP exhibited an AUC of only 0.706 for categorizing HFREF and HFPEF subtypes (Figure 3, C).

[00172] **II. MiRNA measurement**

[00173] Circulating cell-free miRNAs in the blood originate from various organs and blood cells [58]. Therefore the change in the levels of a miRNA caused by heart failure may be partly obscured by the presence of the same miRNA possibly secreted from other sources due to other stimuli. Thus, determining the differences in expression levels of miRNAs found in heart failure and the control group may be challenging. In addition, most of the cell-free miRNAs are of exceptionally low abundance in blood [59]. Therefore, accurate measurement of multiple miRNA targets from limited volume of serum/plasma is critical and highly challenging. To best facilitate the discovery of significantly altered expressions of miRNAs and the identification of multivariate miRNA biomarker panels for the diagnosis of heart failure, instead of using low sensitivity or semi-quantitative screening methods (microarray, sequencing), the inventors of the present study chose to perform qPCR-based assays with an exceptionally well designed workflow (Figure 4).

[00174] All qPCR assays (designed by MiRXES^{TM}, Singapore) were performed at least twice in a single-plex for miRNA targets and at least four repeats for synthetic RNA 'spike-in' controls. To ensure the accuracy of the results in a high-throughput qPCR studies, the study designed and established, after much iteration, a robust workflow for the discovery of circulating biomarkers (Refer to the "METHOD" and Figure 4). In this novel workflow, various designed 'spike-in' controls were used to monitor and correct for technical variations in isolation, reverse transcription, augmentation and the qPCR processes. All spike-in controls were non-natural synthetic miRNAs mimics (small single-stranded RNA with length range from 22-24 bases) which were designed *in silico* to have exceptionally low similarity in the sequence to all known human miRNAs, thus minimizing cross-

hybridization to the primers used in the assays. In addition, the miRNA assays were deliberately divided into a number of multiplex groups *in silico* to minimize non-specific amplifications and primer-primer interactions. Synthetic miRNAs were used to construct standard curves for the interpolation of absolute copy numbers in all the measurements, thus further correcting for technical variations. Predictably, with this highly robust workflow and multiple levels of controls, the study were able to identify low levels of expression of miRNAs in circulation and the approach of the present study is highly reliable and reproducibility of data is ensured.

[00175] Two hundred and three (203) miRNA targets were selected for this study based on the prior-knowledge of highly expressed plasma miRNAs (data not shown) and the expression levels of those miRNAs in all 546 plasma samples (HF and control) were quantitatively measured using highly sensitive qPCR assays (designed by MiRXES^{TM}, Singapore).

[00176] In the current experimental design, total RNA including miRNAs was extracted from 200 µl plasma. Extracted RNA was reversed transcribed and augmented by touch-down amplification to increase the amount of cDNA without changing the total miRNA expression levels (Figure 4). The augmented cDNA was then diluted for qPCR measurement. A simple calculation based on the effect of dilution revealed that a miRNA which is expressed at levels ≤500 copies/ ml in serum will be quantified at levels close to the detection limit of the single-plex qPCR assay (≤10 copies/well). At such a concentration, measurements will be a significant challenge due to the technical limitations (errors in pipetting and qPCR reactions). Thus, miRNAs expressed at concentration of ≤500 copies/ ml were excluded from analyses and considered undetectable.

[00177] About 70% (n=137) of the total miRNA assayed were found to be highly expressed across all the samples. These 137 miRNAs were detected in more than 90% of the samples (expression levels ≥500 copies/ ml; Table 4). As compare to published data (Table 1), the inventors of the present study detected many more miRNAs not previously reported in heart failure, highlighting the importance of the use of the careful and well-controlled experimental design.

[00178] **Table 4. sequence of 137 reliably detected mature miRNA**

Name	Sequence	SEQ ID NO:
hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA	1
hsa-miR-134	UGUGACUGGUUGACCAGAGGGG	2
hsa-let-7b-3p	CUAUACAACCUACUGCCUUCCC	3
hsa-miR-34b-3p	CAAUCACUAACUCCACUGCCAU	4
hsa-miR-101-5p	CAGUUAUCACAGUGCUGAUGCU	5
hsa-miR-550a-5p	AGUGCCUGAGGGAGUAAGAGCCC	6
hsa-miR-576-5p	AUUCUAAUUUCUCCACGUCUUU	7
hsa-miR-181b-5p	AACAUUCAUUGCUGUCGGUGGGU	8
hsa-miR-197-3p	UUCACCACCUUCUCCACCCAGC	9
hsa-miR-369-3p	AAUAAUACAUGGUUGAUCUUU	10

hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG	11
hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA	12
hsa-miR-379-5p	UGGUAGACUAUGGAACGUAGG	13
hsa-miR-579	UUCAUUUGGUAUAAACCGCGAUU	14
hsa-miR-106b-3p	CCGCACUGUGGGUACUUGCUGC	15
hsa-miR-497-5p	CAGCAGCACACUGUGGUUUGU	16
hsa-miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC	17
hsa-miR-19b-3p	UGUGCAAAUCCAUGCAAAACUGA	18
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	19
hsa-miR-424-5p	CAGCAGCAAUUCAUGUUUUGAA	20
hsa-miR-144-3p	UACAGUAUAGAUGAUGUACU	21
hsa-miR-154-5p	UAGGUUAUCCGUGUUGCCUUCG	22
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	23
hsa-miR-30d-5p	UGUAAACAUCCCCGACUGGAAG	24
hsa-miR-30e-3p	CUUUCAGUCGGAUGUUUACAGC	25
hsa-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG	26
hsa-miR-374c-5p	AUAAUACAACCUGCUAAGUGCU	27
hsa-miR-495	AAACAAACAUGGUGCACUUCUU	28
hsa-miR-1275	GUGGGGGAGAGGCUGUC	29
hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU	30
hsa-miR-23a-3p	AUCACAUUGCCAGGGAUUUCC	31
hsa-miR-27a-3p	UUCACAGUGGCUAAGUUCCGC	32
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	33
hsa-miR-133a	UUUGGUCCCCUUCAACCAGCUG	34
hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCU	35
hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG	36
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	37
hsa-miR-30b-5p	UGUAAACAUCCUACACUCAGCU	38
hsa-let-7e-3p	CUAUACGGCCUCCUAGCUUUCC	39
hsa-miR-337-3p	CUCCUAUAUGAUGCCUUUCUUC	40
hsa-miR-363-3p	AAUUGCACGGUAUCCAUCUGUA	41
hsa-miR-421	AUCAACAGACAUUAAUUGGGCGC	42
hsa-miR-335-5p	UCAAGAGCAAUAACGAAAAAUGU	43
hsa-miR-518b	CAAAGCGCUCCCCUUUAGAGGU	44
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	45
hsa-miR-660-5p	UACCCAUUGCAUAUCGGAGUUG	46
hsa-miR-192-5p	CUGACCUAUGAAUUGACAGCC	47
hsa-miR-199b-5p	CCCAGUGUUUAGACUAUCUGUUC	48
hsa-miR-19a-3p	UGUGCAAAUCUAUGCAAAACUGA	49
hsa-miR-493-5p	UUGUACAUGGUAGGCUUUCAUU	50
hsa-miR-377-3p	AUCACACAAAGGCAACUUUUGU	51
hsa-miR-500a-5p	UAAUCCUUGCUACCUGGGUGAGA	52
hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	53

hsa-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU	54
hsa-miR-299-3p	UAUGUGGGAUGGUAAACCGCUU	55
hsa-miR-15b-5p	UAGCAGCACAUCAUGGUUUACA	56
hsa-miR-21-3p	CAACACCAGUCGAUGGGCUGU	57
hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG	58
hsa-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC	59
hsa-miR-22-3p	AAGCUGCCAGUUGAAGAACUGU	60
hsa-miR-23b-3p	AUCACAUUGCCAGGGAUUACC	61
hsa-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA	62
hsa-miR-29b-3p	UAGCACCAUUUGAAAUCAGUGUU	63
hsa-miR-33a-5p	GUGCAUUGUAGUUGCAUUGCA	64
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	65
hsa-miR-124-5p	CGUGUUCACAGCGGACCUUGAU	66
hsa-miR-532-5p	CAUGCCUUGAGUGUAGGACCGU	67
hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA	68
hsa-miR-222-3p	AGCUACAUCUGGCUACUGGGU	69
hsa-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA	70
hsa-miR-451a	AAACCGUUACCAUUACUGAGUU	71
hsa-miR-1226-3p	UCACCAGCCCUGUGUUCCCUAG	72
hsa-miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU	73
hsa-miR-374b-5p	AUAUAAUACAACCUGCUAAGUG	74
hsa-miR-4732-3p	GCCCUGACCUGUCCUGUUCUG	75
hsa-miR-487b	AAUCGUACAGGGUCAUCCACUU	76
hsa-miR-551b-3p	GCGACCCAUACUUGGUUUCAG	77
hsa-miR-23c	AUCACAUUGCCAGUGAUUACCC	78
hsa-miR-183-5p	UAUGGCACUGGUAGAAUUCACU	79
hsa-miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA	80
hsa-miR-425-3p	AUCGGGAAUGUCGUGUCCGCCC	81
hsa-miR-484	UCAGGCUCAGUCCCCUCCCGAU	82
hsa-miR-485-3p	GUCAUACACGGCUCUCCUCUCU	83
hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	84
hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU	85
hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG	86
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG	87
hsa-miR-10b-5p	UACCCUGUAGAACCGAAUUUGUG	88
hsa-miR-130b-3p	CAGUGCAAUGAUGAAAGGGCAU	89
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG	90
hsa-miR-133b	UUUGGUCCCCUUCAACCAGCUA	91
hsa-miR-186-5p	CAAAGAAUUCUCCUUUUGGGCU	92
hsa-miR-193a-5p	UGGGUCUUUGCGGGCGAGAUGA	93
hsa-miR-23a-5p	GGGGUUCCUGGGGAUGGGAUUU	94
hsa-miR-454-3p	UAGUGCAAUAUUGCUUAUAGGGU	95
hsa-miR-501-5p	AAUCCUUUGUCCCUGGGUGAGA	96

hsa-miR-18b-5p	UAAGGUGCAUCUAGUGCAGUUAG	97
hsa-miR-223-5p	CGUGUAUUUGACAAGCUGAGUU	98
hsa-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC	99
hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU	100
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU	101
hsa-miR-452-5p	AACUGUUUGCAGAGGAAACUGA	102
hsa-miR-148a-3p	UCAGUGCACUACAGAACUUUGU	103
hsa-miR-194-5p	UGUAACAGCAACUCCAUGUGGA	104
hsa-miR-29c-5p	UGACCGAUUUCUCCUGGUGUUC	105
hsa-miR-196b-5p	UAGGUAGUUUCCUGUUGUUGGG	106
hsa-miR-345-5p	GCUGACUCCUAGUCCAGGGCUC	107
hsa-miR-503	UAGCAGCGGGAACAGUUCUGCAG	108
hsa-miR-627	GUGAGUCUCUAAGAAAAGAGGA	109
hsa-let-7d-3p	CUAUACGACCUGCUGCCUUUCU	110
hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAG	111
hsa-miR-654-3p	UAUGUCUGCUGACCAUCACCUU	112
hsa-miR-598	UACGUCAUCGUUGUCAUCGUCA	113
hsa-miR-671-3p	UCCGGUUCUCAGGGCUCCACC	114
hsa-miR-132-3p	UAACAGUCUACAGCCAUGGUCG	115
hsa-miR-142-5p	CAUAAAGUAGAAAGCACUACU	116
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU	117
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	118
hsa-miR-185-5p	UGGAGAGAAAGGCAGUUCCUGA	119
hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG	120
hsa-miR-99b-5p	CACCCGUAGAACCGACCUUGCG	121
hsa-miR-128	UCACAGUGAACCGGUCUCUUU	122
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	123
hsa-miR-32-5p	UAUUGCACAUUACUAAGUUGCA	124
hsa-miR-382-5p	GAAGUUGUUCGUGGUGGAUUCG	125
hsa-miR-532-3p	CCUCCCACACCCAAGGCUUGCA	126
hsa-miR-181a-2-3p	ACCACUGACCGUUGACUGUACC	127
hsa-miR-139-5p	UCUACAGUGCACGUGUCUCCAG	128
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	129
hsa-miR-1280	UCCCACCGCUGCCACCC	130
hsa-miR-331-5p	CUAGGUAUGGUCCCAGGGAUCC	131
hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG	132
hsa-miR-101-3p	UACAGUACUGUGAUAACUGAA	133
hsa-miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA	134
hsa-miR-205-5p	UCCUUCAUUCCACCGGAGUCUG	135
hsa-miR-505-3p	CGUCAACACUUGCUGGUUUCCU	136
hsa-miR-136-5p	ACUCCAUUUGUUUUGAUGAUGGA	137

[00179] **III. MiRNA biomarkers**

[00180] Firstly, all measured miRNAs were examined for targets that were only detectable in heart failure samples but not in control samples. Those miRNAs specifically secreted by heart muscles in heart failure patients would be the ideal biomarker for the detection of the disease. As the miRNAs in the blood circulating system are known to be contributed by various organs and/or type of cells (including heart muscles), it was not surprising that these miRNAs may already been represented in the plasma of normal and heart failure patients. However, the differential expression of these miRNAs in the plasma may still serve as useful biomarker during the development of heart failure.

[00181] The global unsupervised analysis (principal component analysis, PCA) was initially performed on the expression levels of all detected plasma miRNAs (137, Table 4) in all 546 samples. The first 15 principal components (PCs) with eigenvalues higher than 0.7 were selected for further analysis, which in total accounted for 85% of the variance (Figure 5, A). To examine the difference between the control and heart failure, the AUCs were calculated for the classification of those two groups at each of the selected PCs (Figure 5, B). Multiple PCs were found to have AUCs significantly higher than 0.5 and the 2nd PC even had an AUC of 0.79 indicating that the differences between those two groups largely contributed to the overall variance of the miRNA expression profile. As the variations between the control and heart failure subjects were found in multiple dimensions (PCs), it was not possible to represent all the information based on single miRNA. Thus a multivariate assay including multiple miRNAs was necessary for optimal classification. Similarly, multiple PCs had AUCs significantly higher than 0.5 for the categorization to either of two heart failure subtypes: HFREF or HFPEF (Figure 5, C) including the 1st PC (AUC = 0.6) although the AUCs were less than those for heart failure detection. Hence, a multivariate assay was necessary to capture the information in multiple dimensions for the classification HFREF and HFPEF as well.

[00182] Plotting the two groups of subjects (C and heart failure (HF)) on a space defined by the two major discriminative PCs for HF detection, showed they were separately located (Figure 6, A). Separation of HFREF and HFPEF groups (Figure 6, B) was less distinct. The global analysis revealed that it was possible to separate control, HFREF and HFPEF subjects based their miRNA profiles. However, using only one or two dimensions was not statistically robust for classification.

[00183] A pivotal step towards identifying biomarkers is to directly compare the expression levels of each miRNA in normal and disease state as well as between disease subtypes. Student's t-test was used for univariate comparisons to assess the significance of between group differences in individual miRNA and multivariate logistic regression was used to adjust for confounding factors including age, gender, BMI, AF, hypertension and diabetes. All p-values were corrected for false discovery rate (FDR) estimation using Bonferroni-type multiple comparison procedures [60]. MiRNAs with p-values lower than 0.01 were considered significant in this study.

[00184] The expressions of the 137 plasma miRNAs were then compared A] Between control (healthy) and heart failure (individual subtypes or both subtypes grouped together), B] Between the two subtypes of heart failure (i.e. HFREF and HFPEF).

[00185] **A] Identification of miRNAs differentially expressed between non-HF Control subjects and HF patients**

[00186] Plasma from patients clinically confirmed to have either subtype of heart failure (HFREF or HFPEF) were grouped together and compared to plasma from healthy non-heart failure donors.

[00187] The comparisons were initially carried out using univariate analysis (Student's t-test) where 94 miRNAs were found to be significantly altered in heart failure patients compared to Control (p-value after FDR < 0.01) (Figure 7, A). Further examining the two subtypes separately, 82 and 94 miRNAs were found to be significant altered in HFREF and HFPEF subjects compared to control respectively (Figure 7, A). In total, 101 unique miRNAs were identified by univariate analysis with 75% (n=76) of them significant for both subtypes (Figure 7A).

[00188] Since the control subjects were recruited from the community, clinical parameters may not be well matched with the heart failure patients including three risk factors for heart failure: AF, hypertension and diabetes where fewer of the control subjects had such conditions. Also, age differed slightly between the analyzed populations. In order to adjust for those possible confounding factors, multivariate analysis (logistic regression) was performed to test the significance of the miRNAs selected by univariate analysis. In total, 86 out of the 101 miRNAs still differed significantly between test populations after multivariate analysis (Figure 7, B). For the detection of all heart failure compared to control, 75 out of the 94 miRNAs (Table 5) were found to be significant (p-value after FDR< 0.01) in the multivariate analysis; while 52 out of 82 (Table 6) were significant for the detection of HFREF comparing to control and 68 out of 94 (Table 7) were significant for the detection of HFPEF comparing to control (Figure 7B). After multivariate analysis, 36 miRNAs were found to remain significantly different between controls and both heart failure subtypes while 16 differed significantly only between Control and HFREF subtype and 32 differed significantly only between Control and HFPEF subtype (Figure 7, B). In the multivariate analysis, many miRNAs were found to differ between Control and only one of the two heart failure subtypes suggesting genuine differences between the two subtypes in terms of miRNA expression.

[00189] **Table 5. miRNAs differentially expressed between control and all heart failure subjects**

Up-regulated (n=37)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-let-7d-3p	8.9E-23	4.1E-09	3.8E-05	1.32	0.78

hsa-miR-197-3p	8.9E-23	2.7E-08	7.9E-05	1.27	0.77
hsa-miR-24-3p	2.8E-22	5.5E-10	6.7E-05	1.30	0.76
hsa-miR-1280	4.3E-16	3.3E-06	3.0E-04	1.41	0.74
hsa-miR-221-3p	5.4E-19	4.9E-09	6.2E-05	1.35	0.73
hsa-miR-503	1.1E-17	1.2E-07	9.7E-04	1.69	0.73
hsa-miR-130b-3p	1.2E-14	3.9E-07	1.3E-03	1.27	0.72
hsa-miR-23b-3p	1.1E-13	2.6E-06	8.9E-04	1.31	0.71
hsa-miR-21-3p	2.4E-14	8.0E-06	>0.01	1.25	0.70
hsa-miR-223-5p	4.4E-13	1.6E-06	9.2E-04	1.23	0.70
hsa-miR-423-5p	5.6E-14	4.9E-09	6.1E-04	1.27	0.70
hsa-miR-34b-3p	9.5E-14	4.6E-04	>0.01	1.84	0.69
hsa-miR-22-3p	1.6E-11	1.5E-04	>0.01	1.24	0.69
hsa-miR-148a-3p	2.0E-12	2.3E-06	1.8E-03	1.28	0.68
hsa-miR-23a-5p	6.2E-11	4.3E-04	>0.01	1.25	0.67
hsa-miR-335-5p	1.3E-10	2.5E-06	2.3E-04	1.33	0.67
hsa-miR-124-5p	3.8E-09	9.8E-04	>0.01	1.54	0.66
hsa-miR-382-5p	6.0E-10	1.7E-05	7.6E-03	1.56	0.66
hsa-miR-134	6.4E-10	2.9E-05	6.7E-03	1.57	0.66
hsa-let-7e-3p	7.6E-07	1.1E-03	>0.01	1.33	0.65
hsa-miR-598	4.9E-08	4.8E-05	>0.01	1.20	0.65
hsa-miR-627	2.8E-08	5.5E-04	>0.01	1.31	0.65
hsa-miR-199a-3p	1.3E-05	4.1E-03	>0.01	1.27	0.64
hsa-miR-27b-3p	1.6E-06	8.7E-04	3.8E-04	1.20	0.64
hsa-miR-146b-5p	6.3E-07	8.7E-04	3.4E-04	1.25	0.64
hsa-miR-146a-5p	3.1E-06	4.3E-03	9.7E-04	1.25	0.64
hsa-miR-331-5p	2.7E-07	2.7E-03	>0.01	1.13	0.64
hsa-miR-654-3p	7.4E-08	2.0E-03	>0.01	1.44	0.63
hsa-miR-375	1.1E-05	7.9E-03	>0.01	1.43	0.63
hsa-miR-132-3p	9.8E-07	7.4E-04	>0.01	1.12	0.63
hsa-miR-27a-3p	2.0E-05	2.4E-03	4.9E-03	1.16	0.63
hsa-miR-128	5.9E-06	8.6E-04	>0.01	1.11	0.63
hsa-miR-299-3p	2.9E-06	3.3E-03	>0.01	1.43	0.62
hsa-miR-424-5p	4.0E-07	1.3E-03	>0.01	1.25	0.62
hsa-miR-154-5p	5.9E-06	1.0E-03	>0.01	1.41	0.62
hsa-miR-21-5p	6.5E-07	4.0E-03	>0.01	1.16	0.61
hsa-miR-377-3p	1.3E-05	3.9E-03	>0.01	1.37	0.60
Down-regulated n=(38)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, BNP**	**Fold change**	**AUC**
hsa-miR-454-3p	3.3E-43	3.0E-14	5.6E-06	0.47	0.85
hsa-miR-103a-3p	1.6E-35	7.2E-12	2.4E-05	0.70	0.82
hsa-miR-30c-5p	8.9E-23	1.9E-10	3.2E-04	0.65	0.75
hsa-miR-30b-5p	1.9E-22	2.2E-09	3.0E-03	0.64	0.75

hsa-miR-17-5p	2.4E-19	1.4E-06	3.4E-04	0.73	0.74
hsa-miR-196b-5p	2.2E-15	7.8E-06	2.0E-04	0.79	0.73
hsa-miR-500a-5p	5.4E-19	1.1E-07	3.8E-04	0.68	0.73
hsa-miR-106a-5p	1.1E-16	1.3E-06	4.7E-05	0.76	0.72
hsa-miR-20a-5p	2.6E-17	1.4E-06	7.9E-05	0.74	0.72
hsa-miR-451a	5.4E-19	9.8E-08	7.9E-05	0.54	0.72
hsa-miR-29b-3p	1.5E-16	4.6E-08	6.7E-05	0.76	0.71
hsa-miR-374b-5p	2.4E-16	1.1E-07	1.8E-03	0.69	0.71
hsa-miR-20b-5p	1.5E-16	2.3E-06	8.1E-05	0.60	0.71
hsa-miR-501-5p	2.2E-14	3.3E-06	1.2E-04	0.71	0.70
hsa-miR-18b-5p	4.4E-13	3.9E-05	4.7E-05	0.78	0.69
hsa-miR-23c	3.1E-12	1.2E-06	>0.01	0.68	0.69
hsa-miR-551b-3p	3.0E-12	3.2E-05	>0.01	0.65	0.69
hsa-miR-26a-5p	4.7E-13	3.9E-05	>0.01	0.74	0.69
hsa-miR-183-5p	1.8E-12	2.8E-05	3.8E-04	0.59	0.68
hsa-miR-16-5p	4.2E-12	1.9E-05	8.4E-04	0.71	0.68
hsa-miR-191-5p	1.8E-12	9.3E-05	>0.01	0.74	0.68
hsa-miR-532-5p	1.2E-11	8.0E-06	4.9E-04	0.77	0.67
hsa-miR-363-3p	3.9E-11	1.7E-04	2.7E-03	0.70	0.67
hsa-miR-374c-5p	4.5E-10	3.7E-04	>0.01	0.71	0.67
hsa-let-7b-5p	3.5E-11	3.8E-04	>0.01	0.80	0.66
hsa-miR-15a-5p	3.8E-09	9.8E-04	4.7E-03	0.82	0.66
hsa-miR-144-3p	3.9E-11	9.4E-05	3.8E-04	0.63	0.66
hsa-miR-93-5p	1.3E-09	3.8E-04	1.3E-03	0.82	0.66
hsa-miR-181b-5p	3.1E-09	1.2E-07	>0.01	0.80	0.66
hsa-miR-19b-3p	2.3E-09	3.4E-05	8.3E-05	0.80	0.65
hsa-miR-4732-3p	2.4E-08	4.7E-04	3.5E-03	0.70	0.64
hsa-miR-484	5.9E-07	9.9E-03	>0.01	0.89	0.64
hsa-miR-25-3p	3.3E-07	4.4E-03	8.8E-03	0.79	0.63
hsa-miR-192-5p	8.9E-06	9.9E-04	>0.01	0.76	0.63
hsa-miR-205-5p	3.2E-05	2.0E-03	>0.01	0.75	0.62
hsa-miR-19a-3p	2.2E-06	1.1E-03	6.9E-04	0.84	0.61
hsa-miR-32-5p	7.5E-06	8.3E-03	>0.01	0.88	0.61
hsa-miR-150-5p	1.2E-05	2.9E-05	>0.01	0.79	0.60

[00190] **Table 6. miRNAs differentially expressed between control and REF subjects**

Up-regulated (n=25)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-miR-423-5p	5.5E-18	1.8E-09	>0.01	1.32	0.75
hsa-let-7d-3p	5.0E-15	1.2E-06	>0.01	1.26	0.75
hsa-miR-24-3p	5.9E-15	5.1E-07	>0.01	1.27	0.74
hsa-miR-503	5.0E-15	1.9E-06	>0.01	1.74	0.74

hsa-miR-197-3p	6.6E-14	6.7E-05	>0.01	1.22	0.73
hsa-miR-1280	4.2E-11	1.9E-04	>0.01	1.34	0.72
hsa-miR-22-3p	7.3E-11	1.1E-04	>0.01	1.27	0.71
hsa-miR-130b-3p	1.6E-10	1.1E-05	>0.01	1.23	0.71
hsa-miR-221-3p	4.4E-12	1.2E-06	>0.01	1.31	0.71
hsa-miR-34b-3p	2.4E-12	7.2E-04	>0.01	1.90	0.70
hsa-miR-30a-5p	2.0E-10	1.1E-03	>0.01	1.39	0.70
hsa-miR-21-3p	1.1E-09	3.6E-04	>0.01	1.22	0.69
hsa-miR-132-3p	7.4E-09	6.8E-04	>0.01	1.16	0.68
hsa-miR-331-5p	2.7E-09	2.2E-03	>0.01	1.18	0.68
hsa-miR-124-5p	3.3E-09	5.6E-04	>0.01	1.62	0.67
hsa-miR-148a-3p	1.2E-08	2.6E-04	>0.01	1.26	0.66
hsa-miR-23b-3p	3.2E-07	2.0E-04	>0.01	1.22	0.66
hsa-miR-375	1.0E-06	3.8E-03	>0.01	1.55	0.65
hsa-miR-134	2.4E-06	4.2E-04	>0.01	1.48	0.64
hsa-miR-627	1.4E-05	2.2E-03	>0.01	1.28	0.64
hsa-miR-382-5p	6.2E-06	6.4E-04	>0.01	1.45	0.63
hsa-miR-598	6.1E-05	2.6E-04	>0.01	1.16	0.63
hsa-miR-23a-5p	1.4E-05	3.3E-03	>0.01	1.17	0.63
hsa-miR-223-5p	3.7E-04	9.3E-03	>0.01	1.12	0.62
hsa-miR-335-5p	1.6E-04	2.6E-04	>0.01	1.19	0.61
Down-regulated (n=27)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-miR-454-3p	2.9E-30	2.0E-11	>0.01	0.48	0.83
hsa-miR-103a-3p	8.8E-23	3.0E-09	>0.01	0.74	0.79
hsa-miR-30b-5p	3.7E-19	3.0E-08	>0.01	0.62	0.76
hsa-miR-30c-5p	3.7E-19	1.1E-08	>0.01	0.64	0.76
hsa-miR-374b-5p	1.1E-15	5.7E-07	>0.01	0.66	0.73
hsa-miR-23c	1.3E-13	2.7E-07	>0.01	0.63	0.72
hsa-miR-551b-3p	6.9E-11	1.5E-04	>0.01	0.63	0.70
hsa-miR-17-5p	1.5E-11	2.6E-04	>0.01	0.80	0.70
hsa-miR-26a-5p	6.9E-11	6.7E-05	>0.01	0.73	0.70
hsa-miR-181b-5p	1.9E-11	1.4E-06	>0.01	0.73	0.70
hsa-miR-500a-5p	2.1E-10	6.7E-05	>0.01	0.73	0.69
hsa-miR-196b-5p	1.0E-07	1.7E-03	>0.01	0.84	0.68
hsa-miR-374c-5p	1.4E-08	6.3E-04	>0.01	0.70	0.68
hsa-miR-451a	2.1E-10	1.1E-04	>0.01	0.62	0.68
hsa-miR-29b-3p	2.8E-09	8.8E-05	>0.01	0.80	0.67
hsa-miR-20a-5p	1.7E-08	7.8E-04	>0.01	0.81	0.67
hsa-miR-191-5p	1.7E-08	4.1E-04	>0.01	0.77	0.66
hsa-miR-106a-5p	3.0E-07	4.7E-03	>0.01	0.85	0.65
hsa-miR-181a-2-3p	1.1E-04	5.9E-03	>0.01	0.84	0.65

hsa-miR-501-5p	4.3E-06	4.3E-03	>0.01	0.80	0.64
hsa-miR-20b-5p	2.0E-06	4.0E-03	>0.01	0.75	0.63
hsa-miR-183-5p	5.8E-06	1.4E-03	>0.01	0.69	0.63
hsa-miR-16-5p	1.6E-05	3.8E-03	>0.01	0.79	0.63
hsa-miR-125a-5p	7.9E-05	6.4E-04	>0.01	0.81	0.62
hsa-miR-150-5p	4.3E-05	2.0E-04	>0.01	0.78	0.61
hsa-miR-205-5p	3.3E-04	5.9E-03	>0.01	0.76	0.61
hsa-miR-532-5p	2.3E-04	9.3E-03	>0.01	0.85	0.61

[00191] **Table 7. MiRNAs differentially expressed between control and HFPEF subjects**

Up-regulated (n=33)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-let-7d-3p	4.40E-22	5.90E-07	4.10E-05	1.39	0.81
hsa-miR-197-3p	4.10E-24	2.10E-07	6.40E-05	1.34	0.81
hsa-miR-223-5p	6.80E-21	2.10E-07	7.80E-05	1.37	0.79
hsa-miR-24-3p	5.80E-19	2.10E-07	4.10E-05	1.33	0.78
hsa-miR-23b-3p	1.60E-16	1.40E-05	2.60E-04	1.41	0.76
hsa-miR-221-3p	5.90E-17	5.90E-07	4.10E-05	1.4	0.76
hsa-miR-1280	3.00E-14	2.20E-04	9.10E-04	1.49	0.75
hsa-miR-130b-3p	2.20E-14	9.00E-05	9.10E-04	1.32	0.74
hsa-miR-335-5p	2.40E-14	5.30E-06	4.10E-05	1.5	0.73
hsa-miR-503	1.40E-11	3.80E-05	5.80E-04	1.64	0.72
hsa-miR-23a-5p	1.90E-12	1.40E-03	3.60E-03	1.34	0.72
hsa-miR-21-3p	8.50E-13	8.20E-05	3.60E-03	1.29	0.72
hsa-miR-148a-3p	4.40E-11	1.10E-04	2.20E-03	1.3	0.7
hsa-miR-199a-3p	2.70E-07	8.40E-04	2.20E-03	1.38	0.69
hsa-miR-146a-5p	3.10E-08	2.50E-04	1.50E-04	1.37	0.69
hsa-miR-382-5p	1.00E-09	1.00E-04	1.10E-03	1.69	0.68
hsa-miR-134	3.90E-09	2.90E-04	1.60E-03	1.67	0.68
hsa-let-7e-3p	4.00E-08	1.30E-03	8.50E-03	1.43	0.68
hsa-miR-146b-5p	9.90E-08	1.60E-04	6.40E-05	1.33	0.67
hsa-miR-27b-3p	5.10E-07	6.70E-04	1.50E-04	1.26	0.67
hsa-miR-598	3.10E-08	1.20E-03	>0.01	1.25	0.67
hsa-miR-27a-3p	1.60E-06	3.40E-04	5.80E-04	1.21	0.67
hsa-miR-128	2.60E-07	2.90E-04	2.50E-03	1.15	0.67
hsa-miR-627	4.20E-07	8.70E-03	>0.01	1.36	0.66
hsa-miR-299-3p	2.30E-07	2.30E-03	>0.01	1.59	0.65
hsa-miR-21-5p	2.50E-07	1.30E-03	>0.01	1.21	0.65
hsa-miR-425-3p	1.30E-04	9.30E-04	1.60E-03	1.15	0.65
hsa-miR-154-5p	1.10E-06	9.10E-04	3.60E-03	1.55	0.64
hsa-miR-377-3p	2.40E-06	8.30E-03	>0.01	1.52	0.64
hsa-miR-424-5p	3.50E-06	3.00E-03	>0.01	1.28	0.63

hsa-miR-423-5p	2.50E-07	1.30E-03	4.10E-03	1.23	0.63
hsa-miR-99b-5p	8.30E-04	9.30E-03	4.60E-03	1.18	0.62
hsa-miR-671-3p	6.80E-04	8.60E-03	9.40E-03	1.18	0.61
Down-regulated (n=35)					
Name	**p-value, t-test**	**p-value, Logistic regression**	**p-value, ln_BNP**	**Fold change**	**AUC**
hsa-miR-454-3p	8.9E-36	5.9E-07	4.1E-05	0.45	0.87
hsa-miR-103a-3p	8.9E-36	1.3E-06	4.5E-05	0.65	0.86
hsa-miR-106a-5p	3.4E-22	5.9E-07	4.1E-05	0.68	0.80
hsa-miR-17-5p	4.0E-21	2.5E-05	2.9E-04	0.67	0.80
hsa-miR-20b-5p	4.1E-24	5.0E-07	4.1E-05	0.47	0.79
hsa-miR-20a-5p	7.0E-21	2.1E-06	6.4E-05	0.66	0.79
hsa-miR-196b-5p	5.6E-18	2.8E-05	1.8E-04	0.75	0.78
hsa-miR-451a	3.3E-21	5.9E-07	7.0E-05	0.46	0.78
hsa-miR-18b-5p	3.0E-17	8.2E-05	9.2E-05	0.69	0.77
hsa-miR-500a-5p	2.1E-19	7.0E-06	1.6E-04	0.62	0.77
hsa-miR-29b-3p	1.0E-18	1.3E-06	6.2E-05	0.72	0.76
hsa-miR-501-5p	5.2E-19	2.4E-06	4.5E-05	0.62	0.76
hsa-miR-532-5p	1.3E-16	1.2E-06	7.0E-05	0.69	0.75
hsa-let-7b-5p	1.1E-16	3.8E-05	9.0E-04	0.71	0.74
hsa-miR-30c-5p	1.3E-15	1.0E-04	2.1E-03	0.67	0.74
hsa-miR-183-5p	2.9E-15	3.8E-05	3.8E-04	0.50	0.74
hsa-miR-30b-5p	3.0E-15	5.7E-04	>0.01	0.66	0.74
hsa-miR-144-3p	1.9E-16	3.6E-06	1.5E-04	0.51	0.73
hsa-miR-93-5p	4.6E-15	2.5E-05	5.0E-04	0.74	0.73
hsa-miR-16-5p	6.5E-15	4.1E-06	2.6E-04	0.62	0.73
hsa-miR-363-3p	1.5E-13	4.5E-05	1.3E-03	0.62	0.73
hsa-miR-25-3p	3.3E-12	8.2E-05	2.5E-03	0.68	0.71
hsa-miR-4732-3p	1.1E-13	1.1E-05	9.1E-04	0.57	0.71
hsa-miR-192-5p	2.2E-09	4.1E-04	>0.01	0.65	0.70
hsa-miR-19b-3p	1.5E-11	1.6E-05	1.0E-04	0.74	0.70
hsa-miR-15a-5p	3.3E-08	3.0E-03	3.6E-03	0.80	0.69
hsa-miR-486-5p	2.2E-10	3.4E-04	>0.01	0.64	0.69
hsa-miR-374b-5p	3.4E-10	5.1E-03	>0.01	0.72	0.68
hsa-miR-484	4.0E-08	9.9E-03	>0.01	0.86	0.67
hsa-miR-194-5p	1.0E-06	4.6E-03	>0.01	0.71	0.67
hsa-miR-101-3p	2.7E-08	5.1E-04	6.7E-03	0.74	0.67
hsa-miR-551b-3p	3.3E-08	8.9E-03	>0.01	0.67	0.67
hsa-miR-185-5p	1.7E-09	1.8E-03	4.6E-03	0.80	0.66
hsa-miR-19a-3p	3.3E-08	1.9E-04	9.1E-04	0.79	0.66
hsa-miR-550a-5p	3.5E-05	3.0E-03	5.7E-03	0.81	0.62

[00192] A number of miRNAs has previously been reported to be up-regulated and down-regulated in HF (Table 1). Interestingly; the miRNAs found to be differentially expressed in the present study were substantially different from these reports. The significant miRNAs in both univariate and multivariate analysis were listed in Table 5 (C vs heart failure (HF)), Table 6 (C vs HFREF) and Table 7 (C vs HFPEF) where 37, 25 and 33 miRNAs were found to be up-regulated and 38, 27 and 35 miRNAs were found to be down-regulated in the three comparisons, respectively. The number of differentially expressed miRNAs validated by qPCR (101 in univariate analysis and 86 in both univariate analysis and multivariate analysis) was substantially higher than previously reported (Table 8, in total 47). Each miRNA or combinations of from these 86 miRNAs can serve as biomarker or as a component of a panel of biomarkers (multivariate index assays) for the diagnosis of heart failure.

[00193] **Table 8. Comparison between the current study and previously published reports**

Name in miRBase V18	Name in literature	No in literature	Regulation in literature	Regulation in this study
hsa-miR-210	miR-210	2	Up & Down	N.A.
hsa-miR-194-5p	miR-194	1	Up	Down
hsa-miR-192-5p	miR-192	1	Up	Down
hsa-miR-185-5p	miR-185	1	Up	Down
hsa-miR-101-3p	miR-101	1	Up	Down
hsa-miR-92b-3p	miR-92b	1	Up	N.A.
hsa-miR-675-5p	miR-675	1	Up	N.A.
hsa-miR-622	miR-622	2	Up	N.A.
hsa-miR-499a-5p	miR-499	1	Up	N.A.
hsa-miR-34a-5p	miR-34a	1	Up	N.A.
hsa-miR-320a	miR-320a	1	Up	N.A.
hsa-miR-200b-5p	miR-200b*	1	Up	N.A.
hsa-miR-18b-3p	miR-18b*	1	Up	N.A.
hsa-miR-17-3p	miR-17*	1	Up	N.A.
hsa-miR-129-5p	miR-129-5p	1	Up	N.A.
hsa-miR-1254	miR-1254	1	Up	N.A.
hsa-miR-1228-5p	miR-1228*	1	Up	N.A.
hsa-miR-92a-3p	miR-92a	1	Up	No Change
hsa-miR-532-3p	miR-532-3p	1	Up	No Change
hsa-miR-29c-3p	miR-29c	1	Up	No Change
hsa-miR-423-5p	miR-423-5p	2	Up	Up
hsa-miR-30a-5p	miR-30a	2	Up	Up
hsa-miR-22-3p	miR-22	1	Up	Up
hsa-miR-21-5p	miR-21	1	Up	Up
hsa-miR-103a-3p	miR-103	1	Down	Down
hsa-miR-30b-5p	miR-30b	1	Down	Down
hsa-miR-191-5p	miR-191	2	Down	Down

hsa-miR-150-5p	miR-150	1	Down	Down
hsa-miR-28-5p	miR-28-5p	2	Down	N.A.
hsa-miR-223-3p	miR-223	2	Down	N.A.
hsa-miR-142-3p	miR-142-3p	3	Down	N.A.
hsa-miR-126-3p	miR-126	1	Down	N.A.
hsa-miR-342-3p	miR-342-3p	1	Down	N.A.
hsa-miR-331-3p	miR-331-3p	2	Down	N.A.
hsa-miR-324-5p	miR-324-5p	1	Down	N.A.
hsa-miR-574-3p	miR-574-3p	2	Down	N.A.
hsa-miR-151a-5p	miR-151-5p	2	Down	N.A.
hsa-miR-744-5p	miR-744	2	Down	N.A.
hsa-miR-23a-3p	miR-23a	1	Down	No Change
hsa-miR-33a-5p	miR-33a	2	Down	No Change
hsa-miR-199b-5p	miR-199b-5p	2	Down	No Change
hsa-miR-1	miR-1	1	Down	No Change
hsa-miR-24-3p	miR-24	2	Down	Up
hsa-miR-27a-3p	miR-27a	2	Down	Up
hsa-miR-199a-3p	miR-199a-3p	1	Down	Up
hsa-miR-27b-3p	miR-27b	3	Down	Up
hsa-miR-335-5p	miR-335	2	Down	Up

[00194] In total, 47 distinct miRNAs have been reported in the literature (Table 1). Hsa-miR-210 had contradictory observations in the direction of change in the heart failure patients (Table 8). In the present study, 22 of the other 46 reported miRNAs were not measurable or fell below the detection limit (N.A. in Table 8) leaving 24 miRNAs to be used for comparison. Comparing the results (p-value after FDR < 0.01 in univariate analysis) with the 24 reported miRNAs, only 4 of these previously reported miRNAs (hsa-miR-423-5p, hsa-miR-30a-5p, hsa-miR-22-3p, hsa-miR-21-5p) were found to be consistently up-regulated and four (hsa-miR-103a-3p, hsa-miR-30b-5p, hsa-miR-191-5 and hsa-miR-150-5p) were found to be consistently down-regulated in the present study (Table 8). Interestingly, in eight of the dysregulated miRNAs the direction of change was opposite to that previously reported while seven of them remained unchanged (Table 8). Thus, the majority miRNAs previously reported to be differentially regulated in heart failure could NOT be confirmed in the present study. Conversely, the current study identified more than 70 novel miRNAs which could be the potential biomarkers for HF detection not previously reported.

[00195] NT-proBNP/BNP is the best studied heart failure biomarker and has exhibited the best clinical performance to date. Thus, the present study aimed to examine whether these significantly regulated miRNAs could provide additional information to NT-proBNP. The enhancement by miRNA of detecting heart failure by NT-proBNP was tested by logistic regression with adjustment for age AF, hypertension and diabetes (p-value, ln_BNP, Table 5-7). Using the p-values after FDR correction

lower than 0.01 as the criterion, 55 miRNAs (p-value, ln_BNP, Table 7) were found to have information complementary to ln_NT-proBNP for HFPEF detection but not for HFREF (p-value, ln_BNP, Table 6) . NT-proBNP used alone clearly had better diagnostic performance for detection of HFREF (AUC = 0.985, Figure 3D) than HFPEF (AUC=0.935, Figure 3E). Combining any or multiple of those 55 miRNAs together with ln_NT-proBNP, in multivariate assay, could potentially improve detection of HFPEF.

[00196] The AUC values for the most up-regulated (hsa-let-7d-3p, Figure 8, A) and most down-regulated (hsa-miR-454-3p, Figure 8, B) miRNA in heart failure (both subtypes) were 0.78 and 0.85, respectively. Both miRNAs have not previously been reported as useful for detection of heart failure. Although the diagnostic power of single miRNA may not be clinically useful, combining multiple miRNAs in a multivariate manner to may well enhance performance for heart failure diagnosis.

[00197] **B] Identification of miRNAs differentially expressed between HFREF and HFPEF**

[00198] Univariate analysis (Student's t-test) indicated that 40 miRNAs were significantly altered between HFREF and HFPEF subjects (p-value after FDR < 0.01), with 10 miRNAs having higher expression levels in HFPEF than HFREF and 30 miRNAs higher expressions in HFREF than HFPEF (Table 9).

[00199] Background clinical characteristics are expected to differ between the two heart failure subtypes (Table 3). HFPEF patients were more frequently female, had higher BMI, were older and more often had AF or hypertension compared with HFREF patients. On multivariate analysis (logistic regression) with adjustment for these characteristics, only 18 out of the 40 miRNAs remained significant (p-value after FDR < 0.01) (p-value, logistic regression, Table 9). However, since the difference between the two subtypes were due to the natural occurrence and characterization of the disease rather than caused by biased sample selection for the study, all the 40 miRNAs in Table 9 (univariate analysis) could be useful for classifying heart failure subtypes.

[00200] The AUC values for discriminating heart failure from Control for the most up-regulated miRNA (hsa-miR-223-5p, Figure 10, A) and most down-regulated miRNA (hsa-miR-185-5p, Figure 10, B) in all heart failure were moderate only at 0.68 and 0.69, respectively. This is the first report of using circulating cell free miRNAs from blood (plasma/ serum) for the classification of heart failure patients into two clinically relevant subtypes. Combining multiple miRNAs in a multivariate index assay provides more diagnostic power for subtype categorization.

[00201] Most of the miRNAs differentially expressed between HFREF and HFPEF (38 out of 40 in univariate analysis and 17 out of 18 in multivariate analysis) were also found to differ from Control reflecting the fact that the degree of dysregulation varied between the two heart failure subtypes (Figure 11).To further examine the 38 overlapped miRNAs that were found to be altered in

either of the HF subtypes as well as between the two subtypes in the univariate analysis (Figure 9, A), they were classified into 6 groups based on the relationship of their expression levels in three subject groups: control, HFREF and HFPEF (Figure 11). If the p-value (FDR) for the comparison between the two groups was higher than 0.01, the relation was then be defined as equal (indicated as"=") while if the p-value (after FDR correction) was lower than 0.01, the relation was then defined by the direction of the change (indicated as higher ">" or lower "<").

[00202] A graded change from control to HFREF to HFPEF was found in most of the miRNAs where 21 miRNAs were gradually decreased (C > HFREF > HFPEF, Figure 11) and 5 miRNAs were gradually increased (C < HFREF < HFPEF, Figure 11). Also, 5 miRNAs were found to be only lower in HFPEF subtype (C = HFREF > HFPEF, Figure 11) and 2 were found to be only higher in HFPEF subtype (C = HFREF < HFPEF, Figure 11) while there was no difference between HFREF and control. Comparing to the control, only 3 miRNAs had more distinct levels in HFREF subtype than in HFPEF (C < HFPEF < HFREF or C = HFPEF > HFREF or C = HFPEF < HFREF, Figure 11). Unlike the LVEF and NT-proBNP, HFPEF had more distinct miRNA profiles than the HFREF subtype compared to the healthy control. This suggested that the miRNAs could complement NT-proBNP to provide better discrimination of HFPEF.

[00203] Analyses of all detectable miRNAs revealed a large number to be positively correlated to one another (Pearson correlation coefficient > 0.5, Figure 12) especially between those miRNA both altered in HF patients and differing between the two heart failure subtypes (miRNAs indicated black in the x-axis, towards right hand side of the x-axis, Figure 12). The change of miRNA levels in plasma is due to heart failure (HFREF and/or HFPEF). These observations demonstrate that many pairs of miRNAs were regulated similarly among all subjects. As a result, a panel of miRNAs could be assembled by substituting one or more specific miRNAs with another to systematically optimize diagnostic performance. All the significantly altered miRNAs were critical for the development of a multivariate index diagnostic assay for heart failure detection or heart failure subtype categorization.

[00204] **IV. Plasma miRNA as prognostic markers**

[00205] At their index admission when recruited to the SHOP cohort study, heart failure patients were sampled after treatment for 3-5 days when symptomatically improved, with resolution of bedside physical signs of HF, and considered fit for discharge. This ensured assessment of marker performance in this study is relevant to the sub-acute or "chronic" phase of HF. The present study assessed the prognostic performance of circulating miRNAs for mortality and heart failure re-hospitalization. 327 of the heart failure patients (176 HFREF and 151 HFPEF) were followed-up for a period of 2 years (Table 10) during which 49 died (15%).

[00206] **Table 10. Clinical information of the subjects included in the prognosis study**

Type	Death or last follow-up (days)	Death	HF related hospitalization or death or last follow-up (days)	HF related hospitalization
REF	650	Yes	650	
PEF	804		804	Yes
PEF	776		776	Yes
REF	989		989	
REF	763		763	Yes
PEF	664		664	
PEF	650		650	Yes
REF	672		672	Yes
REF	678		678	
REF	42		42	
REF	318	Yes	318	
REF	739		739	Yes
REF	722		722	Yes
REF	738		738	
REF	412	Yes	412	Yes
REF	730		730	
REF	728		728	Yes
PEF	734		734	Yes
REF	728		728	Yes
PEF	372		372	
PEF	186		186	
REF	730		730	Yes
REF	731		731	
REF	731		731	
REF	731		731	Yes
REF	391	Yes	391	Yes
REF	731		731	Yes
REF	731		731	
REF	249	Yes	249	Yes
REF	673	Yes	673	Yes
REF	219	Yes	219	
REF	731		731	Yes
REF	731		731	
REF	376	Yes	376	Yes
REF	731		731	
REF	738		738	Yes
REF	175		175	
PEF	731		731	
PEF	731		731	Yes

PEF	365		365	Yes
PEF	733		733	
REF	263		263	Yes
REF	411		411	Yes
REF	365		365	
REF	196		196	
PEF	717		717	
PEF	183		183	
PEF	708		708	Yes
PEF	706		706	
PEF	13	Yes	13	
PEF	712		712	
PEF	736		736	Yes
REF	731		731	
PEF	724		724	Yes
PEF	735		735	
PEF	404		404	
PEF	125		125	
PEF	41		41	
REF	46	Yes	46	Yes
REF	762		762	
PEF	713		713	
REF	441	Yes	441	Yes
REF	715		715	Yes
REF	52	Yes	52	
REF	773		773	
PEF	725		725	Yes
REF	72	Yes	72	
REF	260	Yes	260	
REF	693	Yes	693	Yes
PEF	361		361	
REF	371		371	
PEF	361		361	Yes
PEF	358		358	
REF	731		731	
PEF	742		742	Yes
PEF	174		174	
PEF	179		179	Yes
PEF	731		731	Yes
REF	434	Yes	434	Yes
PEF	53		53	Yes
PEF	742		742	
REF	749	Yes	749	Yes

PEF	733		733	
PEF	379		379	Yes
PEF	365		365	
PEF	1		1	
REF	353		353	
REF	731		731	Yes
REF	365		365	
REF	768		768	
PEF	723		723	
REF	24	Yes	24	
PEF	731		731	Yes
REF	370		370	
REF	665		665	Yes
PEF	468		468	Yes
REF	40		40	
PEF	707		707	
PEF	58		58	Yes
PEF	723		723	
PEF	70	Yes	70	
REF	725		725	
REF	372		372	
PEF	391		391	
PEF	734		734	
PEF	353		353	Yes
REF	392		392	
PEF	56	Yes	56	
PEF	739		739	Yes
PEF	378		378	
PEF	354		354	
REF	733		733	
REF	665		665	
REF	253		253	
REF	707		707	Yes
REF	70	Yes	70	Yes
PEF	382		382	Yes
PEF	365		365	
REF	2		2	
PEF	183	Yes	183	
REF	732		732	
PEF	365		365	Yes
PEF	721		721	
REF	740		740	
PEF	343		343	Yes

REF	348		348	
REF	732		732	Yes
PEF	166		166	
REF	365		365	
PEF	200	Yes	200	Yes
PEF	190		190	
PEF	362		362	
REF	393		393	
REF	1	Yes	1	
REF	811		811	
REF	665		665	
REF	911	Yes	911	Yes
REF	734		734	
PEF	750		750	
PEF	593	Yes	593	
PEF	362		362	Yes
PEF	506	Yes	506	
REF	348		348	
REF	734		734	
PEF	365		365	Yes
PEF	68		68	
PEF	370		370	
PEF	78		78	
REF	752		752	
REF	378		378	
REF	53		53	
PEF	734		734	
REF	353		353	Yes
REF	730		730	
REF	728		728	
PEF	721		721	Yes
PEF	394		394	Yes
PEF	727		727	Yes
PEF	728		728	
PEF	920		920	
REF	732		732	Yes
REF	748		748	
REF	672		672	Yes
PEF	745		745	
REF	747		747	
PEF	358		358	
REF	747		747	
PEF	97		97	

REF	4		4	
PEF	188		188	
PEF	731		731	
REF	835		835	
REF	729		729	
PEF	729		729	
REF	674		674	
PEF	172		172	
PEF	666		666	
REF	731		731	
PEF	274	Yes	274	
PEF	374		374	
REF	351	Yes	351	Yes
REF	966		966	
PEF	722		722	Yes
PEF	730		730	
PEF	734		734	Yes
REF	686		686	
REF	595	Yes	595	
REF	368		368	Yes
PEF	731		731	
PEF	365		365	Yes
REF	741		741	Yes
REF	388		388	Yes
PEF	49		49	
REF	365		365	
REF	385		385	
PEF	672		672	
PEF	731		731	
PEF	741		741	
PEF	343		343	
REF	740		740	
REF	672		672	
PEF	364		364	
PEF	743		743	Yes
REF	398		398	
PEF	668		668	
REF	720		720	
PEF	731		731	Yes
PEF	193	Yes	193	
REF	365		365	
PEF	726		726	
PEF	365		365	

REF	448	Yes	448	Yes
PEF	123	Yes	123	
PEF	752		752	Yes
PEF	399		399	Yes
PEF	657		657	
PEF	770		770	
PEF	357		357	
REF	761		761	
PEF	372		372	
REF	366		366	Yes
REF	730		730	
REF	766		766	
REF	658	Yes	658	Yes
REF	736		736	
REF	372		372	Yes
PEF	334		334	
REF	49		49	
REF	730		730	Yes
REF	730		730	Yes
PEF	360		360	
REF	672		672	
REF	338		338	
REF	196		196	
PEF	322		322	Yes
PEF	731		731	
REF	730		730	Yes
REF	701		701	
REF	364		364	
PEF	742		742	
PEF	365		365	
REF	336		336	
REF	715		715	Yes
REF	747		747	
REF	29	Yes	29	
REF	738		738	
REF	739		739	Yes
REF	365		365	
REF	742		742	
PEF	208	Yes	208	Yes
REF	440	Yes	440	
PEF	731		731	Yes
REF	636	Yes	636	
REF	741		741	

PEF	723		723	Yes
PEF	367		367	Yes
PEF	735		735	
REF	730		730	Yes
REF	366		366	
PEF	728		728	
REF	730		730	Yes
REF	731		731	Yes
REF	731		731	Yes
REF	730		730	
REF	165		165	Yes
PEF	728		728	
PEF	939		939	
REF	674		674	
REF	379		379	Yes
PEF	41		41	
REF	92		92	
REF	125	Yes	125	Yes
REF	367		367	
PEF	862		862	Yes
REF	765		765	
PEF	732		732	Yes
REF	393		393	
REF	731		731	
REF	753		753	
PEF	723		723	
REF	739		739	
PEF	744		744	
REF	13	Yes	13	
REF	730		730	
PEF	379		379	Yes
REF	715		715	Yes
PEF	377		377	
REF	365		365	
PEF	239	Yes	239	
REF	183		183	
PEF	25	Yes	25	
REF	714		714	Yes
REF	729		729	Yes
PEF	357		357	
REF	723		723	Yes
PEF	725		725	
PEF	335		335	

PEF	457	Yes	457	
PEF	390		390	
PEF	726		726	
REF	404		404	Yes
PEF	171		171	
REF	270	Yes	270	Yes
REF	357		357	
REF	99	Yes	99	
REF	732		732	Yes
REF	365		365	
REF	739		739	Yes
PEF	730		730	Yes
REF	168		168	
PEF	367		367	
PEF	334		334	
PEF	377		377	Yes
PEF	361	Yes	361	Yes
PEF	771		771	
REF	484	Yes	484	Yes
REF	190		190	
REF	672		672	Yes
PEF	766		766	
REF	365		365	
REF	389		389	Yes
REF	381		381	Yes
PEF	357		357	
PEF	711		711	Yes
PEF	743		743	Yes
REF	7	Yes	7	
PEF	34	Yes	34	

[00207] Among all the study cases, 115 were re-hospitalized because of heart failure during the follow-up (Table 10) and 49 died. MiRNAs were assessed as potential markers (ie predictors) of both observed (all-cause survival) OS and event free survival (EFS) the composite of all-cause death and/or recurrent admission for decompensated heart failure.

[00208] Anti- heart failure pharmacotherapy prescribed to study participants is summarized in Table 11. Comparing the treatments for HFREF and HFPEF, the frequency of prescription of half the drugs concerned were found to differ (Figure 13). Notably those classes of drugs proven to improve prognosis in HFREF (ACEI's/ARB's, beta blockers and mineralocorticoid antagonists) were more commonly prescribed to HFREF than HFPEF patients. Treatments were according to current clinical practice and were included among clinical variables for the analysis of prognostic markers.

[00209] **Table 11. Treatment of subjects included in the prognosis study**

	Me 1	Me 2	Me 3	Me 4	Me 5	Me 6	Me 7	Me 8	Me 9	Me 10	Me 11	Me 12	Me 13	Me 14	Me 15
1	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes		Yes				
2		Yes	Yes	Yes	Yes	Yes			Yes	Yes					Yes
3	Yes		Yes	Yes		Yes		Yes	Yes						Yes
4	Yes		Yes	Yes		Yes	Yes				Yes				
5			Yes	Yes	Yes	Yes					Yes		Yes		Yes
6			Yes	Yes		Yes	Yes	Yes							
7			Yes	Yes	Yes	Yes			Yes			Yes	Yes		
8	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes		
9	Yes		Yes	Yes	Yes	Yes			Yes		Yes		Yes		
10			Yes	Yes	Yes	Yes			Yes				Yes		
11	Yes		Yes	Yes	Yes	Yes					Yes				
12	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes		
13	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes				
14		Yes	Yes	Yes	Yes	Yes	Yes		Yes						
15	Yes		Yes	Yes		Yes	Yes	Yes			Yes		Yes		Yes
16	Yes			Yes	Yes	Yes	Yes		Yes		Yes				
17	Yes		Yes	Yes	Yes	Yes	Yes				Yes				
18			Yes	Yes	Yes	Yes				Yes					
19		Yes	Yes	Yes		Yes	Yes			Yes	Yes		Yes		
20		Yes		Yes		Yes									
21	Yes			Yes	Yes	Yes			Yes						
22	Yes		Yes	Yes	Yes	Yes			Yes		Yes		Yes		
23		Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes				
24	Yes		Yes	Yes	Yes	Yes				Yes	Yes				
25	Yes		Yes	Yes	Yes	Yes				Yes			Yes		
26	Yes		Yes	Yes	Yes	Yes				Yes	Yes				
27			Yes	Yes	Yes	Yes			Yes	Yes	Yes		Yes		Yes
28	Yes		Yes	Yes	Yes	Yes					Yes				
29	Yes		Yes	Yes	Yes	Yes					Yes		Yes		
30			Yes	Yes	Yes		Yes								Yes
31	Yes		Yes	Yes	Yes	Yes	Yes				Yes				
32	Yes		Yes	Yes	Yes	Yes			Yes	Yes	Yes		Yes	Yes	
33	Yes		Yes	Yes	Yes	Yes	Yes				Yes		Yes		
34	Yes		Yes	Yes	Yes	Yes			Yes		Yes		Yes		Yes

35	Yes		Yes	Yes	Yes	Yes									
36			Yes	Yes	Yes	Yes					Yes		Yes		
37	Yes		Yes	Yes	Yes	Yes							Yes		
38			Yes	Yes	Yes	Yes									
39		Yes	Yes	Yes		Yes	Yes	Yes		Yes			Yes		Yes
40			Yes	Yes	Yes	Yes							Yes		
41		Yes	Yes	Yes	Yes	Yes			Yes				Yes		
42		Yes	Yes	Yes	Yes	Yes		Yes			Yes		Yes		
43	Yes	Yes	Yes	Yes		Yes	Yes	Yes			Yes				
44	Yes		Yes	Yes	Yes	Yes	Yes		Yes		Yes				
45	Yes		Yes	Yes	Yes				Yes		Yes		Yes		
46	Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes		Yes	Yes		
47			Yes	Yes	Yes					Yes			Yes	Yes	
48	Yes		Yes		Yes	Yes				Yes			Yes	Yes	Yes
49			Yes						Yes						
50			Yes	Yes										Yes	
51		Yes		Yes	Yes			Yes		Yes			Yes		
52	Yes		Yes	Yes	Yes	Yes	Yes		Yes	Yes				Yes	Yes
53		Yes				Yes							Yes		Yes
54	Yes	Yes	Yes	Yes	Yes	Yes			Yes						
55		Yes	Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes		
56			Yes	Yes	Yes	Yes			Yes	Yes			Yes	Yes	
57	Yes		Yes	Yes	Yes					Yes					Yes
58		Yes	Yes	Yes		Yes			Yes	Yes			Yes		
59	Yes		Yes	Yes	Yes	Yes	Yes				Yes				
60		Yes	Yes	Yes	Yes	Yes	Yes								
61		Yes	Yes			Yes							Yes		
62	Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes		Yes	Yes	Yes
63	Yes		Yes	Yes	Yes	Yes			Yes		Yes		Yes		
64	Yes		Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes		Yes		Yes
65		Yes	Yes	Yes		Yes				Yes	Yes		Yes		Yes
66		Yes	Yes	Yes	Yes	Yes	Yes		Yes				Yes		
67			Yes	Yes	Yes	Yes			Yes			Yes	Yes	Yes	
68		Yes	Yes		Yes	Yes							Yes		Yes
69	Yes		Yes	Yes	Yes	Yes			Yes			Yes	Yes	Yes	Yes
70	Yes		Yes	Yes	Yes	Yes			Yes	Yes			Yes		
71	Yes		Yes	Yes		Yes									
72	Yes		Yes	Yes		Yes		Yes	Yes	Yes		Yes	Yes		Yes

73	Yes		Yes		Yes	Yes							Yes		
74	Yes		Yes	Yes	Yes	Yes	Yes			Yes	Yes			Yes	
75		Yes	Yes	Yes	Yes	Yes			Yes	Yes					
76	Yes		Yes		Yes				Yes				Yes		
77			Yes	Yes	Yes	Yes			Yes	Yes			Yes	Yes	Yes
78	Yes	Yes	Yes	Yes		Yes							Yes		Yes
79	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes		
80	Yes		Yes	Yes	Yes	Yes			Yes				Yes		Yes
81	Yes		Yes	Yes		Yes	Yes		Yes	Yes	Yes				
82	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes		Yes
83		Yes		Yes	Yes		Yes								
84	Yes		Yes		Yes	Yes							Yes		
85		Yes	Yes	Yes	Yes	Yes				Yes			Yes		
86	Yes			Yes		Yes							Yes		
87	Yes		Yes	Yes	Yes	Yes									Yes
88		Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes		Yes		
89	Yes			Yes	Yes	Yes			Yes		Yes				
90	Yes		Yes	Yes	Yes	Yes			Yes		Yes				
91	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes		
92	Yes		Yes	Yes	Yes	Yes									
93	Yes		Yes	Yes	Yes	Yes							Yes		Yes
94	Yes		Yes	Yes							Yes				
95	Yes		Yes	Yes	Yes	Yes					Yes		Yes		
96	Yes		Yes		Yes	Yes			Yes						
97		Yes	Yes	Yes							Yes				
98		Yes	Yes	Yes	Yes	Yes		Yes		Yes			Yes		
99		Yes		Yes	Yes	Yes	Yes			Yes			Yes		
100			Yes	Yes		Yes	Yes								Yes
101			Yes		Yes	Yes			Yes						
102		Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes			Yes		
103		Yes	Yes	Yes	Yes	Yes			Yes		Yes		Yes		Yes
104	Yes		Yes	Yes			Yes			Yes					Yes
105		Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes				
106		Yes	Yes	Yes	Yes	Yes							Yes		
107	Yes		Yes	Yes	Yes	Yes					Yes		Yes		
108		Yes	Yes	Yes	Yes	Yes			Yes						
109	Yes		Yes	Yes	Yes	Yes			Yes	Yes			Yes	Yes	
110	Yes		Yes	Yes		Yes	Yes	Yes							
111	Yes		Yes	Yes		Yes		Yes	Yes				Yes		

112	Yes		Yes	Yes	Yes	Yes					Yes		Yes		
113	Yes			Yes		Yes	Yes	Yes					Yes		
114				Yes	Yes	Yes			Yes				Yes		
115	Yes		Yes	Yes	Yes	Yes	Yes				Yes		Yes		
116	Yes		Yes	Yes		Yes		Yes	Yes						
117	Yes		Yes	Yes	Yes	Yes			Yes	Yes	Yes		Yes		
118		Yes	Yes	Yes	Yes	Yes			Yes				Yes		
119			Yes	Yes	Yes	Yes			Yes				Yes		
120	Yes		Yes	Yes		Yes			Yes						
121	Yes		Yes	Yes	Yes	Yes	Yes		Yes		Yes				
122			Yes	Yes		Yes		Yes	Yes	Yes				Yes	Yes
123		Yes	Yes			Yes				Yes					
124		Yes	Yes	Yes							Yes				
125			Yes	Yes	Yes	Yes				Yes					
126		Yes	Yes	Yes	Yes	Yes			Yes		Yes		Yes		Yes
127	Yes			Yes	Yes	Yes			Yes		Yes	Yes			
128	Yes		Yes	Yes		Yes									
129			Yes	Yes			Yes	Yes					Yes		Yes
130					Yes	Yes			Yes	Yes			Yes		Yes
131		Yes	Yes	Yes	Yes										
132		Yes	Yes	Yes	Yes	Yes							Yes		Yes
133	Yes		Yes	Yes							Yes				
134															
135	Yes		Yes	Yes	Yes	Yes							Yes		
136		Yes		Yes	Yes	Yes			Yes			Yes	Yes		
137		Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes		Yes		Yes
138	Yes		Yes	Yes	Yes	Yes					Yes				
139	Yes		Yes	Yes	Yes	Yes			Yes				Yes		
140			Yes	Yes	Yes	Yes			Yes				Yes		
141		Yes	Yes	Yes											
142		Yes	Yes		Yes	Yes			Yes				Yes	Yes	Yes
143	Yes		Yes	Yes		Yes					Yes				
144	Yes		Yes	Yes	Yes	Yes					Yes				
145	Yes		Yes	Yes		Yes		Yes	Yes					Yes	Yes
146			Yes			Yes				Yes					
147	Yes	Yes	Yes	Yes	Yes	Yes				Yes			Yes	Yes	Yes
148	Yes	Yes			Yes	Yes				Yes					
149		Yes	Yes	Yes		Yes					Yes				
150	Yes		Yes	Yes	Yes	Yes							Yes		

151	Yes		Yes	Yes	Yes	Yes									
152	Yes			Yes	Yes	Yes			Yes			Yes	Yes		
153		Yes	Yes	Yes			Yes				Yes				
154	Yes		Yes	Yes	Yes	Yes							Yes		
155	Yes	Yes	Yes	Yes	Yes		Yes			Yes					
156	Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes		Yes		Yes
157			Yes	Yes	Yes	Yes			Yes				Yes	Yes	
158		Yes	Yes	Yes	Yes	Yes			Yes	Yes					
159	Yes		Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes		Yes
160			Yes	Yes	Yes	Yes		Yes							
161	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes		Yes
162		Yes	Yes	Yes	Yes	Yes					Yes				
163	Yes		Yes	Yes	Yes	Yes	Yes						Yes		Yes
164			Yes	Yes	Yes	Yes	Yes		Yes	Yes		Yes			
165			Yes	Yes	Yes	Yes	Yes						Yes		Yes
166	Yes	Yes				Yes				Yes					Yes
167	Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes		Yes		
168		Yes			Yes	Yes			Yes				Yes		
169	Yes		Yes	Yes	Yes	Yes			Yes		Yes				
170		Yes	Yes	Yes	Yes	Yes	Yes				Yes				
171	Yes		Yes	Yes		Yes			Yes	Yes			Yes	Yes	
172	Yes	Yes	Yes	Yes							Yes				
173	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes		
174			Yes			Yes				Yes			Yes		Yes
175	Yes	Yes	Yes	Yes	Yes	Yes				Yes			Yes		
176			Yes	Yes		Yes				Yes			Yes		
177	Yes		Yes	Yes	Yes	Yes				Yes	Yes				
178	Yes		Yes	Yes	Yes		Yes			Yes					
179	Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes			Yes	Yes	
180	Yes		Yes	Yes	Yes	Yes			Yes	Yes			Yes		
181			Yes	Yes	Yes	Yes			Yes	Yes	Yes			Yes	
182	Yes		Yes	Yes		Yes					Yes		Yes		
183	Yes		Yes	Yes			Yes	Yes			Yes				
184		Yes	Yes	Yes	Yes	Yes			Yes	Yes					
185			Yes	Yes	Yes	Yes		Yes	Yes	Yes			Yes	Yes	Yes
186	Yes		Yes	Yes									Yes		
187	Yes		Yes	Yes	Yes	Yes					Yes		Yes		
188	Yes		Yes	Yes	Yes	Yes	Yes		Yes		Yes		Yes		
189	Yes	Yes	Yes	Yes	Yes	Yes				Yes		Yes	Yes		

190			Yes	Yes	Yes	Yes							Yes		Yes
191	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes				
192		Yes	Yes	Yes		Yes				Yes	Yes		Yes		
193		Yes	Yes	Yes		Yes							Yes		Yes
194		Yes	Yes	Yes	Yes	Yes									
195	Yes		Yes	Yes	Yes		Yes		Yes		Yes	Yes	Yes		Yes
196	Yes		Yes			Yes				Yes					Yes
197			Yes	Yes		Yes									
198	Yes		Yes	Yes	Yes	Yes			Yes						Yes
199		Yes	Yes	Yes		Yes	Yes	Yes							
200	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes		
201		Yes		Yes	Yes						Yes				
202	Yes		Yes	Yes	Yes	Yes			Yes					Yes	
203	Yes		Yes	Yes	Yes	Yes		Yes	Yes	Yes			Yes	Yes	
204	Yes		Yes	Yes	Yes	Yes			Yes		Yes		Yes		
205	Yes			Yes	Yes	Yes					Yes				
206	Yes		Yes	Yes		Yes	Yes				Yes				
207	Yes		Yes			Yes		Yes					Yes		
208	Yes		Yes	Yes	Yes	Yes			Yes						
209	Yes		Yes	Yes	Yes	Yes					Yes				
210	Yes		Yes	Yes						Yes			Yes		Yes
211	Yes			Yes		Yes							Yes		Yes
212	Yes		Yes		Yes	Yes			Yes	Yes					
213			Yes	Yes	Yes	Yes				Yes					
214		Yes	Yes	Yes		Yes				Yes			Yes		Yes
215		Yes		Yes	Yes	Yes									Yes
216	Yes		Yes	Yes	Yes	Yes			Yes	Yes			Yes		Yes
217	Yes	Yes	Yes	Yes	Yes	Yes			Yes				Yes	Yes	
218		Yes	Yes	Yes	Yes	Yes			Yes	Yes					Yes
219	Yes		Yes	Yes	Yes	Yes	Yes	Yes					Yes		
220	Yes		Yes	Yes		Yes	Yes	Yes	Yes			Yes	Yes		
221			Yes	Yes	Yes	Yes					Yes		Yes		
222	Yes		Yes	Yes	Yes	Yes	Yes		Yes		Yes				Yes
223		Yes	Yes	Yes		Yes					Yes				
224		Yes	Yes	Yes		Yes			Yes	Yes	Yes		Yes		
225	Yes		Yes	Yes	Yes	Yes									
226		Yes	Yes	Yes	Yes	Yes			Yes						
227		Yes		Yes		Yes		Ye							

								s							
228	Yes		Yes			Yes					Yes				
229	Yes	Yes	Yes	Yes	Yes	Yes	Yes				Yes		Yes		
230		Yes	Yes	Yes		Yes	Yes			Yes	Yes	Yes	Yes		
231			Yes	Yes		Yes	Yes					Yes			
232	Yes		Yes	Yes	Yes	Yes	Yes		Yes						Yes
233		Yes	Yes	Yes	Yes	Yes		Ye s			Yes		Yes		
234			Yes			Yes									Yes
235		Yes	Yes	Yes						Yes					
236	Yes		Yes	Yes	Yes	Yes			Yes						Yes
237	Yes		Yes	Yes			Yes				Yes				
238	Yes		Yes	Yes							Yes				
239	Yes		Yes	Yes	Yes	Yes					Yes				
240	Yes		Yes	Yes	Yes	Yes									Yes
241		Yes	Yes	Yes	Yes	Yes				Yes			Yes		
242	Yes			Yes	Yes	Yes					Yes				
243	Yes		Yes	Yes	Yes	Yes				Yes	Yes	Yes			
244	Yes		Yes	Yes	Yes	Yes	Yes				Yes		Yes		
245	Yes			Yes	Yes	Yes	Yes		Yes						Yes
246	Yes		Yes	Yes	Yes	Yes	Yes	Ye s			Yes		Yes		
247	Yes		Yes	Yes	Yes	Yes	Yes						Yes		
248	Yes		Yes	Yes	Yes	Yes		Ye s	Yes				Yes		
249	Yes		Yes	Yes	Yes	Yes			Yes		Yes			Yes	Yes
250		Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes				Yes
251		Yes		Yes	Yes	Yes			Yes		Yes		Yes		
252		Yes	Yes		Yes	Yes	Yes		Yes	Yes					
253	Yes	Yes	Yes	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes	Yes
254	Yes		Yes	Yes		Yes	Yes				Yes		Yes		
255		Yes	Yes	Yes	Yes	Yes			Yes	Yes			Yes		Yes
256			Yes	Yes	Yes	Yes			Yes	Yes					
257		Yes		Yes	Yes	Yes	Yes			Yes			Yes		
258	Yes		Yes	Yes		Yes					Yes				
259	Yes		Yes	Yes		Yes			Yes		Yes				
260	Yes		Yes	Yes	Yes	Yes			Yes	Yes			Yes	Yes	
261		Yes	Yes	Yes	Yes	Yes	Yes	Ye s			Yes		Yes		
262	Yes		Yes	Yes	Yes	Yes			Yes				Yes		
263	Yes	Yes	Yes	Yes	Yes	Yes					Yes		Yes		
264	Yes		Yes	Yes			Yes		Yes		Yes		Yes		Yes
265	Yes		Yes	Yes	Yes	Yes			Yes				Yes		
266	Yes		Yes		Yes	Yes							Yes		

267			Yes	Yes		Yes		Yes					Yes		
268		Yes		Yes											
269	Yes		Yes	Yes	Yes	Yes	Yes		Yes		Yes				
270		Yes	Yes		Yes	Yes			Yes	Yes				Yes	
271	Yes		Yes	Yes	Yes	Yes	Yes								
272		Yes	Yes	Yes	Yes	Yes			Yes	Yes			Yes		
273		Yes	Yes	Yes			Yes				Yes				
274	Yes			Yes	Yes	Yes			Yes	Yes					
275	Yes		Yes	Yes	Yes	Yes	Yes				Yes		Yes		Yes
276	Yes		Yes	Yes	Yes	Yes			Yes	Yes			Yes		
277	Yes		Yes	Yes	Yes	Yes			Yes		Yes				
278	Yes		Yes	Yes	Yes	Yes					Yes		Yes		
279	Yes		Yes	Yes		Yes					Yes				
280		Yes	Yes	Yes	Yes	Yes				Yes					Yes
281		Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes			Yes		Yes
282	Yes		Yes	Yes	Yes	Yes					Yes		Yes		Yes
283		Yes	Yes			Yes				Yes			Yes		
284		Yes	Yes	Yes	Yes	Yes									
285		Yes	Yes	Yes	Yes	Yes			Yes	Yes			Yes		Yes
286			Yes	Yes	Yes	Yes			Yes		Yes		Yes		Yes
287		Yes	Yes	Yes	Yes	Yes			Yes	Yes					
288	Yes		Yes	Yes	Yes	Yes			Yes				Yes		
289	Yes		Yes	Yes	Yes	Yes			Yes						
290			Yes	Yes	Yes	Yes			Yes				Yes		
291	Yes	Yes	Yes	Yes	Yes	Yes							Yes		
292	Yes		Yes	Yes	Yes	Yes					Yes				
293		Yes	Yes	Yes	Yes	Yes	Yes				Yes		Yes		
294		Yes	Yes	Yes	Yes	Yes			Yes	Yes				Yes	
295	Yes		Yes	Yes	Yes	Yes	Yes				Yes				
296		Yes	Yes	Yes	Yes	Yes				Yes	Yes				
297	Yes		Yes	Yes	Yes	Yes									
298	Yes		Yes	Yes	Yes		Yes								
299		Yes		Yes	Yes	Yes									Yes
300	Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes			Yes		Yes
301	Yes		Yes	Yes	Yes	Yes					Yes		Yes		
302			Yes	Yes	Yes	Yes					Yes			Yes	
303	Yes		Yes	Yes	Yes	Yes	Yes		Yes		Yes		Yes		
304	Yes		Yes	Yes	Yes	Yes			Yes		Yes		Yes	Yes	
305	Yes		Yes	Yes		Yes			Yes						
306	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes		
307	Yes		Yes	Yes	Yes	Yes			Yes		Yes				

308		Yes	Yes	Yes	Yes	Yes	Yes		Yes				Yes		
309		Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes		Yes		Yes
310		Yes	Yes	Yes	Yes	Yes					Yes		Yes		
311	Yes		Yes	Yes	Yes	Yes				Yes			Yes		
312	Yes		Yes	Yes	Yes	Yes				Yes					
313				Yes		Yes		Yes							
314	Yes		Yes	Yes	Yes	Yes				Yes			Yes		Yes
315		Yes	Yes			Yes	Yes	Yes	Yes	Yes					
316			Yes	Yes	Yes	Yes			Yes	Yes		Yes	Yes	Yes	Yes
317	Yes		Yes	Yes	Yes	Yes			Yes		Yes		Yes		
318	Yes		Yes	Yes	Yes	Yes		Yes		Yes			Yes		
319	Yes	Yes	Yes	Yes		Yes				Yes			Yes	Yes	
320	Yes		Yes	Yes	Yes	Yes			Yes				Yes		
321	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes				
322	Yes		Yes	Yes		Yes		Yes		Yes	Yes				
323	Yes		Yes		Yes	Yes			Yes	Yes			Yes		Yes
324		Yes	Yes	Yes	Yes	Yes			Yes	Yes		Yes	Yes		
325		Yes	Yes	Yes	Yes	Yes				Yes	Yes		Yes		Yes
326	Yes		Yes	Yes		Yes	Yes			Yes					
327			Yes			Yes	Yes			Yes			Yes		Yes

[00210] Cox proportional hazards (CoxPH) modeling was used for survival analysis and the explanatory variables were individually (univariate analysis) or simultaneously analyzed in the same model (multivariate analysis). In order to have a better comparison between various hazard ratios (HR), all normally distributed variables including the miRNA expression levels (log2 scale), the clinical variables such as BMI, ln_NT-proBNP, LVEF, age as well as the multivariate scores generated by combining multiple variables were scaled to one standard deviation. The hazard ratio (HR) was then used as the indicator for the prognostic power for those variables. A p-value < 0.05 was considered as statistically significant. Patients were classified as high risk and low risk according to presence or absence of categorical variables and by supra or infra-median levels of normally distributed continuous variables. Kaplan-Meier plots (KM plot) were used to illustrate various risk groups' survival over time with inter-curve comparisons tested by log-rank test. Inter-group survival at 750 days (OS750) and/or EFS at 750 days (EFS750) were also compared.

[00211] All the clinical variables were initially assessed for prediction of overall survival (OS). In univariate analyses, five variables (age, hypertension, ln_NT-proBNP, nitrates and hydralazine) were

found to be positively associated with risk of death and two variables (BMI and Beta Blockers) were found to be negatively associated with the risk of death (Table 12). Interestingly, overall survival did not differ between HFREF and HFPEF patients. The KM plots of the subject groups defined by those significant parameters are shown in Figure 14, A and the OS750 were shown in Figure 14, B. All the parameters were able to define high and low risk groups with ln_NT-proBNP the most significant (p-value = 7.2E-07, HR =2.36 (95% CI: 1.69-3.30)). Based on the level of ln_NT-proBNP, the low risk group had OS750 of 92.4% while the value for the high risk group was only 66.0%. In the multivariate analysis with all clinical variables included, 6 variables (gender, hypertension, BMI, ln_NT-proBNP, BetaBlockers and Warfarin) were found to be significant. These 6 variables were later combined with each of the 137 miRNAs in the CoxPH model for the identification of prognostic miRNA markers for overall survival.

[00212] **Table 12. Analysis of clinical variables of observed survival**

	Univariate analysis			**Multivariate analysis**		
Variables:	ln(HR)	SE of ln(HR)	p-value	ln(HR)	SE of ln(HR)	p-value
Type (REF/PEF)	-0.50	0.30	0.10	0.03	0.98	0.98
Gender	0.01	0.29	0.97	**1.03**	**0.50**	**0.040**
Atrial Fibrillation/ Flutter	0.39	0.31	0.20	0.84	0.46	0.07
Hypertension	**0.81**	**0.41**	**0.048**	**1.24**	**0.49**	**0.0119**
Diabetes	0.28	0.30	0.36	1.01	0.53	0.06
Smoking History	-0.08	0.29	0.79	0.16	0.46	0.73
Alcohol History	-0.08	0.32	0.81	-0.70	0.48	0.15
Age	**0.55**	**0.16**	**0.00039**	0.21	0.24	0.38
Body Mass Index	**-0.50**	**0.13**	**0.00019**	**-0.55**	**0.25**	**0.026**
LVEF	-0.17	0.15	0.24	-0.09	0.51	0.87
ln_NT-proBNP	**0.86**	**0.17**	**7.2E-07**	**0.67**	**0.25**	**0.0078**
ACE Inhibitors	-0.01	0.29	0.96	0.07	0.39	0.86
Angiontensin Receptor Blockers	-0.37	0.32	0.25	-0.04	0.43	0.92
Loop/thiazide Diuretics	0.01	0.52	0.99	0.71	0.79	0.37
BetaBlockers	**-0.90**	**0.39**	**0.021**	**-1.72**	**0.57**	**0.0025**
Aspirin or Plavix	0.32	0.36	0.37	-0.27	0.47	0.56
Statins	0.17	0.52	0.75	-0.41	0.59	0.49
Digoxin	-0.25	0.33	0.45	-0.36	0.42	0.38
Warfarin	-0.83	0.52	0.11	**-1.33**	**0.67**	**0.05**
Nitrates	**0.60**	**0.29**	**0.039**	0.09	0.41	0.82
Calcium Channel Blockers	-0.03	0.31	0.92	-0.47	0.41	0.25
Spironolactone	-0.21	0.29	0.48	0.20	0.44	0.65
Fibrate	0.52	0.44	0.23	0.55	0.57	0.34
Antidiabetic	-0.10	0.29	0.73	-0.78	0.52	0.13
Hydralazine	**0.78**	**0.37**	**0.036**	0.11	0.52	0.83

Iron supplements	0.43	0.30	0.15	-0.07	0.39	0.85

[00213] Similar analyses were performed for event free survival (EFS) and seven variables (AF, hypertension, diabetes, age, ln_NT-proBNP, nitrates and hydralazine) were found to be positively correlated with the risk of recurrent admission for decompensated heart failure (Table 13) in univariate analyses. The KM plots of the subject groups defined by those significant parameters were shown in Figure 15, A and the EFS750 were shown in Figure 15, B. Again, there was no difference between HFREF and HFPEF in event free survival and ln_NT-proBNP was the most significant predictor of event free survival (p-value = 1.5E-09, HR =1.79 (95% CI: 1.47-2.17)). Infra-median ln_NT-proBNP was associated with EFS750 of 65.1% and supra-median levels an EFS750 of only 34.1%. By multivariate analysis, only two variables: diabetes and ln_NT-proBNP were found to be significant. These variables were subsequently combined with each of the 137 miRNAs for the identification of prognostic miRNA markers for event free survival.

[00214] **Table 13. Analysis of clinical variables for event free survival (EFS)**

	Univariate analysis			**Multivariate analysis**		
	ln(HR)	SE of ln(HR)	p-value	ln(HR)	SE of ln(HR)	p-value
Type (REF/PEF)	-0.12	0.17	0.48	-0.49	0.50	0.33
Gender	0.04	0.17	0.83	0.27	0.23	0.25
Atrial Fibrillation/ Flutter	**0.38**	**0.18**	**0.035**	0.08	0.25	0.75
Hypertension	**0.60**	**0.22**	**0.0064**	0.44	0.25	0.09
Diabetes	**0.62**	**0.18**	**0.00061**	**1.04**	**0.32**	**0.0011**
Smoking History	-0.10	0.22	0.65	0.09	0.32	0.78
Alcohol History	-0.03	0.25	0.89	0.09	0.33	0.78
Age	**0.26**	**0.09**	**0.0026**	0.10	0.13	0.41
Body Mass Index	-0.13	0.09	0.14	0.05	0.12	0.66
LVEF	-0.02	0.08	0.85	0.38	0.27	0.17
ln_NT-proBNP	**0.58**	**0.10**	**1.5E-09**	**0.66**	**0.13**	**9.0E-07**
ACE Inhibitors	-0.17	0.17	0.32	-0.25	0.22	0.24
Angiontensin Receptor Blockers	-0.11	0.18	0.53	-0.18	0.23	0.43
Loop/thiazide Diuretics	0.43	0.34	0.21	0.38	0.43	0.38
BetaBlockers	-0.11	0.29	0.71	-0.49	0.35	0.17
Aspirin or Plavix	0.32	0.21	0.12	-0.03	0.25	0.90
Statins	0.62	0.34	0.07	0.30	0.38	0.43
Digoxin	0.12	0.18	0.51	0.15	0.23	0.50
Warfarin	0.29	0.22	0.18	-0.03	0.30	0.91
Nitrates	**0.47**	**0.17**	**0.0049**	0.28	0.21	0.18
Calcium Channel Blockers	0.23	0.17	0.176	-0.03	0.23	0.91
Spironolactone	0.07	0.17	0.69	0.27	0.24	0.24

Fibrate	0.23	0.30	0.45	0.12	0.34	0.72
Antidiabetic	0.29	0.17	0.09	-0.53	0.29	0.07
Hydralazine	**0.49**	**0.25**	**0.048**	-0.03	0.29	0.93
Iron supplements	0.30	0.18	0.09	-0.04	0.22	0.87

[00215] To identify miRNA biomarkers for the prediction of overall survival, each of the 137 miRNAs were tested by the univariate CoxPH model as well as in multivariate CoxPH models including 6 additional predictive clinical variables. In total, 40 miRNAs had p-values less than 0.05. Thirty seven (37) were significant in univariate analyses and 29 were significant in multivariate analyses (Table 14). 11 miRNAs found to be significant in the univariate analysis were not able to improve the prediction performance of clinical parameters (multivariate analysis) and 3 miRNAs were only significant when combined with clinical variables (Figure 16, A). Except for hsa-miR-374b-5p (p-value = 0.25), the 2 miRNAs had p-values less than 0.1 in univariate analysis (Table 14).

[00216] The miRNA with the highest hazard ratio (HR) for mortality in both univariate (HR = 1.90 (95% CI: 1.36 – 2.65, p-value = 0.00014)) and multivariate analysis (HR = 1.79 (95% CI: 1.23 – 2.59, p-value = 0.0028)) was hsa-miR-503. Hsa-miR-150-5p had the lowest HR (ie the expression level was negatively correlated with the risk) for both univariate analysis (HR = 0.52 (95% CI: 0.40 – 0.67, p-value = 1.3E-7)) and multivariate analysis (HR = 0.59 (95% CI: 0.45 – 0.78, p-value = 0.00032)) (Table 14). The KM plots for the two miRNAs are shown in Figure 18, A. Good separation between the two risk groups can be observed. Based on a single miRNA, the high risk and low risk group had about 21.3% (hsa-miR-503) or 17.8% (has-miR-150-5p) difference in terms of OS750 (Figure 18, B). With the addition of 6 clinical variables, the combined scores provide better risk predictions where the differences were 25.3% for hsa-miR-503 + 6 clinical variables and 22.4% for has-miR-150-5p + 6 clinical variables (Figure 18, B). Any one or numbers of the 40 miRNAs (Table 14) could be used as the prognostic marker/ panel for risk of death for the chronic HF patients.

[00217] For the prediction of event free survival, 13 miRNAs were found significant in the univariate analysis (p-value < 0.05) where 4 were positively correlated and 9 were negative correlated with the risk of recurrent admission for decompensated heart failure after treatment (Table 15). None of the miRNAs were found to be significant for EFS prediction in the multivariate analysis where 2 additional clinical variables were included in the CoxPH model. However, the top positively correlated miRNA (hsa-miR-331-5p, HR = 1.27 (95% CI: 1.09 – 1.49, p-value = 0.0025)) and the top negatively correlated miRNA (hsa-miR-30e-3p, HR = 0.80 (95% CI: 0.69 – 0.94, p-value = 0.0070)) with EFS in the univariate analysis also had certain levels of significance in the multivariate analysis where the p-values were 0.15 and 0.14 respectively (Table 15). The KM plots of the high and low risk groups for EFS defined by either of the miRNAs with and without additional clinical variables were shown in Figure 19, A and their EFS750 were shown in Figure 19, B. Based on a single miRNA

(either hsa-miR-331-5p or hsa-miR-30e-3p), the high risk group had EFS750 at about 40% and the low risk group had EFS750 at about 60% while the numbers were 33% and 66% with the addition of 2 clinical variables (Figure 19, B). Any one or numbers of the 13 miRNAs (Table 15) could be used as the prognostic marker/ panel for risk of recurrent admission for decompensated HF for the chronic HF patients.

[00218] Fewer miRNA were identified as predictive of event free survival (n=13) than for overall survival (n=43) and only 3 of them overlapped (Figure 16, B). The results suggest differing mechanisms for death and recurrent decompensated heart failure. One important issue to note is that the definition of event free survival in this study involved a less well defined clinical variable – ie hospitalization which could be biased by the patients or the clinicians from case to case. None the less, all the 53 miRNAs could be valuable prognostic markers for chronic heart failure patients.

[00219] The 53 prognostic markers were then compared to the 101 markers for HF detection (Figure 17, A) or the 40 markers for heart failure subtype categorization (Figure 17B). Some overlaps were observed but still a large portion of the prognostic markers were not found in the other two lists indicating that a separate set of miRNAs should be used or combined to form the multivariate index assay for the prognosis.

[00220] **V. Multivariate biomarker panels for HF detection**

[00221] As discussed above, panels consisting of combinations of multiple miRNAs might serve to provide better diagnostic power than the use of a single miRNA.

[00222] An important criterion to assemble such multivariate panel was to include at least one miRNA from the specific list for each subtype of heart failure to ensure all heart failure subgroups were covered. However, the miRNAs defining the two subtypes of heart failure overlapped (Figure 7). At the same time, large numbers of heart failure related or non-related miRNAs were found to be positively correlated (Figure 12) which makes the choice of the best miRNA combinations for heart failure diagnosis challenging.

[00223] In view of the complexity of the task, the inventors of the present study decided to identify panels of miRNA with the highest AUC using sequence forward floating search algorithm [53]. The state-of-the art linear support vector machine, a well utilized and recognized modeling tool for the construction of panels of variables, was also used to aid in the selection of the combinations of miRNAs [54]. The model yields a score based on a linear formula accounting for the expression level of each member and their weightages. These linear models could be readily applied in the clinical practice.

[00224] A critical requirement for the success of such process is the availability of high quality data. The quantitative data of all the detected miRNAs in a large number of well-defined clinical

samples not only improves the accuracy as well as precision of the result but also ensures the consistency of the identified biomarker panels for further clinical application using qPCR.

[00225] To ensure the veracity of the result, multiple (>80) times of hold-out validation (two fold cross validation) were carried out to test the performance of the identified biomarker panel based on the discovery set (half of the samples at each fold) in an independent set of validation samples (the remaining half of the samples at each fold). With the large number of clinical samples (546) the issue of over-fitting of data in modeling was minimized as there were only 137 candidate features to be selected from while at each fold 273 samples was used as the discovery set and the sample to feature ratio is more than two. During the cross validation process, the samples were matched for subtype, gender and race. And the process was carried out to optimize the biomarker panel with 3, 4, 5, 6, 7, 8, 9 or 10 miRNAs separately.

[00226] The boxplots representative of the results (the AUC of the biomarker panel in both discovery phase and validation phase) were shown in Figure 20, A. The AUC values were quite close in the various discovery sets (box size < 0.01) and they approached unity (AUC=1.0) with increasing number of miRNAs in the panel. With 4 or more miRNAs, the size of the box in the validation phase, indicative of a spread of values, was quite small (≤0.01 AUC values) as well. As predicted, there was a decrease in AUC values with the validation set for each search (0.02-0.05 AUC).

[00227] A more quantitative representation of the results was shown in Figure 20, B. Although there was always a gradual increase of the AUC in the discovery phase when increasing the number of miRNA in the biomarker panel, there were no further significant improvements in the AUC values in the validation phase when the numbers of the miRNAs were greater than 8. Although the difference between 6 miRNA and 8 miRNA biomarker panels was statistically significant, the improvement was less than 0.01 in AUC values. Thus, a biomarker panel with 6 or more miRNAs giving AUC value around 0.93 should be useful for heart failure detection.

[00228] To examine the composition of multivariate biomarker panels, the present study counted the occurrence of miRNAs in all the panels containing 6-10 miRNAs, where the panels with the top 10% and bottom 10% AUC were excluded. This was carried out to avoid counting of falsely discovered biomarkers due to fitting of inaccurate data from subpopulations generated by the randomization process in cross-validation analysis. Excluding these miRNAs chosen in less than 2% of the panels, a total of 51 miRNA were selected in the discovery process (Table 16) where the expression of 42 of these were also found to be significantly altered in HF (Table 5-7). The inclusion of 9 others, although not altered in heart failure, were found to significantly improve the AUC values as 39% of the panels included at least one of these miRNA from the list and the most frequently selected miRNA (hsa-miR-10b-5p) presented in 35% of the panels. Without a direct and quantitative measurement of all miRNA targets, these miRNAs would never have been selected in high-through

put screening studies (microarray, sequencing) and would have been excluded for further qPCR validation.

[00229] When comparing the identities of the chosen miRNAs for multivariate panels and single miRNA as diagnostic markers, they were not necessarily the same. For example, the top up - regulated (hsa-let-7d-3p) miRNA was not present in the list while the top down-regulated (hsa-miR-454-3p) was only used in 24.2% of the panels. Hence, it was not possible merely to combine the best single miRNA identified to form the optimal biomarker panel but rather a panel of miRNAs providing complementary information gave the best result.

[00230] All those miRNAs were not randomly selected as 7 of them presented in more than 30% of the panels but it was also difficult to find miRNAs to be critical for a good biomarkers panel as the two most frequently selected miRNAs hsa-miR-551b-3p and hsa-miR-24-3p were only found in 59.7% and 57.3% of the panels respectively. As discussed, a lot of those miRNAs were correlated (Figure 11) which could serve as replacement or substitutes for each other in the biomarker panels. In conclusion, a biomarker panel with at least 6 miRNAs from the frequently selected list (Table 16) should be used for the detection of heart failure.

[00231] To compare the miRNA biomarkers and NT-proBNP, one of the six miRNA biomarker panel was selected to calculate the combined miRNA scores for all subjects, which were plotted against the NT-proBNP levels from the same subjects (Figure 21, A). In general, the inventors of the present study observed a positive correlation where the Pearson correlation coefficient between the miRNA score and ln_NT-proBNP was 0.61 (p-value = 8.2E-56). Applying the suggested cut-off for NT-proBNP (125 pg/mL, dashed line), 35 of the healthy subjects were falsely classified as heart failure patient (false positive, FP, NT-proBNP>125) and 23 heart failure patients had NT-proBNP levels lower than the cut-off (false negative, FN). Predictably, most of the false negative (FN) were HFPEF subjects (n=20). Those false positive (FP) and false negative (FN) subjects with respect to NT-proBNP were selected and results plotted against the miRNA score (Figure 21, B). Based on the separate plot, most of the false positive (FP) and false negative (FN) subjects could be correctly re-classified by the miRNA score with zero as the cut-off (dashed line). The results validated the hypothesis that the miRNA biomarkers carry different information than NT-proBNP. The next step was to explore a multivariate biomarker panel including both miRNA and NT-proBNP.

[00232] The same biomarker identification process (multiple times of two fold cross validation) was performed where NT-proBNP was pre-fixed as one of the predictive variables and the level of ln_NT-proBNP together with the miRNA expression levels (log2 scale) were used to build the classifier using the support-vector-machine. Since there was no significant increase of AUC when more than 8 miRNAs were used to predict heart failure (Figure 20), the process was carried out to optimize the biomarker panel with 2, 3, 4, 5, 6, 7 or 8 miRNAs (together with NT-proBNP).

[00233] The classifier built in the discovery phase approached perfect separation (AUC = 1.00) with increasing numbers of miRNAs. Performance decreased somewhat in the validation phase (Figure 22, A). Nevertheless, the AUC of the panels containing NT-proBNP in the validation phase (mean AUC > 0.96) were always higher than those biomarker panels including only miRNA (mean AUC < 0.94). The quantitative results (Figure 22, B) showed that there were no further significant improvements in the AUC values in the validation phase when the numbers of the miRNAs were greater than 4 and there was only a tiny increase (0.001 AUC) between 4 and 5 miRNA biomarker panels. Thus, when combining with NT-proBNP, a biomarker panel with 4 or more miRNAs giving AUC value around 0.98 can be used for heart failure detection. By combining miRNA and NT-proBNP, the classification efficiency was significantly improved over NT-proBNP (AUC = 0.962, Figure 22, B).

[00234] Excluding the panels with the top 10% and bottom 10% AUC, the composition of multivariate biomarker panels containing 3-8 miRNAs was examined (Table 17). A total number of 49 miRNA were selected in the discovery process with 14 of them having prevalence higher than 10% (Table 17). Forty two (42) of them also carried information additional to NT-proBNP (p-value after FDR lower than 0.01 in the logistic regression). Again, 46% of the panels included at least one of the 13 miRNAs found to be insignificant in addition to NT-proBNP.

[00235] Although more than half of the significant miRNAs (Table 17, significant list) were also frequently selected when searching for miRNA only biomarker panels (Table 16, significant list) (Figure 23, A), the ranking of the prevalence was different. Some of the highly selected miRNA in conjunction with NT-proBNP (hsa-miR-17-5p (11.6%) and hsa-miR-25-3p (11.0%)) were even not chosen when searching for miRNA based biomarker panels (without NT-proBNP). Also there were only two miRNAs overlapped between the insignificant lists (Table 16 and Table 17, insignificant list) (Figure 23, B). Together, the evidences suggested that a different list of miRNAs should be used together with NT-proBNP compared to the list used for the construction of miRNA only biomarker panels.

[00236] **VI. Multivariate biomarker panels for HF subtype categorization**

[00237] The next attempt was made to identify multivariate biomarker panels for distinguishing between HFREF and HFPEF. Again, all the quantitative data for 137 miRNAs on the 338 heart failure patients were used. Due to the constraints of sample size, multiple (>50) times of four fold cross validation were carried out where all the subjects were randomly divided into four even groups and three of the groups were used (discovery group) to build the classifier to predict the last group (validation group) in turn. In this way, 253-254 subjects will be used in the discovery phase ensuring the same size in each subgroup (HFREF or HFPEF) similar to the number of candidate features (137)

to be selected to minimize the over-fitting. Again the process was performed to optimize 3, 4, 5, 6, 7, 8, 9 or 10 miRNA biomarker panels and 2, 3, 4, 5, 6, 7 or 8 miRNA plus NT-proBNP biomarker panels separately.

[00238] The quantitative results showed that there were no improvements in AUC values when the miRNA-only biomarker panel contained more than 5 miRNAs (Figure 24, A). About 0.76 AUC could be achieved with miRNA biomarker panels which is better than NT-proBNP (AUC = 0.706). Counting all the 6-10 miRNA panels (excluding the top 10% and bottom 10% in terms of AUC), 46 miRNAs were frequently selected (in > 2% of the panel) where 22 were found to be significant in the t-test comparing HFREF and HFPEF while 24 were not (Table 18). The panels for heart failure subtype categorization were less diversified than those for heart failure detection as two of the miRNAs presented in more than 80% of the panels (hsa-miR-30a-5p (94.6%) and hsa-miR-181a-2-3p (83.7%), Table 18).

[00239] For the biomarker panels consisting both miRNA and NT-proBNP, fewer miRNAs were needed when compared to the miRNA-only panels as there were no improvements on the AUC values beyond inclusion of 4 miRNAs (Figure 24, B). Even clearer classification could be achieved (AUC~0.82) when compared to miRNA-only panels. Again, miRNAs and NT-proBNP may carry complementary information for heart failure subtype categorization. Examining the composition of the 5-8 miRNA plus NT-proBNP panels, 31 miRNAs were frequently selected (in >2% of the panels) where 14 were found to be significant in the logistic regressions together with ln_NT-proBNP while 17 were not (Table 19). Two different miRNAs were found in more than 80% of the panels: hsa-miR-199b-5p (91.5%) and hsa-miR-191-5p (74.9%). Although, the most frequently selected insignificant miRNA for both the miRNA only and miRNA plus NT-proBNP panel were the same (hsa-miR-199b-5p), remarkable differences could be found between the rest of the significant and insignificant lists in terms of identities and rankings.

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Claims

1. A method of determining whether a subject suffers from heart failure, or is at risk of developing heart failure, the method comprising the steps of

a) measuring the level of at least one miRNA from a list of miRNAs "increased" or at least one from a list of miRNAs "reduced" as listed in Table 25, or Table 20, or Table 21, or Table 22, in a sample obtained from the subject, and

b) determining whether it is different as compared to a control,

wherein altered levels of the miRNA indicates that the subject has heart failure or is at a risk of developing heart failure.

2. The method of claim 1, wherein an increase in the level of miRNAs as listed as "increased" in Table 20 or Table 25, as compared to the control, indicates the subject to have heart failure or is at a risk of developing heart failure.

3. The method of claim 1 or 2, wherein a reduction in the level of miRNAs as listed as "reduced" in Table 20 or Table 25 as compared to the control, indicates the subject to have heart failure or is at a risk of developing heart failure.

4. The method of claim 1, wherein an increase in the level of miRNAs as listed as "increased" in Table 21, as compared to the control, indicates the subject to have heart failure with reduced left ventricular ejection fraction (HFREF) or is at a risk of developing heart failure with reduced left ventricular ejection fraction (HFREF).

5. The method of claim 1 or 4, wherein a reduction in the level of miRNAs as listed as "reduced" in Table 21 as compared to the control, indicates the subject to have heart failure with reduced left ventricular ejection fraction (HFREF) or is at a risk of developing heart failure with reduced left ventricular ejection fraction (HFREF).

6. The method of claim 1, wherein an increase in the level of miRNAs as listed as "increased" in Table 22, as compared to the control, indicates the subject to have heart failure with preserved left ventricular ejection fraction (HFPEF) or is at a risk of developing heart failure with preserved left ventricular ejection fraction (HFPEF).

7. The method of claim 1 or 6, wherein a reduction in the level of miRNAs as listed as "reduced" in Table 22 as compared to the control, indicates the subject to have heart failure with

preserved left ventricular ejection fraction (HFPEF) or is at a risk of developing heart failure with preserved left ventricular ejection fraction (HFPEF).

8. The method of any one of claims 1 to 7, wherein the control is a sample obtained from a non-heart failure subject (normal).

9. A method of determining whether a subject suffers from a heart failure selected from the group consisting of a heart failure with reduced left ventricular ejection fraction (HFREF) and a heart failure with preserved left ventricular ejection fraction (HFPEF), the method comprising the steps of

a) detecting the levels of at least one miRNA as listed in Table 9 in a sample obtained from the subject and

b) determining whether it is different as compared to a control,

wherein altered levels of the miRNA indicates that the subject has, or is at a risk of, developing heart failure with reduced left ventricular ejection fraction (HFREF) or heart failure with preserved left ventricular ejection fraction (HFPEF).

10. The method of claim 9, wherein an increase in the level of miRNAs as listed as "increased" in Table 9, as compared to the control, indicates the subject has heart failure with reduced left ventricular ejection fraction (HFREF) or heart failure with preserved left ventricular ejection fraction (HFPEF).

11. The method of claim 9 or 10, wherein a reduction in the level of miRNAs as listed as "reduced" in Table 9 as compared to the control, indicates the subject has developing heart failure with reduced left ventricular ejection fraction (HFREF) or heart failure with preserved left ventricular ejection fraction (HFPEF).

12. The method of any one of claims 9 to 11, wherein the control is a subject that has either a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

13. The method of claim 12, wherein when the control is a patient with a heart failure with reduced left ventricular ejection fraction (HFREF), differential expression of miRNAs as listed in Table 9 indicates the subject to have a heart failure with preserved left ventricular ejection fraction (HFPEF).

14. The method of claim 12, wherein when the control is a patient with a heart failure with preserved left ventricular ejection fraction (HFPEF), differential expression of miRNAs as listed in Table 9 indicates the subject to have a heart failure with reduced left ventricular ejection fraction (HFREF).

15. A method for determining the risk of a heart failure patient having an altered risk of death, the method comprises the steps of

a) measuring the levels of at least one miRNA as listed in Table 14 in a sample obtained from the subject; and

b) determining whether the levels of at least one miRNAs listed in Table 14 is different as compared to the levels of the miRNAs of a control population, wherein altered levels of the miRNA indicates that the subject is likely to have an altered risk of death compared to the control population.

16. The method of claim 15, wherein an increase in the level of miRNA as listed as "hazard ratio > 1" in Table 14, as compared to the control, indicates the subject has an increased risk of death.

17. The method of claim 15 or 16, wherein a reduction in the level of miRNA as listed as "hazard ratio > 1" in Table 14, as compared to the control, indicates the subject has a decreased risk of death.

18. The method of claim 15, wherein an increase in the level of miRNA as listed as "hazard ratio < 1" in Table 14, as compared to the control, indicates the subject has a decreased risk of death.

19. The method of claim 15 or 18, wherein a reduction in the level of miRNA as listed as "hazard ratio < 1" in Table 14, as compared to the control, indicates the subject has an increased risk of death.

20. A method for determining the risk of a heart failure patient having an altered risk of disease progression to hospitalization or death, the method comprises the steps of

a) measuring the levels of at least one miRNA as listed in Table 15 in a sample obtained from the subject; and

b) determining whether the levels of at least one miRNAs listed in Table 15 is different as compared to the levels of the miRNAs of a control population, wherein altered levels of the miRNA indicates that the subject is likely to have an altered risk of disease progression to hospitalization or death compared to the control population.

21. The method of claim 20, wherein an increase in the level of miRNA as listed as "hazard ratio > 1" in Table 15, as compared to the control, indicates the subject has an increased risk of disease progression to hospitalization or death.

22. The method of claim 20 or 21, wherein a reduction in the level of miRNA as listed as "hazard ratio > 1" in Table 15, as compared to the control, indicates the subject has a decreased risk of disease progression to hospitalization or death.

23. The method of claim 20, wherein an increase in the level of miRNA as listed as "hazard ratio < 1" in Table 15, as compared to the control, indicates the subject has a decreased risk of disease progression to hospitalization or death.

24. The method of claim 20 or 23, wherein a reduction in the level of miRNA as listed as "hazard ratio < 1" in Table 15, as compared to the control, indicates the subject has an increased risk of disease progression to hospitalization or death.

25. The method of any one of claims 15 to 24, wherein the control population is a cohort of heart failure subjects.

26. The method of any one of claims 15 to 25, wherein the heart failure patient is a subject who has had primary diagnosis of heart failure and/or being treated 3-5 days when symptomatically improved, with resolution of bedside physical signs of heart failure and considered fit to discharge.

27. A method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure, comprising the steps of:

(a) measuring the levels of at least three miRNAs listed in Table 16 or Table 23 in a sample obtained from the subject; and

(b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure.

28. The method of claim 27, wherein the method further comprises measuring the levels of at least one miRNA as listed in "insignificant group" in Table 16, or Table 23 and wherein the at least one miRNA is hsa-miR-10b-5p.

29. A method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure, comprising the steps of:

(a) measuring the levels of at least two miRNAs listed in Table 17 in a sample obtained from the subject; and

(b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure.

30. The method of claim 29, wherein the method further comprises the step of determining the level of Brain Natriuretic Peptide (BNP) and/or N-terminal prohormone of brain natriuretic peptide (NT-proBNP).

31. A method of determining the likelihood of a subject to be suffering from a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF), comprising the steps of:

(a) measuring the levels of at least three miRNA listed in Table 18 in a sample obtained from the subject; and

(b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

32. A method of determining the likelihood of a subject having a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF), comprising the steps of:

(a) measuring the levels of at least two miRNAs listed in Table 19 or Table 24 in a sample obtained from the subject; and

(b) using a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

33. The method of claim 32, wherein the method further comprises the step of determining the level of Brain Natriuretic Peptide (BNP) and/or N-terminal prohormone of brain natriuretic peptide (NT-proBNP).

34. The method of any one of claims 27 to 33, wherein the levels of at least one of the miRNAs measured in step (b), when compared to a control, is not altered in the subject.

35. The method of claim 34, wherein the miRNA which levels when compared to a control is not altered in the subject is the miRNAs listed as "insignificant" in the respective tables.

36. The method of any one of claims 27 to 35, wherein the score is calculated using a classification algorithm selected from the group consisting of support vector machine algorithm, logistic regression algorithm, multinomial logistic regression algorithm, Fisher's linear discriminant algorithm, quadratic classifier algorithm, perceptron algorithm, k-nearest neighbors algorithm, artificial neural network algorithm, random forests algorithm, decision tree algorithm, naive Bayes algorithm, adaptive Bayes network algorithm, and ensemble learning method combining multiple learning algorithms.

37. The method of claim 36, wherein the classification algorithm is pre- trained using the expression level of the control.

38. The method of any one of claims 27 to 37, wherein the classification algorithm compares the expression level of the subject with that of the control and returns a mathematical score that identifies the likelihood of the subject to belong to either one of the control groups.

39. A method of determining the risk of developing heart failure in a subject or determining whether a subject suffers from heart failure, comprising the steps of:

(a) measuring the level of miRNAs of a selected panel as listed in Table 26, in a sample obtained from the subject; and

(b) assigning a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to develop or to have heart failure.

40. A method of determining the likelihood of a subject to be suffering from a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF), comprising the steps of:

(a) measuring the level of miRNA of a selected panel as listed in Table 27 in a sample obtained from the subject; and

(b) assigning a score based on the levels of the miRNAs measured in step (a) to predict the likelihood of the subject to be suffering from, a heart failure with reduced left ventricular ejection fraction (HFREF) or a heart failure with preserved left ventricular ejection fraction (HFPEF).

41. The method of claim 39 or 40, wherein the method further comprises the step of determining the level of Brain Natriuretic Peptide (BNP) and/or N-terminal prohormone of brain natriuretic peptide (NT-proBNP).

42. The method of any one of claims 27 to 41, wherein the expression level of the miRNAs is any one of concentration, log(concentration), Ct/Cq number, and two to the power of Ct/Cq number.

43. The method of any one of claims 39 to 42, wherein the score is calculated based on the formula as listed in Table 26 or Table 27.

44. The method of any one of claims 35 to 43, wherein the control is at least one selected from the group consisting of a heart failure free control, a heart failure patient, a HFPEF subtype heart failure patient, and a HFREF subtype heart failure patient.

45. The method of any one of the preceding claims, wherein the sample is bodily fluid.

46. The method of any one of the preceding claims, wherein the subject is of Asian ethnicity.