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(54) Title: CIRCULATING MICRORNAS IN PATIENTS WITH ACUTE HEART FAILURE

(57) Abstract: The invention relates to diagnosis, prognosis or classification of acute heart failure (AHF) using circulating micro RNAs as molecular markers. Provided is a method of determining whether a subject has AHF, is at increased risk of developing AHF, or has AHF that is progressing, comprising measuring in a test sample obtained from the subject at least four mi RNAs selected from the group consisting of mi R-18a-5p, mi R-26b-5p, mi R-27a-3p, mi R-30e-5p, mi R-106a-5p, mi R-199a-3p, and mi R-652-3p. Also provided is a diagnostic kit comprising a plurality of primers and/or probes specific for determining expression levels at least four of mi R-18a-5p, mi R-26b-5p, mi R-27a-3p, mi R-30e-5p, mi R-106a-5p, mi R-199a-3p, and mi R-652-3p.



CIRCULATING MICRORNAS IN PATIENTS WITH ACUTE HEART FAILURE

Title:

5 The invention relates generally to the fields of cardiovascular disease, molecular diagnostics and patient management. More particularly, it concerns diagnosis, prognosis or classification of acute heart failure using circulating microRNAs as molecular markers. Provided are means and methods for determining whether a subject has acute heart failure, is at
10 increased risk of developing acute heart failure, or has acute heart failure that is progressing.

 Heart failure is a leading cause of hospitalization amongst adults in the developed world. Despite improvements in the treatment of patients with chronic heart failure (CHF), evidence-based treatment for patients
15 hospitalized with acute heart failure (AHF) is lacking, and outcome remains poor. Approximately 40% of patients hospitalized with acute heart failure are either re-admitted to the hospital or die within 1 year. Better understanding of the pathophysiology underlying AHF may aid the development of novel, targeted therapies.

20 MicroRNAs (miRNAs) are a class of small (approximately 22 nucleotides in length) non-coding RNAs that are potent regulators of gene expression at the post-transcriptional level. MiRNAs are released into the systemic circulation, are remarkably stable and are thought to reflect miRNA expression in tissue. Thus, circulating miRNAs are potential
25 biomarkers for a variety of pathological conditions, including heart failure.

 The discovery that miRNAs are secreted and can be measured extracellularly in blood and other body fluids has stimulated research on circulating miRNAs. To date, most studies on circulating miRNAs in heart failure have been conducted in small numbers of patients with *chronic* heart
30 failure. See for example Zhao et al., *Cardiovasc Pathol.* 2013;22(6):444-450.; Marfella et al., *Eur J Heart Fail.* 2013;15(11):1277-1288; Vogel et al., *Eur*

Heart J. 2013;34(36):2812-2822; Goren et al., Eur J Heart Fail. 2012;14(2):147-154 or Voellenkle et al., Physiol Genomics. 2010;42(3):420-426.

In contrast, circulating miRNA patterns related to *acute* heart failure are not well described. To identify miRNAs associated with acute heart failure, Tijssen *et al.* (Circ Res. 2010;106(6):1035-1039) studied 50 breathless patients, 30 of whom had acute heart failure, and found miR-423-5p levels to be higher compared to those without acute heart failure. See also WO2010/126370. A similarly designed study measured miRNA levels in 135 dyspnoeic patients, including 44 with acute heart failure. Four miRNAs (miR-103, miR-142-3p, miR-30b and miR-342-3p) differed with lower levels compared to controls and patients with other causes of dyspnea (Ellis *et al.*, KL, Eur J Heart Fail. 2013;15(10):1138-1147) Corsten *et al.* (Circ Cardiovasc Genet. 2010;3(6):499-506) found increased plasma miR-499 levels in patients admitted with acute heart failure (n=33) compared to healthy controls (n=20). However, whereas studies on AHF did provide some miRNA marker patterns, these studies were limited by a small sample size and lack of validation.

Recognizing the clinical need for a reliable miRNA biomarker panel for acute heart failure, preferably a prognostic/predictive panel, the present inventors conducted a largest and comprehensive study on miRNAs in acute heart failure. They identified and validated a panel of miRNAs that were consistently down-regulated in patients with acute heart failure. The panel was compared and validated in different cohorts of chronic heart failure, acute exacerbation of COPD and healthy controls. Surprisingly, the association was further supported by a consistent association between a further down regulation early after hospital admission for acute heart failure, and mortality within 180 days after discharge. This miRNA panel is not only a signature to diagnose a subject suffering from acute heart failure,

but it also allows for the early identification of patients at-risk of poorer outcomes, thus improving patient treatment by identifying those who need a more intense follow-up and management.

Accordingly, in one embodiment the invention provides a method
5 of determining whether a subject has acute heart failure, is at increased risk of developing acute heart failure, or has acute heart failure that is progressing, comprising

measuring the level of a plurality of miRNA biomarkers in test
sample obtained from the subject, wherein said plurality of miRNA
10 biomarkers comprises at least four miRNAs selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, wherein a reduced level of said at least four miRNAs relative to a normal control indicates that the subject has
acute heart failure, is at increased risk of developing acute heart failure, or
15 has heart failure that is adversely progressing.

As is exemplified herein below, the expression of each of the above miRNA biomarkers was found to be significantly decreased (downregulated) in AHF patients. Accordingly, a reduced level of said at least four miRNAs
20 relative to a normal control indicates that the subject has acute heart failure, is at increased risk of developing acute heart failure, or has heart failure that is adversely progressing. In one aspect, a reduced level refers to an expression of the biomarker that is decreased by at least 2-fold, 3-fold, 4-fold, 5-fold, or 6-fold relative to control sample. In a preferred embodiment,
25 there is at least a 10-fold reduction. For example, levels of miR-18a-5p, miR-26-5p, miR-27a-3p, miR-106a-5p, miR-199a-3p and miR-625-30 were each found to be at least 10-fold lower in plasma from AHF patients compared to controls.

Exemplary combinations of miRNAs present in a AHF biomarker panel of the invention include one or more of miR-18a-5p, miR-27a-3p and miR-199a-3p. In one aspect, a method of the invention comprises measuring the level of miR-18a-5p, miR-27a-3p and miR-199a-3p, with at least one
5 additional marker selected from the group consisting of miR-26b-5p, miR-30e-5p, miR-106a-5p, and miR-652-3p. Specific exemplary panels include the following biomarker combinations:

(i) miR-18a-5p, miR-27a-3p and miR-199a-3p, with at least two additional marker selected from the group consisting of miR-26b-5p, miR-
10 30e-5p, miR-106a-5p, and miR-652-3p.

(ii) miR-18a-5p, miR-27a-3p, with at least two additional markers selected from the group consisting of miR-199a-3p, miR-26b-5p, miR-30e-5p, miR-106a-5p, and miR-652-3p.

(iii) miR-27a-3p, miR-199a-3p, with at least two additional
15 markers selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-30e-5p, miR-106a-5p, and miR-652-3p.

(iv) miR-18a-5p, miR-199a-3p, with at least two additional markers selected from the group consisting of miR-27a-3p, miR-26b-5p, miR-30e-5p, miR-106a-5p, and miR-652-3p.

20

Further exemplary combinations of miRNAs present in a AHF biomarker panel of the invention include one or more of miR-106a-5p, miR-199a-3p, and miR-652-3p. In one aspect, a method of the invention comprises measuring the level of miR-106a-5p, miR-199a-3p, and miR-652-
25 3p, with at least one additional marker selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p and miR-30e-5p. Specific exemplary panels include the following biomarker combinations:

(v) miR-106a-5p, miR-199a-3p, and miR-652-3p, with at least two additional markers selected from the group consisting of miR-18a-5p, miR-
30 26b-5p, miR-27a-3p and miR-30e-5p;

(vi) miR-106a-5p, miR-199a-3p, and at least two additional markers selected from the group consisting of miR-652-3p, miR-18a-5p, miR-26b-5p, miR-27a-3p and miR-30e-5p;

(vii) miR-199a-3p, miR-652-3p, and at least two additional markers selected from the group consisting of miR-106a-5p, miR-18a-5p, miR-26b-5p, miR-27a-3p and miR-30e-5p;

(viii) miR-106a-5p, miR-652-3p, and at least two additional markers selected from the group consisting of miR-199a-3p miR-18a-5p, miR-26b-5p, miR-27a-3p and miR-30e-5p.

10

“Acute heart failure”, abbreviated to “AHF”, as used herein refers to a gradual or rapid change in heart failure signs and symptoms resulting in a need for urgent therapy. These symptoms are primarily the result of severe pulmonary congestion due to elevated left ventricular (LV) filling pressures (with or without low cardiac output). Thus, AHF is distinct from the general term “heart failure” that describes the final common endpoint of many disease processes. Notably, AHF is to be distinguished from other heart conditions such as heart failure caused by coronary artery disease, which can lead to a myocardial infarction (heart attack), often resulting in death of cardiac cells. Valve disease or high blood pressure can lead to heart failure by increasing the workload of the heart. Less frequent causes of heart failure, which primarily involve cardiac muscle, are classed as cardiomyopathy. AHF is of course also distinct from chronic obstructive coronary artery disease, which can also cause heart failure.

25

“miRNA” and “miR” are used interchangeably herein and is meant to refer to the microRNAs described herein.

“miR-18a-5p” as used herein indicates a mature miR-18a-5p, i.e. a nucleic acid having at least 85% (e.g. at least 90%, 95%, 98%, or 100%) identity to the sequence UAAGGUGCAUCUAGUGCAGAUAG (Sequence 1).

30

‘miR-26b-5p ‘ as used herein indicates a mature miR-26b-5p i.e. a nucleic acid having at least 85% (e.g. at least 90%, 95%, 98%, or 100%) identity to the sequence UUCAAGUAAUUCAGGAUAGGU (Sequence 2).

5 ‘miR-27a-3p ‘ as used herein indicates a mature miR-27a-3p i.e. a nucleic acid having at least 85% (e.g. at least 90%, 95%, 98%, or 100%) identity to the sequence AGGGCUUAGCUGCUUGUGAGCA (Sequence 3).

‘miR-30e-5p ‘ as used herein indicates a mature miR-30e-5p i.e. a nucleic acid having at least 85% (e.g. at least 90%, 95%, 98%, or 100%) identity to the sequence UGUAAACAUCCUUGACUGGAAG (Sequence 4).

10 ‘miR-106a-5p ‘ as used herein indicates a mature miR-106a-5p i.e. a nucleic acid having at least 85% (e.g. at least 90%, 95%, 98%, or 100%) identity to the sequence CUGCAAUGUAAGCACUUCUUAC (Sequence 5).

‘miR-199a-3p ‘ as used herein indicates a mature miR-199a-3p i.e. a nucleic acid having at least 85% (e.g. at least 90%, 95%, 98%, or 100%) identity to the sequence ACAGUAGUCUGCACAUUGGUUA (Sequence 6).

‘miR-652-3p ‘ as used herein indicates a mature miR-652-3p i.e. a nucleic acid having at least 85% (e.g. at least 90%, 95%, 98%, or 100%) identity to the sequence AAUGGCGCCACUAGGGUUGUG (Sequence 7).

20 WO 2008/043521 discloses a large number of miRNAs, only one of which overlaps with those of the present invention, for evaluating and treating a cardiac disease. Di Stefano, Valeria et al Vascular Pharmacology, Vol. 55, no. 4, sp. ISS. Si, (2011-10), pages 111-118 discloses various miRNAs as markers. WO 2008/042231 discloses a list of microRNAs, including miR-25 27a, as suitable markers for evaluating heart diseases. WO 2013/127782 and DE102012101557 relate to the use of certain microRNAs and/or genes, both individually and as a combination of several, in the form of profiles as markers for identifying individual forms of non-ischemic cardiomyopathies or storage diseases of the heart. WO2013/107459 relates to methods for 30 improving the diagnosis and prognosis of patients with pancreatic

carcinoma by making use of specific mi RNA biomarkers associated with pancreatic carcinoma that may be identified based on a blood sample from an individual. Corsten *et al.* (Circ. Cardiovas. Genet. 2010; 3:499-506) disclose the isolation of miRNAs from plasmas from well-characterized

5 patients with varying degrees of cardiac damage: (1) acute myocardial infarction, (2) viral myocarditis, (3) diastolic dysfunction, and (4) acute heart failure. In patients with AHF, only miR-499 was significantly elevated. WO2008/042231 discloses the analysis of several miRNAs in a myocardium sample. WO 2014/083081 discloses an miRNA panel that contains miR-16,

10 miR-27a, miR-101 and miR-150 to aid in the prognostication of patients having suffered from acute myocardial infarction. Importantly, the specific combination of miRNAs present in a AHF biomarker panel of the invention is not disclosed or suggested in the art.

15 Whereas a biomarker panel of the invention can contain only at least four miRNA biomarkers, the skilled person will appreciate that the reliability generally increases with increasing the number of biomarkers. Accordingly, in one embodiment the measuring comprises measuring the level of at least five, preferably at least six, of said miR-18a-5p, miR-26b-5p, miR-27a-3p,

20 miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p. In a specific aspect, the level of each of said miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p is measured. Hence, also provided is a circulating miRNA profile for AHF, comprising at least four, preferably at least five, more preferably at least six, most preferably all

25 of said miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p. Still further useful markers include one or more selected from the group consisting of let-7i-5p, miR-16-5p, miR-18b-5p, miR-128, miR-223-3p, miR-301a-3p, miR-423-3p and miR-423-5p.

In one aspect, it comprises measuring the level of let-7i-5p, miR-16-5p, miR-18a-5p, miR-18b-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-128, miR-199a-3p, miR-223-3p, miR-301a-3p, miR-423-3p, miR-423-5p and miR-652-3p.

5

A method of the invention comprises measuring the level of a plurality of miRNAs in a test sample obtained (isolated) from the subject. The invention thus relates to a method of determining *in vitro* whether a subject has acute heart failure, is at increased risk of developing acute heart failure, or has acute heart failure that is progressing. The subject is preferably a human subject.

"Test sample" is used in its broadest sense as containing nucleic acids. More specifically, any biological test sample comprising or suspected to comprise circulating miRNAs of the subject can be used. A sample may comprise a bodily fluid such as blood or urine. A suitable sample for detection of a is a blood sample. A blood sample may comprise a whole blood sample, or a sample that is obtained by centrifugation and/or filtration such as, for example, plasma, serum, platelets, red blood cell, white blood cells, as is known to the skilled person. A blood sample may be obtained by venepuncture, arteripuncture and/or capillary puncture such as, for example, a finger prick. The sample, preferably a blood sample, may be collected in a tube comprising an anticoagulant such as EDTA, as is known to the skilled person.

In a preferred embodiment, the biological sample is a plasma sample. "Plasma" as used herein indicates the straw-colored/pale-yellow liquid component of blood that normally contains blood cells in whole blood in suspension. Blood plasma is prepared by spinning a tube of fresh blood containing an anti-coagulant in a centrifuge until the blood cells fall to the bottom of the tube. The blood plasma is then poured or drawn off.

30

The amount of miRNAs associated with acute heart failure may be determined after isolation of RNA from a sample. Methods for isolating RNA are known in the art, including the use of commercial RNA isolation kits such as, for example, mirVana PARIS kit (Ambion), miRCURY RNA
5 Isolation Kits - Biofluids (Exiqon) or Trizol LS (Invitrogen). The isolation of RNA is typically performed in the presence of a strong denaturant such as GITC, LiCl, SDS and/or phenol in order to inactivate RNase, if present. Alternatively, a biological sample can be processed for detection of miRNA sequences without prior isolation of RNA, for example by isolating vesicles
10 such as microvesicles from a sample.

With regard to the determination of the panel of miRNAs of the invention, the expression of these miRNAs can be measured separately or simultaneously. In addition to determining the level of at least four biomarkers from the panel described herein above, the method may
15 comprise measuring additional miRNAs that can provide useful biological information, in particular relating to the disease progress of AHF. As shown herein below, the present inventors identified each of let-7i-5p, miR-16-5p, miR-18b-5p, miR-223-3p, miR423-3p and miR-423-5p to be significantly down-regulated in patients suffering from AHF. The method therefore may
20 further comprise measuring the level of at least one of let-7i-5p, miR-16-5p, miR-18b-5p, miR-223-3p, miR423-3p and miR-423-5p, wherein a reduced level relative to a normal control indicates an increased risk of or the presence of acute heart failure in the subject.

In one embodiment, the method comprises detecting the level of
25 each of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, supplemented with at least one, preferably at least two, more preferably at least three of let-7i-5p, miR-16-5p, miR-18b-5p, miR-223-3p, miR423-3p and miR-423-5p.

The present inventors also investigated the relation between circulating miRNAs and the clinical outcome of acute heart failure. Considering the association between increasing miRNA levels and decreasing heart failure acuity, it was hypothesized that short-term (further) decreases in miRNAs of the above mentioned AHF miRNA panel is associated with poor outcome. Indeed, decreased levels of 7 (let-7i-5p, miR-18a-5p, miR-18b-5p, miR-223-3p, miR-301a-5p, miR-423-5p, miR-652-3p) of the selected 15 miRNAs during the first 48 hours after admission for acute heart failure were associated with an increased risk of 180 day mortality on univariable analysis. At least two out of the seven (miR-18a-5p and miR-652-3p) passed Bonferroni correction in the validation cohort.

Accordingly, in a specific aspect, the invention provides a method for monitoring the progression of acute heart failure, comprising:

- (a) obtaining a first biological sample from the subject;
- (b) subsequently, preferably within 5 days of said first biological sample, obtaining a second biological sample from the subject; and
- (c) detecting the level of at least four of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p in said first and second biological samples, wherein a decrease in the expression of said at least four of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p in the second sample relative to the first sample indicates an adverse disease progression of the acute heart failure.

The second sample is preferably obtained within 4 days, more preferably 3 days, e.g. within 48 or 24 hours. Hence, also provided is a method for predicting and/or monitoring the prognosis of acute heart failure in a patient, wherein the patient has suffered from an acute heart failure, comprising determining in successive samples obtained from the same subject the levels of at least four of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p wherein a

decrease in levels of said miR markers is indicative of an adverse disease progression.

In one aspect, the invention provides a method for monitoring the progression of acute heart failure in a subject, comprising detecting the level
5 of at least four of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p in a first and a second sample obtained from the subject, wherein the second sample is obtained within 5 days, preferably 4 days, more preferably 3 days, e.g. within 48 or 24, of said first sample; and wherein a decrease in the expression of said least four of
10 miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, in the second sample relative to the first sample indicates an adverse disease progression of the acute heart failure. For example, an adverse disease progression of the acute heart failure comprises an increased risk of 180 day mortality. Preferably, the method comprises detecting at least miR-
15 18a-5p and miR-652-3p.

Classification of a patient in the “increased” or “high” risk group using a method of the invention, alone or in conjunction with other test results, aids the healthcare provider in determining a treatment plan. Once the health
20 care provider knows to which disease class (i.e. being at risk for 180 day mortality or not) the sample, and therefore, the individual belongs, the health care provider can determine an adequate treatment plan for the individual. For example, different assessments of heart failure acuity left reduction often require differing treatments. Properly diagnosing and
25 understanding the seriousness of acute heart failure of an individual allows for a better, more successful treatment and prognosis. Other applications of the invention include classifying persons who are likely to have successful treatment with a particular drug or therapeutic regiment. In certain embodiments, the treatment is the administration of a drug, such as an
30 ACE inhibitor, an angiotensin II receptor blocker, a Beta-blocker, a

vasodilator, a cardiac glycoside, an antiarrhythmic agent, a diuretic, statins, or an anticoagulant, an inotropic agent; an immunosuppressive agent, use of a pacemaker, defibrillator, mechanical circulatory support, or surgery.

5 Some methods according to the present invention also involve comparing the expression level of the at least one biomarker miRNA to the expression level of a comparative marker from the sample. In other embodiments, methods involve comparing the expression level of at least one biomarker miRNA to the expression level of that biomarker miRNA in a standardized sample,
10 such as a sample known to be isolated from a AHF patient. A "comparative marker" refers to a gene product (such as a protein, RNA transcript, miRNA, or unprocessed miRNA) whose expression level is used to evaluate the level of an miRNA in the sample; in some embodiments, the expression level of a comparative miRNA is used to evaluate a biomarker miRNA
15 expression level. In specific embodiments, the comparative marker is an miRNA. Moreover, in certain embodiments, a comparative marker may also be a biomarker miRNA. In particular cases, a comparative marker is one whose expression level appears to change in the opposite direction as a biomarker miRNA against which it is compared. In some embodiments, the
20 comparative marker is miR-30a-5p, miR-194-5p, miR-627, let7a-5p, miR-378a.

Quantification of the relevant miRNAs in a sample may be performed using any method known in the art for quantification of miRNA or other small
25 RNAs. In one embodiment, the method comprises measuring the level of said miRNAs using RT-PCR, a biochip, quantitative PCR, serial analysis of gene expression (SAGE), or a microarray. All of the following methods are applicable to each of the embodiments described herein for diagnosing AHF or predicting AHF progression. A first example of such methods is miRNA
30 quantitation by RT-PCR using stem-loop primers for reverse transcription

followed by real-time quantitative PCR using a TaqMan® probe. In this method, stem-loop reverse transcription (RT) primers are annealed to the miRNA targets and extended using reverse transcriptase. Generation of the cDNA is followed by real-time PCR with an miRNA- specific forward primer, a TaqMan probe, and a reverse primer. Quantities of the targeted miRNAs are estimated based on measurement of CT values. These methods are known in the art and described, for example, in publications and gene expression assay product bulletins of Applied Biosystems, Foster City, CA. Primers for reverse transcriptase-mediated cDNA synthesis may be provided by the provision of a shared sequence to all miRNA sequences such as, for example, a poly(A)-tail by ligation or through action of a Terminal Transferase, followed by annealing of an adapter-oligo(dT) primer. Further methods comprise the use of a stem-loop primer, and/or the use of a miRNA-specific primer. The quantitative amplification of the RNA sequences, preferably by real-time PCR, preferably comprises a universal primer and a miRNA- specific primer.

The primers used for detection, cDNA synthesis and/or amplification preferably comprise RNA nucleotides, DNA nucleotides or modified nucleotides such as Locked Nucleic Acid (LNA) nucleotides, Peptide Nucleic Acid (PNA) nucleotides, and/or 2'-O-alkyl modifications, 2'-fluoro modifications, 4'-thio modifications, a phosphorotioate linkage, a morpholino linkage, a phosphonocarboxylate linkage. In a preferred embodiment, the length of a primer, preferably a miRNA- specific primer, is identical to the length of the specific miRNA. In a further preferred embodiment, the length of the miRNA- specific primer is shorter than the length of the miRNA, for example 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, or 23 nucleotides, depending on the length of the specific miRNA. The sequence of a primer, preferably a miRNA- specific primer, preferably

comprises one or two mismatches compared to the sequence of the miRNA or the adapter sequence that is added to the miRNA, more preferably is identical to the sequence of the miRNA.

5 Another example of an miRNA quantitation method for use in the embodiments of the invention is SYBR Green detection method using locked nucleic acid (LNA)-based primers (miRCURY™ LNA microRNA PCR system, Applied Biosystems, Foster City, CA; See M. Lunn, et al. Nature Methods, February 2008) or Exiqon's microRNA qPCR system. In this
10 method, miRNAs are reverse transcribed from total RNA in a sample using miRNA- specific RT primers, and the reverse- transcribed miRNAs are amplified using an LNA-enhanced PCR primer anchored in the miRNA sequence together and a universal PCR primer. Amplified miRNAs are quantitated by detection of fluorescence in the SYBR Green assay.

15 In yet another embodiment, the measuring may be performed by hybridization on a chip or microarray having the miRNAs of the AHF panel according to the invention as features thereon. The quantity of an miRNA in the sample being tested is typically determined by measurement of the fluorescence intensity of hybridization to the corresponding feature.

20 Another assay that can be used in the embodiments of the invention for quantitation of the relevant miRNAs is the Luminex® branched DNA (bDNA) assay (Panomics, Fremont, CA). This is a high-throughput multiplex bead-based assay based on the xMAP® technology of Luminex Corporation. Specific miRNAs are captured on their respective
25 beads by hybridization with a capture probe, followed by sequential hybridization of pre- amplifier, amplifier and biotinylated label probes. Binding with streptavidin-conjugated phycoerythrin and analysis of individual beads for level of fluorescence quantifies the amount of miRNA captured by the bead. This assay is described in the Luminex product
30 bulletins published by Panomics.

In still a further embodiment, a method of the invention comprises the use of a next-generation sequencing (NGS) platform for small RNA sequences. (NGS) is gaining popularity and has successfully been used to characterize miRNA profiles in various tissues as well as bio-fluids including blood and cerebral spinal fluid. See for example Wu *et al.* Clinica Chimica Acta 2012, 413(13–14):1058-1065; Burgos *et al.* RNA 2013, 19(5):712-722.

Methods and means for detection of the circulating miRNA signature of the present invention are preferably provided as a kit. Accordingly, the invention also provides a kit comprising a plurality of primers and/or probes specific for determining expression levels at least four, preferably at least five of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p. Preferred miRNAs and combinations thereof are discussed herein above. In one aspect, the kit comprises primers and/or probes designed to detect each of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p. The kit may further comprise primers and/or probes designed to detect at least one of let-7i-5p, miR-16-5p, miR-18b-5p, miR-223-3p, miR423-3p and miR-423-5p. Said kit preferably comprises one or more reagents for RT-PCR or reverse transcription RT-PCR. For example, the kit comprises a set of primers, preferably at least one specific set of primers, enzymes such as a RNA-dependent DNA polymerase and/or a DNA-dependent DNA polymerase, and at least one buffer for performing the reaction or reactions. The kit components may be provided as dried material, for example after lyophilisation, or as a liquid. In one embodiment, the kit contains reagent(s) for small RNA isolation from a (plasma) sample, such as one or more of lysis buffer, binding columns, wash and elution buffers.

A still further aspect relates to a biochip (e.g. microarray) comprising a panel of isolated nucleic acids, wherein said panel comprises at least four of

miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, 196a, or a complement thereof. The biochip preferably comprises each of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, or its complement.

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Any of the diagnostic/predictive methods described herein above may be implemented on tangible computer-readable medium comprising computer-readable code that, when executed by a computer, causes the computer to perform one or more operations. Provided herein is a tangible, computer-readable medium comprising computer-readable code that, when executed by a computer, causes the computer to perform operations comprising: a) receiving information corresponding to the level of expression of at least four miRNA's selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p and miR-652-3p, in a sample of a patient suspected of having or determined to have acute heart failure; and b) determining a difference value in the expression level using the information corresponding to the expression level in the sample compared to a control or reference level. In some embodiments, there is a tangible computer-readable medium comprising computer-readable code that, when executed by a computer, causes the computer to perform operations comprising: a) receiving information corresponding to a level of expression in a biological sample from a subject, at least four of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p; and b) determining a biomarker panel value using information corresponding to the at least four biomarker miRNAs and information corresponding to the level of expression of a comparative microRNA panel, the biomarker panel value being indicative of whether the subject has acute heart failure, is at increased risk of developing acute heart failure, or has acute heart failure that is progressing. In additional embodiments the medium further comprises computer-readable code that, when executed by a

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computer, causes the computer to perform one or more additional operations comprising: sending information corresponding to the biomarker value to a tangible data storage device. In even further embodiments, the tangible computer-readable medium has computer-readable code that, when
5 executed by a computer, causes the computer to perform operations further comprising: c) calculating a diagnostic score for the biological sample, wherein the diagnostic score is indicative of the probability that the patient suffering from acute heart failure has a poor outcome. It is contemplated that any of the methods described above may be implemented with tangible
10 computer readable medium that has computer readable code, that when executed by a computer, causes the computer to perform operations related to the measuring, comparing, and/or calculating a diagnostic score related to the severity of acute heart failure, e.g. the risk of 180 day mortality.

15 In yet another embodiment, the invention relates to a system for drug discovery, comprising detecting the level of one or more of the miRNAs of the AHF biomarker panel of the present invention. For example, provided is a method for identifying a candidate drug e.g. for the treatment of AHF, by evaluating its capacity to affect the pathway(s) responsible for modulating
20 the level of one or more of these miRNAs. The screening may involve detecting at least one miRNA selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p. Especially preferred miRNAs for use in a screening method include

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Preferably, the screening involves measuring the level of multiple miRNAs, for example at least three miRNAs, preferably at least four, or five, selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p.

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Suitable screening systems include cell-based assays, for example cardiomyocytes derived from embryonic stem (ES) cell lines.

The invention thus also relates to a method for screening a
5 pharmaceutically active compound for the treatment and/or prophylaxis of AHF, wherein the method comprises the steps of (a) providing a cell comprising an expression vector comprising at least miRNA sequence selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p; (b) bringing a
10 candidate for a pharmaceutically active compound into contact with the cell, (c) determining the effect of the candidate on the expression of said at least one miRNA sequence, wherein a change in the expression of said at least one miRNA sequence indicates a pharmaceutically active compound. Preferably, the screening method comprises determining the effect on at
15 least three, preferably at least four, more preferably at least five miRNA sequences selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p.

Particularly preferred combinations of miRNAs for use in a screening method of the invention include:

- 20 (i) miR-18a-5p, miR-27a-3p and miR-199a-3p, with at least two additional marker selected from the group consisting of miR-26b-5p, miR-30e-5p, miR-106a-5p, and miR-652-3p.
- (ii) miR-18a-5p, miR-27a-3p, with at least two additional markers selected from the group consisting of miR-199a-3p, miR-26b-5p, miR-30e-5p,
25 miR-106a-5p, and miR-652-3p.
- (iii) miR-27a-3p, miR-199a-3p, with at least two additional markers selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-30e-5p, miR-106a-5p, and miR-652-3p.

(iv) miR-18a-5p, miR-199a-3p, with at least two additional markers selected from the group consisting of miR-27a-3p, miR-26b-5p, miR-30e-5p, miR-106a-5p, and miR-652-3p.

(v) miR-106a-5p, miR-199a-3p, and miR-652-3p, with at least two
5 additional markers selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p and miR-30e-5p;

(vi) miR-106a-5p, miR-199a-3p, and at least two additional markers selected from the group consisting of miR-652-3p, miR-18a-5p, miR-26b-5p, miR-27a-3p and miR-30e-5p;

10 (vii) miR-199a-3p, miR-652-3p, and at least two additional markers selected from the group consisting of miR-106a-5p, miR-18a-5p, miR-26b-5p, miR-27a-3p and miR-30e-5p;

(viii) miR-106a-5p, miR-652-3p, and at least two additional markers selected from the group consisting of miR-199a-3p miR-18a-5p,
15 miR-26b-5p, miR-27a-3p and miR-30e-5p.

LEGEND TO THE FIGURES

Figure 1: Schematic outline of the study design.

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Figure 2: miRNA levels in plasma samples of AHF patients at various time points. (panel A) Circulating levels of miRNAs of interest in plasma samples of acute heart failure patients at admission (PROTECT), at day 7 after admission (PROTECT), at discharge (COACH), at 6 months after
25 hospitalization (COACH), chronic heart failure patients and healthy controls were quantified by qRT-PCR assay. Values are plotted as geometrical mean $\pm$ SD, **P<0.001. Intercept shows Volcano plot illustration a cluster of the 15 circulating miRNAs that changed most significantly between AHF patients and healthy controls. Log2 ratio of fold change (x-axis) is plotted against statistical significance based on $-\log_{10}$ (y-axis) for
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each miRNA. miRNAs plotted in green passed the Bonferonni correction (based on $p \leq 0.00022$; represented by green dash line) and changed more than 2-fold (represented by two black vertical lines). Both biologically and statistically insignificant miRNAs are plotted in grey.

- 5 (panel B) miRNA levels per cohort (median [IQR]) including p for trend. MiRNAs shown to be significantly changed for both discovery and validation cohorts are highlighted in grey.

Figure 3: Profiling of circulating miRNAs in validation cohorts.

- 10 (Panel A) Volcano plots showing miRNAs levels in plasma samples of AHF patients from the validation cohort compared to healthy controls. 44% of the significantly lowered miRNAs detected in the validation cohort overlap with the decreased miRNAs found in the discovery phase. (panel B) 7 miRNAs out of 15 were consistently and significantly changed in the discovery and
- 15 validation cohorts. (panel C) Volcano plots showing miRNAs levels in plasma samples of COPD patients compared to healthy controls. (panel D) There were no differences in miRNAs level between COPD patients and healthy subjects. (panel E) Graph of principal components analysis (PCA), showing partial differentiation between patients with AHF, COPD patients
- 20 and healthy subjects. (panel F) miRNAs levels in plasma samples of CHF patients of the discovery phase (CHF T, Telosophy, discovery cohort) compared to CHF patients of the validation phase (CHF B, Beneficial, validation cohort).

EXPERIMENTAL SECTION

MATERIALS AND METHODS

Study design and procedures

- 5 Study subjects originated from six separate cohorts in various states of heart failure, ranging from acutely decompensated heart failure to stable chronic heart failure, and healthy controls. Furthermore, a validation cohort of patients with acute exacerbation of chronic obstructive pulmonary disease (COPD) and matching controls were included (Figure 1).
- 10 The acute heart failure cohort was selected from the Placebo-controlled Randomized Study of the Selective A1 Adenosine Receptor Antagonist Rolofylline for Patients Hospitalized with acute heart failure and Volume Overload to Assess Treatment Effect on Congestion and Renal Function (PROTECT) trial. PROTECT was a multicenter, randomized, double-blind,
- 15 placebo-controlled study in patients hospitalized for acute heart failure with mild to moderate renal impairment (Weatherley et al., J Card Fail. 2010;16(1):25-35). MiRNA profiles were measured in blood samples from 100 PROTECT patients collected at four different time points: admission for acute heart failure (AHF- admission), after 24 hours (AHF-24h admission),
- 20 48 hours (AHF-48h admission) and day 7 after admission (AHF-7d admission).

- MiRNA patterns at discharge (AHF-discharge) and 6 months after hospitalization (AHF-6m follow up) for acute heart failure were measured in plasma samples from 18 patients from the Coordinating Study Evaluating
- 25 Outcomes of Advising and Counseling in Heart Failure (COACH) study. COACH was a multicenter, randomized trial on the effects of nurse-led therapy in patients after admission for acute heart failure. Design and results of this trial have been described elsewhere (Jaarsma *et al.*, Eur J Heart Fail. 2004;6(2):227-233). Stable chronic heart failure patients and

healthy control subjects originated from the Telosophy study (Wong *et al.*, PLoS One. 2011;6(8):e23118). The Telosophy study included patients with chronic heart failure of at least 6 months duration who had ischemic heart disease receiving stable guideline-indicated therapy for at least 4 weeks and
5 age and sex matched healthy controls recruited from the outpatient clinic of the University Medical Center, Groningen, The Netherlands. Key exclusion criteria for healthy controls were known atherosclerotic disease, heart failure or a family history of premature cardiovascular disease. For the miRNA profiling study, 10 plasma samples from chronic heart failure
10 patients and 24 plasma samples from age and sex matched healthy controls were analyzed.

For validation purposes, the full panel of 375 miRNAs were validated in 3 independent cohorts; acute heart failure (Wroclaw, Poland), chronic heart failure (Beneficial) and acute exacerbation of chronic obstructive pulmonary
15 disease (Biomarcoeurs, Paris, France). Briefly, the Wroclaw cohort comprised patients admitted to the hospital with a diagnosis of acute heart failure in all cases based on the presence of signs and symptoms of acute heart failure requiring intravenous treatment (loop diuretics, nitroglycerin and/or inotropes). Patients with acute coronary syndrome as underlying
20 cause of acute heart failure were excluded. The Beneficial study was a double-blind, placebo-controlled, randomized trial evaluating the efficacy and safety of alagebrium in stable, chronic heart failure patients. The Biomarcoeurs cohort consisted of consecutive patients arriving for shortness of breath at the Lariboisière hospital, which were prospectively included
25 (NCT01374880). Furthermore, 17 plasma samples from healthy controls (8 originating from Paris and 9 from Wroclaw), which were age and sex matched, were analyzed. Informed consent of all patients was obtained.

MiRNA profiling: isolation, cDNA synthesis and qRT-PCR.

The plasma fraction of the collected EDTA blood was prepared as previously described and stored at -80°C (Weatherly *et al.*, supra). RNA was isolated from 200 µl of plasma using the miRCURY RNA isolation kit – Biofluids
5 (Exiqon, Vedbaek, Denmark). Reverse transcription reactions were performed using the Universal cDNA Synthesis Kit (Exiqon, Vedbaek, Denmark). For each reaction, 4 µl of RNA was used. All the procedures were performed according to the manufacturer's instructions.

During the discovery and the validation phases, expression of 375 human
10 miRNAs was measured using a Serum/Plasma Focus microRNA PCR panel (V2.M) (Exiqon, Vedbaek, Denmark). In the discovery phase, 30 plasma samples from 10 patients hospitalized with acute heart failure (AHF-admission, PROTECT cohort), 10 patients with chronic heart failure and 10 healthy controls (Telosophy cohort) were analyzed. In the validation study,
15 miRNA levels of 9 samples from patients with acute heart failure (Wroclaw), 10 from chronic heart failure patients (Beneficial), 8 from acute exacerbation of chronic obstructive pulmonary disease patients and a total of 17 matching controls were measured. Validation cohort samples were treated and analyzed the same way as discovery cohort samples.
20 Amplification was performed on the *LightCycler® 480* (Roche Applied Science, Rotkreuz, Switzerland) using cycling parameters recommended by Exiqon. Of these 375 miRNAs measured in the discovery cohorts, 15 miRNAs that were statistically and biologically different (miRNAs whose expression profile showed at least a 4-fold change) from the control samples
25 were selected for further analysis in extended cohorts. Relative expression was calculated using the comparative delta- delta- Ct method in the GenEx Professional software (MultiD Analyses, Sweden). MiR-30a-5p, miR-627 and miR-194-5p were used as reference genes. These endogenous miRNAs were selected based on calculations by GeNorm and NormFinder (GenEx

Professional software, MultiD Analyses, Sweden). Expression levels of miR-30a-5p, miR-627 and miR-194-5p remained the same in all analyzed cohorts. The customized panel selected for the extended study consisted of 15 quantitative real-time PCR (qRT-PCR) assays of interest, manufactured by
5 Exiqon. These 15 miRNAs were measured in samples from the patients with acute heart failure (PROTECT and COACH cohorts) and controls (Telosophy cohort). qRT-PCR data were analyzed using the GenEx Professional software (MultiD Analyses, Sweden). Threshold cycle (Ct) values greater than 36 were considered to be below the detection level of the
10 assay. The qRT-PCR data set was normalized against reference genes miR-30a-5p and miR-194-5p. MiR-627 was excluded due to the poor performance.

Statistical methods

Differences in miRNA expression between different groups were determined by a two-tailed unpaired t-test. Bonferroni correction was applied to P-
15 values to adjust for multiple testing. The significance threshold was set to a ≥ 2 -fold change of with a corrected P-value ≤ 0.00022 (discovery and validation cohorts, dataset with 226 detected miRNAs) or ≤ 0.0033 (extension of the study, dataset with 15 detected miRNAs) for the comparison of plasma miRNA expression between studied conditions and time points.
20 Unsupervised hierarchical clustering analysis of miRNA expression profiles was performed to create a heat map. Cox proportional hazards regression was performed to examine associations with outcome. Survival analysis included Harrell's C index calculation. The exact binomial test was used to estimate the likelihood of the occurrence of multiple miRNAs being
25 significant predictors of outcome. Correlation between miRNAs was measured using Spearman rank correlation. P-values ≤ 0.05 were considered significant. All statistical analyses were performed using GenEx Professional version and R: A language and Environment for

Statistical Computing, version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

5 Patient characteristics

Table 1 shows the demographic and clinical characteristics of all cohorts used in this study. Plasma NT-proBNP concentrations differed markedly between the cohorts in the discovery and extended study being lowest in healthy controls and highest in patients with acute heart failure at the time of admission (Table 1).

Table 1. Clinical characteristics of the cohorts

Discovery and extended study				
	PROTECT cohort	COACH cohort	Telosophy cohort	Telosophy cohort
	acute heart failure (AHF) at admission n = 100	acute heart failure (AHF) at discharge n = 18	stable chronic heart failure (CHF) n = 10	healthy controls n = 24
Demographics				
Age (years)	68.9±11.4	69.6±9.9	67±6.1	65.4 ±6.6
Sex (% Male)	50 (50)	55.5 (10)	70 (7)	83.3 (20)
Measurements				
LVEF (%)	34.1±12.6	30.3±9.1	27.6±7	
Systolic Blood Pressure (mmHg)	119.4±17.2	111.4±19.1	112.9±13.3	133.1±17.8
Diastolic Blood Pressure (mmHg)	71.3±11.8	66.2±15.8	66±7.4	80.3±10.1
Heart Rate (beats/min)	78.7±15.6	81.3±10.9	67.6±8.1	66.9±9.3
NYHA class (%)				
II	15 (15)	61.1 (11)	50 (5)	-
III	54 (54)	38.9 (7)	50 (5)	-
IV	27 (27)	0 (0)	0 (0)	-
Medical history (%)				
Myocardial infarction	49 (49)	55.5 (10)	100 (10)	0 (0)
Hypertension	83 (83)	50 (9)	10 (1)	20.8 (5)
Diabetes Mellitus	44 (44)	27.8 (5)	20 (2)	4.2 (1)
Ischemic Heart Disease	73 (73)	-	60 (6)	-
Atrial Fibrillation	58 (58)	33.3 (6)	40 (4)	0 (0)
COPD	15 (15)	16.7 (3)	0 (0)	-
Laboratory values				
BNP (pg/mL)	565 [96-994.5]	456 [197-911]	-	-
NT-proBNP (pg/mL)	3000 [3000-5779.2]	2070.1 [1466.3-4443]	1153.5 [231-1792]	52 [35-63]
Creatinine (mg/dL)	1.4 [1.2-1.9]	1.15 [0.9-1.4]	-	-
Blood Urea Nitrogen (mg/dL)	30 [25-45.2]	-	-	-
eGFR (ml/min/1.73m ²)	45.3 [35.1-62.2]	59.8 [51.9-71.3]	-	-

Validation study

	Wroclaw cohort	Wroclaw cohort	Beneficial cohort	Paris cohort	Paris cohort
	acute heart failure (AHF) at admission	healthy controls	stable chronic heart failure (CHF)	acute exacerbation of COPD (AECOPD)	healthy controls
	n = 9	n = 9	n = 10	n = 8	n = 8
Demographics					
Age (years)	68.5±8.5	68.2±7.8	68.9±4.9	69.1±10.4	71.0±8.7
Sex (% Male)	55.6 (5)	44.4 (4)	50 (5)	62.5 (5)	75 (6)
Measurements					
LVEF (%)	32.2±14.4	-	33.5±9.1	-	-
Systolic Blood Pressure (mmHg)	119.2±19.4	123.1±13.1	119.6±19.1	130.3±28.1	-
Diastolic Blood Pressure (mmHg)	71.6±12.1	73.8±10.6	74.2±9.2	79.9±14.3	-
Heart Rate (beats/min)	88.2±19.3	73.2±10.1	67.3±15.4	108.1±22.6	-
NYHA class (%)					
II	0 (0)	-	70 (7)	-	-
III	44.4 (4)	-	30 (3)	-	-
IV	55.6 (5)	-	0 (0)	-	-
Medical history (%)					
Myocardial infarction	44.4 (4)	-	80 (8)	0 (0)	0 (0)
Hypertension	44.4 (4)	-	40 (4)	0 (0)	75 (6)
Diabetes Mellitus	55.6 (5)	-	0 (0)	0 (0)	12.5 (1)
Ischemic Heart Disease	66.7 (6)	-	-	0 (0)	0 (0)
Atrial Fibrillation	22.2 (2)	-	-	25 (2)	12.5 (1)
COPD	11.1 (1)	-	-	75 (6)	0 (0)
Laboratory values					
NT-proBNP (pg/mL)	7714 [2666.8-15443.3]	-	344 [80.0-525.3]	-	-

Circulating miRNA profiling in acute heart failure patients

Of the 266 miRNAs detected during initial screening, 44 remained significantly different in acute heart failure patients compared to healthy controls after the Bonferroni correction (Table 2).

- 5 **Table 2A.** List of all significantly changed circulating miRNAs in plasma of acute heart failure patients at admission (PROTECT) compared to healthy controls.

miRNAs	Fold change (AHF vs. controls)	P-value	miRNAs	Fold change (AHF vs. controls)	P-value
miR-16-5p	-686,218	1,39E-07	miR-24-3p	-1,285,864	6,11E-05
let-7i-5p	-643,378	4,63E-07	miR-342-3p	-73,649	6,24E-05
miR-18a-5p	-1,965,285	1,09E-06	miR-421	-711,403	7,76E-05
miR-30e-5p	-897,347	2,51E-06	miR-15b-5p	-76,405	7,91E-05
miR-26b-5p	-1,120,186	2,79E-06	miR-126-5p	-875,233	7,95E-05
miR-425-5p	-886,221	3,20E-06	miR-106b-5p	-653,264	8,02E-05
miR-106a-5p	-1,211,453	3,99E-06	miR-103a-3p	-635,841	9,23E-05
miR-301a-3p	-1,379,111	6,96E-06	miR-361-5p	-1,336,758	9,44E-05
miR-652-3p	-134,326	8,86E-06	miR-142-5p	-1,267,283	0,00010
miR-128	-4,468,339	9,16E-06	miR-486-5p	-291,737	0,00011
miR-223-3p	-204,307	9,89E-06	miR-23b-3p	-780,645	0,00012
miR-18b-5p	-1,442,667	1,23E-05	miR-324-5p	-1,045,171	0,00012
miR-532-5p	-63,982	1,63E-05	let-7f-5p	-770,966	0,00012
miR-185-5p	-1,082,029	1,68E-05	miR-92a-3p	-336,048	0,00014
miR-199a-3p	-1,637,777	1,80E-05	miR-200c-3p	-335,117	0,00016
miR-191-5p	-906,098	2,35E-05	miR-30d-5p	-611,633	0,00017
miR-101-3p	-543,269	4,32E-05	miR-23a-3p	-601,126	0,00019
miR-423-3p	-1,361,067	4,60E-05	miR-140-5p	-809,857	0,00019
miR-27a-3p	-1,707,326	5,14E-05	miR-27b-3p	-1,755,325	0,00020
miR-19b-3p	-786,075	5,37E-05	miR-199a-5p	-1,468,902	0,00020

Table 2B. List of all significantly changed circulating miRNAs in plasma of acute heart failure patients at admission (Wroclaw, validation study) compared to healthy controls

miRNAs	Fold change (AHF vs. controls)	P-value	miRNAs	Fold change (AHF vs. controls)	P-value
miR-29b-3p	-14.31795	1.22E-07	miR-103a-3p	-9.41727	4.98E-05
miR-874	-26.84666	1.60E-07	miR-130a-3p	-10.04665	5.22E-05
miR-29a-3p	-6.99008	2.89E-07	miR-376a-3p	-12.65096	5.61E-05
miR-652-3p	-88.49469	9.68E-07	miR-328	-10.31508	5.71E-05
miR-142-5p	-22.03882	1.80E-06	miR-136-5p	-27.48737	6.06E-05
miR-199a-5p	-73.39584	2.03E-06	miR-32-5p	-7.97403	6.33E-05
miR-106a-5p	-5.56515	2.91E-06	miR-148b-3p	-8.29347	6.46E-05
miR-93-5p	-5.43385	4.25E-06	miR-126-5p	-3.48697	6.67E-05
miR-29c-3p	-5.37558	4.63E-06	miR-18a-5p	-4.96945	6.95E-05
miR-20a-5p	-6.83042	5.66E-06	miR-146a-5p	-7.15898	7.03E-05
miR-27a-3p	-6.24186	1.18E-05	let-7f-5p	-4.00548	8.05E-05
miR-26b-5p	-4.5834	1.87E-05	let-7g-5p	-4.81869	8.35E-05
miR-33a-5p	-14.46885	1.90E-05	miR-374b-5p	-5.22053	9.03E-05
miR-101-3p	-4.47527	2.01E-05	miR-24-3p	-5.13283	9.48E-05
miR-23b-3p	-3.81871	2.10E-05	miR-22-3p	-8.44168	0.000100088
miR-342-3p	-5.29341	2.33E-05	miR-143-3p	-10.20817	0.00010738
miR-23a-3p	-3.48966	2.66E-05	miR-107	-82.4325	0.000131569
miR-185-5p	-6.83569	2.91E-05	miR-30b-5p	-4.60463	0.000140483
miR-15b-5p	-4.10226	2.99E-05	miR-30e-5p	-4.65455	0.000144772
miR-199a-3p	-12.48371	3.57E-05	miR-766-3p	-13.29896	0.000146944
miR-331-3p	-9.6091	4.43E-05	miR-451a	-5.45482	0.000166655
miR-106b-5p	-5.88699	4.73E-05	miR-144-3p	-7.14245	0.000172204

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Figure 2A provides an overview of the initial screening data depicted in a volcano plot, with the P-value on the Y axis versus the fold change on the X axis. In addition to examining statistical significance, miRNAs that exhibited the greatest biological differences were selected for further analysis. A panel of 14 miRNAs (let-7i-5p, miR-16-5p, miR-18a-5p, miR-18b-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-128, miR-199a-3p, miR-223-3p, miR-301a-3p, miR-423-3p and miR-652-3p) with a >4 fold change was selected for further analysis, supplemented by miR-423-5p as

10

one of the miRNAs reported most consistently with different levels in heart failure patients. The fold changes and p-values of each of the selected miRNAs are presented in Table 3 .

- 5 **Table 3.** List of 15 circulating miRNAs with reduced levels in plasma samples of acute heart failure patients at admission (PROTECT) compared to healthy controls. In the validation cohort, significantly lower levels in acute heart failure patients compared to healthy controls were confirmed for 12 miRNAs out of 15. Lower levels of miR-301a-3p were not replicated and miR-128 was undetectable.

miRNA	Discovery cohort		Validation cohort	
	Fold change (AHF vs. controls)	P- value	Fold change (AHF vs. controls)	P-value
let-7i-5p	-6,4	4,63E-07	-3,4	0,00042
miR-16-5p	-6,9	1,39E-07	-3,9	0,000565
miR-18a-5p	-19,7	1,09E-06	-4,97	6,95E-05
miR-18b-5p	-14,4	1,23E-05		
miR-26b-5p	-11,2	2,79E-06	-4,58	1,87E-05
miR-27a-3p	-17,1	5,14E-05	-6,24	1,2E-05
miR-30e-5p	-9,0	2,51E-06	-4,65	0,000145
miR-106a-5p	-12,1	3,99E-06	-5,6	2,9E-06
miR-128	-44,7	9,16E-06		
miR-199a-3p	-16,4	1,8E-05	-12,5	3,57E-05
miR-223-3p	-20,4	9,89E-06	-7,7	0,000539
miR-301a-3p	-13,8	6,96E-06		
miR-423-3p	-13,6	4,6E-05	-3,9	0,000934
miR-423-5p	-2,3	1,37E-02	-2,0	0,027
miR-625-3p	-13,4	8,86E-06	-88,4	9,68E-07

Differential miRNA levels in various stages of heart failure

In order to determine the profile of circulating miRNA levels from acute heart failure admission to post discharge and 6 month follow-up status, the 15 miRNAs selected during the discovery phase were measured in the
5 extended cohort described in the methods section. The association of the selected 15 miRNAs with acute heart failure was further supported by a highly consistent pattern of decreased miRNA levels with increased acuity of heart failure (Figure 2A). The lowest levels of miRNAs were observed in patients from admission for acute heart failure to day 7. The miRNAs of the
10 panel gradually increased in COACH acute heart failure patients at discharge (AHF-discharge) and converged at 6 months towards the chronic heart failure levels (AHF-6m follow up). A significant trend over the different time points was observed for all miRNAs (all $p < 0.001$), shown in Figure 2B. The results were confirmed using unsupervised hierarchical
15 cluster analysis, which showed a clear separation of patients with acute heart failure from the healthy controls and chronic heart failure patients (data not shown).

Circulating miRNAs profiles in the validation cohorts

The obtained results were validated in three independent cohorts: a
20 validation cohort consisting of 9 acute heart failure patients at admission (Wroclaw cohort) and 9 matching healthy controls (Wroclaw cohort), 10 patients with chronic heart failure and a dyspnoeic control cohort comprising 8 patients with acute exacerbation of chronic obstructive pulmonary disease (Paris cohort) and 8 matching healthy control subjects
25 (Paris cohort). Reassuringly, plasma levels of 12 miRNAs out of 15 that were identified during the discovery phase were shown to be significantly decreased in acute heart failure compared to chronic heart failure patients and healthy controls (Table 3), of which seven crossed the false discovery rate, settled to correct for multiple testing; miR-18a-5p, miR-26b-5p, miR-

27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, respectively.

The validation results are summarized in Figure 3. In the validation cohort the levels of these 7 miRNAs were significantly lower in acute heart failure patients compared with healthy controls (Figure 3A,B). There were no differences in miRNA levels between patients admitted with an acute exacerbation of chronic obstructive pulmonary disease and healthy controls (Figure 3C,D). The differentiation between acute heart failure patients from both discovery and validation cohorts and healthy controls is clearly shown by the result of a principal component analysis (PCA) depicted in Figure 3E. Patients admitted with an acute exacerbation of chronic obstructive pulmonary disease were clustered together with healthy controls.

Circulating miRNAs related to clinical outcome

Associations between miRNA levels and 180 day mortality were examined in the PROTECT extended cohort. Considering the association between increasing miRNAs levels and decreasing heart failure acuity, we hypothesized that short-term decreases in miRNAs levels might be associated with poor outcome. Table 4 shows the results of the univariable Cox regression analysis with a Harrell's C-index to assess the predictive accuracy of the Cox model. In Cox regression models, decreased levels of 7 (let-7i-5p, miR-18a-5p, miR-18b-5p, miR-223-3p, miR-301a-5p, miR-423-5p, miR-652-3p) of the selected 15 miRNAs during the first 48 hours after admission for acute heart failure were associated with an increased risk of 180 day mortality on univariable analysis (Table 4). At least two out of the seven (miR-18a-5p and miR-652-3p) passed Bonferroni correction in the validation cohort. The result of the exact binomial test was highly significant ($p < 3.518 \times 10^{-6}$), thus the likelihood of the occurrence of multiple miRNAs predicting outcome by chance is highly improbable. Furthermore,

the directionality of the Hazard Ratios of all 15 miRNAs (HR >1) supports our hypothesis.

Table 4. Cox analysis for 180 day mortality.

- 5 Univariable associations of miRNA changes during the first 48 hours after hospitalization for AHF and 180 day mortality. Predictive performance was quantified with the C-index. MiRNAs depicted in grey represent the 2 miRNAs validated in the additional independent cohorts.

Delta miRNA	Hazard Ratio (95% CI)	P-value	Harrell's C-index
let-7i-5p	1.536 (1.122-2.103)	0.007	0.657
miR-16-5p	1.263 (0.964-1.654)	0.091	0.617
miR-18a-5p	1.303 (1.056-1.607)	0.014	0.664
miR-18b-5p	1.353 (1.066-1.717)	0.013	0.669
miR-26b-5p	1.049 (0.829-1.328)	0.69	0.515
miR-27a-3p	1.141 (0.964-1.352)	0.125	0.596
miR-30e-5p	1.26 (0.987-1.609)	0.063	0.609
miR-106a-5p	1.264 (0.993-1.608)	0.057	0.638
miR-128	1.094 (0.917-1.306)	0.32	0.576
miR-199a-3p	1.097 (0.93-1.294)	0.273	0.572
miR-223-3p	1.232 (1.016-1.494)	0.034	0.649
miR-301a-3p	1.326 (1.092-1.611)	0.004	0.697
miR-423-3p	1.145 (0.943-1.391)	0.173	0.585
miR-423-5p	1.332 (1.046-1.696)	0.02	0.64
miR-652-3p	1.252 (1.001-1.566)	0.049	0.633

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- In summary, the above study identifies a distinct panel of 15 circulating miRNAs associated with acute heart failure that were consistently decreased compared both to patients with chronic heart failure and healthy controls. A gradual increase of all 15 miRNAs with decreasing acuity of heart failure was clearly demonstrated. A further early drop in 7 out of 15 miRNAs during hospitalization was associated with a higher mortality at 180 days. Validation in independent cohorts in patients with acute heart failure confirmed the above findings and led to a panel of 7 heart failure

specific miRNAs of which miR-18a-5p and miR-652-3p were predictive for 180 day mortality. The data also show that another cause of breathlessness, i.e. an acute exacerbation of chronic obstructive pulmonary disease, did not change circulating miRNAs profiles of the invention when compared to
5 controls.

In conclusion, the present invention provides a heart failure specific panel of circulating miRNAs that showed decreased levels in acute heart failure patients. Notably, 7 of those (miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p) were validated to a
10 statistically significant extent in an independent cohort of acute heart failure patients. Of note, none of these miRNAs were differentially regulated in patients with acute chronic obstructive pulmonary disease exacerbation.

Claims

1. A method of determining whether a subject has acute heart failure, is at increased risk of developing acute heart failure, or has acute heart failure that is progressing, comprising:
 - measuring the level of a plurality of miRNAs in a test sample obtained from the subject, wherein said plurality of miRNAs comprises at least four miRNAs selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p,
 - wherein a reduced level of said at least four miRNAs relative to a normal control indicates that the subject has acute heart failure, is at increased risk of developing acute heart failure, or has heart failure that is adversely progressing.
2. The method according to claim 1, wherein said measuring comprises measuring the level of at least five, preferably at least six, of said miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p.
3. The method according to claim 2, wherein said measuring comprises measuring the level of each of said miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p.
4. The method according to any one of the preceding claims, wherein the test sample is a blood sample, preferably a plasma sample.
5. The method according to any one of the preceding claims, wherein said measuring comprises measuring the level of said miRNAs using RT-

PCR, a biochip, quantitative PCR, serial analysis of gene expression (SAGE), or a microarray.

6. The method according to any one of claims 1-5, further comprising
5 measuring the level of at least one of let-7i-5p, miR-16-5p, miR-18b-5p, miR-223-3p, miR423-3p and miR-423-5p, wherein a reduced level relative to a normal control indicates an increased risk of or the presence of a acute heart failure in the subject.
- 10 7. The method according to any one of claims 1-6, for monitoring the progression of acute heart failure in a subject comprising:
detecting the level of at least four of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p in a first and a second sample obtained from the subject, wherein the second
15 sample is obtained within 5 days of said first sample; and
wherein a decrease in the expression of said least four of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p in the second sample relative to the first sample indicates an adverse disease progression of the acute heart failure.
- 20 8. Method according to claim 7, comprising detecting at least miR-18a-5p and miR-652-3p.
9. The method of claim 7 or 8, wherein said adverse disease
25 progression of the acute heart failure comprises an increased risk of 180 day mortality.
10. A kit comprising a plurality of primers and/or probes specific for determining expression levels at least four, preferably at least five of miR-

18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p,
and miR-652-3p.

11. The kit of claim 10, comprising primers and/or probes designed to
5 detect each of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-
5p, miR-199a-3p, and miR-652-3p.

12. The kit of claim 10 or 11, further comprising primers and/or
probes designed to detect at least one of let-7i-5p, miR-16-5p, miR-18b-5p,
10 miR-223-3p, miR423-3p and miR-423-5p.

13. The kit according to any one of claims 10-12, wherein said kit
further comprises one or more reagents for RT-PCR or reverse transcription
RT-PCR.

14. A biochip comprising a panel of isolated nucleic acids, wherein
said panel comprises at least four of miR-18a-5p, miR-26b-5p, miR-27a-3p,
miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p, 196a, or a
complement thereof.

15. The biochip of claim 14, comprising each of miR-18a-5p, miR-26b-
5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p,
or its complement.

16. A tangible, computer-readable medium comprising computer-
readable code that, when executed by a computer, causes the computer to
perform operations comprising: a) receiving information corresponding to
the level of expression of at least four miRNA's selected from the group
consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-
25 5p, miR-199a-3p and miR-652-3p, in a sample of a patient suspected of
30

having or determined to have acute heart failure; and b) determining a difference value in the expression level using the information corresponding to the expression level in the sample compared to a control or reference level.

5

17. A method for screening a pharmaceutically active compound for the treatment and/or prophylaxis of AHF, wherein the method comprises the steps of :

(a) providing a cell comprising an expression vector comprising at least miRNA sequence selected from the group consisting of miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, and miR-652-3p;

(b) bringing a candidate for a pharmaceutically active compound into contact with the cell, and

(c) determining the effect of the candidate on the expression of said at least one miRNA sequence, wherein a change in the expression of said at least one miRNA sequence indicates a pharmaceutically active compound.

Figure 1

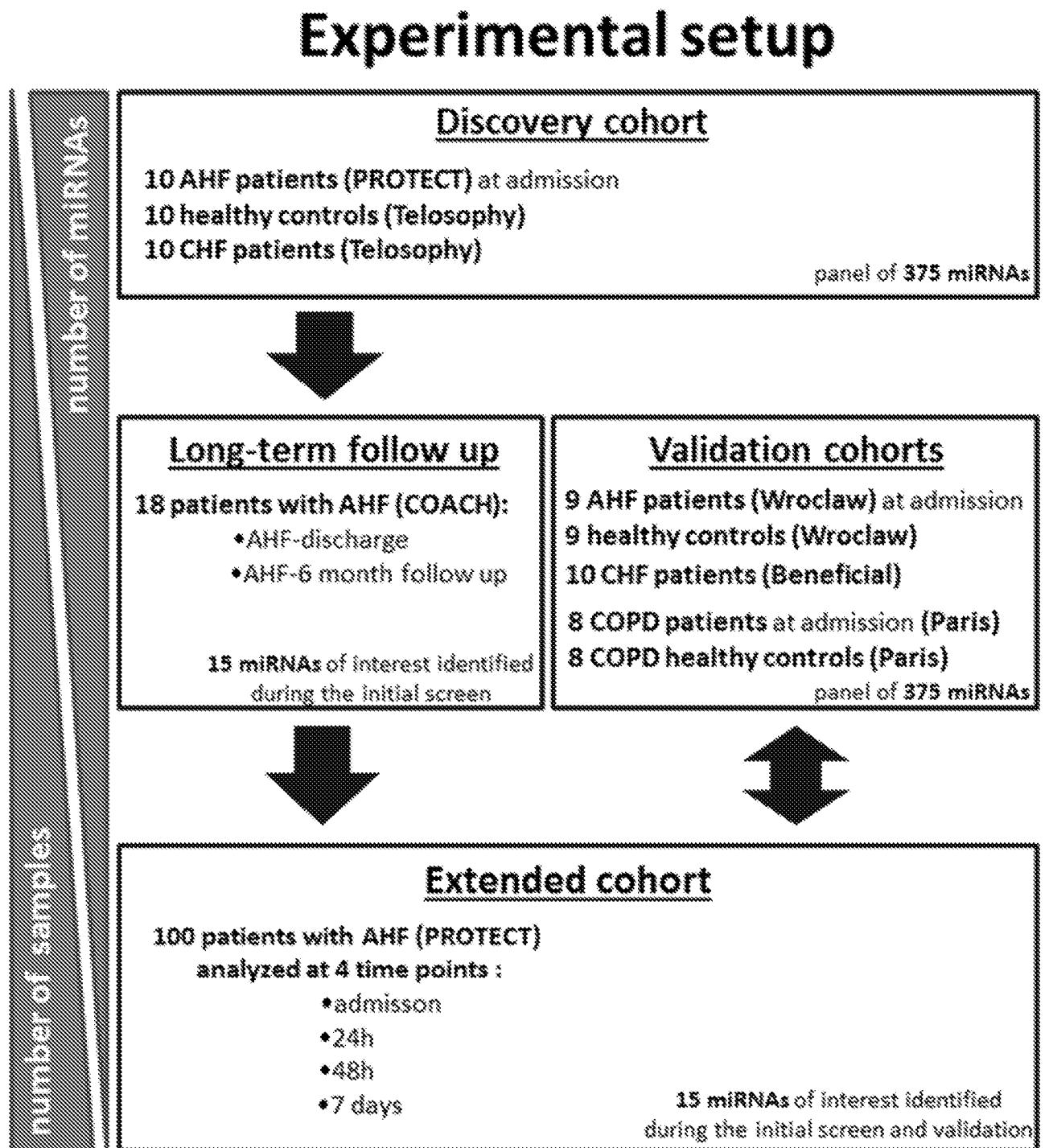


Figure 2

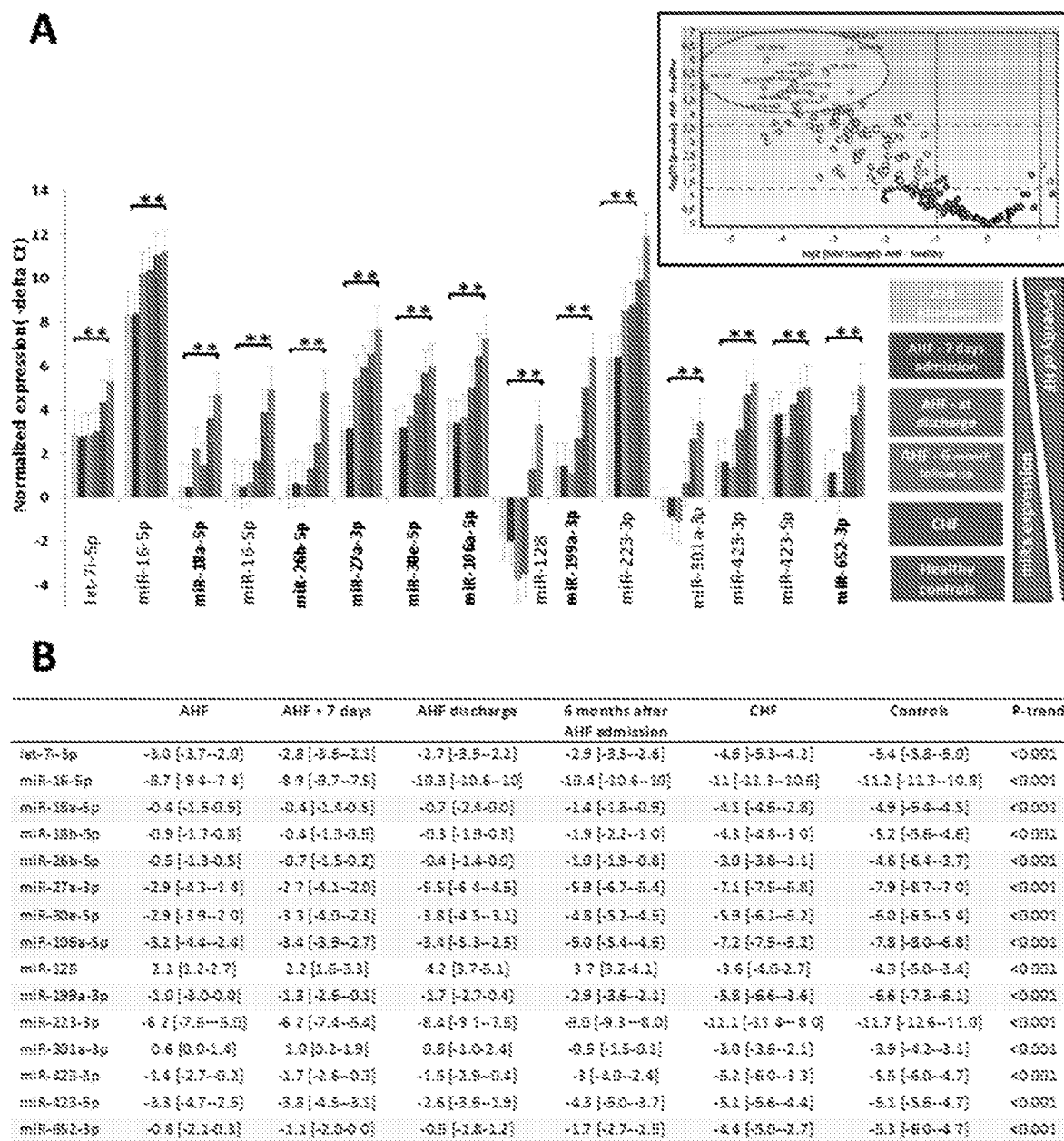
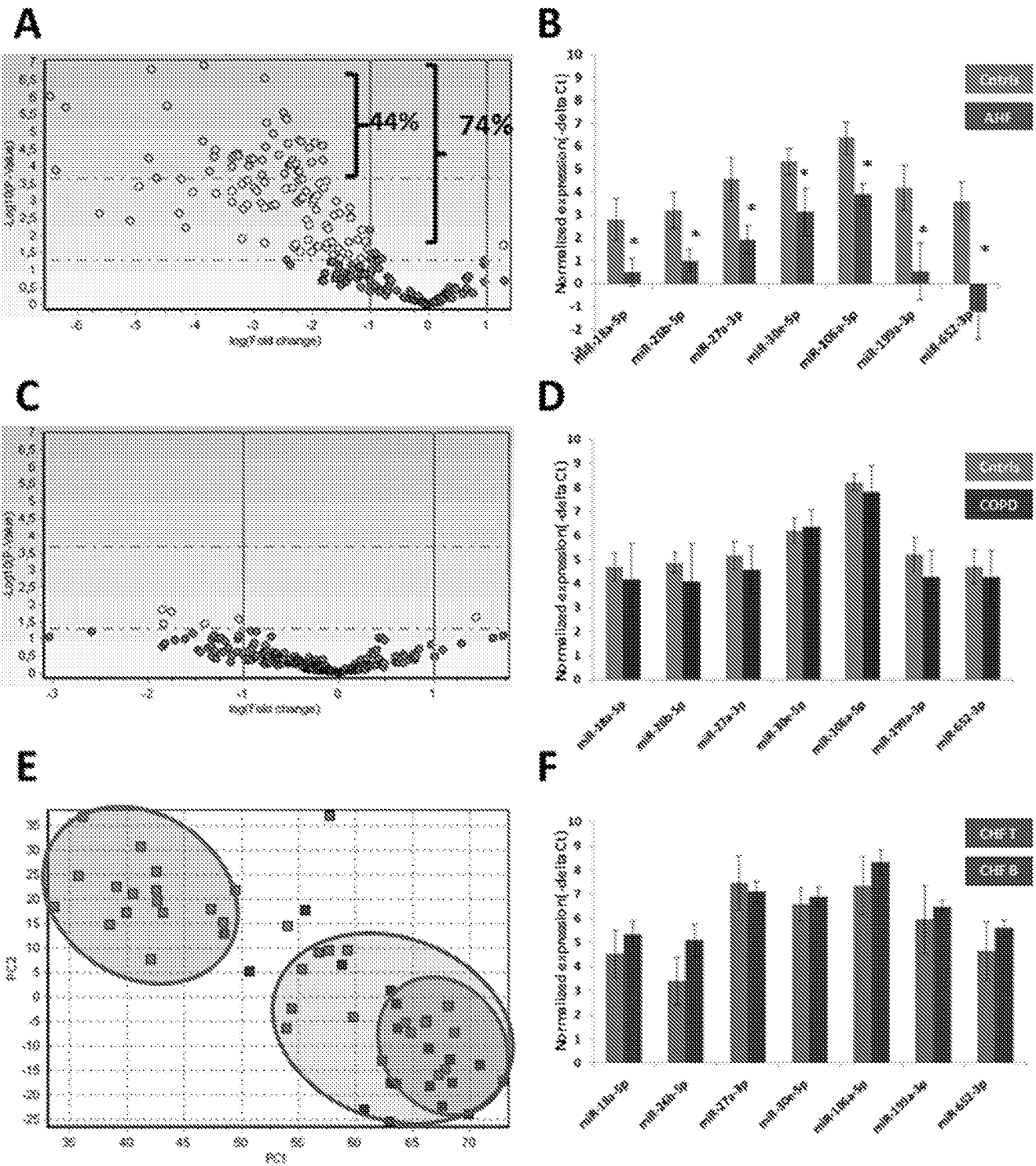


Figure 3



INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2016/050123

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal

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Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

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Celler, Jakub

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2016/050123

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International application No

PCT/NL2016/050123

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