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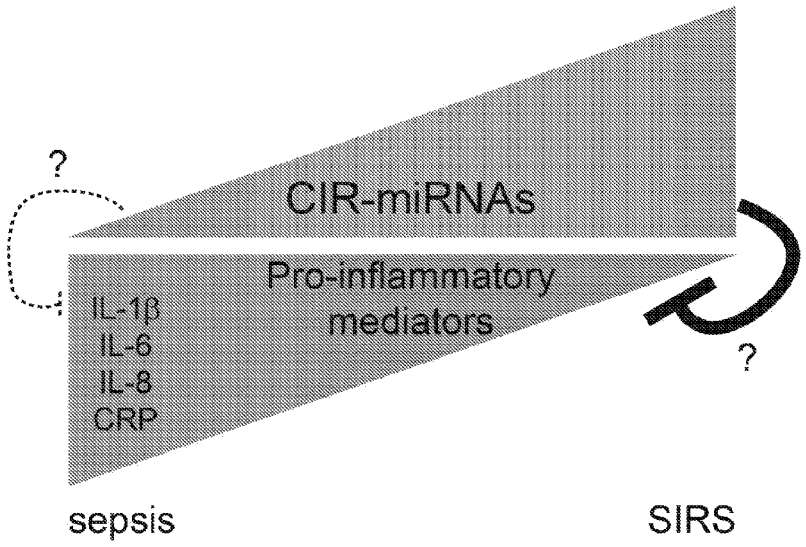
[Continued on next page]

(54) **Title**: SEPSIS BIOLOGICAL MARKER

Figure 6



(57) **Abstract**: The invention relates to biological markers for distinguishing between sepsis and SIRS, and in particular to the use of micro RNAs as diagnostic markers that may be used to distinguish between sepsis and SIRS. The invention extends to methods and kits which detect for such diagnostic micro RNAs for distinguishing between sepsis and SIRS, and to methods of treatment.

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EPSIS BIOLOGICAL MARKER

The present invention relates to biological markers for distinguishing between sepsis and SIRS, and in particular to the use of microRNAs as diagnostic markers that may be used to distinguish between sepsis and SIRS. The invention extends to methods and kit which detect for such diagnostic microRNAs for distinguishing between sepsis and SIRS.

Sepsis is defined as the systemic inflammatory response syndrome (SIRS) initiated by infection[1]. Severe sepsis (sepsis accompanied by acute organ dysfunction) is a leading cause of death worldwide (~19 million deaths/year) and the most common cause of death (30% mortality rate) among patients on Intensive Care Units (ICUs)[2]. Research over the past three decades has focused primarily on the inflammatory responses that underlie sepsis. Biomarkers of inflammation[3] have been identified but investigational treatments which attempt to 'switch-off' inflammation in sepsis have uniformly failed to improve patient outcomes. More recently there has been a growing recognition that anti-inflammatory, regulatory mechanisms accompany sepsis[4]. These are physiological in that they terminate inflammation during recovery, and pathological in that they cause sepsis-related immunosuppression. Ultimately, understanding how pro- and anti-inflammatory pathways are regulated may be key to understanding the pathology of sepsis[5] and developing accurate biomarkers and novel interventions.

MicroRNAs (miRNAs) are small (~22nt) regulatory RNAs that function as post-transcriptional gene regulators[6,7]. In 2003, only 255 human miRNAs were predicted to exist[8,9], but the number of known human miRNAs had risen dramatically to 2588 by 2014 (miRBase.org[10]). In 2008, it was demonstrated for the first time that miRNAs could be identified circulating in blood[11-13]. It is now thought 100-200 miRNAs may be detectable in the circulation[13-15]. The impact of disease on circulating miRNAs has been assessed principally in the context of cancer[11,16,17]. A handful of studies have measured miRNAs present in blood of sepsis patients, and reported conflicting findings[18-25]. Early studies looked for specific candidate miRNAs based on leukocyte inflammatory responses in experimental models of sepsis[26-30]. Advances in array and sequencing technologies now allow screening for miRNAs with genome-wide approaches, directly in human serum and plasma[31,32]. However, previous studies which have taken this approach have been hampered by lack of robust normalization to properly quantify miRNA levels, small sample sizes and heterogeneous patient populations[18,21] .

Thus, there is a need to provide miRNA biomarkers that are capable of distinguishing between sepsis and SIRS.

According to a first aspect of the present invention, there is provided a method for distinguishing between sepsis and SIRS in a subject, the method comprising analysing the concentration of one or more type of microRNA molecule in a bodily sample from a test subject and comparing this concentration with:-

(a) a reference for the concentration of the one or more type of microRNA molecule in an individual who suffers from sepsis, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference suggests that the subject is suffering from SIRS, and wherein if there is no difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference then this suggests that the subject is suffering from sepsis; and/or

(b) a reference for the concentration of the one or more type of microRNA molecule in an individual who suffers from SIRS, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference suggests that the subject is suffering from sepsis, and wherein if there is no difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference then this suggests that the subject is suffering from SIRS.

According to a second aspect of the invention, there is provided a kit for distinguishing between sepsis and SIRS in a subject, the kit comprising:-

(i) means for determining the concentration of one or more type of micro RNA molecule in a sample from a test subject; and

(ii) a reference for the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers sepsis, wherein the kit is used to identify a difference in the concentration of the one or more type of microRNA molecule in the sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from SIRS, or wherein the kit is used to determine that there is no difference in the

concentration of the one or more type of microRNA molecule in the sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from sepsis, and/or

(iii) a reference for the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers from SIRS, wherein the kit is used to identify a difference in the concentration of the one or more type of microRNA molecule in the sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from sepsis, or wherein the kit is used to determine that there is no difference in the concentration of the one or more type of microRNA molecule in the sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from SIRS.

According to a third aspect of the invention, there is provided a method of treating an individual suffering from sepsis, said method comprising the steps of:

(i) determining the concentration of one or more microRNA molecule in a sample having been obtained from a test subject, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers from SIRS, suggests that the test subject suffers from sepsis; and

(ii) administering, to the test subject, a therapeutic agent that prevents, reduces or delays progression of sepsis.

According to a fourth aspect of the invention, there is provided a method of treating an individual suffering from SIRS, said method comprising the steps of:

(i) determining the concentration of one or more microRNA molecule in a sample having been obtained from a test subject, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers from sepsis, suggests that the test subject suffers from SIRS; and

(ii) administering, to the test subject, a therapeutic agent that prevents, reduces or delays progression of SIRS.

Surprisingly, the inventors have found that there is a general increase in the levels of circulating miRNAs present in the blood of patients with sepsis or SIRS compared with controls, and that this increase is more marked for SIRS than sepsis patients. This indicates that a previously unanticipated number of circulating miRNAs are affected by systemic inflammation. These circulating miRNAs are referred to as circulating inflammation-related miRNAs (CIR-miRNAs). The inventors have identified six CIR-miRNAs that that are highly discriminatory for sepsis from SIRS having AUCs by ROC analysis comparable or better than clinical biomarkers, C-reactive protein (CRP) and procalcitonin (PCT). Notably, they found that CIR-miRNA levels correlate inversely with pro-inflammatory biomarkers. More importantly, the inventors have found that the levels of some CIR-miRNAs are differentially affected in sepsis and non-infective SIRS. Surprisingly, among sepsis and SIRS patients, the blood levels of CIR-miRNAs inversely correlate with the plasma levels of key pro-inflammatory mediators such as IL-1, IL-6, IL-8 and C-reactive protein (CRP), which have previously identified as increased in systemic inflammation and sepsis[3,34].

The method according to the first aspect and the kit according to the second aspect are useful for enabling a clinician to make decisions with regards to the best course of treatment for a subject who is currently or who may suffer from either sepsis or SIRS in the future. It is preferred that the method of the first aspect or the kit according to the second aspect is useful for enabling a clinician to decide how to treat a subject who is suffering from sepsis or SIRS, according to the methods of the third and fourth aspects. The methods and the kit are therefore very useful for guiding a SIRS or sepsis treatment regime for the clinician. The clinician may use the kit of the invention in conjunction with existing diagnostic tests to improve the accuracy of diagnosis.

MicroRNA molecules are non-coding, post-transcriptional regulators that normally bind to complementary sequences in the 3' untranslated regions (3' UTRs) of target messenger RNA transcripts (mRNAs), usually resulting in gene silencing. miRNAs are short ribonucleic acid (RNA) molecules, on average only about 22 nucleotides long. Thus, the miRNA detected in the methods and kit of the invention may be about 15 to 30 nucleotides long, or about 18 to 25 nucleotides long, or about 21 to 23 nucleotides long.

The methods and kit of the invention may comprise analysing the concentration of any of the known miRNAs, and which can be found on the miRBase website

(http://www.mirbase.org/). The miRBase (release 21) currently includes 2588 mature human miRNAs, all of which are processed from longer precursors and differ from each other in nucleotide sequence. The current understanding is that each miRNA is expressed in one or more human tissues and binds to one or more target RNA sequences expressed in particular tissues. The binding of this single miRNA, by itself or in combination with other miRNAs and/or proteins to a particular mRNA, leads to down-regulation of gene expression, usually by degradation of the target mRNA or repression of protein translation.

It is preferred that the methods according to the invention may comprise analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p, and the kit according to the invention may comprise a means for analysing the concentration of the one or more type of microRNA molecule.

Hence, in a fifth aspect, there is provided use of one or more type of microRNA, as a biomarker for distinguishing between sepsis and SIRS in a subject, wherein the one or more type of microRNA molecule is selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

The inventors have found that three of the miRNA molecules (miRNA-30d-5p, miRNA-30a-5p and miRNA-192-5p) described herein act as particularly robust biomarkers for distinguishing between sepsis and SIRS, and form preferred embodiments of the invention.

Hence, in a sixth aspect, there is provided use of miRNA-30d-5p, as a biomarker for distinguishing between sepsis and SIRS in a subject, optionally wherein one or more additional microRNA molecule is used and is selected from the group of miRNA molecules consisting of miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

Hence, in a seventh aspect, there is provided use of miRNA-30a-5p, as a biomarker for distinguishing between sepsis and SIRS in a subject, optionally wherein one or more additional microRNA molecule is used and is selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

Hence, in an eighth aspect, there is provided use of miRNA-192-5p as a biomarker for distinguishing between sepsis and SIRS in a subject, optionally wherein one or more additional microRNA molecule is used and is selected from the group of miRNA molecules consisting of miRNA-30a-5p, miRNA-30d-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

In one preferred embodiment, the methods and uses of the invention may comprise analysing the concentration of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p and miRNA-191-5p, and optionally at least one of miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p, and the kit may comprise a means for analysing the concentration of the one or more type of microRNA molecule.

In one preferred embodiment, the methods and uses of the invention may comprise analysing the concentration of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, and optionally at least one of miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p, , and the kit may comprise a means for analysing the concentration of the one or more type of microRNA molecule.

In an alternative preferred embodiment, the methods and uses of the invention may comprise analysing the concentration of miRNA-30d-5p, and optionally at least one of miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p, and the kit may comprise a means for analysing the concentration of the one or more type of microRNA molecule.

In an alternative preferred embodiment, the methods and uses of the invention may comprise analysing the concentration of miRNA-30a-5p, and optionally at least one of miRNA-30d-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p, and the kit may comprise a means for analysing the concentration of the one or more type of microRNA molecule.

In yet an alternative preferred embodiment, the methods and uses of the invention may comprise analysing the concentration of miRNA-192-5p, and optionally at least one of miRNA-30d-5p, miRNA-30a-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p, and the kit may comprise a means for analysing the concentration of the one or more type of microRNA molecule.

As described in the Examples, the best normalizer for the dataset was a combination of miR320a and miR486-5p. Hence, preferably the methods and uses of the invention may further comprise analysing the concentration of miR320a and/or miR486-5p and then adjusting the concentration of the one or more type of microRNA molecule being detected in the bodily sample before comparing the detected concentration against the reference value, and the kit according to the invention may comprise a means for normalising the concentration of the one or more type of microRNA molecule in a test sample with respect to miR320a and/or miR486-5p.

The inventors have found that miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p provide a very robust and reliable diagnosis when distinguishing between SIRS and sepsis. Accordingly, most preferably the methods and uses of the invention may comprise analysing the concentration of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p, and the kit may comprise a means for analysing the concentration of the one or more type of microRNA molecule. Indeed, these miRNAs are very useful for generating a circulating inflammation-related miRNAs (CIR-miRNA) score.

Hence, in a ninth aspect, there is provided use of miRNA-30d, miRNA-30a, miRNA-192, miRNA-26a, miRNA-23a, and miRNA-191, for generating a CIR-miRNA score.

The CIR-miRNA score means a score generated as a linear combination of the top performing 6 miRNA measurements in severe sepsis and SIRS patients (n=41) and interpolated using IBM SPSS Statistics 22 by binary logistic regression to predict SIRS vs sepsis. In particular, the CIR-miRNA score (S) is mathematically defined as: $S = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6 + k$ where $x_{1\text{-}6}$ are the measurements of the top 6 miRNAs in a specific individual and the variables, $a_{1\text{-}6}$, and the constant, k, are the coefficients returned by the binary logistic regression model. In mathematical terms, the CIR-miRNA score is the natural logarithm of the odds of having SIRS vs sepsis given the measurements of the 6 top miRNAs, that is $ODDS=e^S$. Correlations between the interpolated CIR-miRNA scores and plasma levels of inflammatory mediators are evaluated using the Spearman rho co-efficient.

In one embodiment, the miRNA being detected is miRNA-30a-5p. The sequence of miRNA-30a-5p is 22 nucleotides long, and is referred to herein as SEQ ID No.1, as follows:

5'-uguaaacauccucgacuggaag-3'

[SEQ ID No.1]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.1, or the complementary sequence thereof, or a variant or fragment thereof.

In another embodiment, the miRNA being detected is miRNA-30d-5p. The sequence of miRNA-30d is, 22 nucleotides long, and is referred to herein as SEQ ID No.2, as follows:

5'-uguaaacaucccgacuggaag-3'

[SEQ ID No.2]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.2or a variant or fragment thereof.

In another embodiment, the miRNA being detected is miRNA-192-5p. The sequence of miRNA-192 is 21 nucleotides long, and is referred to herein as SEQ ID No.3, as follows:

5'-cugaccuaugaauugacagcc-3'

[SEQ ID No.3]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.3, or a variant or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-26a-5p. The sequence of miRNA-26a-5p is 22 nucleotides long, and is referred to herein as SEQ ID No.4, as follows:

5'-uucaaguaauccaggauaggcu-3'

[SEQ ID No.4]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.4 or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-23a. The sequence of miRNA-23a-3p is 21 nucleotides long, and is referred to herein as SEQ ID No.5, as follows:

5'-aucacauugccagggauuucc-3'

[SEQ ID No.5]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.5, or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-191-5p. The sequence of miRNA-191-5p is 23 nucleotides long, and is referred to herein as SEQ ID No.6, as follows:

5'-caacggaaucccaaaagcagcug-3'

[SEQ ID No.6]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.6, or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-101-3p. The sequence of miRNA-101-3p is 21 nucleotides long, and is referred to herein as SEQ ID No.7, as follows:

5'-uacaguacugugauaacugaa-3'

[SEQ ID No.7]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.7, or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-122-5p. The sequence of miRNA-122-5p is 22 nucleotides long, and is referred to herein as SEQ ID No.8, as follows:

5'-uggagugugacaaugguguuug-3'

[SEQ ID No.8]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.8, or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-378a-3p. The sequence of miRNA-378a-3p is 21 nucleotides long, and is referred to herein as SEQ ID No.9, as follows:

5'-acuggacuuggagucagaaggc-3'

[SEQ ID No.9]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.9, or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-151a-3p. The sequence of miRNA-151a-3p is 21 nucleotides long, and is referred to herein as SEQ ID No.10, as follows:

5'-cuagacugaagcuccuugagg-3'

[SEQ ID No.10]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.10, or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is miRNA-146a-5p. The sequence of miRNA-146a-5p is 22 nucleotides long, and is referred to herein as SEQ ID No.11, as follows:

5'-ugagaacugaauuccauggguu-3'

[SEQ ID No.11]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.11, or a variant, or fragment thereof.

In one embodiment, the miRNA being detected is let-7f-5p. The sequence of let-7f-5p is 22 nucleotides long, and is referred to herein as SEQ ID No.12, as follows:

5'-ugagguaguagauuguauaguu-3'

[SEQ ID No.12]

Therefore, the miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.12, or a variant or fragment thereof.

The sequence of miR320a is 22 nucleotides long, and is referred to herein as SEQ ID No.13, as follows:

5'-aaaagcuggguugagagggcga-3'

[SEQ ID No.13]

The sequence of miR486-5p is 22 nucleotides long, and is referred to herein as SEQ ID No.14, as follows:

5'-uccuguacugagcugccccgag-3'

[SEQ ID No.14]

It will be appreciated that miRNAs of the invention have a hairpin loop structure. As defined above, the nucleotide sequence of certain microRNAs according to the invention are located 5' (-5p) of the hairpin loop (i.e. miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-191-5p, miRNA-122-5p, miRNA-146a-5p, let-7f-5p and miR486-5p), whereas the nucleotide sequence of the remaining microRNAs is located 3' (-3p) of the hairpin loop (i.e. miRNA-23a-3p, miRNA-378a-3p and miRNA-151a-3p).

The methods uses of the invention may comprise determining the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules comprising a nucleotide sequence substantially as set out in any or SEQ ID No.1 to 12, or variants or fragments thereof, and the kit according to the invention may comprise a means for determining the concentration of the one or more type of microRNA

molecule. The methods and uses of the invention may comprise determining the concentration of miRNA320a (SEQ ID No:13) and/or miR486-5p (SEQ ID No:14) in a bodily sample and then adjusting the concentration of the one or more type of microRNA molecule being detected in the bodily sample with respect to the level of expression of miR320a and/or miR486-5p before comparing the detected concentration against the reference value. The pattern of expression of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and/or let-7f-5p are found at either significantly higher or significantly lower levels in a bodily sample compared to a test subject (e.g. plasma or serum from peripheral blood) may be termed the "miRNA signature".

In a tenth aspect, there is provided use of miR320a (SEQ ID No:13) and/or miR486-5p (SEQ ID No:14) as a microRNA for normalizing the expression of levels of a biomarker.

Preferably, the biomarker is one or more microRNAs selected from miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

Variants and fragments of any of the miRNA molecules that may be detected may include truncations or additions of nucleotides of the miRNA molecule, for example SEQ ID No.1-12. A truncation may comprise the miRNA molecule having been reduced in size by the removal of at least one nucleotide from the 5' and/or 3' end of the miRNA, or by deletion of one of more nucleotides from within the core or centre of the miRNA. The truncation may comprise deletion of at least 2, 3, 4 or 5 nucleotides from the miRNA molecule. An addition may comprise the miRNA molecule having been increased in size by the addition of at least one nucleotide to the 5' and/or 3' end of the miRNA, or by the introduction of one of more nucleotides into the core or centre of the miRNA. The addition may comprise addition of at least 2, 3, 4, 5, or up to 10 nucleotides to the miRNA molecule.

The concentration of the at least one type of miRNA molecule may act as a diagnostic and/or prognostic marker for sepsis or SIRS. The inventors investigated the expression levels of a large number of miRNA molecules in sepsis and SIRS patients, and were surprised to observe that a number of miRNAs (i.e. miRNA-30d-5p, miRNA-30a-5p,

miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p) exhibited increased levels in SIRS than in sepsis patients. This pattern of increased expression can be used to form a miRNA signature. The inventors therefore realised that these miRNA molecules, which together form a miRNA signature, represents a useful and robust physiological marker for distinguishing between a patient suffering from sepsis and a patient suffering from SIRS. Accordingly, each of these biomarkers can be robustly used for prognostic and diagnostic purposes.

The inventors have established that circulating levels of miRNAs in a test subject is highly suggestive of whether the subject suffers from either sepsis or SIRS, and is sufficiently sensitive to detect the disorder at an early stage. Accordingly, the kit and methods the invention provides a very reliable prognostic marker for monitoring conditions, both before and after treatment. Accordingly, assaying for miRNA molecules is a substantial improvement over assaying for other markers, because it is more sensitive and also provides enhanced specificity. In addition, assaying for miRNA molecules also provides far more information to the clinician, and will help stratify the disease, be that either sepsis or SIRS.

It will be appreciated that detecting one particular type of miRNA molecule may be of use by itself as a biomarker for distinguishing between sepsis and SIRS. Further, detecting more than one type of miRNA molecule, may provide a more robust diagnosis or prognosis of the disease. In addition, the biomarker may also be used in combination with an assay of another biological marker indicative of sepsis or SIRS. Hence, assaying for one or more miRNA molecules may be used to complement the use of another marker to provide even more information to the clinician.

The subject may be any animal of veterinary interest, for instance, a cat, dog, horse etc. However, it is preferred that the subject is a mammal, such as a human, either male or female.

Preferably, a sample is taken from the subject, and the concentration of the one or more type of miRNA molecule may be measured. The kit of the second aspect may comprise sample extraction means for obtaining the sample from the test subject. The sample extraction means may comprise a needle or syringe or the like. Preferably, the kit comprises one or more microRNAs for normalising the expression of levels of the

biomarker in the sample. More preferably, the microRNA for normalising the expression levels of the biomarker in the sample is miRNA320a and/or miR486-5p.

It has been demonstrated that miRNAs occur in body and organ fluids, such as cerebrospinal fluid or follicular fluid. However, the sample may be any bodily sample into which miRNA molecules are secreted, e.g. it may be lymph or interstitial fluid. The sample may be a urine sample or a blood sample. It is preferred that the miRNA molecule is measured or assayed in a blood sample. The blood sample may be venous or arterial.

The kit may comprise a sample collection container for receiving the extracted sample. Blood samples may be assayed for miRNA molecule levels immediately. Alternatively, the blood may be stored at low temperatures, for example in a fridge or even frozen before the miRNA assay is conducted. Measurement of miRNA may be made on whole blood.

However, the blood may be further processed before the assay is performed. For instance, an anticoagulant, such as citrate (such as sodium citrate), hirudin, heparin, PPACK, or sodium fluoride may be added. Thus, the sample collection container may contain an anticoagulant in order to prevent the blood sample from clotting. Alternatively, the blood sample may be centrifuged or filtered to prepare a plasma or serum fraction, which may be used for analysis. Hence, it is preferred that the miRNA is analysed or assayed in a blood plasma or a blood serum sample. It is preferred that miRNA concentration is measured *in vitro* from a blood serum sample or a plasma sample taken from the subject.

It will also be appreciated that "fresh" bodily samples may be analysed immediately after they have been taken from a subject. Alternatively, the serum or plasma samples may be frozen and stored. The sample may then be de-frosted and analysed at a later date.

As described in the examples, the inventors monitored the concentration of various miRNAs in numerous patients who suffered from either sepsis or SIRS, and compared them to the concentration of the same miRNAs in individuals who did not suffer from either condition. They demonstrated that there was a statistically significant increase or decrease in the concentration of certain miRNA molecules described herein in the

patients suffering from sepsis or SIRS. Thus, the difference in concentration may be an increase or a decrease compared to the reference taken from individuals who do not suffer from either condition. It will be appreciated that the concentration of a certain miRNA molecule in sepsis or SIRS patients is highly dependent on a number of factors, for example how far the disease has progressed, and the age and gender of the subject. It will also be appreciated that the concentration of miRNAs in individuals who do not suffer from sepsis or SIRS may fluctuate to some degree, but that on average over a given period of time, the concentration tends to be substantially constant. In addition, it should be appreciated that the concentration of miRNA in one group of individuals who do not suffer from, for example, sepsis may be different to the concentration of those miRNAs in another group of individuals who do not suffer sepsis. However, the skilled technician will know how to determine the average concentration of certain miRNAs in individuals who suffer from either sepsis or SIRS, and this is referred to as the 'normal' concentration of miRNA for the disease. The normal concentration corresponds to the reference values discussed above in the first to third aspects.

The miRNAs may be extracted from the bodily sample by a variety of techniques. Briefly, these may comprise addition of a protein denaturant (such as Trizol or guanidine thiocyanate) to the sample, centrifugation to remove protein debris, addition of DNaseI to remove DNA, and extraction of RNA using a suitable column. RNA samples may be further concentrated by ethanol/isopropanol precipitation and/or centrifugal concentration. In one embodiment, the preferred extraction kit is supplied by Ambion, but other extraction kits could be used, depending on availability and/or suitability in subsequent downstream reactions.

PCR may be used to amplify the one or more type of miRNA molecule. The PCR technology may be selected from the group consisting of real-time PCR, reverse transcriptase PCR, multiplex PCR or molecular beacon PCR. It will be appreciated that PCR involves the use of two primers which are substantially complementary to the miRNA molecule being assayed in the sample. The kit according to the second aspect comprises means for determining the concentration of one or more type of miRNA molecule in a sample from a test subject. The kit may comprise a container in which the means for determining the concentration of one or more type of miRNA molecule in a sample from a test subject may be contained. The kit may also comprise instructions for use.

Thus, the kit may comprise detection means for determining the concentration of the one or more type of miRNA in the sample once this has been obtained from the subject. For example, the detection means may comprise one or more primer, for use in a PCR method for amplifying the miRNA. In one embodiment, detection of the one or more type of miRNA molecule may be achieved by TaqMan quantitative RT-PCR using primer and probe sets specific for particular human miRNAs, as described on the Applied Biosystems website (http://www.appliedbiosystems.com/absite/us/en/home.html). This method makes use of looped Reverse Transcriptase primers to generate the cDNA and then forward and reverse primers for the PCR amplification. Quantification is achieved by use of a fluorescently labelled probe, situated between the two primers, where fluorescence is activated upon the PCR reaction (for method see http://www.appliedbiosystems.com/absite/us/en/home.html).

In another embodiment, detection may be achieved using an Exiqon microRNA detection kit. (http://www.exiqon.com/ls). However, other PCR-based and microarray-based detection methods are also applicable to this invention. The primers may comprise at least partial sequence identity with the miRNA molecule being detected, for example, miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and/or let-7f-5p.

In another embodiment, the Reverse Transcriptase and PCR reactions may comprise the procedure as set out in Examples.

The reference values may be obtained by assaying a statistically significant number of control samples (i.e. samples from subjects who suffer from sepsis but do not suffer from SIRS or vice versa). Accordingly, the reference (ii) according to the kit of the second aspect of the invention may be a control sample (for assaying).

The kit may comprise a positive control (preferably provided in a container), which corresponds to total RNA extracted from a sample (e.g. the plasma) of a subject having, for example, sepsis where it has been established that the relevant miRNAs (for example, miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p) are present at statistically higher or lower levels than those present in a subject suffering from SIRS. Similarly, where the positive control

may be total RNA extracted from a sample of a subject having SIRS, where it has been established that the relevant miRNAs (for example, miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p) are present at statistically higher or lower levels than those present in a subject suffering from sepsis. Hence, the positive control miRNA may comprise a nucleotide sequence substantially as set out in SEQ ID No.1-12, or a variant, or fragment thereof.

The kit may comprise a negative control (preferably provided in a container), which corresponds to total RNA extracted from a sample (e.g. the plasma) of a subject without sepsis or SIRS where it has previously been established that the above miRNAs are detectable at significantly lower or higher levels.

In a preferred embodiment, the kit may comprise the reference, a positive control and a negative control. The kit will also comprise further controls, as necessary, such as "spike-in" controls to provide a reference for concentration, and further positive controls for each of the "signature" microRNAs.

Hence, by way of example only, the blood plasma concentration of the signature miRNA in sepsis patient may not be detectable, whereas the concentration of certain signature miRNAs in a patient with SIRS may be at least 1.5-, 5-, 10, 15- or 20-fold higher (or vice versa, in terms of sepsis and SIRS). Also, by way of example, the decrease in concentration of certain signature miRNAs in sepsis may be at least 1.5- 5-, 10, 15- or 20-fold lower than a SIRS patient (or vice versa, in terms of sepsis and SIRS).

The skilled technician will appreciate how to measure the concentrations of miRNAs in a statistically significant number of control individuals, and the concentration of miRNA in the test subject, and then use these respective figures to determine whether the test subject has a statistically significant increase or decrease in miRNA concentration, and therefore infer whether that subject is suffering from sepsis or SIRS. In one embodiment, statistical significance is found at 10%. The preferred statistical significance value is 5%.

By way of example, the increase in concentration of miRNA compared to the 'sepsis or SIRS' concentration may be at least 1.5-, 5-, 10-, 15- or 20-fold higher than the 'normal' or reference concentration. By way of example, the decrease in concentration of miRNA

compared to the 'normal' concentration may be at least 1.5-, 5-, 10-, 15- or 20-fold lower than the 'normal' or reference concentration. Such changes in miRNA concentration infer that the test subject is suffering from either SIRS or sepsis. Accordingly, a clinician would be able to make a decision as to the preferred course of treatment required, for example the type and dosage of the therapeutic agent according to the third aspect to be administered.

It will be appreciated that the invention extends to any nucleic acid or variant, derivative or analogue thereof, which comprises substantially the nucleic acid sequences of any of the sequences referred to herein, including functional variants or functional fragments thereof. The terms "substantially the nucleotide sequence", "functional variant" and "functional fragment", can be a sequence that has at least 40% sequence identity with the nucleotide sequences of any one of the sequences referred to herein, for example 40% identity with the nucleotide identified as SEQ ID No:1 (i.e. miRNA-30a) or SEQ ID No.2 (i.e. miRNA-30d), and so on, for all of the miRNAs described herein.

Nucleotide sequences with a sequence identity which is greater than 65%, more preferably greater than 70%, even more preferably greater than 75%, and still more preferably greater than 80% sequence identity to any of the sequences referred to are also envisaged. Preferably, the nucleotide sequence has at least 85% identity with any of the sequences referred to, more preferably at least 90% identity, even more preferably at least 92% identity, even more preferably at least 95% identity, even more preferably at least 97% identity, even more preferably at least 98% identity and, most preferably at least 99% identity with any of the sequences referred to herein.

The skilled technician will appreciate how to calculate the percentage identity between two nucleotide sequences. In order to calculate the percentage identity between two nucleotide sequences, an alignment of the two sequences must first be prepared, followed by calculation of the sequence identity value. The percentage identity for two sequences may take different values depending on:- (i) the method used to align the sequences, for example, ClustalW, BLAST, FASTA, Smith-Waterman (implemented in different programs), or structural alignment from 3D comparison; and (ii) the parameters used by the alignment method, for example, local vs global alignment, the pair-score matrix used (e.g. BLOSUM62, PAM250, Gonnet etc.), and gap-penalty, e.g. functional form and constants.

Having made the alignment, there are many different ways of calculating percentage identity between the two sequences. For example, one may divide the number of identities by: (i) the length of shortest sequence; (ii) the length of alignment; (iii) the mean length of sequence; (iv) the number of non-gap positions; or (iv) the number of equivalenced positions excluding overhangs. Furthermore, it will be appreciated that percentage identity is also strongly length dependent. Therefore, the shorter a pair of sequences is, the higher the sequence identity one may expect to occur by chance.

Hence, it will be appreciated that the accurate alignment of protein or DNA sequences is a complex process. The popular multiple alignment program ClustalW (Thompson et al., 1994, Nucleic Acids Research, 22, 4673-4680; Thompson et al., 1997, Nucleic Acids Research, 24, 4876-4882) is a preferred way for generating multiple alignments of proteins or DNA in accordance with the invention. Suitable parameters for ClustalW may be as follows: For DNA alignments: Gap Open Penalty = 15.0, Gap Extension Penalty = 6.66, and Matrix = Identity. For protein alignments: Gap Open Penalty = 10.0, Gap Extension Penalty = 0.2, and Matrix = Gonnet. For DNA and Protein alignments: ENDGAP = -1, and GAPDIST = 4. Those skilled in the art will be aware that it may be necessary to vary these and other parameters for optimal sequence alignment.

Preferably, calculation of percentage identities between two nucleotide sequences may then be calculated from such an alignment as (N/T)*100, where N is the number of positions at which the sequences share an identical residue, and T is the total number of positions compared including gaps but excluding overhangs. Hence, a most preferred method for calculating percentage identity between two sequences comprises (i) preparing a sequence alignment using the ClustalW program using a suitable set of parameters, for example, as set out above; and (ii) inserting the values of N and T into the following formula:- Sequence Identity = (N/T)*100.

Alternative methods for identifying similar sequences will be known to those skilled in the art. For example, a substantially similar nucleotide sequence will be encoded by a sequence which hybridizes to the sequences shown in SEQ ID No's: 1-12, or their complements under stringent conditions. By stringent conditions, we mean the nucleotide hybridises to filter-bound DNA or RNA in 3x sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in 0.2x SSC/0.1%

SDS at approximately 20-65°C. Alternatively, a substantially similar polypeptide may differ by at least 1, but less than 5, 10, 20, 50 or 100 amino acids from the sequences described herein.

Due to the degeneracy of the genetic code, it is clear that any nucleic acid sequence described herein could be varied or changed without substantially affecting the sequence of the protein encoded thereby, to provide a functional variant thereof. Suitable nucleotide variants are those having a sequence altered by the substitution of different codons that encode the same amino acid within the sequence, thus producing a silent change. Other suitable variants are those having homologous nucleotide sequences but comprising all, or portions of, sequence, which are altered by the substitution of different codons that encode an amino acid with a side chain of similar biophysical properties to the amino acid it substitutes, to produce a conservative change. For example small non-polar, hydrophobic amino acids include glycine, alanine, leucine, isoleucine, valine, proline, and methionine. Large non-polar, hydrophobic amino acids include phenylalanine, tryptophan and tyrosine. The polar neutral amino acids include serine, threonine, cysteine, asparagine and glutamine. The positively charged (basic) amino acids include lysine, arginine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It will therefore be appreciated which amino acids may be replaced with an amino acid having similar biophysical properties, and the skilled technician will know the nucleotide sequences encoding these amino acids.

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying diagrammatic drawings, in which:-

Figure 1 is a series of 12 graphs showing the results of patients whose plasma was tested for miRNAs in Illumina next generation sequencing (NGS). Plasma total RNA was extracted from 10 pools (representative of 89 ICU patients, as in Table 1) using the miRVana PARIS technology and then human miRNAs were sequenced using the

Illumina next generation sequencing (NGS) platform. **A.** Representative plots show the number of blood miRNAs (x-axis, sorted based on their abundance in the first duplicate of SIRS) and relative NGS counts (y-axis), in SIRS, sepsis and no-SIRS patients. Many miRNA were expressed below $1/10^5$ NGS counts (orange shadowed areas) consistently across all pools and were excluded from further analysis. **B.** Prolife of miRNA distribution after miRNAs with $<1/10^5$ counts (orange areas) in all pools were excluded. **C.** miRNA counts in 2 identical replicates are shown in scatter plots for SIRS, sepsis and no-SIRS patients. Reproducible results were obtained for miRNAs with NGS counts$>10/10^5$ (red lines) and miRNA in the grey area were excluded. **D.** The average miRNA counts (shortlisted in A-C, n=116) from SIRS and Sepsis groups was expressed as a ratio against no-SIRS controls (left and middle panels) or in between each others (right panel), resulting in fold differences (fd) for each blood miRNA (histograms). Green and red areas, respectively, represent miRNA decrease and increase, separated by fd=1 (left dotted line) and fd=+2 (right dotted line). Compared to no-SIRS, many CIR-miRNAs had fd>+2 in SIRS (left), but not in sepsis (fd<+2, middle). When Sepsis/SIRS are compared CIR-miRNAs are mostly downregulated (right).

Figure 2 is two graphs that show the results of shortlisted internal normalizers in NGS and miRNA Q-PCR arrays. Plasma total RNA extracted and analyzed in NGS as described in Figure 1, in 10 duplicate plasma pools (5 groups representative of 89 individuals) (A) or in 89 individuals samples using the miRCURY LNA Universal RT microRNA PCR technology (B). **A.** Among the finally shortlisted miRNAs (miR320a, miR92-3p and miR486-5p), the fold-differences (fd) of average NGS counts seen in severe and non-severe sepsis and SIRS groups (8 pools representative of 73 individuals) relative to no-SIRS controls (2 duplicate pools, n=16) are shown. **B.** In miRNA qPCR arrays, normalizer miRNAs were tested for 89 individual patients in 5 groups: severe sepsis (n=21); non-severe sepsis (n=8); severe SIRS (n=23); non-severe SIRS (n=21); and patients without SIRS (no-SIRS controls, n=16), in two independent technical repeat experiments. Because miR92b-3p was below the level of detection in 22/89 patients, it was excluded from further analysis. The mean Cp of the miR320a and miR486-5p is shown in each group and was selected as an internal normalizer for the miRNA qPCR array dataset.

Figure 3 show the results of shortlisted CIR-miRNAs measured with Exiqon miRNA qPCR arrays. In miRNA qPCR arrays, within each patient's specimen, Cp of a single miRNA is compared to the mean Cp of 2 normalizers (as from Figure 2) to give delta-

Cp (dCp). dCp of all patients are analyzed, comparing severe Sepsis (D, n=21) and SIRS (A, n=23). **A.** Volcano plot shows fold changes (log2, D/A) relative to p values (-log10) in each miRNA assay. In the upper left quadrant of the plot, around 20 miRNAs are significantly (red and yellow dots above the horizontal black line, which indicates a level of significance $p \leq 0.05$) downregulated in D/A ($fd < -1.5$, left vertical line), see Table 2. Orange and red dots represent significant differences by t-test ($p < 0.05$) with red dots representing miRNA that also passed the Benjamini-Hochberg correction. No CIR-miRNA significantly increased in D/A. **B.** Heatmap shows the top 12 significant miRNA clustering with opposite patterns in D/A. **C.** Principal component analysis (PCA) transforms the top 5 significant miRNAs to maximize the visualization of differences across the severe sepsis and SIRS groups. The PCA plot shows that within the dataset it is possible to discriminate patients with SIRS (blue dots, mostly in the lower right quadrant of the PCA plot) away from patients with sepsis (Fig. 3C, green dots falling in other quadrants).

Figure 4 is a series of 14 graphs which show that CIR-miRNAs are good-to-excellent biomarkers of sepsis. In miRNA qPCR arrays, data was analyzed as in Figure 3 and the top-6 differentially expressed miRNA in sepsis compared to SIRS (after the Benjamini-Hochberg correction) are shown. **A.** Left dot plots show dCp values in severe SIRS vs sepsis in individual samples (n=21 and n=23 for sepsis and SIRS respectively) together with the level of significance. The relative receiver operator curve (ROC, right) is shown with the Area Under the Curve (AUC). Each of the top 6 significant CIR-miRNAs is a good-to-excellent biomarker and CIR-miRNAs were mostly downregulated in Sepsis compared to SIRS in Exiqon miRNA qPCR arrays. **B.** A model combines the top-6 significant CIR-miRNAs to maximize distinction between SIRS and sepsis. The CIR-miRNA score is directly related to the odds of having SIRS or sepsis given the measurements of the 6 top miRNAs (see Material and Methods for further details). Left dot plot shows the model interpolation of the experimental cohort: SIRS patients -that have high CIR-miRNA levels (in A)- tend to score>0, whilst sepsis patients tend to score<0. ROC (and AUC, right) shows that the 6 CIR-miRNAs combined outperformed single miRNAs.

Figure 5 are a series of eight graphs which show the correlation of the model scores with pathology scores and plasma levels of immune mediators relevant in sepsis and SIRS. The model scores that combine the top-6 CIR-miRNA measurements in Severe SIRS and Severe Sepsis patients were plotted against the pathology score (SOFA,

sequential organ failure assessment); markers of disease and inflammation such as Hb (free hemoglobin), CRP (C-reactive protein), and PSP (pancreatic soluble protein); and markers of immune cell activation: soluble CD25 (sCD25), IL-6 IL-8 and IL-1. Correlation trends are shown with the linear regression model including Spearman rho and the significances of the correlations. Negative scores -typical of sepsis patients with lower plasma CIR-miRNAs (as in Figure 3)- correspond to individuals with increased levels of inflammatory mediators. Positive scores -more often seen in SIRS patients and reflective of high plasma CIR-miRNAs- are found in individuals with low levels of inflammatory cytokines. Thus, CIR-miRNA levels negatively correlate with pro-inflammatory cytokines critical in systemic inflammatory conditions.

Figure 6 shows the proposed model for CIR-miRNA and inflammatory mediator plasma levels. The triangular shapes represent plasma levels of CIR-miRNA (circulating miRNA, top) and pro-inflammatory mediators (bottom). Based on our results, in Sepsis we found low levels of CIR-miRNAs correlating with increasing levels of pro-inflammatory mediators (dark red). In contrast, in SIRS patients CIR-miRNAs are more abundant than what is found for sepsis patients correlating with lower levels of pro-inflammatory markers (blue). We speculate that immunologically relevant CIR-miRNA may exist that act as regulators of inflammatory processes especially during systemic inflammatory diseases. This hypothesis is consistent with recent data showing that regulatory cells secrete exosomes which exert miRNA-mediated immune-suppression.

Figure 7 is two graphs that show the average hemoglobin levels in an experimental cohort in which of hemolyzed samples have been excluded. **A.** Hemolysis, which is marked in plasma if free hemoglobin (Hb) levels >0.6g/L, was measured by Harboe spectrophotometric method in any sample (n=91). Shown measurements are the average of 3 technical replicates/patient. Hemolytic samples (red; 1 in the severe SIRS groups and 1 in the non-severe SIRS) were excluded from the study. **B.** Hb levels are shown in any experimental group used in NGS and miRNA Q-PCR array, after the exclusion of outliers. Importantly, average Hb did not differ significantly across groups, suggesting that RBC lysis is equally represented across the experimental groups prior to the NGS analysis. RBCs may be responsible for miRNA presence in the blood. Balancing the levels of miRNAs across experimental groups may also positively affect normalization. In fact in our analysis, the internal normalizer miR486-5p is one of the most abundant miRNA circulating in blood and is mostly derived from RBC.

Figure 8 is a graph that showing an independent validation of hemolysis levels in miRNA qPCR arrays. In Exiqon miRNA qPCR arrays, hemolysis is scored as a ratio of the Cp of miR23a/miR451a assays in two independent, technical repeat experiments - shown respectively in the black and in the red lines for each individual sample (x-axis, n=89). If the miR23a/miR451a ratio cut off >7 is reached samples are excluded from any further analysis. In agreement with our previous spectrophotometric analysis (Figure 7), the qPCR platform confirmed similar levels of hemolysis across the groups and only 1 patient sample in the severe sepsis group was deemed to be excluded from further analysis.

Examples

The inventors set out to measure miRNAs present in blood of patients with critical illness categorized on the basis of having sepsis or non-infective systemic SIRS, in comparison with control patients having critical illness without a systemic inflammatory response (no-SIRS patients). In Example 1, the different patient populations were established. In Example 2, next generation sequencing (NGS[33]) was used to identify normalizer miRNAs (present at consistent levels between patient groups) and then to identify a long-list of candidate miRNAs differentially present in the blood of patients with sepsis, non-infective SIRS and without SIRS. In Example 3, miRNAs stably expressed in sepsis, SIRS and normal individuals were identified. In Examples 4 and 5, the inventors used miRNA RT-qPCR to validate the most differentiating miRNAs and explore their performance in distinguishing sepsis from non-infective SIRS used singly and in combination.

Materials and Methods

Study Population and Ethics approval

Recruitment of the patients whose samples were used in this study has been described previously[34]. Briefly, the patients comprised unselected adult admissions to the intensive or high-dependency care units at an English acute hospital (Brighton and Sussex University Hospitals NHS Trust). For each enrolled patient, data was gathered that describes demographics, severity of illness (Sequential Organ Failure Assessment (SOFA) score in the first 24 hours), comorbidities, focus of infection, and routine clinical blood test results. Study blood samples were collected in Na-citrate tubes from patients within 6 hours of ICU admission and centrifuged. Plasma was stored at -80°C until the day of analysis, thawed on ice and kept at 4°C until the RNA extraction.

Patients were categorized as having sepsis, non-infective SIRS or no-SIRS using standard criteria[34]. To minimize heterogeneity within groups, only patients with abdominal sepsis and defined distinct levels of severity were included: severe (SOFA ≥ 6) and non-severe (SOFA ≤3); patients with intermediate SOFA scores of 4-5 were excluded. Thus five patient groups were analyzed (Table 1). This study was approved by the North Wales Research Ethics Committee (Central and East) reference number 10/WN003/19. Written informed consent or consultee approval to enroll was secured for all study participants. All data were anonymized.

Sample handling and normalization of hemolysis

Red blood cell (RBC) lysis during sample handling has the potential to bias microRNA content in plasma[35-38]. The concentration of free hemoglobin ([Hb]) in plasma reflects the degree of any hemolysis[39]. Free [Hb] in patient samples was assessed by the Harboe spectrophotometric method[40,41] and samples with [Hb]>0.6g/L were excluded from further analysis[42]. Briefly, the total [Hb] in a freshly prepared Hb standard was validated using SysMex SLS-technology[43] to detect any Hb form in the human blood. Standard dilutions and plasma samples (1:10) were tested in triplicate to determine the A415, A380, and A450 and the Harboe [oxy-Hb][39] was: [oxy-Hb] (g/l)=167x(A415) – 84x(A380) – 84x(A450). Harboe oxy-Hb and total Hb content of the standards were linearly interpolated to quantitate total Hb in each sample. In qPCR miRNA arrays, further assessment of hemolysis in individual samples was made by calculating the ratio of miR23a to miR451a and using a cut-off >7 to indicate significant hemolysis[44].

Table 1 - Demographics, severity of illness and key inflammatory biomarkers of the patients

Group Name	n	Age	SOFA score	CRP	PCT	F:M ratio (F%)	[Hb] mg/l plasma
Severe SIRS	23	58.5±17.0	7.56±2.31	19.8±32.7	1.73±3.85	12:11 (52.2%)	155±110
Severe Sepsis	21	67.7±12.9	8.19±2.68	156±76.9	21.9±28.7	13:8 (61.9%)	215±145
Non-severe SIRS	21	61.0±14.0	1.29±0.96	20.6±34.3	0.29±0.34	12:9 (57.1%)	216±159
Non-severe Sepsis	8	58.1±23.3	1.13±0.99	159±69.5	5.77±9.66	3:5 (37.5%)	115±96.3
NO SIRS	16	64.9±14.6	4.19±3.41	19.2±20.3	0.77±1.00	11:5 (68.7%)	160±133

Plasma RNA extraction and NGS of plasma miRNA

After exclusion of hemolysis specimens, plasma pools were formed by combining equal volumes of patients' plasma in the groups of Table 1. Total RNA was extracted from 2.5 ml plasma using the miRVana™ PARIS™ technology kit (Life Technologies)[17]. Briefly, each sample was denatured and processed according to manufacturer's instructions to extract RNA with Acid-Phenol:$CHCl_3$; the recovered aqueous phase was mixed with ethanol (molecular biology grade; SIGMA; 1:1.25) and loaded onto replicate columns to bind RNA. After multiple column washes, RNA was eluted in 95°C DEPC-treated H_2O (Life Technologies) from replicate columns, pooled and quantified using a Nanodrop spectrophotometer. Typically, 679±165 pg RNA/µl of plasma (mean±SD) was recovered. On the same day, an average RNA input of 849±206 ng (mean±SD) was created for technical duplicates of NGS and stored at -80°C. Before cDNA library preparation for NGS, RNA preparations were validated for the presence of miRNA using a Taqman miRNA assay (Life Technologies) for human miR-16. NGS cDNA libraries were prepared and validated from plasma RNA by ARK Genomics (University of Edinburgh, UK), following manufacturer instructions, with specific barcodes for each cDNA library (Illumina TruSeq Small RNA sample protocol). Briefly, samples were ligated with an adapter (3' end) and a primer (5' end) before being reversely transcribed. The cDNA obtained was used as a template for PCR to add sample specific barcodes and extend adapters. Thereafter, samples were purified by electrophoresis (6% polyacrylamide gels) and bands corresponding to ~22 nucleotides in the original sample were size-selected (correct insert size: 146bp) after band staining and visualization under UV-light. The amplified size selected DNA was extracted from

the gel by overnight soaking (H_2O) and concentrated. The final preparation was checked for size and potential adapter-dimer contamination by electrophoresis. The libraries were finally eluted from gels and run on the High Sensitivity D1K ScreenTape (Agilent Technologies) to determine size and purity prior to final quantification by qPCR and sequenced on a HiSeq™ 2500 Illumina instrument by loading duplicate libraries on separate lanes. In each lane, ~10^8 NGS reads were acquired and, after filtering and sorting by library barcodes, sequences in any sample were mapped to the miRBase (release 20) database. The resulting mapped reads (called counts) were arbitrarily normalized as miRNA counts/10^5.

Identification of miRNA normalizers in plasma

To identify miRNAs stably expressed in sepsis and SIRS individuals relative to no-SIRS controls, for each average NGS miRNA count (technical duplicates, 5 groups representative of 89 individuals) the following were calculated: (i) percentage residual counts relative to average counts across groups and (ii) fold-differences (fd) between sepsis and SIRS (both severe and non-severe). Normalizer candidates were selected on the basis of: (i) percentage residual counts within ±20% when comparing any inflammatory disease group to no-SIRS and (ii) fd=1.00±0.20 (i.e. not more than 20% differential NGS counts in severe/non-severe sepsis vs SIRS).

qPCR miRNA array sample preparation

Total RNA was extracted from plasma of individual patients using the miRCURY™ RNA isolation - biofluids kit (Exiqon, Vedbaek, Denmark). Plasma was thawed on ice and centrifuged (3000g, 5 min, 4°C). For each sample, plasma (200 µL) was mixed with 60 µl of Lysis solution BF containing 1 µg carrier-RNA per 60µl Lysis Solution BF and RNA spike-in template mixture (UniSp4, UniSp3 and UniSp6). Samples were vortexed briefly and incubated 3 min at room temperature, before adding 20 µL Protein Precipitation solution BF. Samples were vortexed, incubated 1 min at room temperature and centrifuged (11000g, 3 min). Clear supernatants were mixed with isopropanol (270 µL, SIGMA), briefly vortexed and loaded onto binding columns. After multiple washes, RNA was eluted in RNase-free H_2O by centrifugation (11000g) and stored at -80°C.

microRNA real-time qPCR array and analysis

RNA (2 µl) was reverse transcribed using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). cDNA (1:50) was

assayed in qPCR as by the miRCURY LNA™ Universal RT microRNA PCR protocol. Each microRNA was assayed once by qPCR (on the microRNA Ready-to-Use PCR, Pick-&-Mix using ExiLENT SYBR® Green master mix) in 2 independent technical repeat experiments including negative controls (no-template from the reverse transcription reaction). In each experimental group, ≥8 biological replicates were included. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384-well plates. The amplification curves were analyzed using the Roche LC software, both for determination of Cq (2nd derivative method) and for melting curve analysis. Amplification efficiency was calculated using a linear regression method. All assays were inspected for distinct melting curves and the Tm was confirmed to be within known specifications for the assay. Assays returning 3 Cq less than the negative control and Cq<37 were accepted and sample runs not matching these criteria were omitted from further analysis (e.g., miR-92b-3p).

The stability values of candidate normalizers were assessed using the 'NormFinder' software[45]. Any qPCR data was normalized to the average Cp of internal normalizers detected in all samples (delta Cp; dCp=normalizer Cp–assay Cp). All miRNA analyses were conducted blind to the clinical data.

Cytokine and inflammatory biomarker measurements

Cytokine levels (IL-6, IL-8, IL-1β) were measured on a Luminex LX200 using Invitrogen's Human Inflammatory 5-Plex panel (Invitrogen/Life Technologies, Darmstadt, Germany) and Millipore filter plates (VWR Darmstadt) as per manufacturers' instructions. PCT was measured on a Kryptor instrument (Brahms, Henningsdorf, Germany). Levels of sCD25 were measured on commercially available microplate assays (Human IL-2 sRa (sCD25) OptEIA Set, Becton Dickenson, San Diego, CA). All biomarker analyses were conducted blind to the clinical data as previously shown[34].

Statistical analyses.

Unless specified, datasets were analyzed and plotted (including receiver operator curves, ROC) using the GraphPad Prism 6 and/or IBM SPSS Statistics 22 software. The D'Agostino and Pearson omnibus and/or Shapiro-Wilk tests were used to test normal data distribution. If not normally distributed, medians with interquartile ranges (IQR, rather than means and standard deviation, SD) are shown and Mann-Whitney U Test (rather than t-) tests were used to calculate p-values in 2-group comparisons.

Significances across more than 2 groups were assessed by ANOVA (Kruskal-Wallis test). For the qPCR miRNA array dataset, a multiple testing correction was used to adjust ordinary p-values in order to control for the number of false positives (Benjamini-Hochberg adjusted p-values[46]). The CIR-miRNA score was generated as a linear combination of the top performing 6 miRNA measurements in severe sepsis and SIRS patients (n=41) and interpolated using IBM SPSS Statistics 22 by binary logistic regression to predict SIRS vs sepsis. In particular, the CIR-miRNA score (S) was mathematically defined as: $S = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6 + k$ where x_{1-6} are the measurements of the top 6 miRNAs in a specific individual and the variables, a_{1-6}, and the constant, k, are the coefficients returned by the binary logistic regression model. In mathematical terms, the CIR-miRNA score is the natural logarithm of the odds of having SIRS vs sepsis given the measurements of the 6 top miRNAs, that is $ODDS=e^S$. Correlations between the interpolated CIR-miRNA scores and plasma levels of inflammatory mediators were evaluated using the Spearman rho and significances of the correlations in GraphPad Prism 6.

Example 1 - Patients

Free Hb concentrations measured by the Harboe method for 91 available patient plasma samples are shown in Fig. 7A. Two samples from the SIRS groups had Hb>0.6g/L (hemolysis limit) and were excluded from further analysis, as potentially containing miRNAs biased by RBC lysis. The remaining 89 patients were analyzed in 5 groups: severe sepsis, 21 patients; non-severe sepsis, 8 patients; severe SIRS, 23 patients; non-severe SIRS, 21 patients; and patients without SIRS (no-SIRS controls), 16 patients. Data describing demographics, severity of illness and key inflammatory biomarkers are shown in Table 1.

The median age of the patients was 66 years (IQR 54-75 years), 38 and 51 patients (43% and 57%) were male and female, respectively. The patient groups were well matched for age and gender (p=0.229 and p=0.638 respectively). Patients with severe sepsis and severe SIRS had similar SOFA scores (Mean±SD:8.19±2.68 and 7.56±2.31 respectively, p=1.00) which were markedly higher than in patients with non-severe disease 1.13±0.99 and 1.29±0.96 (p<0.0001 both for sepsis and SIRS). Patients with severe sepsis had markedly higher levels of C-reactive protein (CRP: 161.8 ng/ml; IQR 109.5-215.7 ng/ml) and procalcitonin (PCT: 8.8 ng/ml; IQR 2.15-39.2 ng/ml) than patients with severe SIRS (CRP: 5.50 ng/ml; IQR 1.90-18.2 ng/ml, p<0.0001 and PCT: 0.20 ng/ml; IQR 0.10-1.10 ng/ml, p=0.0002). PCT levels also tended to be lower among

patients with mild rather than severe sepsis (1.40 ng/ml; IQR 0.32-8.82 ng/ml) but this was not statistically significant (p=1).

Example 2 - Identification of plasma inflammation-related miRNAs by NGS

In order to find out which of the currently known 2588 human miRNAs are found in the blood of sepsis patients the inventors used next generation sequencing (NGS) to sequence and differentially quantitate miRNAs in plasma pools comprising all patients in each of the five groups shown in Table 1. Plasma pools were preferred to individual samples because they decrease the impact of individual outliers on the analysis. To minimize the contribution of miRNAs derived from RBC in differential analysis, the inventors compiled pools in such a way that average levels of hemolysis were comparable (Fig. 7B and Table 1). Total RNA was then extracted from equal volumes of plasma pools and technical duplicates of cDNA libraries for Illumina NGS created. Results from 10 pools representative of 89 individuals are shown in Figure 1. On average, NGS reads/library were 7.94±(SD)1.36 x10^6 and miRBase-mapped reads (counts) were 43.5±(SD)7.2%. Just below half of human microRNAs listed in miRBase.org (1097) returned counts in NGS (Fig 1A) and were similarly distributed in each group. miRNAs present at low levels (<1 read per 10^5 NGS counts across all five groups; Fig.1A, orange area) showed high variability between the technical replicates and were excluded from further analysis. 244 miRNAs were expressed above >1/10^5 counts (Fig. 1B) in at least one group and among these concordance between replicates was poor if average counts were <15/10^5 (Fig. 1C, grey area) consistently across groups, leaving 116 miRNAs for subsequent analysis (Fig. 1D).

For each miRNA, fold differences (fd) were calculated comparing average counts in SIRS and sepsis with no-SIRS patients (Fig. 1D). A significant difference was seen when comparing pooled plasma from patients with non-infective SIRS and patients with sepsis relative to no-SIRS controls, with the median fd for miRNAs being 2.64 (IQR: 2.10-3.29) and 1.52 (IQR: 1.15-1.92) respectively ($p<0.0001$ and n=116 for each comparison). When miRNA levels in plasma from sepsis and SIRS patients were compared, miRNA levels were lower in patients with sepsis (median fd=0.53; IQR: 0.45-0.74) (Fig. 1D, right panel). Blood miRNAs that are reduced in sepsis compared to SIRS are henceforth referred to as circulating inflammation-related miRNAs (CIR-miRNAs).

Example 3 - Identification of normalizer miRNAs

To allow for robust comparison of miRNA levels in blood between individual samples, endogenous miRNA normalizers must be established (i.e. similar to "housekeeping" miRNAs in blood). Previous studies have used different and inconsistent approaches to miRNA normalization in blood[18,21,47]. The inventors first used NGS to identify potential normalizers present at consistent levels across the four other pools (inflammatory disease groups, Fig. 2A) relative to the no-SIRS controls. We shortlisted 3 candidate normalizers: miR320a, miR92b-3p and miR486-5p that were expressed at stable levels across all inflammatory patient groups (sepsis and SIRS), with their expression oscillating no more than ±20% (Fig. 2A).

The inventors then independently validated the NGS normalizers using qPCR miRNA arrays on individual patient samples (n=89, Fig. 2B) which demonstrated that miR92b-3p was in fact below the level of detection in 22/89 patients. As a result, the optimal normalizer (by NormFinder stability value[45]) was the mean Cp of miR320a and miR486-5p, which performed better than any other single miRNA detected and with levels consistent across 89 patients (Fig. 2B).

Example 4 - CIR-miRNA quantification in the blood of individual patients with Sepsis and SIRS

The inventors then asked whether they could (i) validate and (ii) use the general CIR-miRNA decrease detected in NGS to distinguish sepsis and SIRS. To maximize the possibility to detect reliable candidates CIR-miRNAs with high levels of detection in blood were selected (by excluding CIR-miRNAs with consistently less than $35/10^5$ NGS counts in any group) and with fd≤0.66 or fd≥1.5 (when comparing sepsis to SIRS), leaving a panel of 47 CIR-miRNAs to be validated in 89 individuals -including 3 potential normalizers (miR320a, miR92b-3p and miR486-5p) - in RT-qPCR miRNA arrays. Within each patient's specimen, Cp of single miRNAs were compared to the mean Cp of internal normalizers, to give delta-Cp (dCp). dCp of all patients were analyzed, comparing severe Sepsis (D, n=21) and SIRS (A, n=23). In parallel, the inventors scored hemolysis in qPCR miRNA arrays as miR23a/miR451a ratio to exclude one sample that had scored >7[44] from further analysis (Figure 8). Confirming our NGS analysis (Fig. 3 and Table 2), the majority (~94%) of miRNAs had negative fold changes in qPCR analysis, marking a clear trend towards reduced CIR-miRNA levels in sepsis compared to SIRS (Volcano plot in Fig. 3A). Table 2 summarizes the CIR-miRNA species that were significantly different between these groups (t-test, p<0.05, Benjamini-Hochberg correction[46]). 20 CIR-miRNAs were significantly

decreased (Fig. 3A; red and yellow dots above horizontal black line, p≤0.05, and with fd≤-1.5, left vertical line) but no CIR-miRNAs were significantly increased in sepsis compared to SIRS. When shown as a heatmap (Fig. 3B), the top-12 significantly different CIR-miRNAs showed inverse patterns in sepsis and SIRS. Principal component analysis demonstrated that a combination of the top 5 significantly different CIR-miRNAs (including miR30d-5p, miR30a-5p, miR192-5p, miR26a-5p and miR23a-5p) was able to discriminate severe sepsis from SIRS, as patients with SIRS (Fig. 3C, blue dots) tended to group in a different quadrant from sepsis patients (Fig. 3C, green dots).

Table 2 – Significant differentially-expressed miRNAs in severe sepsis (D) and SIRs compared to SIRS (A)

miRname	SD D	SD A	Average dCp D	Average dCp A	Fold change	P-value	Bh adj p-value
hsa-miR-30d-5p	1.1	0.76	-4.9	-3.5	-2.7	0.000014	0.00061
hsa-miR-30a-5p	1.1	1.7	-6.9	-5.1	-3.5	0.00030	0.0064
hsa-miR-192-5p	1.4	1.5	-4.6	-2.9	-3.2	0.00072	0.010
hsa-miR-26a-5p	1.5	1.1	-2.9	-1.5	-2.5	0.0018	0.017
hsa-miR-23a-3p	1.3	0.77	0.90	2.0	-2.1	0.0020	0.017
hsa-miR-191-5p	0.96	0.98	-1.6	-0.60	-1.9	0.0026	0.018
hsa-let-7i-5p	1.5	0.96	-4.2	-3.2	-2.1	0.0099	0.061
hsa-miR-122-5p	2.5	3.2	-2.9	-0.59	-4.8	0.014	0.073
hsa-miR-101-3p	1.0	0.81	-2.9	-2.2	-1.6	0.018	0.084
hsa-miR-30c-5p	1.6	1.1	-4.1	-3.1	-2.0	0.023	0.085
hsa-miR-151a-3p	1.3	0.82	-4.2	-3.4	-1.7	0.024	0.085
hsa-miR-378a-3p	1.4	1.3	-4.2	-3.2	-2.0	0.025	0.085
hsa-miR-223-3p	1.5	1.3	3.3	4.3	-2.0	0.027	0.085
hsa-miR-27b-3p	1.8	0.92	-2.9	-1.9	-2.0	0.030	0.085
hsa-let-7a-5p	1.5	0.81	-0.69	0.14	-1.8	0.030	0.085
hsa-miR-146a-5p	1.2	0.84	-1.7	-1.0	-1.6	0.033	0.089
hsa-miR-107	0.97	0.87	-2.4	-1.9	-1.5	0.043	0.10
hsa-let-7b-5p	0.67	0.63	-0.96	-0.54	-1.3	0.044	0.10
hsa-miR-30e-3p	1.0	1.1	-6.7	-6.0	-1.6	0.046	0.10
hsa-miR-143-3p	1.7	1.3	-4.5	-3.6	-2.0	0.048	0.10

After normalization dCp values in severe SIRS were significantly higher than in sepsis (Fig. 4A), indicating that CIR-miRNAs are more abundant in SIRS than in sepsis patients. When the data were plotted as receiver operator curves (ROC) each of the top 6 significantly different CIR-miRNAs provided good to excellent discrimination with areas under the curve (AUC) between 0.742 to 0.861 (Fig. 4A).

The inventors further created a model combining the top 6 CIR-miRNA levels into a score that maximized the distinction between SIRS and sepsis (Fig. 4B). In the model interpolation (binary logistic regression) of the cohort, SIRS and sepsis patients tended to score respectively >0 and <0; hence the higher the model score the more likely

patients are to have non-infective SIRS rather than sepsis, as described by a concomitant increase of multiple CIR-miRNAs (CIR-miRNA score). The ROC curve with AUC 0.917 for the model interpolation data (Fig. 4B, right) shows that the top-6 significant CIR-miRNAs combined together outperformed any single miRNA. Thus, CIR-miRNAs are excellent biomarkers to distinguish SIRS from sepsis.

Example 5 - Correlations between inflammatory cytokines and CIR-miRNA scores

The inventors obtained CIR-miRNA scores as a mathematical function of the plasma levels of 6 CIR-miRNAs found to be consistently reduced in sepsis (and preferentially leading to score<0). The CIR-miRNA scores were then correlated to plasma levels of pro-inflammatory mediators, and SOFA severity scores, across sepsis and SIRS patients (Figure 5). CIR-miRNA scores did not correlate with SOFA scores (Fig. 5). However, CIR-miRNA scores negatively correlated with levels of pro-inflammatory mediators, suggesting that a marked increase of multiple CIR-miRNAs is significantly associated with low levels of pro-inflammatory cytokines (IL-1, IL-8 and IL-6, Fig. 5) and mediators (CRP and sCD25, Fig. 5). Thus, in severe inflammatory disease CIR-miRNAs change in the opposite direction to pro-inflammatory mediators.

Discussion

These findings demonstrate for the first time that a wide range of blood miRNAs are affected during systemic inflammation humans. The inventors have found a general upregulation of circulating inflammation-related miRNAs (CIR-miRNAs) in both sepsis and non-infective SIRS patients when compared with no-SIRS controls. However, the same CIR-miRNAs were higher in *non-infective* SIRS than in *sepsis*, indicating that CIR-miRNAs is differentially affected in systemic inflammatory conditions depending on etiology. The inventors have identified six CIR-miRNAs that that are highly discriminatory for sepsis from SIRS having AUCs by ROC analysis comparable or better than clinical biomarkers, CRP and PCT. Notably, they found that CIR-miRNA levels correlate inversely with pro-inflammatory biomarkers.

Previous studies have explored whether circulating miRNAs can be used as sepsis biomarkers. Such studies have typically identified one or two miRNAs discriminating sepsis patients from healthy controls[18,22,23] (rather than SIRS patients[19,20,23,25]) or have tested the potential of miRNAs to predict the outcome of sepsis (survivors versus non survivors of sepsis[21,22,24]), frequently with contrasting results. Early studies used miRNA microarrays[18] which are less sensitive than NGS.

Furthermore, most studies have involved small patient numbers analyzed as individuals and have not accounted for the nature of the underlying infection in sepsis patients or for severity of illness[18-25]. In many studies (except[18,21]) circulating miRNAs were not sought primarily with a genome-wide approach. Rather, previous candidates were shortlisted based on preliminary analyses of miRNAs in mouse models of sepsis or in mouse/human cells[25], eventually stimulated with LPS[26-30]. Also, the expression of blood miRNAs was not usually rigorously normalized[21,47] in qPCR experiments. Finally, although hemolysis may release miRNAs from erythrocytes[35-38], its contribution had not been balanced across experimental groups in any of the previous sepsis studies[18-25].

In order to address these issues, the inventors undertook an experimentally robust evaluation of blood miRNAs during systemic inflammation. They recruited robustly, and prospectively, clinically characterized patient groups. The focus of infection and causative organism may influence the inflammatory response in sepsis[48] and thus they enrolled specifically patients with abdominal sepsis where infection will predominantly be caused by Gram negative enterobacteriaceae. The sepsis and non-infective SIRS groups were strictly stratified and matched for severity of illness. They used critically ill patients without SIRS as their controls. This is a particularly important feature of the study, since it is the distinction of sepsis from non-infective SIRS among critically ill patients with is crucial in research and clinical practice. The inventors used NGS to screen miRNA species using pooled samples representative of many individuals, hence minimizing inter-individual variability. They rigorously normalized blood miRNAs and accounted for variation in hemolysis.

The best normalizer for the dataset was a combination of miR320a and miR486-5p, while miR92b-3p was excluded because its levels fell below the detection limit of qPCR in many individuals. Thus, the results highlight the importance of choosing plasma miRNAs (either as normalizers or biomarkers) that are expressed at detectable levels within a relatively large cohort of individuals rather than miRNA species (or other small RNAs, including nuclear RNAs[21,47]), the presence of which had not been validated across all the individuals in the cohort in the blood[18,21].

The extent of CIR-miRNA change that the inventors report in systemic inflammation was surprising. These findings confirm modulation of several previously reported miRNAs biomarkers of sepsis, including miR146, miR23a, miR122 and miR-223 and

the Let-7 family- which collectively showed a tendency to decrease in sepsis compared to SIRS in this study. However, in contrast to previous reports, the inventors do not find increased levels of miR223 and miR146[18,21,22,49,50] associated with sepsis. This is likely to be because previous studies compared sepsis patients with healthy individuals[18,21,22] (or in animal models[49,50]). Indeed, in this study, relative to internal control patients (no-SIRS), CIR-miRNAs are up-regulated in sepsis (including miR-223), thus reconciling this study with previous literature. Furthermore, this study is compatible with a previous report[20] in which miR223 and miR146a are both downregulated in sepsis compared to SIRS. Interestingly, 6/7 miRNAs investigated in the same study also showed a tendency to decrease in sepsis compared to SIRS. However, the inventors' results do not validate other miRNAs previously proposed as "biomarkers of sepsis", including miR15 and miR16[23], miR150[18,25] (in agreement with[19]), and miR4772[25].

The six CIR-miRNAs which were most discriminatory between sepsis and SIRS when used individually all performed comparably with established sepsis biomarkers. A combination of 6 CIR-miRNAs however outperformed any of the single CIR-miRNAs. Interestingly none of the previously proposed "biomarkers of sepsis" was included in the best CIR-miRNAs except for miR23a (also found in[18]).

Here the inventors do not directly address the cellular origin of CIR-miRNAs, except for the exclusion of RBC as a source of differentially expressed CIR-miRNAs. Still, they revealed a vast change in CIR-miRNA levels in systemic inflammatory disease. Correlations with inflammatory cytokines (together with the fact that many CIR-miRNAs were found in specific immune cell-types[51-54]) may point at the immune origin of CIR-miRNAs.

It is known that miRNAs traffic in the blood inside exosomes (or other lipidic vesicles) or in complexes with Argonaute (Ago) proteins[12,13]. Currently, it is not known whether the CIR-miRNAs found in this study are associated to exosomes or Ago and future research will be needed to address this question.

Cellular miRNAs may regulate ~30% of human genes[55], yet it is unclear whether CIR-miRNAs are a means of intercellular communication[54,56,57]. According to recent research, Blimp-1[58], p53/MDM2[59] and PTEN[60] may be targets of the top

3 CIR-miRNAs downregulated in sepsis in our study. Interestingly, these are kinases or transcription factors important in immune-cell differentiation and regulation. Pro-inflammatory protein biomarkers are predominantly acute phase reactants which are upregulated in sepsis[3-5,34]. The inventors have found that levels of CIR-miRNAs inversely correlate with levels of inflammatory cytokines that are typically elevated in sepsis such as IL-1b, IL-6, and IL-8, and CRP[3,61]. This opens up the possibility that CIR-miRNAs may be part of the anti-inflammatory response[4] suppressing immune cell activation in severe sepsis and inflammation (illustrated in Figure 6). This hypothesis is compatible with the recent discovery that (murine) regulatory T cells which suppress inflammatory responses can secrete a number of miRNAs analogous to the human CIR-miRNAs found in this study[54].

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CLAIMS

1. A method for distinguishing between sepsis and SIRS in a subject, the method comprising analysing the concentration of one or more type of microRNA molecule in a bodily sample from a test subject and comparing this concentration with:-
   (a) a reference for the concentration of the one or more type of microRNA molecule in an individual who suffers from sepsis, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference suggests that the subject is suffering from SIRS, and wherein if there is no difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference then this suggests that the subject is suffering from sepsis; and/or
   (b) a reference for the concentration of the one or more type of microRNA molecule in an individual who suffers from SIRS, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference suggests that the subject is suffering from sepsis, and wherein if there is no difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the reference then this suggests that the subject is suffering from SIRS.

2. A method of treating an individual suffering from sepsis, said method comprising the steps of:
   (i) determining the concentration of one or more microRNA molecule in a sample having been obtained from a test subject, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers from SIRS, suggests that the test subject suffers from sepsis; and
   (ii) administering, to the test subject, a therapeutic agent that prevents, reduces or delays progression of sepsis.

3. A method of treating an individual suffering from SIRS, said method comprising the steps of:

(i) determining the concentration of one or more microRNA molecule in a sample having been obtained from a test subject, wherein a difference in the concentration of the one or more type of microRNA molecule in the bodily sample from the test subject compared to the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers from sepsis, suggests that the test subject suffers from SIRS; and

(ii) administering, to the test subject, a therapeutic agent that prevents, reduces or delays progression of SIRS.

4. The method according to any one of the preceding claims, wherein the method comprises analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

5. The method according to any one of claims 1-3,wherein the method comprises analysing the concentration of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p and miRNA-191-5p, and optionally at least one of miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

6. The method according to any one of claims 1-3, wherein the method comprises analysing the concentration of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, and optionally at least one of miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

7. The method according to any one of claims 1-3, wherein the method comprises analysing the concentration of miRNA-30d-5p, and optionally at least one of miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

8. The method according to any one of claims 1-3, wherein the method comprises analysing the concentration of miRNA-30a-5p, and optionally at least one of

miRNA-30d-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

9. The method according to any one of claims 1-3, wherein the method comprises analysing the concentration of miRNA-192-5p, and optionally at least one of miRNA-30d-5p, miRNA-30a-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

10. The method according to any one of the preceding claims, wherein the sample is a urine sample or a blood sample.

11. Use of miRNA-30d, miRNA-30a, miRNA-192, miRNA-26a, miRNA-23a, and miRNA-191, for generating a CIR-miRNA score.

12. Use of miR320a (SEQ ID No:13) and/or miR486-5p (SEQ ID No:14) as a microRNA for normalizing the expression of levels of a biomarker.

13. The use according to claim 12, wherein the biomarker is one or more microRNAs selected from miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

14. A kit for distinguishing between sepsis and SIRS in a subject, the kit comprising:-

(i) means for determining the concentration of one or more type of micro RNA molecule in a sample from a test subject; and

(ii) a reference for the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers sepsis, wherein the kit is used to identify a difference in the concentration of the one or more type of microRNA molecule in the sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from SIRS, or wherein the kit is used to determine that there is no difference in the concentration of the one or more type of microRNA molecule in the

sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from sepsis, and/or

(iii) a reference for the concentration of the one or more type of microRNA molecule in a sample from an individual who suffers from SIRS, wherein the kit is used to identify a difference in the concentration of the one or more type of microRNA molecule in the sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from sepsis, or wherein the kit is used to determine that there is no difference in the concentration of the one or more type of microRNA molecule in the sample from the test subject compared to the reference concentration, thereby suggesting that the test subject suffers from SIRS.

15. The kit according to claim 14, wherein the kit comprises a means for analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

16. The kit according to claim 14, wherein the kit comprises a means for analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p and miRNA-191-5p, and optionally at least one of miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

17. The kit according to claim 14, wherein the kit comprises a means for analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, and optionally at least one of miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

18. The kit according to claim 14, wherein the kit comprises a means for analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-30d-5p, and optionally at least one of

miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

19. The kit according to claim 14, wherein the kit comprises a means for analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-30a-5p, and optionally at least one of miRNA-30d-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

20. The kit according to claim 14, wherein the kit comprises a means for analysing the concentration of one or more type of microRNA molecule selected from the group of miRNA molecules consisting of miRNA-192-5p, and optionally at least one of miRNA-30d-5p, miRNA-30a-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

21. The kit according to any one of claims 14 to 20, wherein the kit comprises one or more microRNAs for normalising the expression of levels of the biomarker in the sample.

22. The kit according to claim 21, wherein the microRNA for normalising the expression levels of the biomarker in the sample is miR320a and/or miR486-5p.

23. The kit according to any one of claims 14 to 22, wherein the kit comprises sample extraction means for obtaining the sample from the test subject.

24. The kit according to claim 23, wherein the sample extraction means comprises a needle or syringe or the like.

25. The kit may according to any one of claims 14 to 24, wherein the kit comprises a sample collection container for receiving the extracted sample.

26. Use of one or more type of microRNA, as a biomarker for distinguishing between sepsis and SIRS in a subject, wherein the one or more type of microRNA molecule is

selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

27. Use of miRNA-30d-5p, as a biomarker for distinguishing between sepsis and SIRS in a subject, optionally wherein one or more additional microRNA molecule is used and is selected from the group of miRNA molecules consisting of miRNA-30a-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

28. Use of miRNA-30a-5p, as a biomarker for distinguishing between sepsis and SIRS in a subject, optionally wherein one or more additional microRNA molecule is used and is selected from the group of miRNA molecules consisting of miRNA-30d-5p, miRNA-192-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

29. Use of miRNA-192-5p as a biomarker for distinguishing between sepsis and SIRS in a subject, optionally wherein one or more additional microRNA molecule is used and is selected from the group of miRNA molecules consisting of miRNA-30a-5p, miRNA-30d-5p, miRNA-26a-5p, miRNA-23a-3p, miRNA-191-5p, miRNA-101-3p, miRNA-122-5p, miRNA-378a-3p, miRNA-151a-3p, miRNA-146a-5p and let-7f-5p.

Figure 1A – 1B

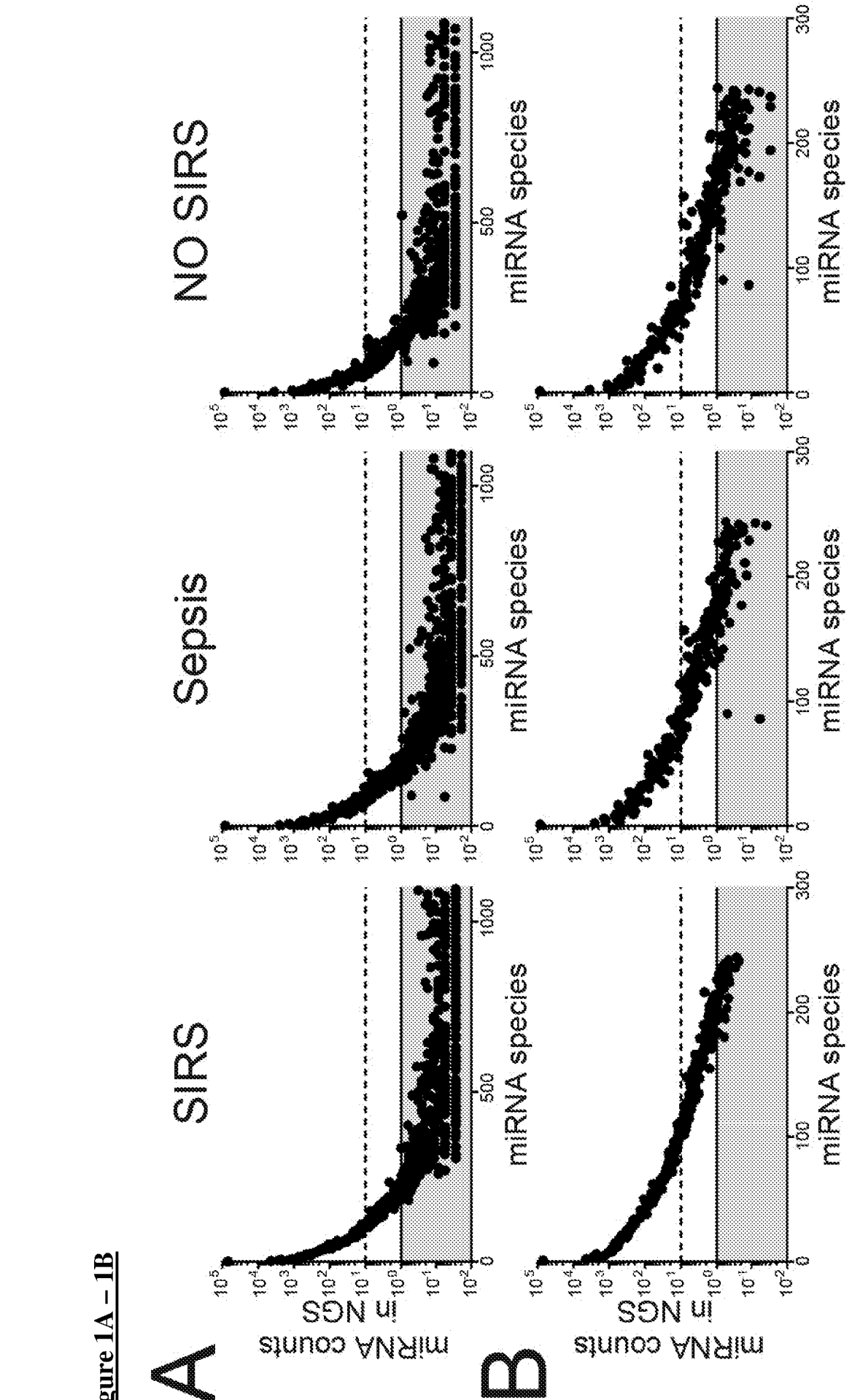


Figure 1C – 1D

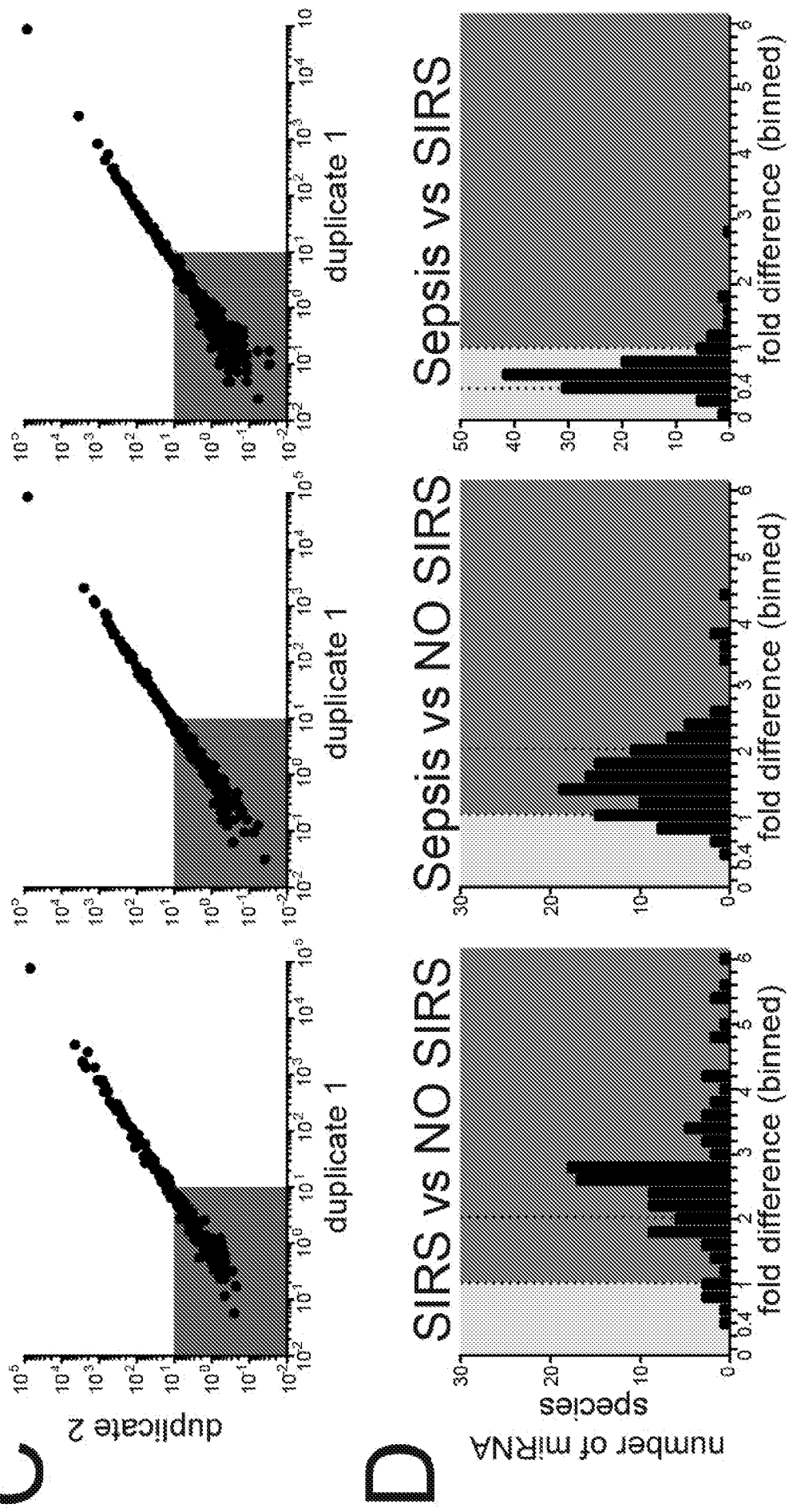
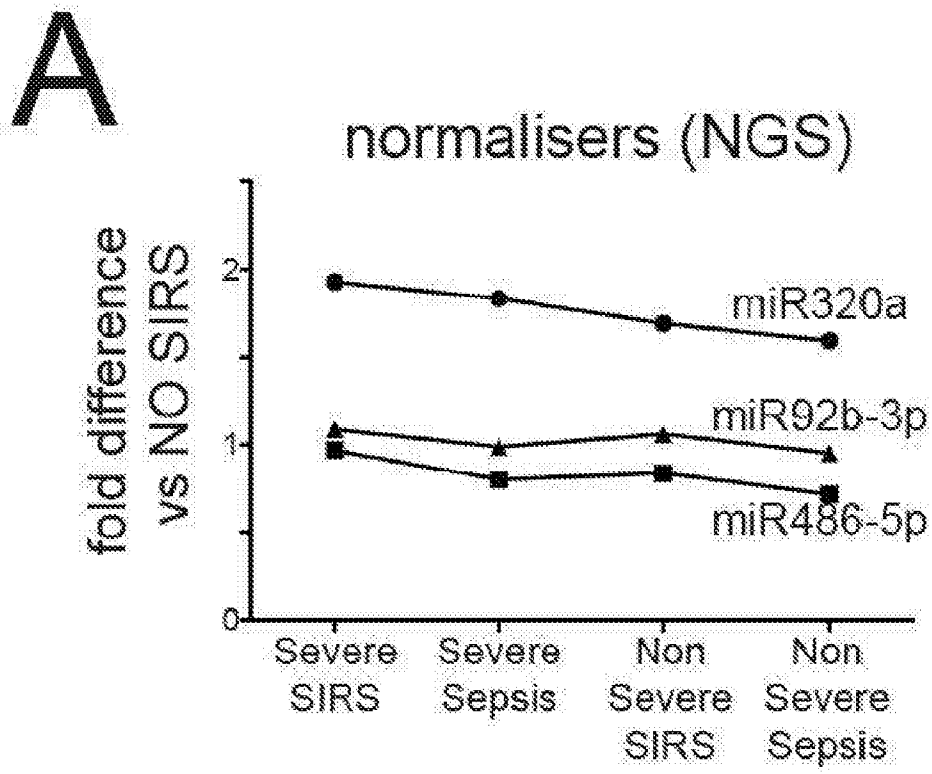

C
duplicate 2
duplicate 1
duplicate 1
duplicate 1
D
SIRS vs NO SIRS
Sepsis vs NO SIRS
Sepsis vs SIRS
number of miRNA species
fold difference (binned)
fold difference (binned)
fold difference (binned)

Figure 2A and 2B



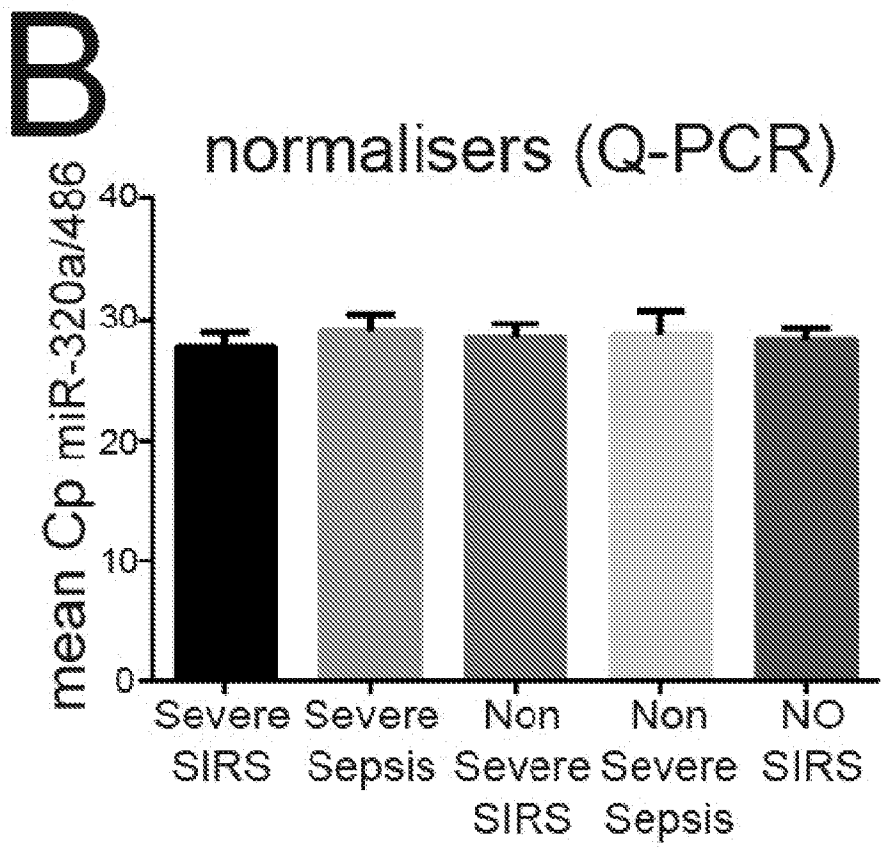
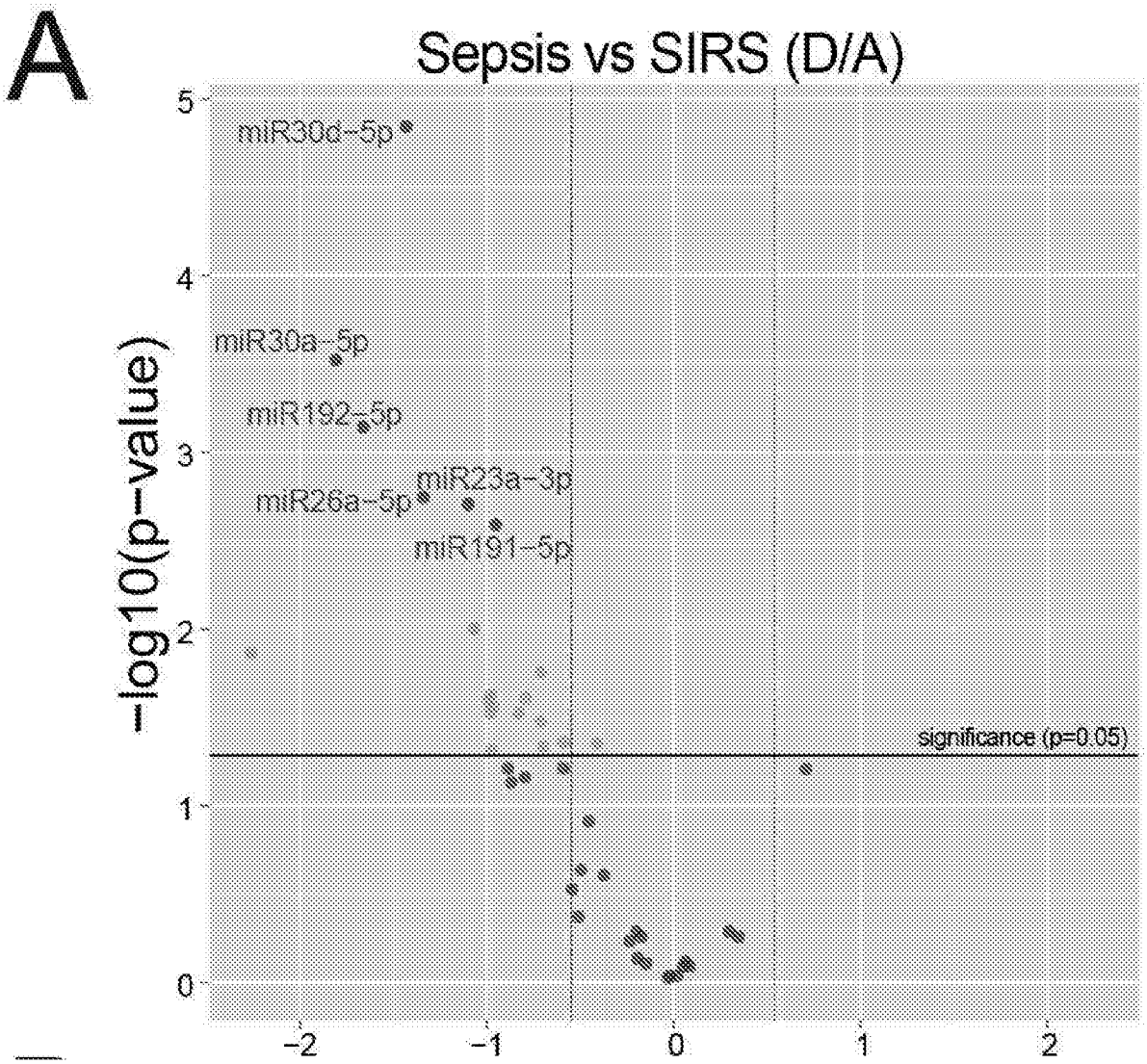


Figure 3A


A
Sepsis vs SIRS (D/A)
−log10(p−value)
miR30d−5p
miR30a−5p
miR192−5p
miR26a−5p
miR23a−3p
miR191−5p
significance (p=0.05)
5
4
3
2
1
0
−2
−1
0
1
2

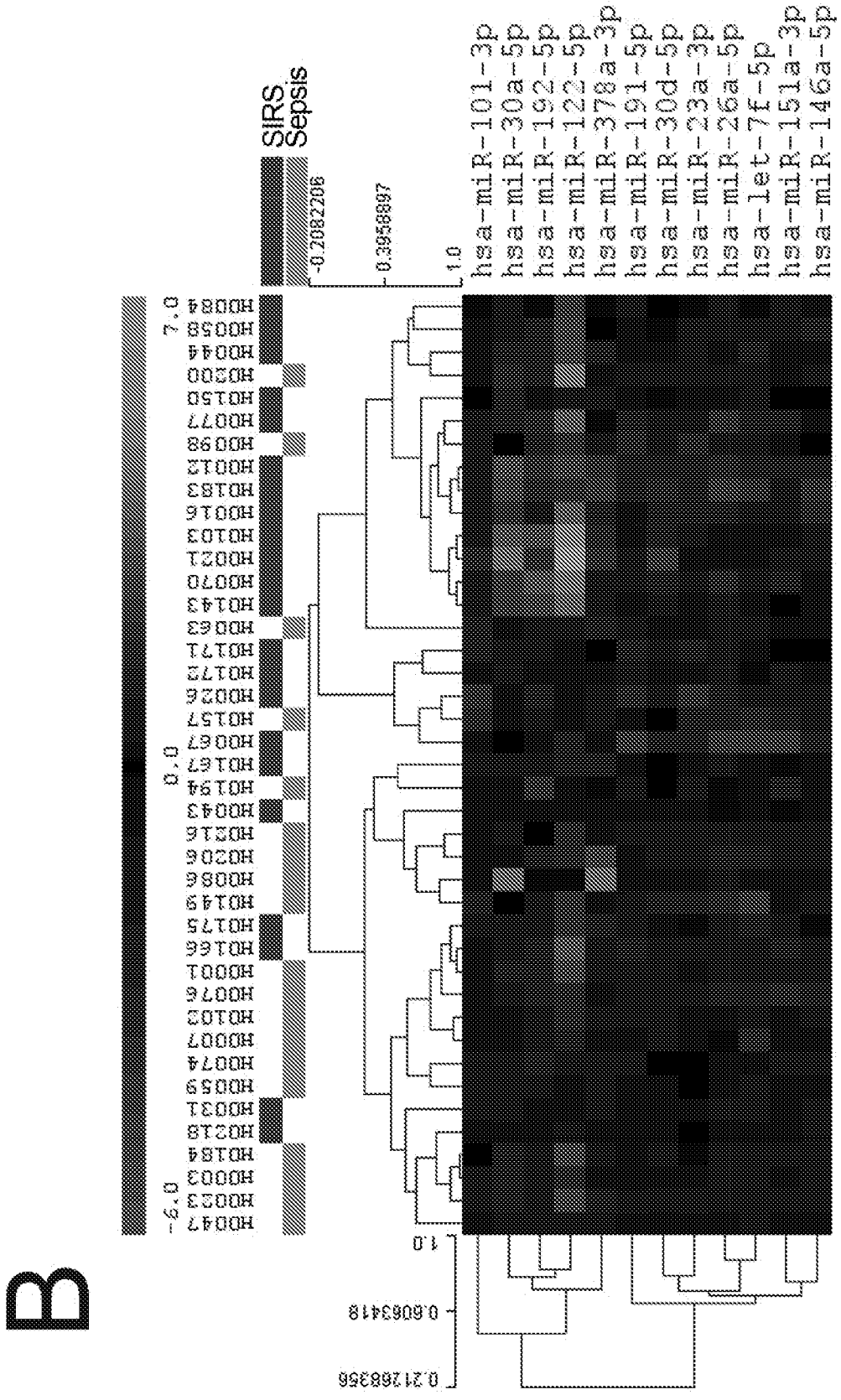

B
SIRS
Sepsis
-0.2082206
0.3958897
1.0
hsa-miR-101-3p
hsa-miR-30a-5p
hsa-miR-192-5p
hsa-miR-122-5p
hsa-miR-378a-3p
hsa-miR-191-5p
hsa-miR-30d-5p
hsa-miR-23a-3p
hsa-miR-26a-5p
hsa-let-7f-5p
hsa-miR-151a-3p
hsa-miR-146a-5p
7.0
0.0
-6.0
H0084
H0058
H0044
H0200
H0150
H0077
H0098
H0013
H0183
H0016
H0103
H0021
H0070
H0143
H0063
H0171
H0173
H0026
H0157
H0067
H0167
H0194
H0043
H0216
H0206
H0086
H0149
H0175
H0166
H0001
H0076
H0102
H0007
H0074
H0059
H0031
H0218
H0184
H0003
H0053
H0047
1.0
0.6063418
0.2126356

Figure 3B

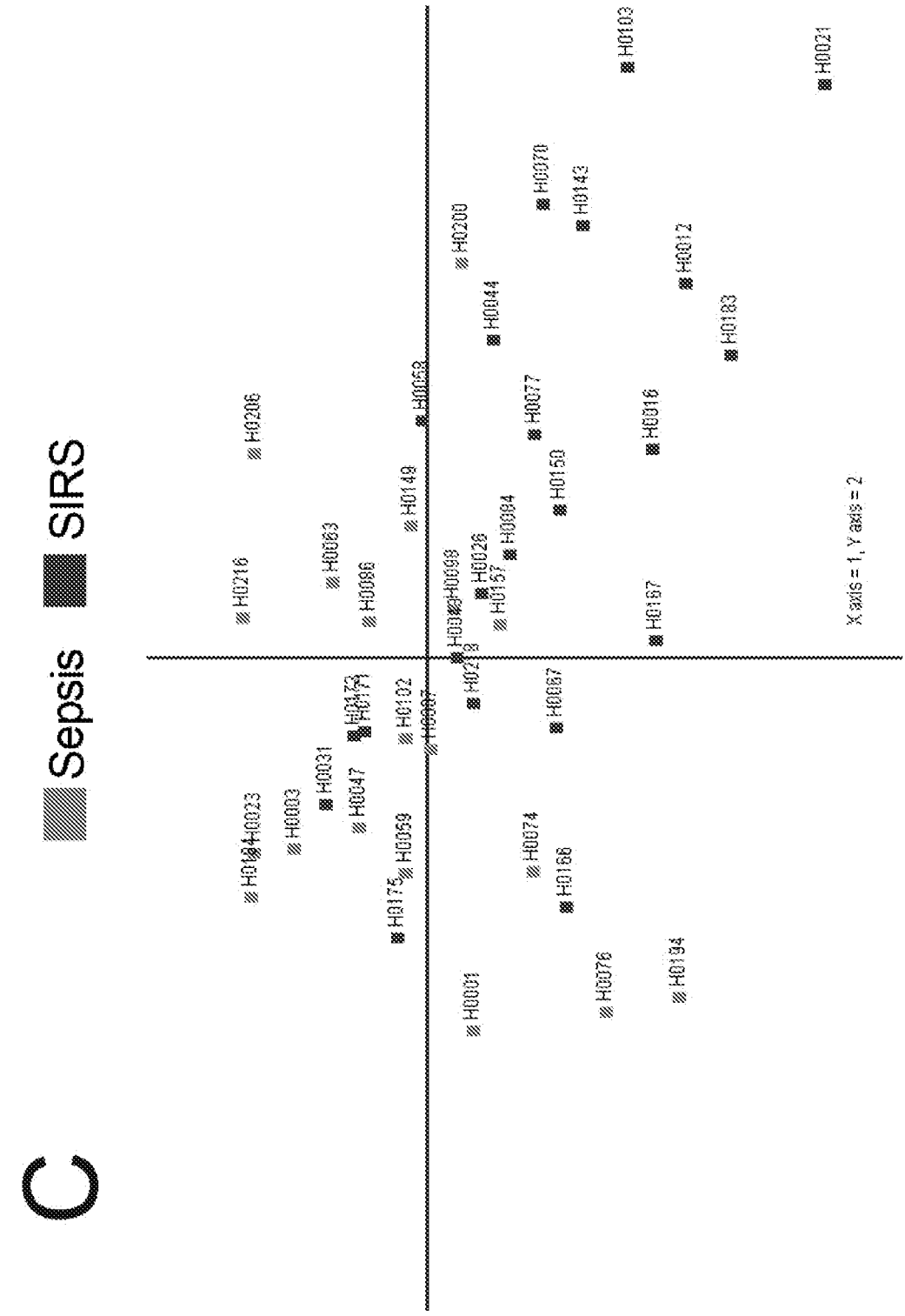

C
Sepsis
SIRS
H0216
H0206
H0063
H0086
H0149
H0053
H0200
H0044
H0070
H0143
H0103
H0012
H0183
H0021
H0077
H0150
H0016
H0167
H0026
H0167
H0094
H0102
H0067
H0074
H0166
H0076
H0194
H0001
H0059
H0175
H0047
H0031
H0003
H0023
X axis = 1, Y axis = 2

Figure 3C

Figure 4A

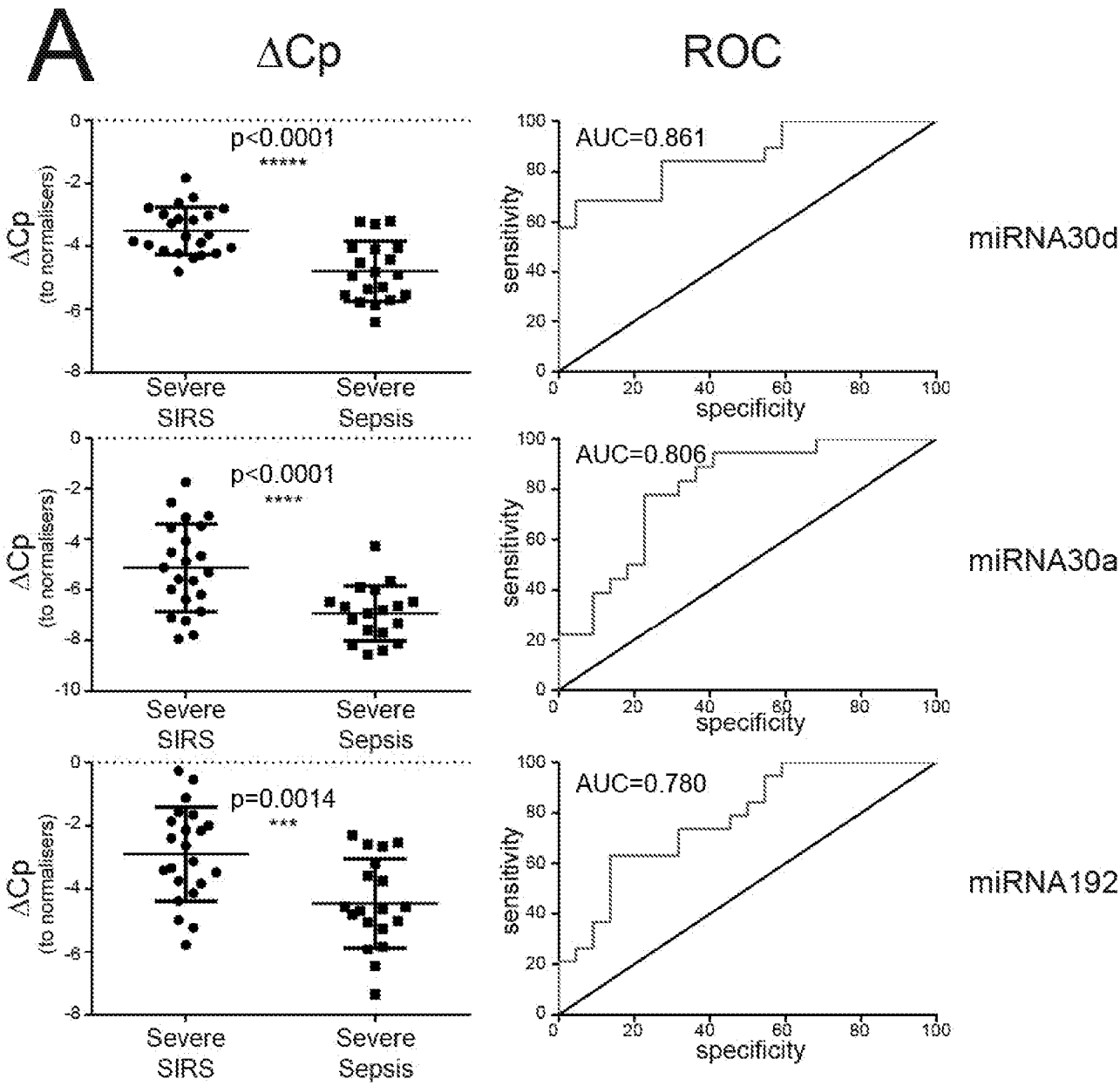
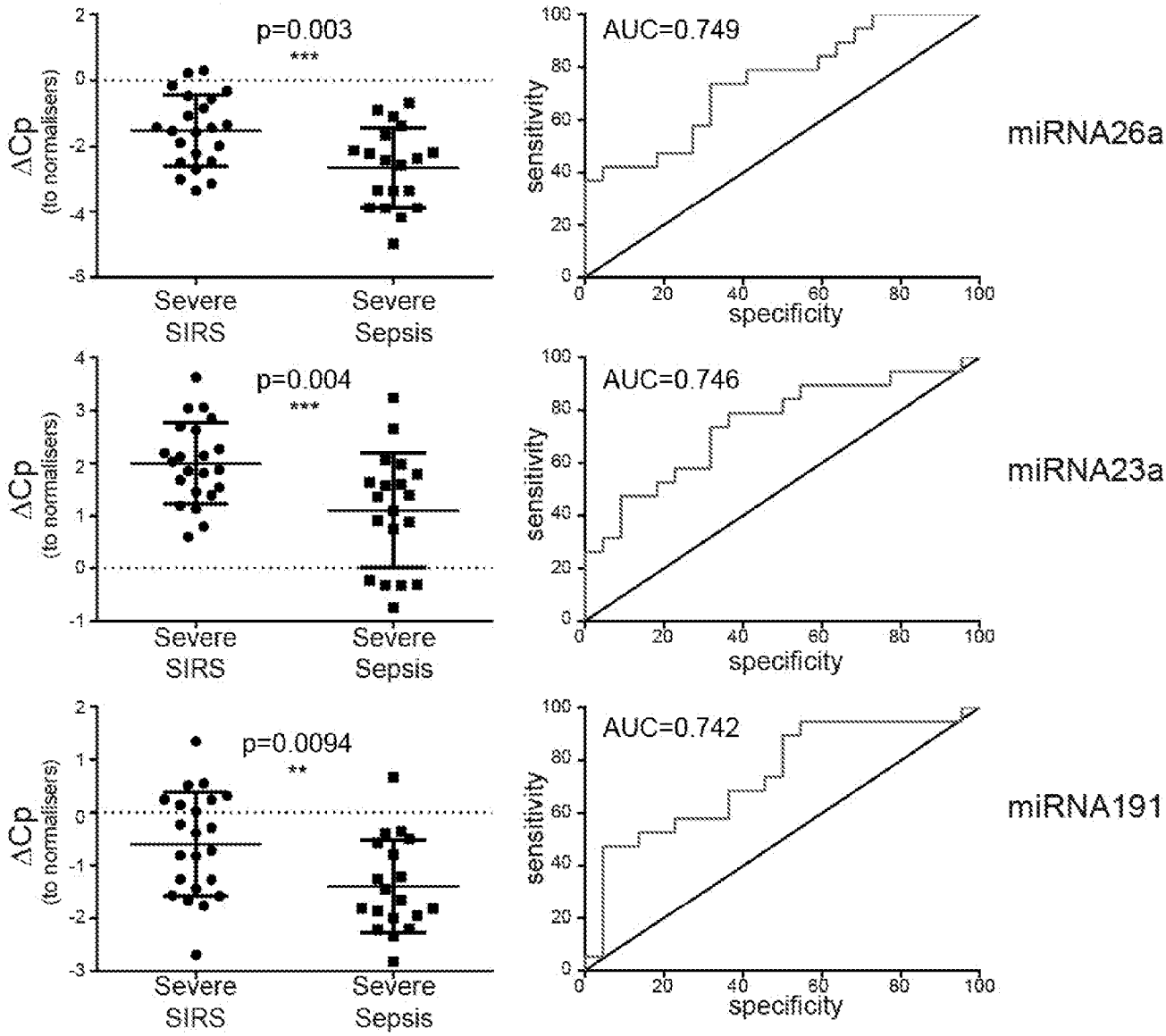

A
ΔCp
ROC
p<0.0001
*****
ΔCp (to normalisers)
Severe SIRS
Severe Sepsis
AUC=0.861
sensitivity
specificity
miRNA30d
p<0.0001
****
AUC=0.806
miRNA30a
p=0.0014
***
AUC=0.780
miRNA192

Figure 4A (continued)



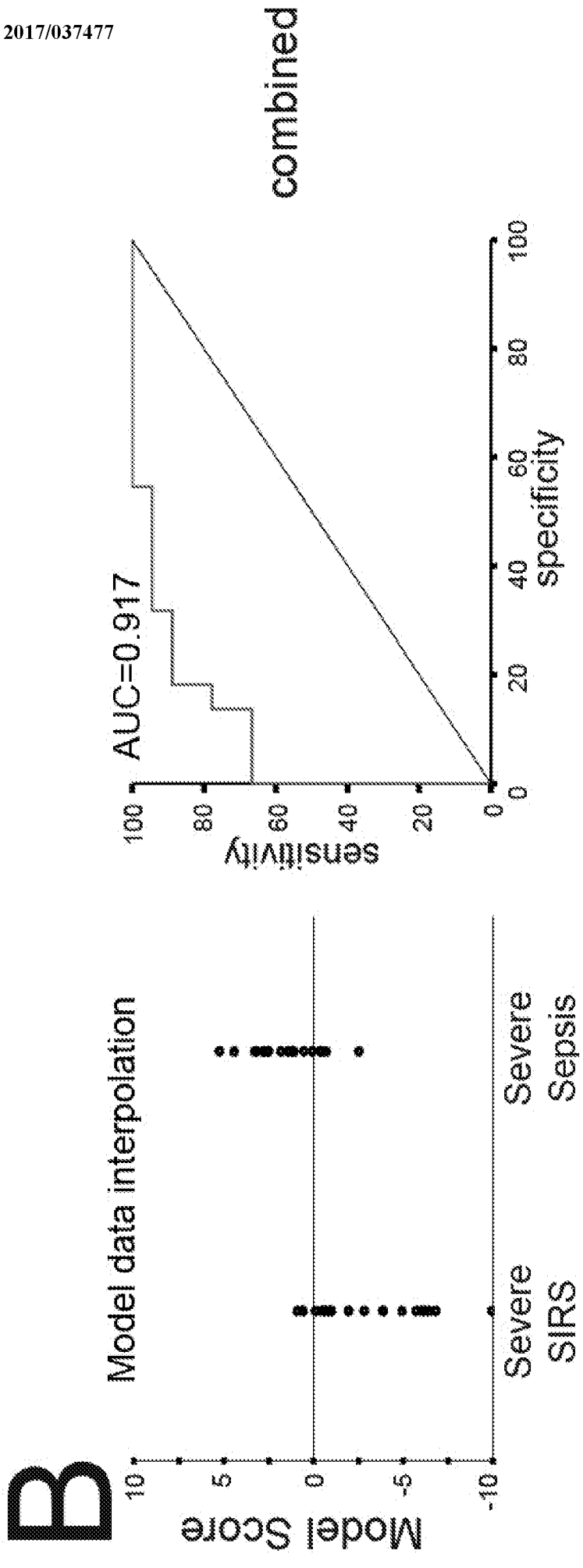

B
Model Score
10
5
0
-5
-10
Model data interpolation
Severe SIRS
Severe Sepsis
combined
AUC=0.917
sensitivity
specificity
100
80
60
40
20
0
0
20
40
60
80
100

Figure 4B

Figure 5

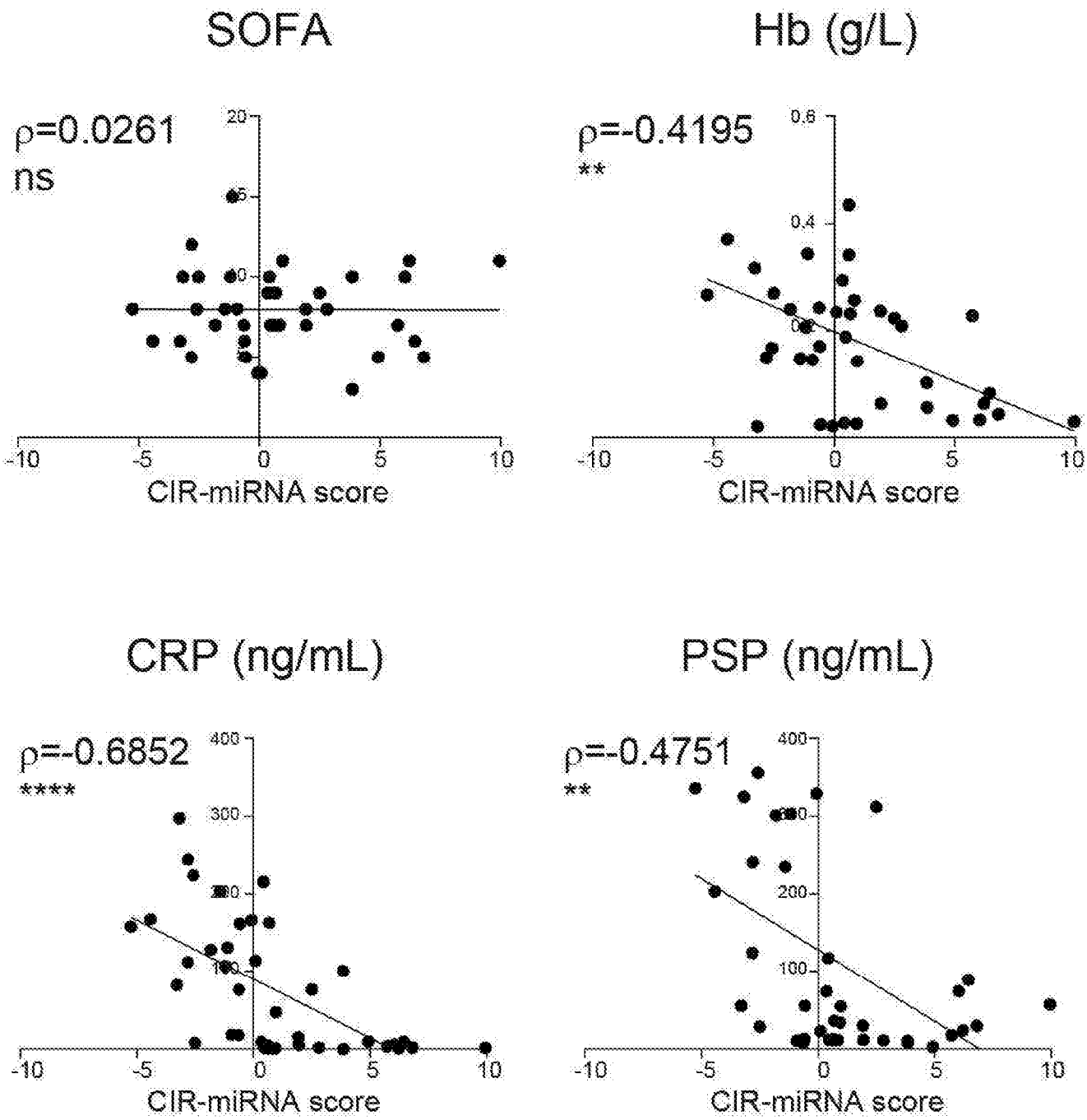

SOFA
ρ=0.0261
ns
CIR-miRNA score
Hb (g/L)
ρ=-0.4195
**
CIR-miRNA score
CRP (ng/mL)
ρ=-0.6852
****
CIR-miRNA score
PSP (ng/mL)
ρ=-0.4751
**
CIR-miRNA score

Figure 5 (continued)

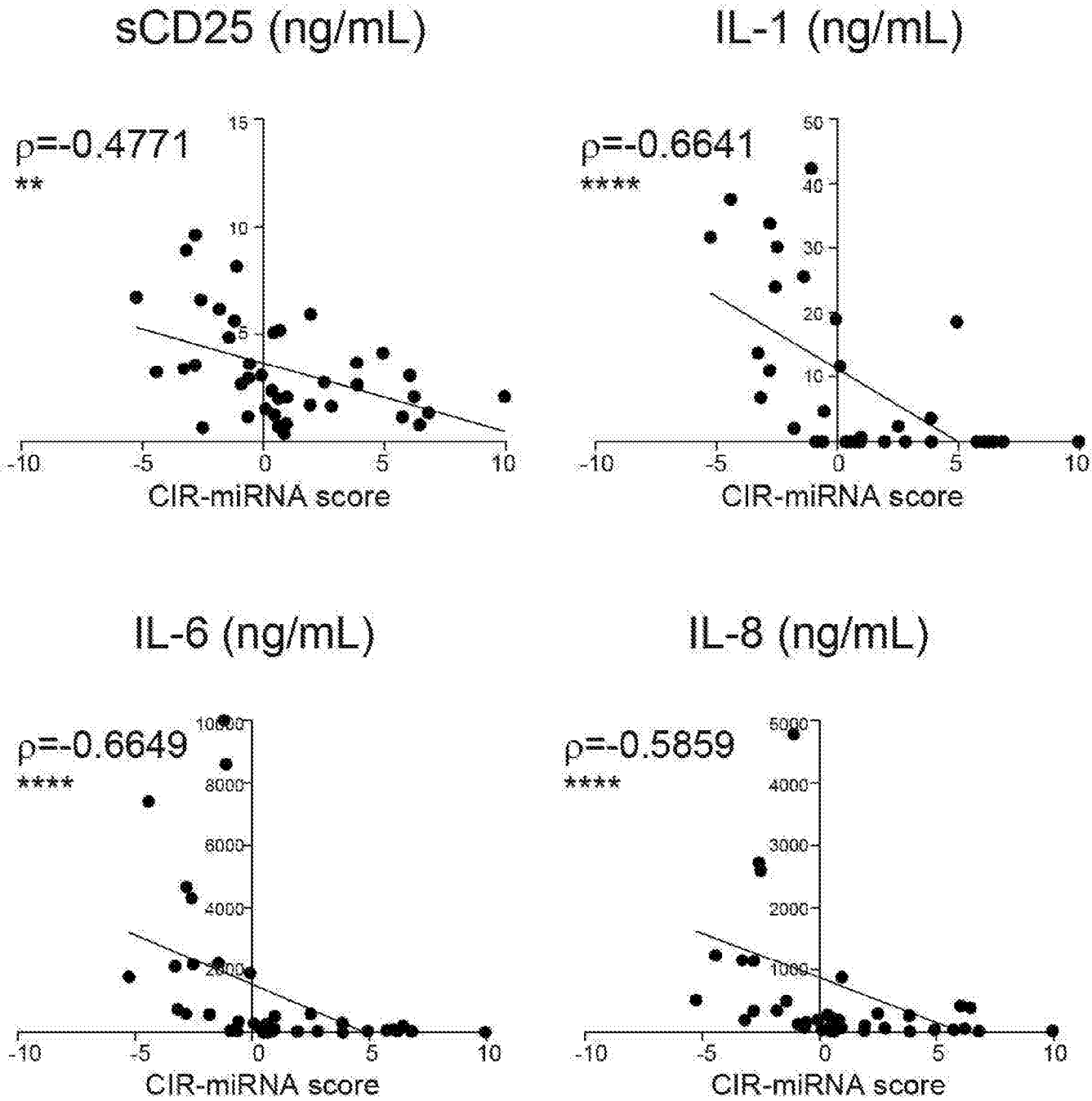


Figure 6

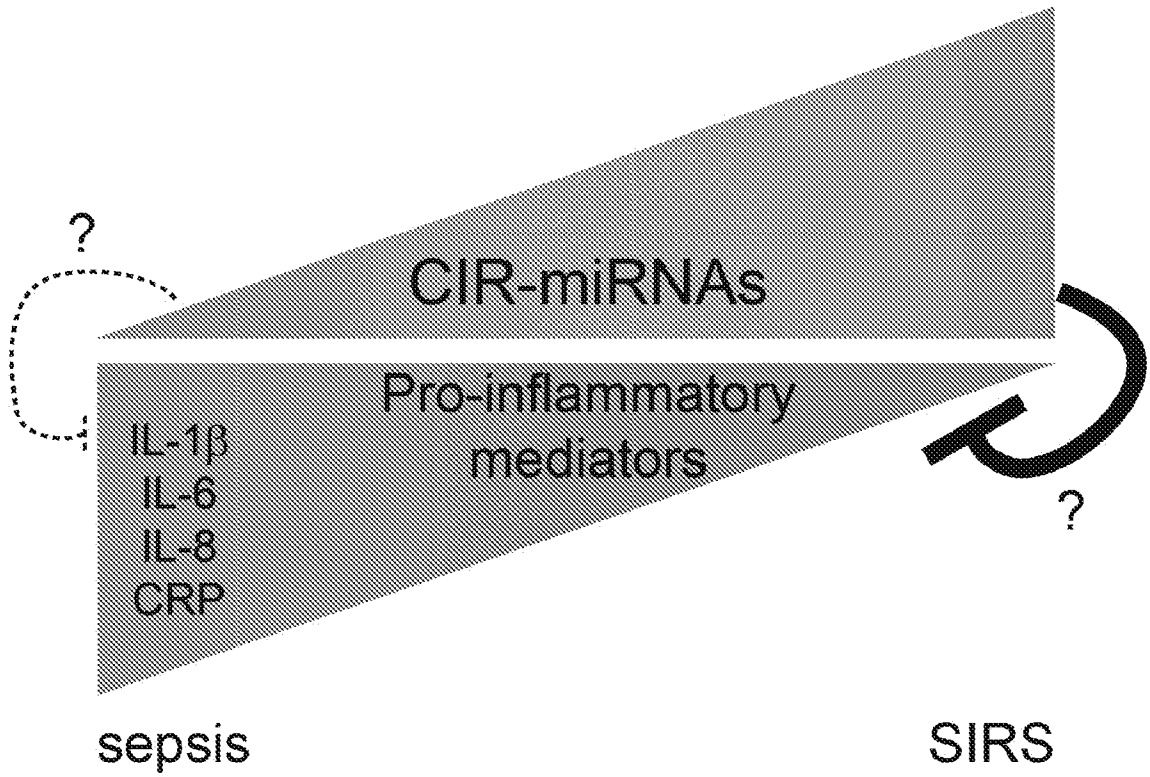

?
CIR-miRNAs
Pro-inflammatory mediators
IL-1β
IL-6
IL-8
CRP
?
sepsis
SIRS

Figure 7A

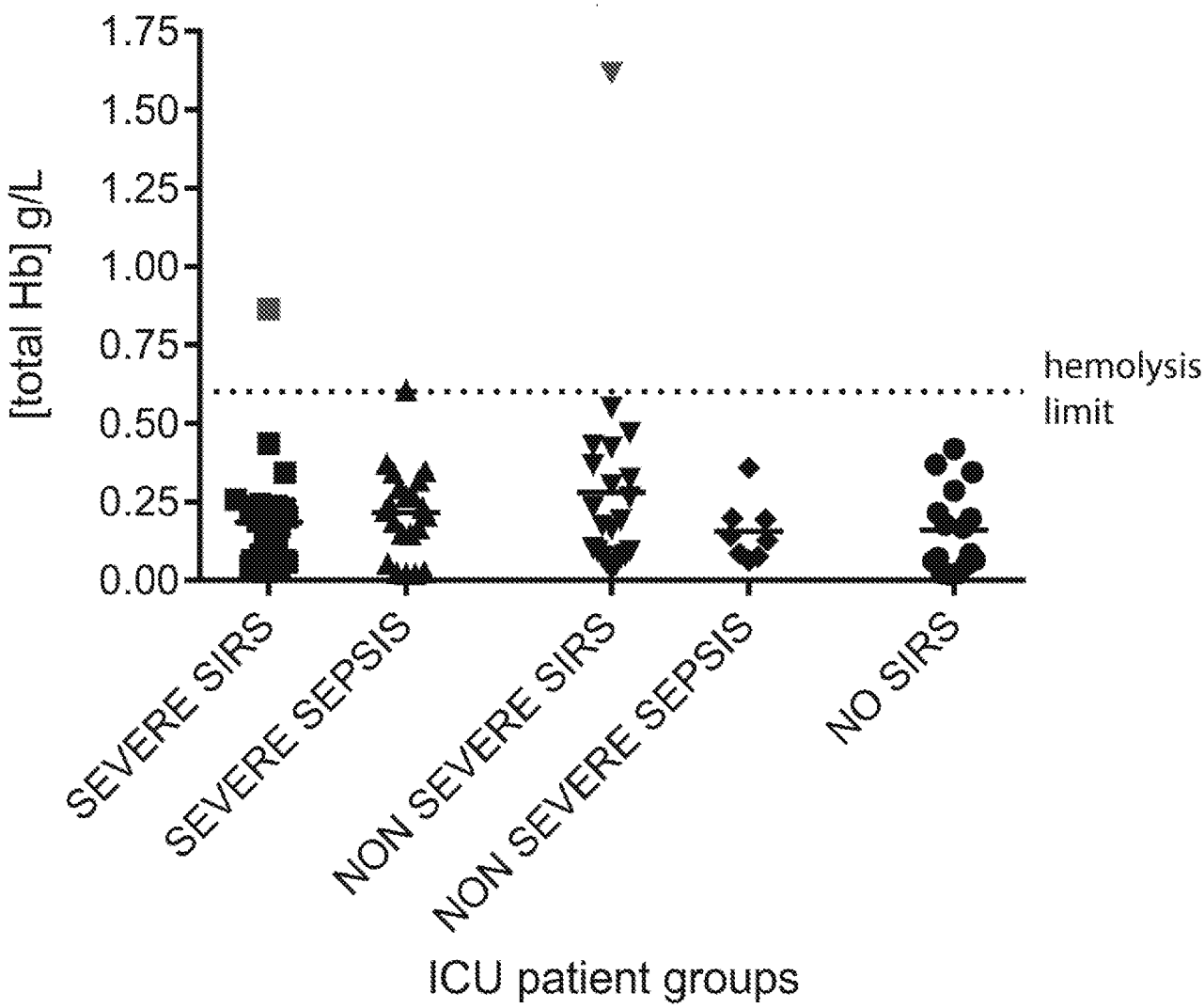

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
[total Hb] g/L
hemolysis limit
SEVERE SIRS
SEVERE SEPSIS
NON SEVERE SIRS
NON SEVERE SEPSIS
NO SIRS
ICU patient groups

Figure 7B

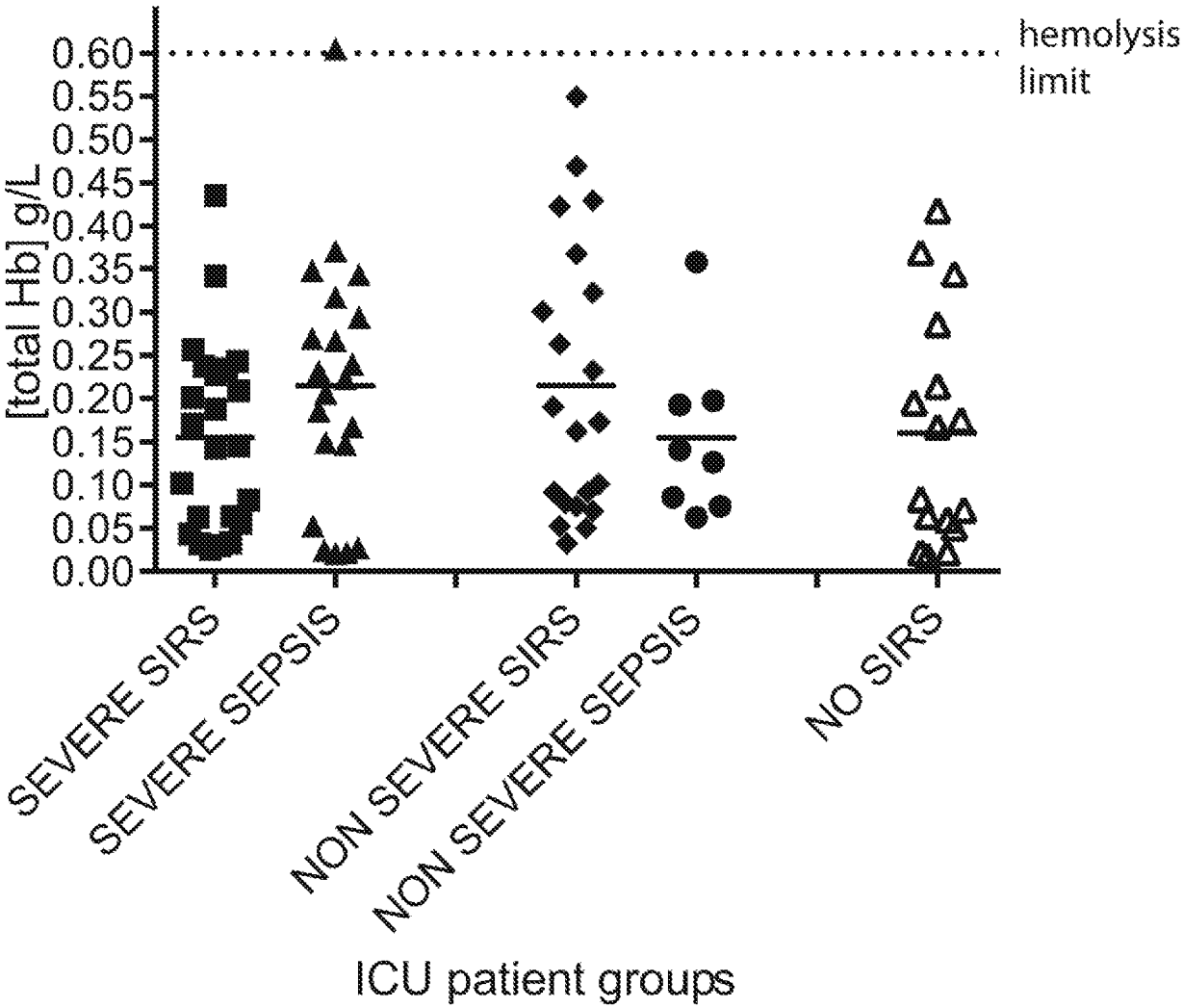
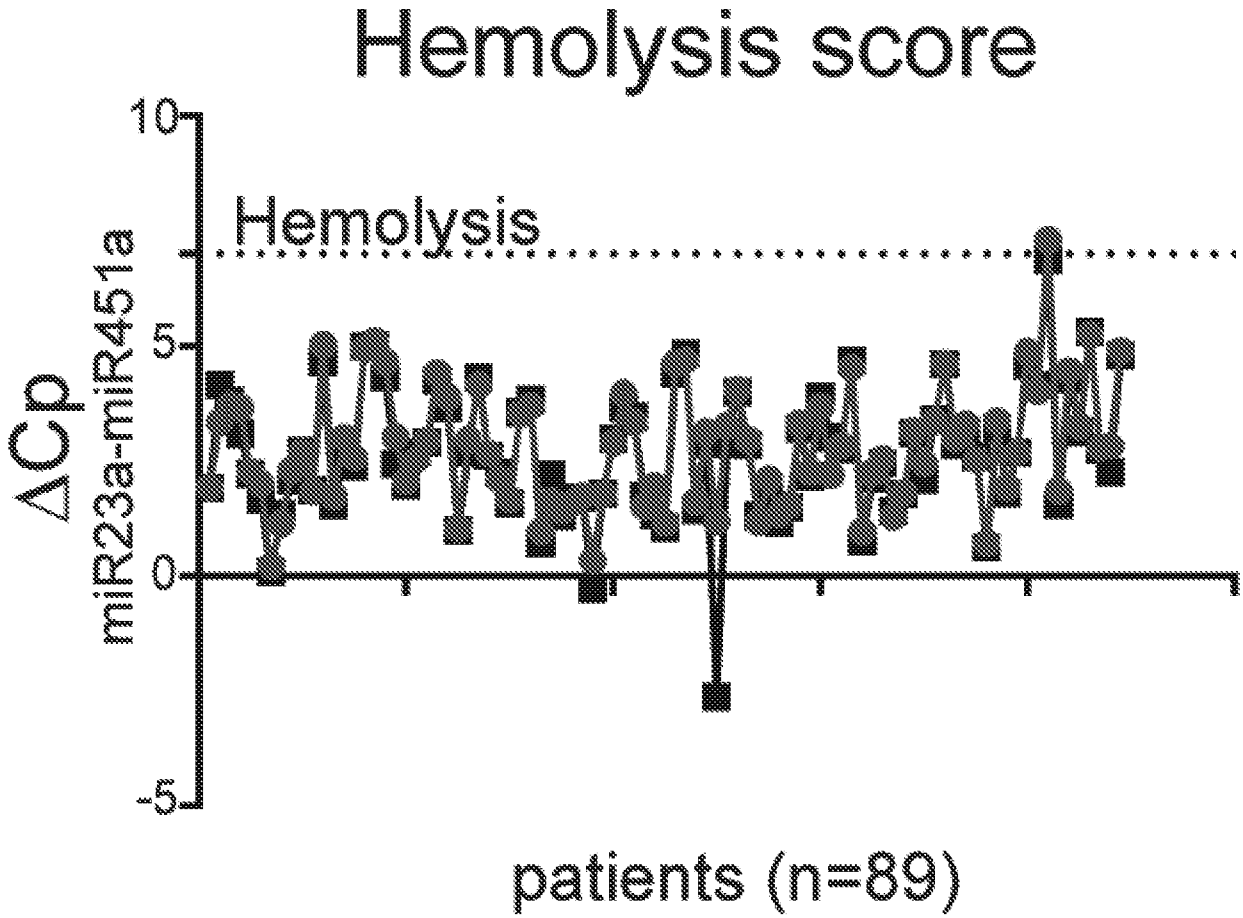

hemolysis limit
[total Hb] g/L
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
SEVERE SIRS
SEVERE SEPSIS
NON SEVERE SIRS
NON SEVERE SEPSIS
NO SIRS
ICU patient groups

Figure 8


Hemolysis score
10
5
0
-5
Hemolysis
ΔCp
miR23a-miR451a
patients (n=89)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/052724

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)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YUQIAN MA ET AL: "Genome-Wide Sequencing of Cellular microRNAs Identifies a Combinatorial Expression Signature Diagnostic of Sepsis", PLOS ONE, vol. 8, no. 10, 16 October 2013 (2013-10-16), page e75918, XP055183318, DOI: 10.1371/journal.pone.0075918	1-26
A	pages 5, 9; table 1	27-29
X	WO 2015/117205 A1 (IMMUNEXPRESS PTY LTD [AU]) 13 August 2015 (2015-08-13)	1-26
A	paragraphs [0134], [0153]	27-29

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2016	05/12/2016

Name and mailing address of the ISA/	Authorized officer
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Liqiong Yao ET AL: "Original Article Clinical evaluation of circulating microRNA-25 level change in sepsis and its potential relationship with oxidative stress", Int J Clin Exp Pathol, 1 January 2015 (2015-01-01), XP055316965, Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4555662/pdf/ijcep0008-7675.pdf	1-26
A	figures 1-2	27-29

X	CHA CHEN: "Differential expression of plasma miR-146a in sepsis patients compared with non-sepsis-SIRS patients", EXPERIMENTAL AND THERAPEUTIC MEDICINE, 30 January 2013 (2013-01-30), XP055316969, GR ISSN: 1792-0981, DOI: 10.3892/etm.2013.937	1-26
A	figures 1-3	27-29

X	US 2009/075258 A1 (LATHAM GARY J [US] ET AL) 19 March 2009 (2009-03-19) paragraph [0022]	12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2016/052724

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015117205 A1	13-08-2015	NONE	
US 2009075258 A1	19-03-2009	NONE	