frontiers in Immunology

ORIGINAL RESEARCH
published: 05 February 2018
doi: 10.3389/fimmu.2017.01977

Severity of Systemic Inflammatory Response Syndrome Affects the Blood Levels of Circulating Inflammatory-Relevant MicroRNAs

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The systemic inflammatory response syndrome (SIRS) is a potentially lethal response triggered by diverse forms of tissue injury and infection. When systemic inflammation is triggered by infection, the term sepsis is used. Understanding how inflammation is mediated and regulated is of enormous medical importance. We previously demonstrated that circulating inflammatory-relevant microRNAs (CIR-miRNAs) are candidate biomarkers for differentiating sepsis from SIRS. Here, we set out to determine how CIR-miRNA levels reflect SIRS severity and whether they derive from activated immune cells. Clinical disease severity scores and markers of red blood cell (RBC) damage or immune cell activation were correlated with CIR-miRNA levels in patients with SIRS and sepsis. The release of CIR-miRNAs modulated during SIRS was assessed in immune cell cultures. We show that severity of non-infective SIRS, but not sepsis is reflected in the levels of miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p. These CIR-miRNA levels positively correlate with levels of the redox biomarker, peroxiredoxin-1 (Prdx-1), which has previously been shown to be released by immune cells during inflammation. Furthermore, *in vitro* activated immune cells produce SIRS-associated miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p. Our study furthers the understanding of the origin, role, and trafficking of CIR-miRNAs as potential regulators of inflammation.

Keywords: systemic inflammatory response syndrome, sepsis, microRNA, inflammation, immune cells, sequential organ failure assessment score, miR-378, miR-30

OPEN ACCESS

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *15 September 2017*
Accepted: *20 December 2017*
Published: *05 February 2018*

Citation:
Caserta S, Mengozzi M, Kern F, Newbury SF, Ghezzi P and Llewelyn MJ (2018) Severity of Systemic Inflammatory Response Syndrome Affects the Blood Levels of Circulating Inflammatory-Relevant MicroRNAs. Front. Immunol. 8:1977. doi: 10.3389/fimmu.2017.01977

INTRODUCTION

The systemic inflammatory response syndrome [SIRS (1)] can be triggered by diverse forms of injury including burns, ischemia, autoimmune diseases, injuries including surgery and infection. The severity of illness seen in SIRS varies widely but severe SIRS accompanied by multiple organ dysfunction syndrome (MODS), especially in the setting of sepsis (a condition which shares common clinical

Abbreviations: miRNA, microRNA; CIR-miRNA, circulating inflammatory-relevant microRNA; SIRS, systemic inflammatory response syndrome; SOFA, sequential organ failure assessment; APACHE II, acute physiology and chronic health evaluation II; MODS, multiple organ dysfunction syndrome; CAR, compensatory anti-inflammatory responses; DAMPs, damage-associated molecular pattern molecules; PAMPs, pathogen-associated molecular pattern molecules; TLR, toll-like receptor; ROS, reactive oxygen species; TNF, tumor necrosis factor; TGF, tumor growth factor; CRP, C-reactive protein; sCD25, soluble CD25; Prdx-1, peroxiredoxin-1; Hb, hemoglobin; IL-, interleukin; PCT, pro-calcitonin; SAg, superantigen; SPE-K/L, streptococcal pyrogenic exotoxin K/L; PBMCs, peripheral blood mononuclear cells; RBCs, red blood cells; PBS, phosphate-buffered saline; FCS, fetal calf serum; RPMI, Roswell Park Memorial Institute medium; RT, reverse transcription/transcribed; IQR, interquartile ranges.

manifestations with SIRS), may be lethal (2). Even with optimal medical care, mortality rates in severe sepsis increase to around 50% (3). In this respect, clinical scores, such as the sequential organ failure assessment [SOFA (4)] and the acute physiology and chronic health evaluation II [APACHE II (5)], are useful to evaluate SIRS severity and patient mortality risk.

During SIRS, immune cell activation spreads to the whole body driving severe immunopathology (1, 6, 7). Damage- and/or pathogen-associated molecular pattern molecules (respectively, DAMPs and PAMPs) released after injury (or infection) initiate, *via* toll-like receptor (TLR) signals (6, 8), an activation cascade in immune and endothelial cells leading to inflammatory cytokine production [e.g., tumor necrosis factor (TNF) α, IL-1, IL-6, and IL-8], in a so-called "cytokine storm" (9, 10). Activated inflammatory cells migrate to affected organs and release secondary inflammatory mediators (1), including reactive oxygen species (ROS) and nitrogen species (11–15), resulting in oxidative stress (16, 17). Inflammatory cell migration to tissues and endothelial cell activation (18) lead to disseminated intravascular coagulation (19) aggravating organ damage. Increased levels of TNFα, IL-1, and IL-6 have been reported to occur more often in sepsis than SIRS (1, 20–22). Anti-inflammatory mediators (e.g., TGFβ and cytokine antagonists) are also present in plasma during SIRS, with soluble cytokine receptors often found at concentration higher than the respective cytokines (20, 23), hence compensatory anti-inflammatory responses [CAR (24)] may be activated during SIRS. Understanding the responses which occur in non-infective inflammation and sepsis and how they are regulated will aid development of diagnostics and therapeutics in these major inflammatory diseases.

MicroRNAs (miRNAs) are small (~23 nt) RNAs that function as post-transcriptional gene regulators (25, 26). By annealing to complementary sequences in the 3′ untranslated region (3′ UTR) of target mRNAs, they reduce expression of specific proteins by promoting target degradation or inhibition of translation (26). The role of miRNAs in SIRS remains unclear, although many inflammatory cytokines, mediators, and their regulators are miRNA targets (27). Approximately 100–200 miRNAs are found in human blood in health and disease (28–30). Previous studies (31, 32) suggest that exogenous miRNAs may be detected by TLRs (33, 34), particularly TLR7 (32), as potential DAMPs leading to inflammatory signals. We previously identified a pool of circulating inflammatory-relevant miRNA (CIR-miRNAs) that robustly distinguish sepsis from non-infective SIRS (35). We were the first to find that, in these conditions, CIR-miRNA levels inversely correlate with those of inflammatory cytokines (35), suggesting that they may be part of the CAR.

To extend these observations, we set out to determine how CIR-miRNAs reflect SIRS severity and whether they are likely to arise directly from immune activation. In this study, we investigated the correlation of plasma levels of CIR-miRNAs with disease severity and other inflammatory parameters. We found that SIRS severity significantly impacts on the levels of miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p. These CIR-miRNAs are unlikely to derive from red blood cell (RBC) damage consequent to hemolysis or coagulopathy. Instead their levels positively correlate with levels of the redox biomarker, Prdx-1, which is released by immune cells during inflammation (36, 37). Finally, we show that activated immune cells produce SIRS-associated miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p, *in vitro*. Our study has implications for furthering the understanding of the origin and role and trafficking of CIR-miRNAs in SIRS, sepsis, and potentially other inflammatory disease.

MATERIALS AND METHODS

Patients and Healthy Donors

The patient population used in this study was previously described in detail (35, 38). Briefly, patients comprised unselected adult admissions to a mixed medical/surgical intensive/high-dependency care unit (ICU/HDU) at an English acute hospital (Brighton and Sussex University Hospitals NHS Trust). Patients were categorized as having non-infective SIRS ($n = 44$) or sepsis ($n = 29$), following standard criteria (38). SIRS severity was defined as: severe (SOFA ≥ 6) and non-severe (SOFA ≤ 3); patients with intermediate SOFA scores of 4–5 were excluded. Only patients with abdominal sepsis were included in this study. Blood samples were collected within <6 h from admission and time of sample collection did not affect levels of CIR-miRNAs (35). Healthy donors were recruited at Brighton and Sussex Medical School (BSMS, University of Sussex, Falmer, Brighton, UK). Refer to Materials and Methods in Supplementary Material for further details, including details of any medication targeting inflammation that patients were taking at the time of admission.

Hemoglobin, Prdx-1, and Cytokine Analysis in Plasma Samples

Red blood cell lysis can bias miRNA content in plasma (39, 40). The concentration of free hemoglobin (Hb) was independently measured in patient plasma by the Harboe spectrophotometric method (41, 42) and hemolytic samples (43–45) were excluded, as carried out previously (35). Prdx-1 plasma levels were independently measured using an ELISA kit for human Prdx-1 (#ABIN418422) from Antibodies Online (Aachen, Germany). Plasma cytokines (IL-6, IL-8) and anti-/inflammatory mediators [pro-calcitonin (PCT), C-reactive protein (CRP), and soluble CD25 (sCD25)] were measured on Luminex LX200 and ELISA (sCD25) as previously described (38).

Human Samples and Primary Cell Cultures

Human peripheral blood mononuclear cells (PBMCs) from healthy donors were freshly isolated by centrifugation over Ficoll–Hypaque density gradient as previously described (46). Cells were washed in sterile PBS (ThermoFisherScientific) and counted with 0.1% Trypan Blue cell viability exclusion dye (Sigma). Thereafter, 20×10^6 viable PBMCs were resuspended in 10 ml (2×10^6 cells/ml) of complete media: RPMI containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from ThermoFisherScientific) and 10% of exosome-depleted, heat-deactivated fetal calf serum (System Biosciences). PBMCs were stimulated with the bacterial superantigen (SAg), streptococcal pyrogenic exotoxin K/L [SPE-K/L (47)] as described before (46),

in parallel to unstimulated control cultures (24-well plates, 1 ml/well). After 5 days, cells were harvested and assayed for viability with Trypan-blue (0.1%) (Figure S7 in Supplementary Material). Equal volumes of culture supernatants were then recovered, frozen at −80°C and total RNA was then extracted as described below.

RNA Extraction and MicroRNA Real-Time qPCR Array

Plasma and supernatant RNA was extracted using the miRCURY™ RNA isolation—biofluids kit (Exiqon, Denmark). After thawing on ice, an RNA spike-in template mixture was added to the samples. Eight milliliters of supernatant per sample was mixed with 2.4 ml of Lysis solution BF containing 16.67 µg/ml of MS2 bacteriophage RNA (UniSp6), prior to RNA purification, as specified by the manufacturer's instructions. Extracted total RNA was stored in a −80°C freezer. RNA was reverse transcribed (RT) and cDNA analyzed using the miRCURY LNA™ Universal RT miRNA PCR, Polyadenylation, and cDNA synthesis method. For plasma samples derived from patients (≥8 biological replicates), each microRNA was assayed by qPCR (miRNA Ready-to-Use PCR, Pick-&-Mix using ExiLENT SYBR® Green master mix) in two independent technical repeats, including negative controls (no-template from the RT reaction) using a LightCycler® 480 Real-Time PCR System (Roche). SAg stimulation experiments were performed as 10 independent biological replicates. Assays returning three crossing point (Cp) values less than the negative control and Cp < 37 were accepted. The stability values of candidate normalizers were assessed using the "NormFinder" software (48). Any qPCR data were normalized to the average Cp of internal normalizers [miR-320a and miR-486-5p (35)] in plasma samples and, in the case of culture supernatants, the Cp of normalizer spike-in (UniSp6); (delta Cp, dCp = normalizer Cp − assay Cp). Refer to the Materials and Methods in the Supplementary Material for further details.

Statistical Analyses

Datasets were analyzed using the GraphPad Prism 6 and/or IBM SPSS Statistics 22 software. The D'Agostino and Pearson omnibus and Shapiro–Wilk tests were used to test normal data distribution; data were considered normally distributed only if they passed both tests. If not normally distributed, medians with interquartile ranges (IQRs, rather than means and SD) are shown and Mann–Whitney *U* Test (rather than *t*-tests) was used to calculate *p* values in 2-group comparisons. Correlations between SOFA and APACHE II scores and plasma levels of CIR-miRNAs and inflammatory cytokines/mediators were evaluated using the Spearman rho (ρ) and significances of the correlations determined as indicated. Generally, $0.35 \leq \rho \leq 1$ or $-1 \leq \rho \leq -0.35$ were considered as moderate-to-strong correlation trends (49, 50). The confidence in the predictive value of each correlation was assessed by individual *p* values; in general, correlations trends were considered significant only if $p \leq 0.05$ (51). In addition, for the qPCR miRNA array dataset, a Benjamini–Hochberg multiple comparison correction (52) was used to control for the number of false positives, using false discovery rates of 15% (**Tables 1** and **2**; Tables S1 and S2 in Supplementary Material) and 5% (**Tables 3** and **4**) which correspond to a ~1/6 and 1/20 chance of false positives, respectively. Such correction excludes that the significance of correlation of any parameter (such as disease severity, free Hb and Prdx-1) with any of the 43 miRNA tests run in parallel was not simply due to the chance of multiple testing. Unless stated differently in figure legends, levels of significance were assigned as: $*p \leq 0.05$; $**p \leq 0.005$; and $***p \leq 0.0005$. MiRNA and cytokine, Hb, and Prdx-1 analyses were conducted blind to the clinical data.

RESULTS

Severity of Non-Infective SIRS Positively Correlates with the Levels of CIR-miRNAs

To determine whether CIR-miRNA levels are regulated by the severity of SIRS, Cp of single miRNAs were compared to the mean Cp of internal normalizers [as previously identified (35)] to give dCp values that increase with the abundancy of specific miRNAs. Severity of disease, according to the SOFA score, was then correlated with dCp in non-infective SIRS patients. Thirteen CIR-miRNAs showed generally positive trends correlating with SIRS severity (**Table 1**). Significantly, 8 miRNAs showed a positive correlation with the disease severity (**Table 1**, black bold), with miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p further passing the Benjamini–Hochberg correction for multiple comparisons (52) (**Figure 1A**). We further determined whether CIR-miRNA levels correlate with disease severity in sepsis patients (**Figure 1B**). None of the CIR-miRNAs correlating with

TABLE 1 | Correlations of circulating inflammatory-relevant microRNAs (CIR-miRNAs) with severity of systemic inflammatory response syndrome (SIRS) as detected by sequential organ failure assessment (SOFA).

MicroRNA species[a]	SOFA Spearman correlation (ρ)[b]	Correlation significance *p*[c]	Benjamini–Hochberg (BH) rank	BH critical value (FDR 15%)
miR-378a-3p	0.491	0.00084	1*	0.00349
miR-30a-5p	0.433	0.00370	2*	0.00698
miR-30d-5p	0.412	0.00609	3*	0.01047
miR-192-5p	0.378	0.01253	4*	0.01395
miR-122-5p	0.359	0.01799	5	0.01744
miR-101-3p	0.351	0.02092	6	0.02093
miR-21-5p	0.336	0.02769	7	0.02442
miR-148a-3p	0.309	0.04357	8	0.02791
miR-10b-5p	0.283	0.06601	9	0.03140
miR-532-5p	0.285	0.07054	10	0.03488
miR-22-3p	0.268	0.08248	11	0.03837
miR-143-3p	0.266	0.08468	12	0.04186
miR-23a-3p	0.255	0.09926	13	0.04535
miR-320a	0.251	0.10514	14	0.04884
miR-486-5p	−0.251	0.10514	15	0.05233

The top 15 performing miRNAs are shown. Significant correlations with disease severity are highlighted in bold black (SIRS) or red (sepsis). Refer to Table S4 in Supplementary Material for the full dataset.

[a]Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

[b]Blue- and violet-shadowed cells indicate miRNAs that returned positive and negative correlations ($\rho \geq 0.2$ or $\rho \leq -0.2$), respectively.

[c]Brown- and red-shadowed cells indicate miRNAs that returned significant correlations ($p \leq 0.05$) and additionally passed the correction for multiple comparisons, respectively.

TABLE 2 | Correlations of circulating inflammatory-relevant microRNAs (CIR-miRNAs) with severity of sepsis as detected by sequential organ failure assessment (SOFA).

MicroRNA species[a]	SOFA Spearman correlation (ρ)[b]	Correlation significance *p*[c]	Benjamini–Hochberg (BH) rank	BH critical value (FDR 15%)
miR-22-3p	0.447	0.01942	1	0.00349
miR-191-5p	−0.432	0.02436	2	0.00698
miR-375	0.590	0.02633	3	0.01047
miR-151a-3p	−0.354	0.06990	4	0.01395
miR-146a-5p	−0.333	0.08966	5	0.01744
miR-103a-3p	−0.299	0.12992	6	0.02093
miR-378a-3p	0.282	0.16305	7	0.02442
let7b-5p	−0.269	0.17521	8	0.02791
miR-122-5p	0.262	0.19539	9	0.03140
let7i-5p	−0.252	0.20556	10	0.03488
miR-192-5p	0.228	0.25220	11	0.03837
miR-23a-3p	−0.224	0.26149	12	0.04186
miR-92b-3p	−0.304	0.27048	13	0.04535
miR-10a-5p	−0.275	0.28483	14	0.04884
miR-28-3p	−0.192	0.34735	15	0.05233

The top 15 performing miRNAs are shown. Significant correlations with disease severity are highlighted in bold black (SIRS) or red (sepsis). Refer to Table S5 in Supplementary Material for the full dataset.

[a]Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

[b]Blue- and violet-shadowed cells indicate miRNAs that returned positive and negative correlations ($\rho \geq 0.2$ or $\rho \leq -0.2$), respectively.

[c]Brown- and red-shadowed cells indicate miRNAs that returned significant correlations ($p \leq 0.05$) and additionally passed the correction for multiple comparisons, respectively.

TABLE 3 | Correlations of circulating inflammatory-relevant microRNAs (CIR-miRNAs) with free hemoglobin levels in non-infective systemic inflammatory response syndrome (SIRS).

MicroRNA species[a]	Hb Spearman correlation (ρ)[b]	Correlation significance *p*[c]	Benjamini Hochberg (BH) rank	BH critical value (FDR 5%)
miR-21-5p	−0.6308	5.78E-06	1	0.00116
miR-10b-5p	−0.5297693	2.59E-04	2	0.00233
miR-320a	−0.5080983	0.000504483	3	0.00349
miR-486-5p	0.5080983	0.000504483	4	0.00465
miR-375	−0.5562014	0.000521672	5	0.00581
miR-451a	0.5024351	0.000596273	6	0.00698
miR-30e-3p	−0.4792892	0.001521716	7	0.00814
miR-30a-5p	−0.4631706	0.001761638	8	0.00930
miR-92b-3p	−0.4986164	0.001966918	9	0.01047
miR-22-3p	−0.4430098	0.002929346	10	0.01163
miR-122-5p	−0.4416506	0.00302822	11	0.01279
miR-28-3p	−0.4402159	0.003135754	12	0.01395
miR-146a-5p	−0.4328161	0.00374532	13	0.01512
miR-192-5p	−0.4219428	0.004828522	14	0.01628
miR-101-3p	−0.4151471	0.005636129	15	0.01744

The top 15 performing miRNAs are shown. miRNAs significantly correlating with disease severity (as by sequential organ failure assessment) are highlighted in bold black (SIRS) or red (sepsis). Refer to Table S6 in Supplementary Material for the full dataset.

[a]Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

[b]Blue- and violet-shadowed cells indicate miRNAs that returned positive and negative correlations ($\rho \geq 0.2$ or $\rho \leq -0.2$), respectively.

[c]Brown- and red-shadowed cells indicate miRNAs that returned significant correlations ($p \leq 0.05$) and additionally passed the correction for multiple comparisons, respectively.

TABLE 4 | Correlations of circulating inflammatory-relevant microRNAs (CIR-miRNAs) with peroxiredoxin-1 levels in non-infective systemic inflammatory response syndrome (SIRS).

MicroRNA species[a]	Prdx1 Spearman correlation (ρ)[b]	Correlation significance *p*[c]	Benjamini Hochberg (BH) rank	BH critical value (FDR 5%)
miR-192-5p	0.590909	3.02E-05	1	0.00116
miR-30a-5p	0.548626	1.39E-04	2	0.00233
miR-22-3p	0.536243	2.10E-04	3	0.00349
miR-122-5p	0.505436	0.000545917	4	0.00465
miR-148a-3p	0.485956	0.00095437	5	0.00581
miR-378a-3p	0.485503	0.000966472	6	0.00698
miR-532-5p	0.465157	0.002181351	7	0.00814
miR-320a	0.453186	0.002274844	8	0.00930
miR-486-5p	−0.45319	0.002274844	9	0.01047
miR-21-5p	0.437632	0.003337895	10	0.01163
miR-101-3p	0.413168	0.005892309	11	0.01279
miR-423-5p	0.340985	0.02524517	12	0.01395
miR-30d-5p	0.318571	0.03733837	13	0.01512
miR-375	0.342017	0.04432343	14	0.01628
miR-10b-5p	0.266687	0.08386032	15	0.01744

The top 15 performing miRNAs are shown. miRNAs significantly correlating with disease severity (as by sequential organ failure assessment) are highlighted in bold black (SIRS) or red (sepsis). Refer to Table S7 in Supplementary Material for the full dataset.

[a]Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

[b]Blue- and violet-shadowed cells indicate miRNAs that returned positive and negative correlations ($\rho \geq 0.2$ or $\rho \leq -0.2$), respectively.

[c]Brown- and red-shadowed cells indicate miRNAs that returned significant correlations ($p \leq 0.05$) and additionally passed the correction for multiple comparisons, respectively.

disease severity in SIRS showed similar significant trends in sepsis (**Figure 1B**; **Table 2**). Although miR-22-3p, miR-375, miR-122-5p, miR-192-5p, and miR-378a-3p maintained modestly positive non-significant trends, multiple CIR-miRNAs showed generally inverse trends with sepsis severity (**Table 2**).

In comparison to pro-inflammatory cytokines (IL-8 and IL-6) and soluble mediators of inflammation and stress such as CRP, PCT, free hemoglobin (Hb), and Prdx-1, CIR-miRNAs showed more robust correlations with disease severity (compare **Figures 1A** and **2A**). None of these inflammatory mediators showed a significant correlation with SIRS severity, although some non-significant trends were apparent (**Figure 2A**). While levels IL-8, IL-6, CRP, and PCT increased in sepsis compared to non-infective SIRS, only IL-6 positively correlated with sepsis severity (**Figure 2B**).

Similar analyses were performed using the APACHE II in alternative to the SOFA severity score. In agreement with our previous analysis, CIR-miRNA levels positively and significantly correlated with the APACHE II score in non-infective SIRS (Figure S1A and Table S1 in Supplementary Material), but not in sepsis (Figure S1B and Table S2 in Supplementary Material). In addition, CIR-miRNAs in comparison to IL-6, CRP, PCT, and free Hb, generally showed more robust correlations with the APACHE II score, while IL-8 and Prdx-1 had significant positive correlation with the APACHE II score in non-infective SIRS (Figure S2A in Supplementary Material), but not in sepsis (Figure S2B in Supplementary Material).

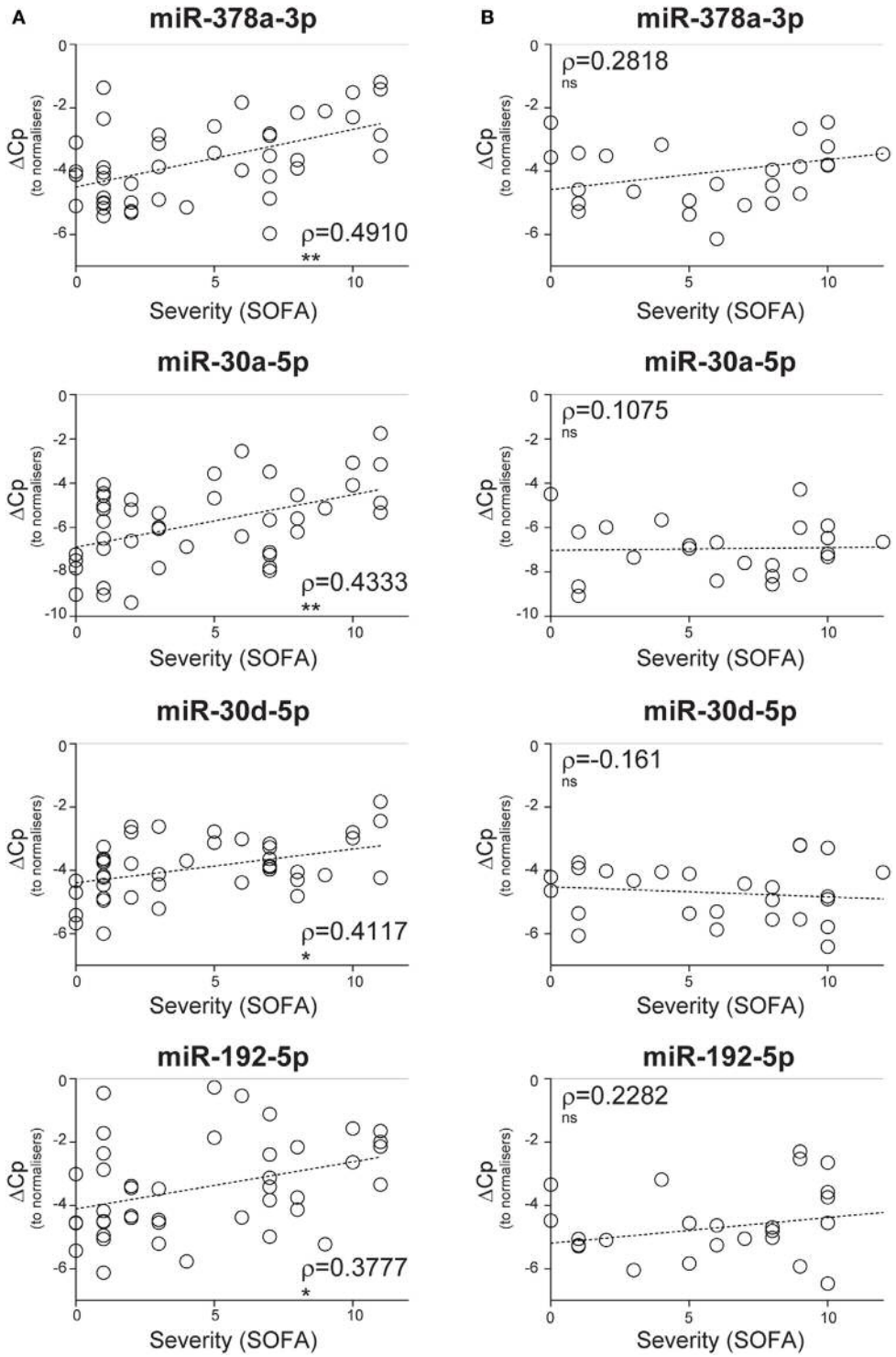

FIGURE 1 | Circulating inflammatory-relevant microRNA levels correlate with the severity of non-infective systemic inflammatory response syndrome (SIRS). In miRNA qPCR arrays, within each patient's specimen, crossing point (Cp) of a single miRNA is compared to the mean Cp of 2 normalizers (miR-486-5p and miR-320a) to give delta Cp (dCp). dCp of non-infective SIRS **(A)** and sepsis **(B)** patients were analyzed in correlation analyses with disease severity, as determined by the sequential organ failure assessment (SOFA) score. **(A)** Non-parametric correlation of SOFA scores with the plasma levels of miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p in non-infective SIRS patients ($n = 43$ for all miRNAs). **(B)** Non-parametric correlation of SOFA scores with the plasma levels of miR-378a-3p ($n = 26$), miR-30a-5p ($n = 24$), miR-30d-5p ($n = 27$), and miR-192-5p ($n = 27$) in infective SIRS (sepsis) patients. Each symbol represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*$p \leq 0.05$; **$p \leq 0.005$; and ***$p \leq 0.0005$ or ns, non-significant).

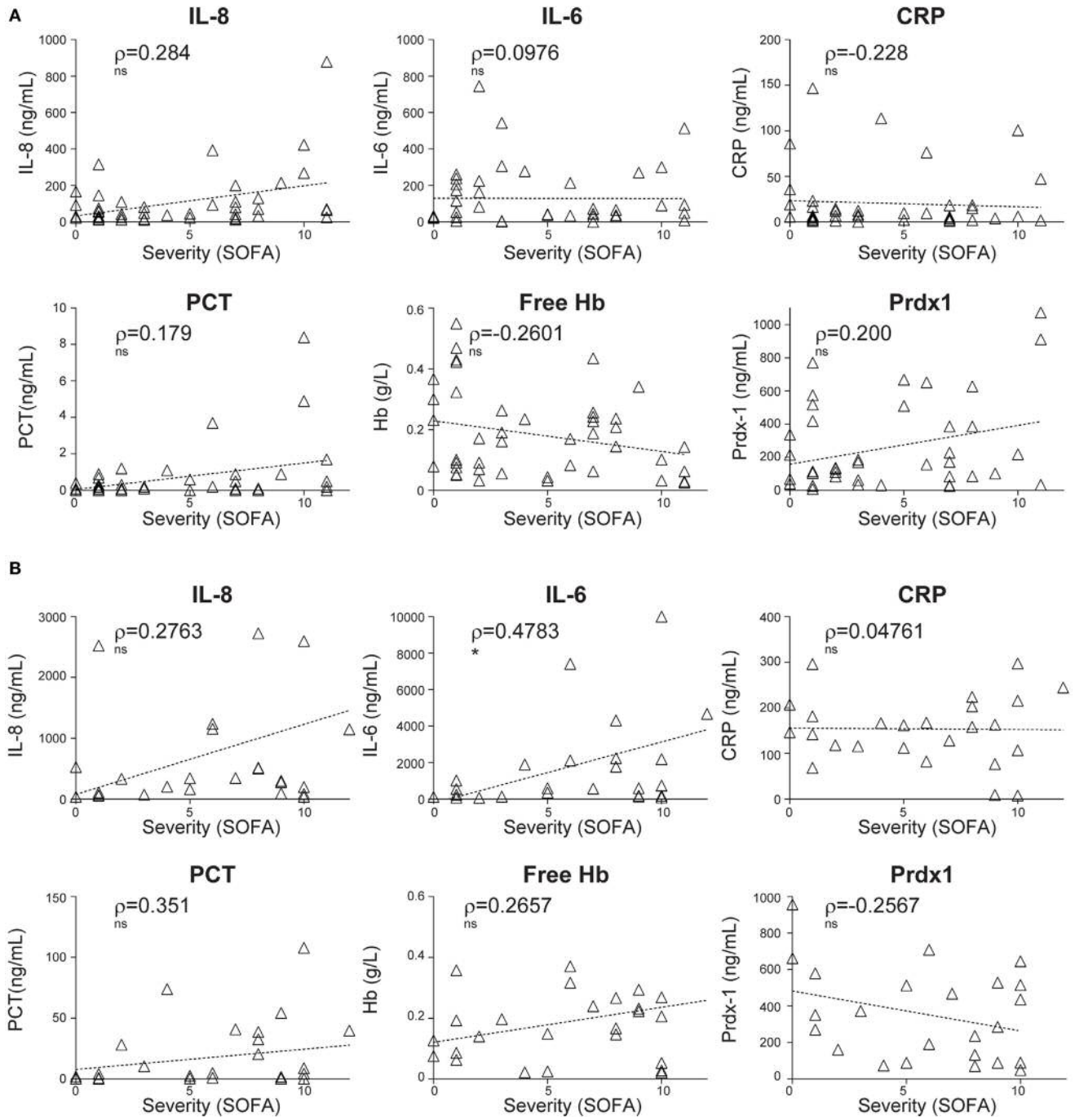

FIGURE 2 | Correlations of plasma levels of inflammatory cytokines and stress mediators with the severity of non-infective systemic inflammatory response syndrome and sepsis. The levels of inflammatory cytokines (interleukin-IL-8 and IL-6) and stress mediators: C-reactive protein (CRP), pro-calcitonin (PCT), free hemoglobin (Hb), and peroxiredoxin-1 (Prdx-1) were measured by ELISA in the plasma of non-infective SIRS **(A)** and sepsis **(B)** patients. Thereafter, the correlation with the severity of disease, as determined by the sequential organ failure assessment (SOFA) score was investigated. **(A)** Non-parametric correlation of SOFA scores with the plasma levels of IL-8 (n = 43), IL-6 (n = 43), CRP (n = 43), PCT (n = 42), free Hb (n = 43), and Prdx-1 (n = 41) in non-infective SIRS patients. **(B)** Non-parametric correlation of SOFA scores with the plasma levels of IL-8 (n = 26), IL-6 (n = 27), CRP (n = 27), PCT (n = 27), free Hb (n = 27), and Prdx-1 (n = 24) in sepsis patients. Each triangle represents an individual patient. Correlation trends are shown with the linear regression model, including Spearman rho (ρ) and the significances of the correlations (* $p \leq 0.05$; ** $p \leq 0.005$; and *** $p \leq 0.0005$ or ns, non-significant).

CIR-miRNAs Significantly Discriminate Severe from Non-Severe SIRS

Consistent with a steady accumulation of CIR-miRNAs in more severe disease, dCp values were higher in severe rather than non-severe SIRS (**Figure 3A**) for the most significantly affected CIR-miRNAs. Hence, miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p distinguished non-severe from severe SIRS patients (**Figure 3A**). Also miR-122-5p, miR-101-3p, miR-21-5p, miR-148a-3p, and miR-532-5p (which all had non-significant positive trends to increase with SIRS severity, **Table 1**) significantly discriminated severe from non-severe SIRS (Figure S3 in Supplementary Material). By contrast, none of the inflammatory cytokines and stress mediators we measured in our cohort discriminated SIRS severity groups, including IL-8 (**Figure 3B**) and IL-6 (Figure S3B in Supplementary Material).

SIRS-Relevant CIR-miRNAs Are Unlikely to Derive from RBCs

We next investigated where the CIR-miRNAs regulated by SIRS severity may originate. We reasoned that if they derived from RBCs that contain multiple miRNAs (released in the blood after physiological hemolysis or coagulopathy), then CIR-miRNA levels would positively correlate with the amount of free Hb. Instead, around 95% of the analyzed CIR-miRNAs (40/43) showed inverse correlation trends with free Hb (**Tables 3**; Table S6 in Supplementary Material). Many CIR-miRNAs, including miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p (**Figure 4A**) and miR-101-3p, miR-21-5p, miR-22-3p, miR-423-5p, and miR-122-5p (Figure S4 in Supplementary Material) significantly inversely correlated with free Hb levels even after a stringent Benjamini–Hochberg correction (52), supporting that CIR-miRNAs relevant in severe SIRS do not derive from RBCs. By contrast, free Hb levels positively correlated with miRNAs abundant in RBCs (29), miR-486-5p and miR-451a (**Figure 4B**; **Table 3**), consistent with a RBC origin.

CIR-miRNAs Positively Correlate with Redox Biomarker Prdx-1

We sought to investigate whether SIRS-relevant CIR-miRNAs derive from immune cells (i.e., white blood cells). To do this, we used Prdx-1, a marker that we had previously found to be released by immune cells after inflammatory stimuli (36, 37). Consistently, in the entire SIRS cohort, Prdx-1 levels correlated with markers of immune activation (IL-8 and sCD25), rather than free Hb (Figure S5 in Supplementary Material), demonstrating that Prdx-1 did not increase due to RBC hemolysis. We then analyzed the correlation of Prdx-1 with CIR-miRNA dCp (**Figure 5**; **Table 4**). Around 40% (18/43) of CIR-miRNAs showed moderate-to-strong correlation trends with Prdx-1 levels, with a prevalence of positive relationships (**Tables 4**; Table S7 in Supplementary Material). Levels of 10 CIR-miRNAs, including miR-378a-3p, miR-30a-5p, and miR-192-5p (**Figure 5A**) and miR-101-3p, miR-21-5p, miR-22-3p, and miR-122-5p (Figure S6 in Supplementary Material) significantly positively correlated with those of Prdx-1, suggesting that the amounts of CIR-miRNAs modulated in non-infective SIRS mirror those of this inflammatory biomarker. By contrast, miRNAs abundant in RBCs, miR-486-5p (**Figure 5B**) and miR-451a (Table S7 in Supplementary Material), did not show positive correlation with Prdx-1.

Stimulated Immune Cells Produce CIR-miRNAs Affected by Severity of SIRS

To determine whether immune cells could produce miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p, PBMCs of 10 healthy donors were stimulated *in vitro* with bacterial SAg, known to drive massive inflammatory cell activation. After 5 days, viability of the cell cultures was determined using the Trypan-blue dye exclusion assay (Figure S7 in Supplementary Material). Relative to a normalizer spike-in, we found increased amounts of miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p in culture supernatants of stimulated PBMCs compared to unstimulated controls (**Figure 6A**). The increase of miRNAs in the supernatants varied across the miRNA species between 10- and 100-fold (**Figure 6B**), with the notable exception of miR-122-5p that was not significantly increased (**Figures 6A,B**). In cultures derived from four donors, levels of miR-10b-5p, which did not discriminate non-severe from severe SIRS (Figure S3 in Supplementary Material), did not increase upon activation (**Figures 6A,B**).

DISCUSSION

We report here that, during non-infective SIRS, the blood levels of CIR-miRNAs increase in parallel with the severity of inflammation. Levels of CIR-miRNAs do not correlate with those of free Hb, indicating that they do not derive from RBCs during SIRS. Instead, CIR-miRNAs positively correlate with levels of the inflammatory mediator and redox enzyme, Prdx-1 which is released by immune cells in inflammation. Consistently miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p affected by severity of SIRS are produced by immune cells upon activation. As CIR-miRNAs are increasingly proposed as biomarkers for sepsis, cancer, and other disease (35, 53–55), their inflammatory cell origin should be considered in future research.

In our study, CIR-miRNAs distinguish non-severe from severe SIRS better than inflammatory cytokines and mediators. Inflammatory cytokines are thought to be released during SIRS as part of the cytokine storm, during MODS (1). As in our current study, plasma levels of cytokines have not previously been found to reliably increase in severe SIRS (1), perhaps due to low cytokine concentration and short half-life in samples (56, 57). By contrast, regulators of inflammatory mediators are abundant in the blood of SIRS patients (20), as we found to be the case for the CIR-miRNAs affected by SIRS severity (**Figure 3**; Figure S3 in Supplementary Material). In addition, it should be noted that blood levels of miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p were regulated by disease severity, irrespectively

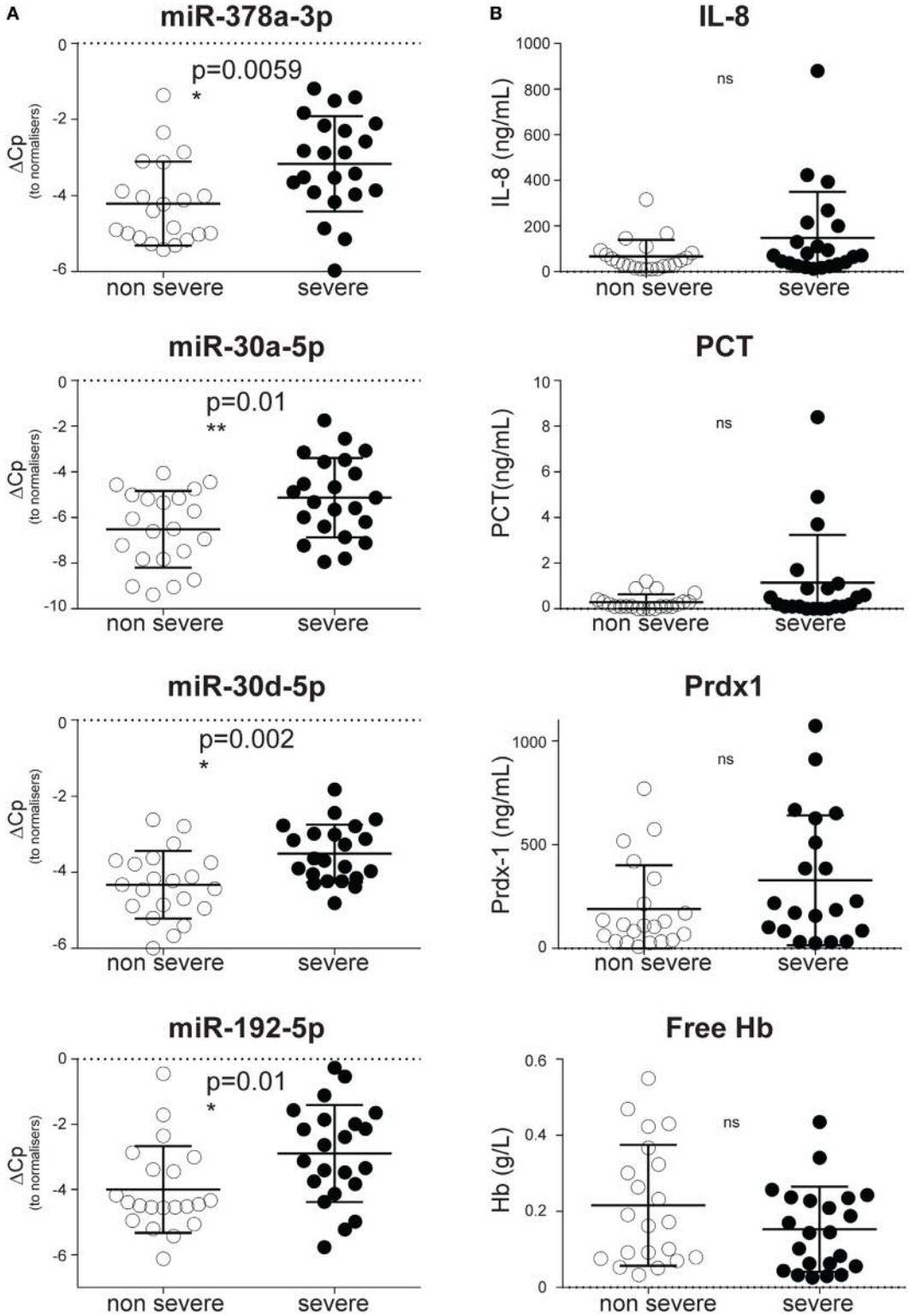

FIGURE 3 | Circulating inflammatory-relevant microRNA biomarkers discriminate the severity of systemic inflammatory response syndrome (SIRS) better than inflammatory and stress mediators. In miRNA qPCR arrays, within each patient's specimen, crossing point (Cp) of individual miRNAs were normalized as in **Figure 1** and analyzed in patients with non-severe (open circles) and severe (filled circles) non-infective SIRS. Each symbol represents an individual patient. **(A)** Dot plots show delta Cp values for miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p in non-severe ($n = 21$) and severe ($n = 22$) non-infective SIRS patients, together with the level of significance. **(B)** Dot plots show concentration of IL-8, pro-calcitonin (PCT), peroxiredoxin-1 (Prdx-1) and free hemoglobin (Hb) in non-severe ($n = 21$) and severe ($n = 22$, apart from Prdx-1 in which $n = 20$) non-infectious SIRS patients, together with the level of significance.

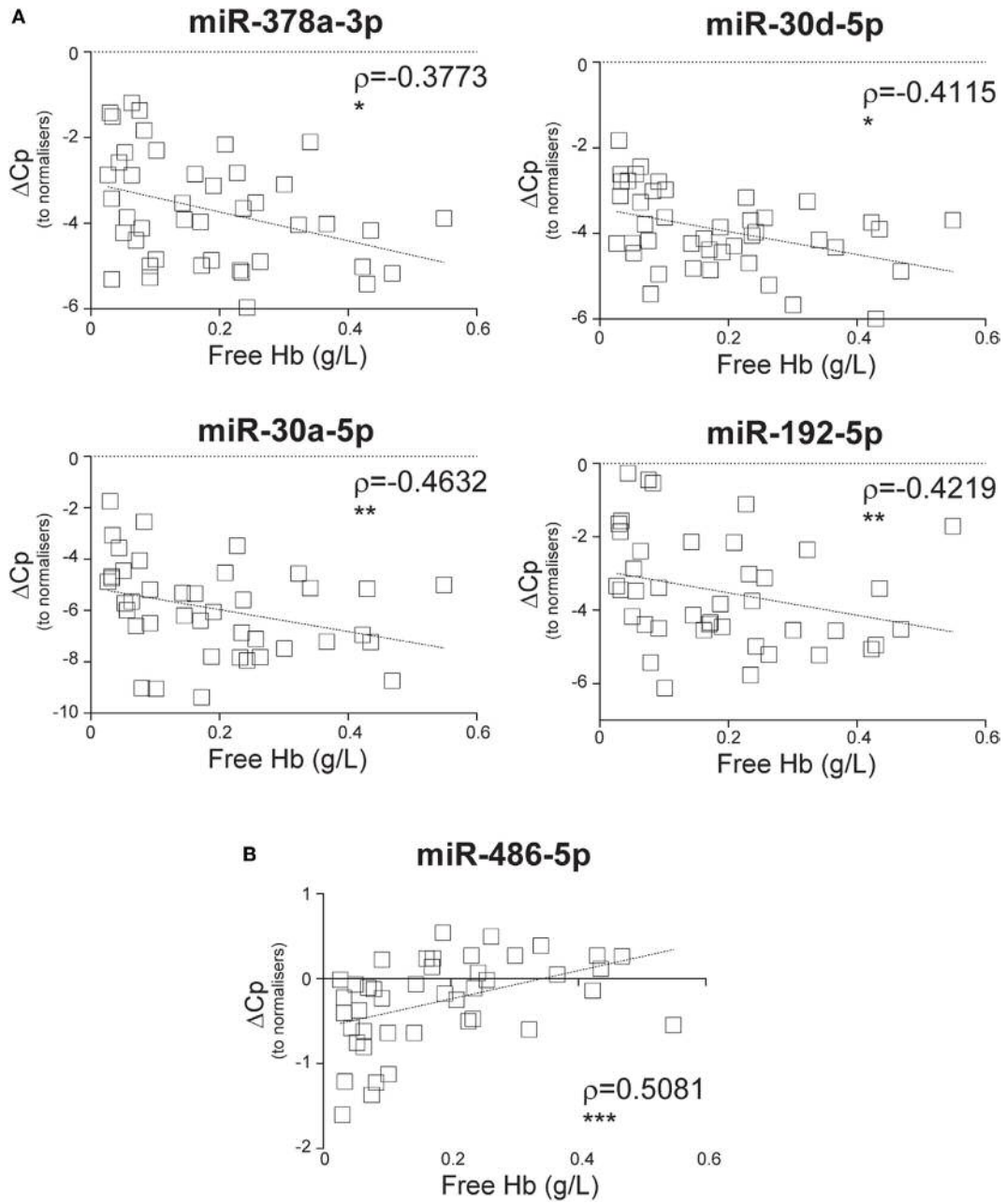

FIGURE 4 | Circulating inflammatory-relevant microRNA levels inversely correlate with markers of red-blood cell lysis. In miRNA qPCR arrays, within each patient's specimen, crossing point (Cp) of individual miRNAs (open squares) were normalized as in **Figure 1** and analyzed in correlation with levels of free Hb, which is derived from the lysis of red blood cells. Each square represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*$p \leq 0.05$; **$p \leq 0.005$; and ***$p \leq 0.0005$ or ns, non-significant) for **(A)** miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p and **(B)** miR-486-5p in non-infective systemic inflammatory response syndrome patients ($n = 43$).

from whether patients were taking anti-inflammatory drugs (at the time of admission) (Figure S8 and Table S8 in Supplementary Material).

CIR-miRNAs may act as regulators of inflammation (35) by targeting the 3′UTR of mRNAs encoding pro-inflammatory cytokines/mediators (58–61). Many miRNAs are involved in inflammation/immune function (27), including miR-378 (62, 63), miR-30a/d (64, 65), miR-192 (64–68), and others found in this study (69). Interestingly, the more severe SIRS is, the more CIR-miRNAs are released, potentially counteracting systemic inflammation as part of the CAR (24). Significantly, direct or indirect targets of miR-378, miR-30a/d, and miR-192 include, among

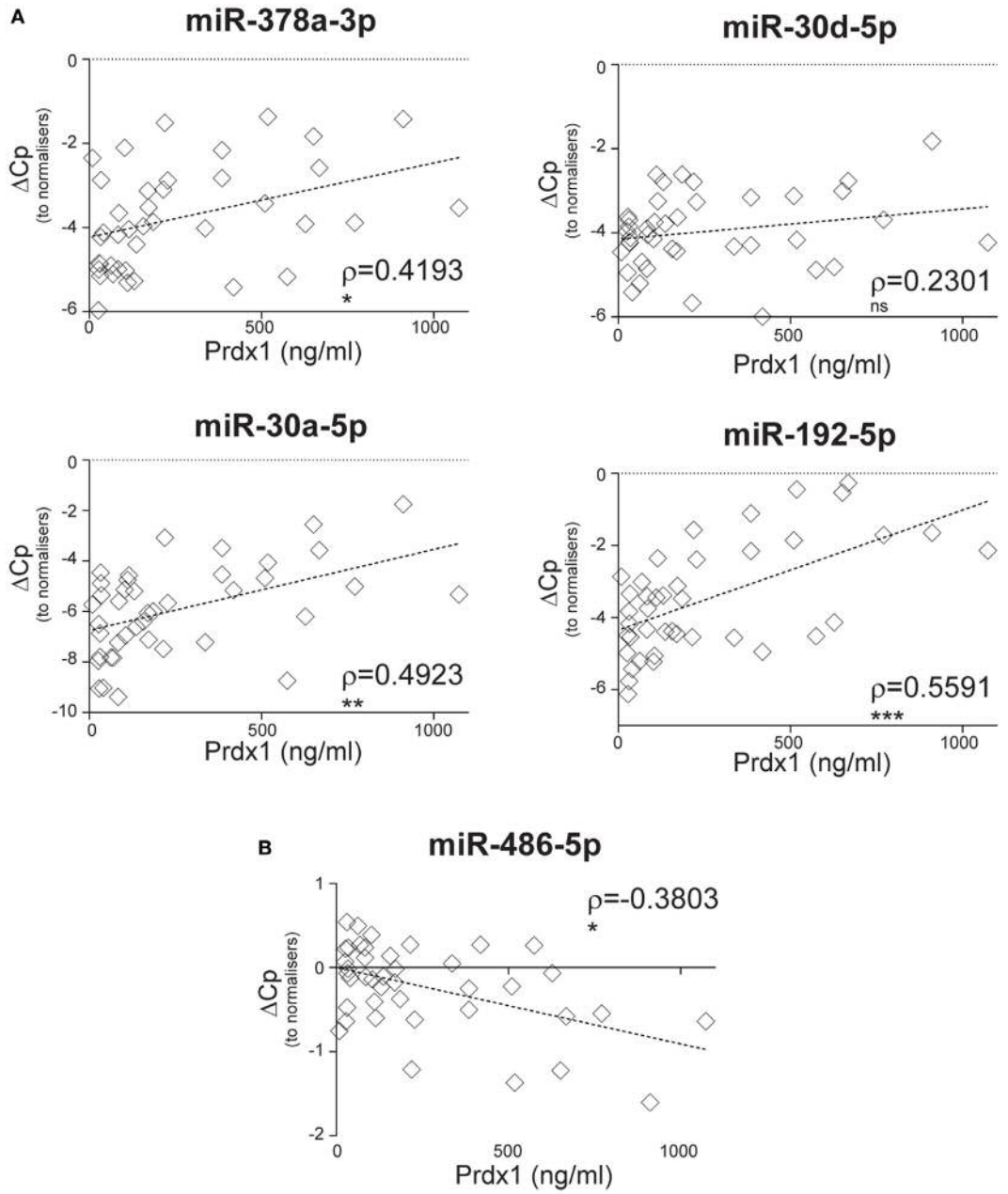

FIGURE 5 | Circulating inflammatory-relevant microRNA levels positively correlate with markers of immunological stress, peroxiredoxin-1 (Prdx1). In miRNA qPCR arrays, within each patient's specimen, crossing point (Cp) of individual microRNAs (miRNAs) (open rhombi) were normalized as in **Figure 1** and analyzed in correlation with levels of plasma inflammatory stress marker, Prdx-1. Each symbol represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*$p \leq 0.05$; **$p \leq 0.005$; and ***$p \leq 0.0005$ or ns, non-significant) for **(A)** miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p, and **(B)** miR-486-5p in non-infective systemic inflammatory response syndrome patients ($n = 41$).

other genes, IL-1A (70), IL-1R (71), IL-17 (72), IL-17RA, and IL-17RE (73). In contrast, CIR-miRNA levels did not correlate with sepsis severity (compare **Tables 1** and **2**; Tables S1 and S2 in Supplementary Material), suggesting that inflammatory pathways leading to CIR-miRNA accumulation in blood are dysregulated in sepsis. Consequently, upon sepsis progression, the negative regulation exerted by CIR-miRNAs may be restrained, thus boosting immunopathology. Inflammatory cytokine (IL-1, IL-6, etc.) levels increased consistently much more in sepsis than in SIRS (35), therefore inflammatory mRNAs may act as a "sponge" to sequestrate miRNAs in sepsis, but not in other trauma including SIRS (74). Although miR-30a was recently associated with

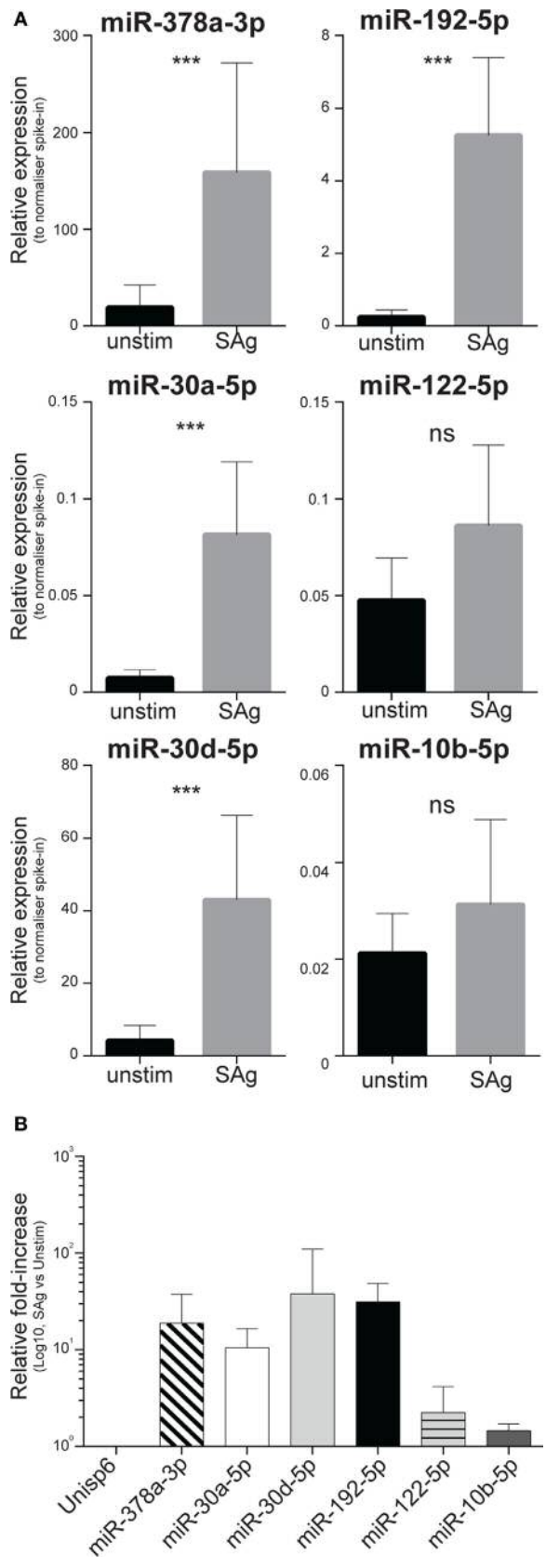

FIGURE 6 | Continued

FIGURE 6 | Levels of circulating inflammatory-relevant microRNAs affected by the severity of systemic inflammatory response syndrome (SIRS) are increased in supernatants of blood-derived immune cell cultures. PBMCs derived from 10 individuals were freshly purified from blood and equal cell numbers were then incubated in complete media in the presence of exosome-free bovine serum, strictly at the concentration of 2×10^6 cells/ml, in replicate wells. Half of the cultures were stimulated with the SPE-KL bacterial superantigen (SAg) from 5 days, in comparison to unstimulated controls (unstim). On day 5, cells were harvested and equal volumes of supernatants were recovered after two sequential spins prior to RNA extraction as detailed in the Section "Materials and Methods." The presence of microRNAs regulated (miR-378a-3p, miR-30a-5p, miR-30d-5p, miR-192-5p, and miR-122-5p) or unaffected (miR-10b-5p) by the severity of non-infective SIRS was assessed in Q-PCR array in multiple biological replicates. **(A)** Within each donor's specimen, crossing point (Cp) of a single miRNA is compared to the Cp of a spike-in RNA added to supernatants just prior to the RNA-purification to give delta Cp (dCp). dCp were then linearized to give the relative expression of individual miRNAs in supernatants from unstimulated (black bars; n = 10, 7, 5, 7, 10, 4, respectively, for miR-378a-3p, miR-30a-5p, miR-30d-5p, miR-192-5p, miR-122-5p, and miR-10b-5p) compared to SAg-activated cells (gray bars; n = 10, 10, 8, 9, 10, and 9, respectively, for miR-378a-3p, miR-30a-5p, miR-30d-5p, miR-192-5p, miR-122-5p, and miR-10b-5p). **(B)** Average miRNA fold-induction in 4–10 individuals were calculated as the average ratio of miRNA levels detected in supernatants from SAg-activated cells compared to unstimulated cultures.

induction of myeloid derived suppressor cells in cancer (75), the question of whether CIR-miRNAs are anti-inflammatory and ameliorate sepsis outcome remains to be further investigated.

Alternatively, CIR-miRNAs may act as modulators of inflammation *via* indirect effects (i.e., by not targeting directly inflammatory cytokine expression). For instance, at least in mice, miR-378 is known to target phosphoinositide 3 kinase (PI3K) expression in liver cells, affecting liver metabolism with systemic effects which may be relevant during systemic inflammatory disease and MODS (76). Furthermore, PI3K pathway is also crucially regulated during immune cell proliferation, differentiation, and apoptosis (77), all of which may affect immune cell function during SIRS. Similarly, miR-30a has been shown to target the expression of Blimp-1 (78) (an important differentiation factor in immune cells) and various members of the calcineurin signaling pathway, including NFATc3 (79) at least in podocytes and cardiomyocytes, which might have implications for MODS and inflammation. Finally, miR-192 has been shown to suppress cell-proliferation pathways in myeloma (80) and other leukemic cells (81). However for all abovementioned cases, whether the same targets are also regulated in normal immune cells remains so far elusive.

Altogether, our results have implications for clinical practice and future therapeutic interventions. In particular in ICU/HDU, using miRNAs to distinguish non-infective SIRS from sepsis and severe from non-severe SIRS could help guide patient management, for example, triaging patients based on severity, informing decisions based on prognosis, and helping target therapy including the need for and choice of antibiotics. In future, miRNAs may become markers and/or targets for novel immunotherapy for acute inflammatory conditions, potentially providing a non-antibiotic alternative intervention.

The origin of circulating miRNAs is unclear. RBCs contain miRNAs that they release upon hemolysis (39, 40). To exclude

artifacts from sample processing, we removed hemolytic samples and used plasma rather than serum, as the latter contains more Hb (and RBC miRNAs) due to coagulation damage (82, 83). Coagulopathy may drive pathophysiological levels of hemolysis and release of CIR-miRNAs, as seen for miR-486-5p and miR-451a [abundant in RBCs (29)]. Although free Hb levels did not significantly increase with SIRS severity, levels of multiple CIR-miRNAs inversely correlate with free Hb, indicating that these do not derive from RBCs in SIRS as in sepsis.

Our data suggest that CIR-miRNAs affected in SIRS may derive from inflammatory cells activated during disease. In particular, CIR-miRNAs may be released in the blood in association with inflammation-induced oxidative stress, as suggested by the positive correlation between Prdx-1 and multiple CIR-miRNAs. Prdx-1 is an anti-oxidant enzyme that mediates the elimination of H_2O_2 and can be secreted as a dimer by macrophages, upon TLR triggering (36, 37). Its expression is regulated mainly by the transcription factor Nrf2, activated by ROS and various other reactive, electrophilic species (84). Its induction may be an indicator of a protective response to oxidative stress (85, 86). Because Prdx-1 is associated with immune cells activation (37), we asked whether SIRS-relevant CIR-miRNAs were derived from circulating immune cells upon activation. We found that miRNAs affected by SIRS severity were indeed increased >10-fold in supernatants of PBMC cultures after activation with bacterial SAg that drove ~1.5-fold cell-expansion. Thus, sustained immune cell activation may explain the massive release of miRNAs regulated during severe SIRS. Macrophages/monocytes, T and NK cells could be possible candidates for the release of CIR-miRNAs.

Of note, activated PBMCs did not produce significant increase in the supernatant levels of miR-10b-5p, which was not affected by SIRS severity (Figure S3 in Supplementary Material). Unlike other miRNAs, the modest (<2-fold) increase of miR-122-5p upon SAg stimulation may be explained simply by cell proliferation, suggesting that PBMCs contribute little to miR-122-5p blood levels. As hepatocytes highly express miR-122 and increased blood levels of miR-122 were previously associated with liver pathology (87), epithelial cell release of miR-122 and potentially other CIR-miRNAs during SIRS should be further investigated in future.

In conclusion, our work opens up a number of questions about CIR-miRNAs for future investigation. Currently, the exact function of circulating miRNAs in inflammatory conditions remains unknown. CIR-miRNAs released by cells in the body after inflammatory damage may act as DAMPs that bind to TLR ligands (33), a property that is shared by Prdx-1 (86). For instance, small RNAs contained in exosomes can be phagocytosed in myeloid-origin, antigen-presenting like cells and trigger TLR7 signaling, *in vitro* (31). In this context, miRNAs may act as pro-inflammatory DAMPs to drive inflammatory cytokines (including IL-6) production downstream of intracellular TLRs (31). However, we previously found that pro-inflammatory cytokine levels inversely correlate with blood levels of CIR-miRNAs, suggesting that CIR-miRNAs may negatively regulate inflammation *in vivo* (35). In mice, regulatory T cells (Tregs) have been shown to dampen the activity of conventional T cells by miRNA transfer (88). Yet, it is unclear whether miRNAs from Tregs can exert suppression at distance. Furthermore, beyond targeting cytokine and inflammatory mediator expression, CIR-miRNAs may regulate inflammation also *via* indirect mechanisms, or a complex combination of direct and indirect mechanisms, by acting in concert on distinct targets, in multiple cell types, and in different organs. The dynamic contribution of immune cells to blood miRNAs has been frequently overlooked, despite inflammation clearly affecting them. Thus, while differences in miRNA transfer may exist *in vivo* compared to *in vitro*, future research is needed to clarify the role of CIR-miRNAs in immunopathological and homeostatic conditions. In particular, more research is needed to address which immune cells specifically produce CIR-miRNAs to be released in the blood, including when and how. Also the mode of trafficking and the cellular and molecular targets of CIR-miRNAs need to be identified yet. In future, such research may allow to harness CIR-miRNAs as immunomodulatory drugs useful for therapeutic purposes in inflammatory conditions as in other diseases.

ETHICS STATEMENT

Written informed consent or consultee approval to enroll was secured for all study participants (patients and healthy donors). This study was approved by the North Wales Research Ethics Committee (Central and East, reference 10/WNo03/19) and the BSMS Research Governance and Ethics Committee (reference: 13/182/LLE) and carried out in accordance with the approved guidelines. All data were anonymized.

AUTHOR CONTRIBUTIONS

SC and MM performed experiments and analyzed the data; SC, PG, SN, FK, and ML conceived and designed experiments; SC, SN, and ML wrote the first manuscript draft. All authors critically contributed to the final version of the manuscript.

ACKNOWLEDGMENTS

We thank staff and patients who participated in the clinical study. We thank Dr. Helen Stewart for reading the manuscript and other BSMS members at the University of Sussex for participating in useful discussions. We thank Dr. Antonio Sorrentino and Dr. Michael Thorsen (Exiqon), for assistance.

FUNDING

SC is supported by a Brighton and Sussex Medical School Internal Fellowship (University of Sussex). This work was funded by the University of Sussex Research Development Fund Round 3 (RDF 3-021). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01977/full#supplementary-material.

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Conflict of Interest Statement: The authors have no conflict of interest directly related to the current manuscript. However, SC, FK, SN, and ML are inventors in a patent submitted by the University of Sussex for the use of CIR-miRNAs as biomarkers of sepsis.

Severity of systemic inflammatory response syndrome (SIRS) affects the blood levels of circulating inflammatory-relevant miRNAs

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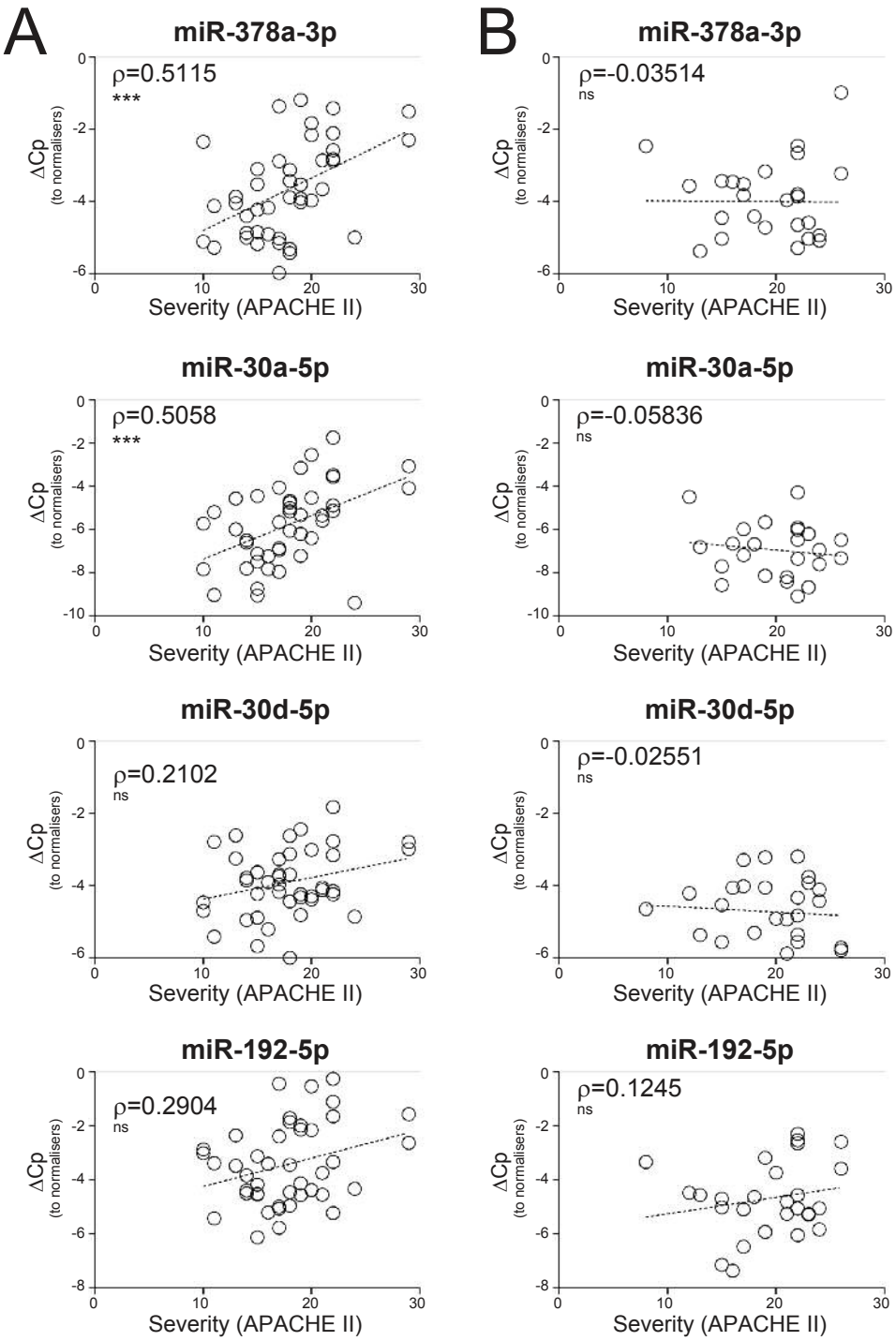
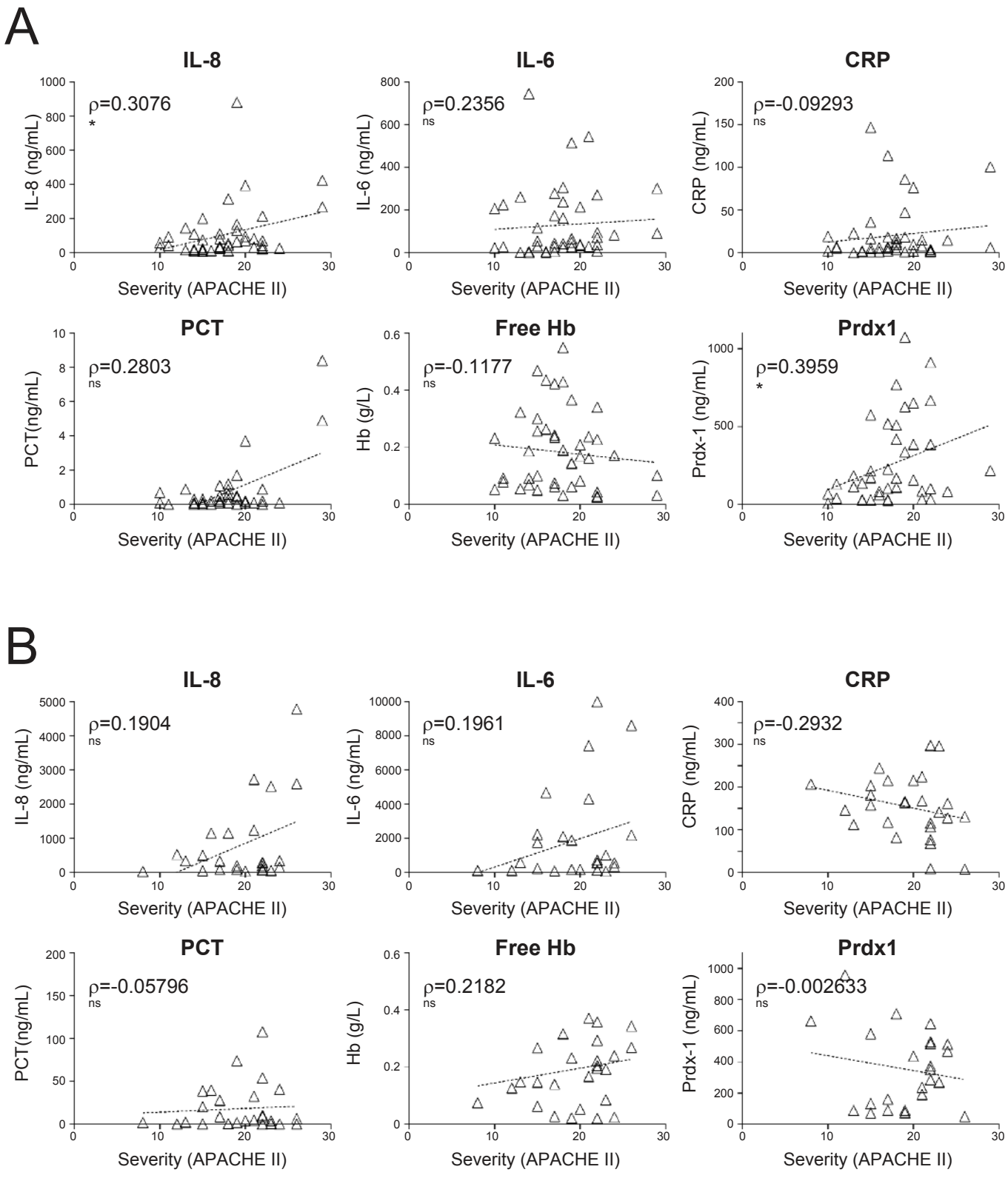
Supplementary Material Contents:



Supplementary Figure 1



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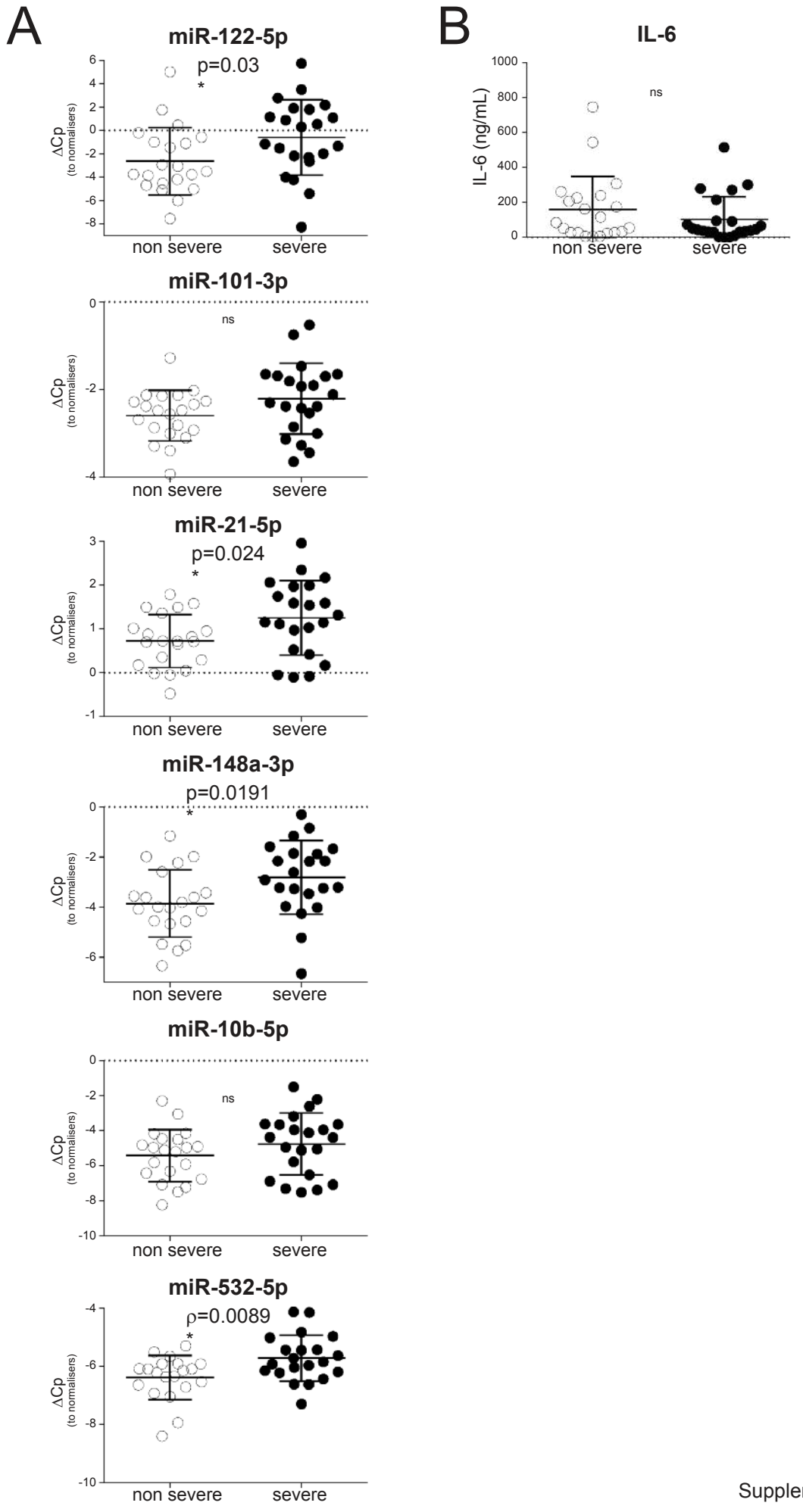

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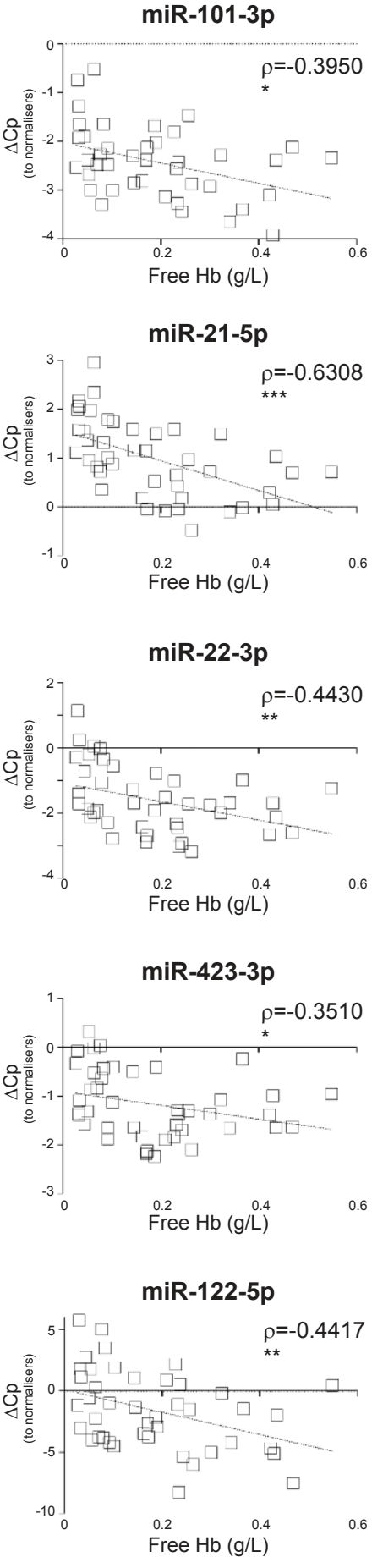
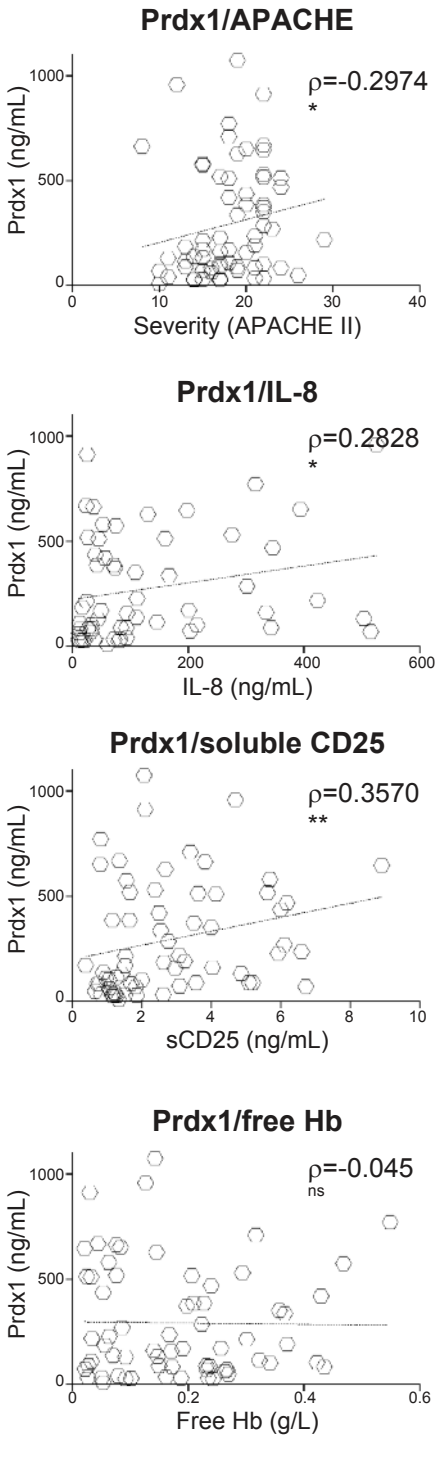


Supplementary Figure 4



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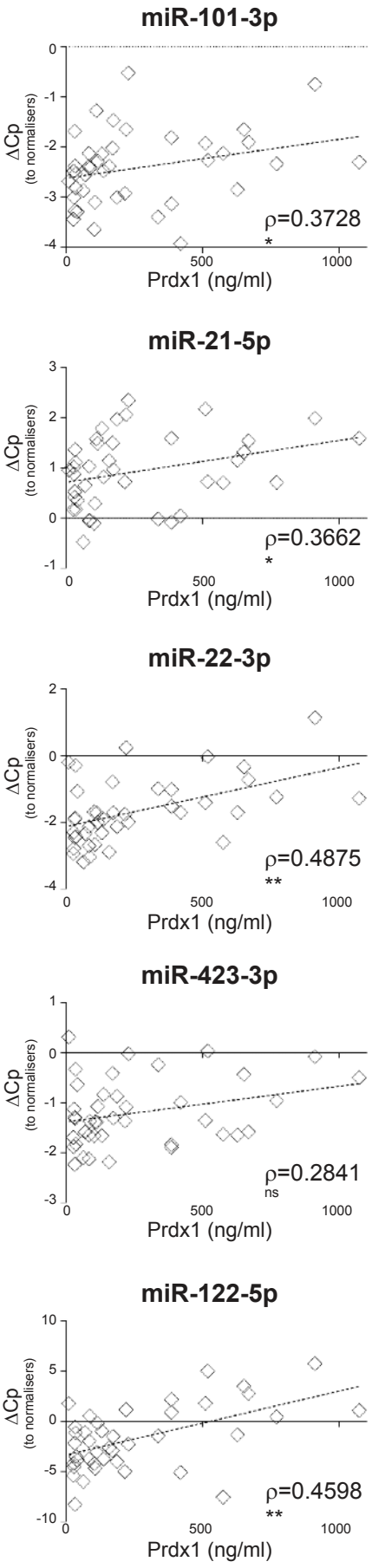

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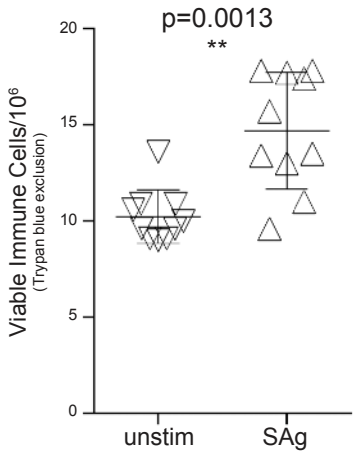

Supplementary Figure 7

miR-378a-3p

Hierarchical multiple regression levels	Variables considered	R square (% variance of dependent variable)	p (level of significance)
1st	Steroids; NSAIDs; Immuno-suppressants	0.127 (12.7%)	0.147 (ns)
2nd	SOFA (severity)	0.377(37.7%)	0.001 (***)

miR-30a-5p

Hierarchical multiple regression levels	Variables considered	R square (% variance of dependent variable)	p (level of significance)
1st	Steroids; NSAIDs; Immuno-suppressants	0.147 (14.7%)	0.098 (ns)
2nd	SOFA (severity)	0.367 (36.7%)	0.001 (***)

miR-30d-5p

Hierarchical multiple regression levels	Variables considered	R square (% variance of dependent variable)	p (level of significance)
1st	Steroids; NSAIDs; Immuno-suppressants	0.056 (5.6%)	0.517 (ns)
2nd	SOFA (severity)	0.225 (22.5%)	0.042 (*)

miR-192-5p

Hierarchical multiple regression levels	Variables considered	R square (% variance of dependent variable)	p (level of significance)
1st	Steroids; NSAIDs; Immuno-suppressants	0.094 (9.4%)	0.375 (ns)
2nd	SOFA (severity)	0.200 (20.0%)	0.070(ns)

Supplementary Figure 8

Supplementary Figure Legends

Supplementary Figure 1. CIR-miRNA levels correlate with the severity of non-infective SIRS as detected by the APACHE II score. In miRNA qPCR arrays, within each patient's specimen, Cp of a single miRNA is compared to the mean Cp of 2 normalizers (miR-486-5p and miR-320a) to give delta-Cp (dCp). dCp of non-infective SIRS **(A)** and sepsis **(B)** patients were analyzed in correlation analyses with disease severity, as determined by acute physiology and chronic health evaluation II (APACHE II) score as an alternative to the SOFA score used in Figure 1. **(A)** Non-parametric correlation of APACHE II scores with the plasma levels of miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p in non-infective SIRS patients. **(B)** Non-parametric correlation of APACHE II scores with the plasma levels of miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p in infective SIRS (sepsis) patients. Each symbol represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*, $p \leq 0.05$; **, $p \leq 0.005$; and ***, $p \leq 0.0005$ or ns, non-significant).

Supplementary Figure 2. Correlations of plasma levels of inflammatory cytokines and stress mediators with the severity of non-infective SIRS and sepsis as detected by the APACHE II score. The levels of inflammatory cytokines (interleukin-IL-8 and IL-6) and stress mediators: C-reactive protein (CRP), pro-calcitonin (PCT), free hemoglobin (Hb) and peroxiredoxin-1 (Prdx-1) were measured by ELISA in the plasma of non-infective SIRS **(A)** and sepsis **(B)** patients. Thereafter, the correlation with the severity of disease, as detected by acute physiology and chronic health evaluation II (APACHE II) score (as an alternative to the SOFA score used in Figure 1) was investigated. **(A)** Non-parametric correlation of APACHE II scores with the plasma levels of IL-8, IL-6, CRP, PCT, free Hb and Prdx-1 in non-infective SIRS patients. **(B)** Non-parametric correlation of APACHE II scores with the plasma levels of IL-8, IL-6, CRP, PCT, free Hb and Prdx-1 in sepsis patients. Each triangle represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*, $p \leq 0.05$; **, $p \leq 0.005$; and ***, $p \leq 0.0005$ or ns, non-significant).

Supplementary Figure 3. Unlike IL-6, other CIR-miRNA biomarkers discriminate the severity of non-infective SIRS. In miRNA qPCR arrays, within each patient's specimen, Cp of individual miRNAs were normalized as in Figure 1 and analyzed in patients with non-severe (open circles) and severe (filled circles) non-infective SIRS. Each symbol represents an individual patient. **(A)** Dot plots show dCp values for miR-122-5p, miR-101-3p, miR-21-5p, miR-148a-3p, miR-10b-5p and miR-532-5p in non-severe (n=21) and severe (n=22) non-infective SIRS patients, together with the level of significance. Beyond miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p (Figure 3), also miR-122-5p, miR-21-5p, miR-148a-3p and miR-532-5p significantly discriminate the severity of non-infective SIRS. **(B)** Dot plots show concentration of IL-6 in non-severe (n=21) and severe (n=22) non-infectious SIRS patients, together with the level of significance.

Supplementary Figure 4. The plasma levels of many other CIR-miRNAs inversely correlate with free Hb levels. In miRNA qPCR arrays, within each patient's specimen, Cp of individual miRNAs (open squares) were normalized as in Figure 1 and analyzed in correlation with levels of free Hb, which is derived from the lysis of red blood cells (RBCs). Each square represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*, $p \leq 0.05$; **, $p \leq 0.005$; and ***, $p \leq 0.0005$ or ns, non-significant). Beyond miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p (Figure 4), also miR-101-3p, miR-21-5p, miR-22-3p, miR-423-3p and miR-122-5p, like many others shown in Table 3, significantly correlate with free Hb.

Supplementary Figure 5. Correlations of plasma levels of Prdx-1 with IL-8, soluble CD25 and free Hb in Sepsis/SIRS. The levels of: the stress mediator, Prdx1; the inflammatory cytokine, IL-8; the soluble decoy receptor, sCD25; and free hemoglobin (Hb) were measured by ELISA in the plasma of infective and non-infective SIRS. Thereafter, the correlation of levels of Prdx-1 with IL-8, sCD25, and free Hb in parallel to the severity of disease, as detected by acute physiology and chronic health evaluation II (APACHE II) score was investigated. From the top to the bottom, graphs show the non-parametric correlation of the plasma levels of Prdx-1 with: APACHE II scores, IL-8, sCD25, and free Hb within the entire SIRS cohort. Each symbol represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*, $p \leq 0.05$; **, $p \leq 0.005$; and ***, $p \leq 0.0005$ or ns, non-significant). Prdx-1 levels tended to correlate with disease severity and levels of IL-8 and sCD25, but not free Hb.

Supplementary Figure 6. The plasma levels of many other CIR-miRNAs positively correlate with Prdx-1 levels. In miRNA qPCR arrays, within each patient's specimen, Cp of individual miRNAs (open rhombi) were normalized as specified in Figure 1 and analyzed in correlation with levels of plasma inflammatory stress marker, Prdx-1. Each symbol represents an individual patient. Correlation trends are shown with the linear regression model including Spearman rho (ρ) and the significances of the correlations (*, $p \leq 0.05$; **, $p \leq 0.005$; and ***, $p \leq 0.0005$ or ns, non-significant). Beyond miR-378a-3p, miR-30a-5p and miR-192-5p (Figure 5), many other miRNAs including miR-101-3p, miR-21-5p, miR-22-3p, and miR-122-5p significantly correlated with levels of Prdx-1 (refer to Table 4 for full list).

Supplementary Figure 7. Assessment of cell viability in cultures of PBMCs producing CIR-miRNAs. PBMCs derived from 10 individuals were freshly purified from blood and equal cells numbers were then cultured in complete media in the presence of exosome-free bovine serum, at the concentration of $2x10^6$ cells/ml, in replicate wells. Half of the cultures were stimulated with the SPE-KL bacterial superantigen (SAg) from 5 days, in comparison to unstimulated controls (unstim). On day 5 cells were harvested and counted with the Trypan Blue exclusion dye. Approximately the same numbers of cells were recovered in unstimulated cultures across 10 independent biological repeats as also seen in the case of SAg activated cultures (each triangle represents a culture derived from individual donors). However, in SAg activated cultures there was approximately 1.5-fold cell-expansion.

Supplementary Figure 8. Significant effect of severity on levels of CIR-miRNAs during non-infective SIRS, after correction for medication. Hierarchical multiple regression models of the blood levels of miR-378a-3p, miR-30a-3p, miR-30d-5p and miR-192-5p were generated based on SIRS severity (SOFA), taking into account whether patients were under anti-inflammatory medication (steroids, NSAIDs and other immunosuppressants), at the time of admission (only 21% patients were taking such drugs, Table S8). In this relatively small cohort (n=43), medication dichotomous variables behaved essentially as confounding variables after they were introduced at the first level (block 1, 1st) of the regression. Thereafter, in the 2^{nd} level (block 2, 2nd) of the regression, disease severity (SOFA) was assessed for the capacity to significantly affect the levels of blood miRNAs in SIRS. For each miRNA, tables list the variables introduced at each step (1^{st} and 2^{nd}) of the regression together with the resulting R square values (the percentages of the variance affected) with levels of significance after the first and the second regression steps (i.e., the total model significance). R squares values show that, in any case, medication (introduced at step 1) did not affect significantly blood levels of miRNAs ($p>0.05$, 1^{st}). At the 2^{nd} step, blood levels of miR-378a-3p, miR-30a-3p, and miR-30d-5p were significantly affected by severity of SIRS, accounting for 37%-20% of the variance. This suggests that severity of disease is a major factor driving the increase of CIR-miRNAs, irrespective of anti-inflammatory drug medication.

Table S1. Correlations of CIR-miRNAs with SIRS severity, as detected by APACHE II. Significant correlations with disease severity detected using the SOFA score are highlighted in bold black (SIRS) or red (sepsis) for comparison.

Micro-RNA Species§	APACHE II Spearman (ρ)*	Correlation significance p#	Benjamini Hochberg (BH) rank	BH critical value (15%)
miR-378a-3p	0.51145	4.56E-04	1	0.00349
miR-30a-5p	0.505765	0.000541	2	0.00698
miR-122-5p	0.365376	0.015984	3	0.01047
miR-22-3p	0.352489	0.020434	4	0.01395
miR-106b-3p	-0.32683	0.042274	5	0.01744
miR-30c-5p	-0.29776	0.052472	6	0.02093
miR-192-5p	0.290406	0.058873	7	0.02442
let7i-5p	-0.26782	0.082509	8	0.02791
miR-423-3p	-0.26607	0.084603	9	0.03140
miR-143-3p	0.248183	0.108559	10	0.03488
miR-532-5p	0.251794	0.11226	11	0.03837
miR-101-3p	0.217103	0.161983	12	0.04186
miR-30d-5p	0.210175	0.176132	13	0.04535
miR-320a	0.205505	0.186155	14	0.04884
miR-486-5p	-0.2055	0.186155	15	0.05233
miR-10b-5p	0.199895	0.198725	16	0.05581
miR-148a-3p	0.195878	0.208087	17	0.05930
miR-744-5p	-0.1866	0.236724	18	0.06279
miR-26a-5p	-0.17147	0.27158	19	0.06628
miR-103a-3p	-0.16791	0.281814	20	0.06977
miR-451a	-0.16343	0.295013	21	0.07326
miR-191-5p	-0.15138	0.332536	22	0.07674
miR-181a-5p	-0.15009	0.336716	23	0.08023
let7f-5p	-0.14994	0.33721	24	0.08372
miR-130a-3p	-0.14403	0.356821	25	0.08721
miR-941	-0.16401	0.361749	26	0.09070
miR-107	-0.12811	0.412961	27	0.09419
miR-146a-5p	-0.11204	0.474428	28	0.09767
miR-127-3p	-0.1134	0.49784	29	0.10116
miR-223-3p	-0.09476	0.545575	30	0.10465
let7a-5p	-0.09294	0.55335	31	0.10814
miR-151a-3p	-0.0921	0.55693	32	0.11163
miR-21-5p	0.079519	0.612242	33	0.11512
let7b-5p	0.062842	0.688911	34	0.11860
miR-10a-5p	0.04732	0.76892	35	0.12209
miR-182-5p	0.043627	0.786516	36	0.12558
miR-30e-3p	-0.04067	0.800679	37	0.12907
miR-375	0.033198	0.849843	38	0.13256
miR-27b-3p	0.018799	0.904757	39	0.13605

miR-423-5p	-0.01296	0.93425	40	0.13953
miR-28-3p	-0.00523	0.973445	41	0.14302
miR-92b-3p	-0.00491	0.977333	42	0.14651
miR-23a-3p	0.002577	0.986913	43	0.15000

Table S2. Correlations of CIR-miRNAs with sepsis severity, as detected by APACHE II. Significant correlations with disease severity detected using the SOFA score are highlighted in bold black (SIRS) or red (sepsis) for comparison.

Micro-RNA species§	APACHE II Spearman (ρ)*	Correlation significance p#	Benjamini Hochberg (BH) rank	BH critical value (FDR 15%)
miR-532-5p	-0.35297	0.07694	1	0.00349
miR-101-3p	-0.34272	0.080122	2	0.00698
miR-10a-5p	-0.40887	0.103193	3	0.01047
miR-30e-3p	-0.33633	0.108072	4	0.01395
miR-941	-0.34139	0.129881	5	0.01744
miR-122-5p	0.301722	0.134137	6	0.02093
miR-107	-0.29016	0.142063	7	0.02442
miR-106b-3p	-0.29653	0.159423	8	0.02791
miR-30c-5p	-0.25788	0.194052	9	0.03140
miR-182-5p	-0.32705	0.234107	10	0.03488
miR-146a-5p	-0.22776	0.253216	11	0.03837
miR-10b-5p	-0.21358	0.29481	12	0.04186
miR-92b-3p	-0.27644	0.318577	13	0.04535
miR-26a-5p	-0.19764	0.323077	14	0.04884
miR-423-5p	-0.19487	0.330032	15	0.05233
miR-27b-3p	-0.18442	0.357119	16	0.05581
miR-23a-3p	-0.1835	0.35957	17	0.05930
miR-744-5p	-0.17074	0.425044	18	0.06279
miR-181a-5p	-0.14877	0.458954	19	0.06628
miR-148a-3p	0.146615	0.465548	20	0.06977
hsalet7a-5p	-0.1334	0.507118	21	0.07326
miR-192-5p	0.124484	0.53615	22	0.07674
hsalet7f-5p	-0.11465	0.569068	23	0.08023
miR-423-3p	-0.10604	0.598592	24	0.08372
miR-191-5p	-0.10512	0.601794	25	0.08721
miR-21-5p	0.103583	0.607145	26	0.09070
miR-375	0.149403	0.610212	27	0.09419
miR-127-3p	-0.11457	0.630531	28	0.09767
miR-223-3p	-0.09405	0.640762	29	0.10116
miR-130a-3p	-0.09313	0.644053	30	0.10465
miR-103a-3p	-0.09067	0.652863	31	0.10814
miR-151a-3p	-0.08606	0.669503	32	0.11163
hsalet7b-5p	0.084526	0.675084	33	0.11512
miR-320a	-0.06117	0.761833	34	0.11860

miR-486-5p	0.061166	0.761833	35	0.12209
miR-30a-5p	-0.05836	0.786484	36	0.12558
miR-378a-3p	-0.03514	0.864668	37	0.12907
miR-30d-5p	-0.02551	0.899487	38	0.13256
miR-451a	-0.02152	0.915169	39	0.13605
miR-143-3p	0.019672	0.922418	40	0.13953
hsalet7i-5p	0.019364	0.923626	41	0.14302
miR-28-3p	-0.00206	0.992024	42	0.14651
miR-22-3p	0.001844	0.992716	43	0.15000

Table S3. Correlations of CIR-miRNAs with free Hb levels in sepsis. MiRNAs significantly correlating with disease severity (as by SOFA) are highlighted in bold black (SIRS) or red (sepsis).

Micro-RNA species§	Hb Spearman correlation (ρ)*	Correlation significance p#	Benjamini Hochberg (BH) rank	BH critical value (FDR 5%)
miR-106b-3p	-0.66957	3.46E-04	1	0.00116
miR-744-5p	-0.65217	0.000554	2	0.00233
miR-151a-3p	-0.57143	0.001849	3	0.00349
miR-532-5p	-0.58085	0.001862	4	0.00465
miR-92b-3p	-0.68571	0.004772	5	0.00581
miR-130a-3p	-0.51954	0.005481	6	0.00698
miR-146a-5p	-0.50672	0.006991	7	0.00814
miR-941	-0.55065	0.009687	8	0.00930
miR-191-5p	-0.46642	0.014191	9	0.01047
miR-10b-5p	-0.46462	0.016788	10	0.01163
miR-10a-5p	-0.55882	0.019709	11	0.01279
miR-181a-5p	-0.35287	0.071015	12	0.01395
miR-451a	0.350427	0.073129	13	0.01512
miR-103a-3p	-0.34982	0.073665	14	0.01628
miR-28-3p	-0.35453	0.075554	15	0.01744
miR-320a	-0.34249	0.080333	16	0.01860
miR-486-5p	0.342491	0.080333	17	0.01977
miR-30c-5p	-0.29548	0.134559	18	0.02093
miR-107	-0.28999	0.142305	19	0.02209
miR-27b-3p	-0.2851	0.149455	20	0.02326
miR-21-5p	-0.27656	0.162581	21	0.02442
let7b-5p	0.257631	0.194502	22	0.02558
miR-143-3p	-0.25458	0.200028	23	0.02674
miR-30a-5p	-0.26609	0.208836	24	0.02791
let7a-5p	-0.24847	0.2114	25	0.02907
miR-30d-5p	-0.23016	0.248116	26	0.03023
let7f-5p	-0.21673	0.277553	27	0.03140
let7i-5p	-0.21062	0.291641	28	0.03256
miR-26a-5p	-0.21062	0.291641	29	0.03372
miR-423-5p	-0.20574	0.303231	30	0.03488

miR-423-3p	-0.1978	0.322667	31	0.03605
miR-22-3p	-0.18681	0.350806	32	0.03721
miR-122-5p	0.186325	0.362103	33	0.03837
miR-23a-3p	-0.17277	0.388811	34	0.03953
miR-378a-3p	-0.15487	0.44999	35	0.04070
miR-30e-3p	-0.1513	0.480346	36	0.04186
miR-101-3p	-0.10989	0.585311	37	0.04302
miR-375	-0.13846	0.636885	38	0.04419
miR-182-5p	-0.13214	0.638744	39	0.04535
miR-127-3p	-0.10677	0.654142	40	0.04651
miR-223-3p	0.043956	0.827663	41	0.04767
miR-148a-3p	-0.03358	0.867949	42	0.04884
miR-192-5p	0.029915	0.88225	43	0.05000

Table S4. Full-length version of Table 1 (SIRS). Significant correlations of CIR-miRNA levels with SIRS severity as detected by SOFA are highlighted in bold black (SIRS) or red (sepsis).

Micro-RNA species§	SOFA Spearman correlation (ρ)*	Correlation significance p#	Benjamini-Hochberg (BH) Rank	BH critical value (FDR 15%)
miR-378a-3p	0.491	0.00084	1*	0.00349
miR-30a-5p	0.433	0.00370	2*	0.00698
miR-30d-5p	0.412	0.00609	3*	0.01047
miR-192-5p	0.378	0.01253	4*	0.01395
miR-122-5p	0.359	0.01799	5	0.01744
miR-101-3p	0.351	0.02092	6	0.02093
miR-21-5p	0.336	0.02769	7	0.02442
miR-148a-3p	0.309	0.04357	8	0.02791
miR-10b-5p	0.283	0.06601	9	0.03140
miR-532-5p	0.285	0.07054	10	0.03488
miR-22-3p	0.268	0.08248	11	0.03837
miR-143-3p	0.266	0.08468	12	0.04186
miR-23a-3p	0.255	0.09926	13	0.04535
miR-320a	0.251	0.10514	14	0.04884
miR-486-5p	-0.251	0.10514	15	0.05233
miR-106b-3p	-0.191	0.24514	16	0.05581
let7b-5p	0.178	0.25436	17	0.05930
miR-423-3p	-0.148	0.34376	18	0.06279
let7i-5p	-0.134	0.39056	19	0.06628
miR-28-3p	0.117	0.45378	20	0.06977
miR-27b-3p	0.103	0.51175	21	0.07326
miR-130a-3p	-0.091	0.56033	22	0.07674
let7a-5p	0.089	0.57090	23	0.08023
miR-451a	-0.083	0.59466	24	0.08372
miR-10a-5p	-0.080	0.61864	25	0.08721

let7f-5p	0.077	0.62539	26	0.09070
miR-941	-0.082	0.64920	27	0.09419
miR-30e-3p	0.071	0.65837	28	0.09767
miR-744-5p	-0.066	0.67700	29	0.10116
miR-181a-5p	0.063	0.68622	30	0.10465
miR-26a-5p	0.061	0.69698	31	0.10814
miR-92b-3p	-0.067	0.69714	32	0.11163
miR-182-5p	-0.060	0.70734	33	0.11512
miR-375	-0.054	0.75931	34	0.11860
miR-151a-3p	0.040	0.79890	35	0.12209
miR-146a-5p	0.038	0.80829	36	0.12558
miR-223-3p	0.038	0.80829	37	0.12907
miR-30c-5p	-0.037	0.81583	38	0.13256
miR-103a-3p	-0.035	0.82375	39	0.13605
miR-191-5p	0.025	0.87127	40	0.13953
miR-127-3p	0.026	0.87822	41	0.14302
miR-423-5p	-0.020	0.90078	42	0.14651
miR-107	0.006	0.96827	43	0.15000

Table S5. Full-length version of Table 2 (sepsis). Significant correlations of CIR-miRNA levels with sepsis severity as detected by SOFA are highlighted in bold black (SIRS) or red (sepsis).

Micro-RNA species§	SOFA Spearman correlation (ρ)*	Correlation significance p#	Benjamini Hochberg (BH) rank	BH critical value (FDR 15%)
miR-22-3p	0.447	0.01942	1	0.00349
miR-191-5p	-0.432	0.02436	2	0.00698
miR-375	0.590	0.02633	3	0.01047
miR-151a-3p	-0.354	0.06990	4	0.01395
miR-146a-5p	-0.333	0.08966	5	0.01744
miR-103a-3p	-0.299	0.12992	6	0.02093
miR-378a-3p	0.282	0.16305	7	0.02442
let7b-5p	-0.269	0.17521	8	0.02791
miR-122-5p	0.262	0.19539	9	0.03140
let7i-5p	-0.252	0.20556	10	0.03488
miR-192-5p	0.228	0.25220	11	0.03837
miR-23a-3p	-0.224	0.26149	12	0.04186
miR-92b-3p	-0.304	0.27048	13	0.04535
miR-10a-5p	-0.275	0.28483	14	0.04884
miR-28-3p	-0.192	0.34735	15	0.05233
miR-107	-0.188	0.34852	16	0.05581
miR-423-3p	-0.178	0.37562	17	0.05930
miR-30d-5p	-0.161	0.42252	18	0.06279
miR-182-5p	0.220	0.43072	19	0.06628
miR-941	-0.161	0.48520	20	0.06977

miR-223-3p	-0.139	0.48880	21	0.07326
miR-130a-3p	-0.135	0.50148	22	0.07674
miR-423-5p	-0.135	0.50246	23	0.08023
miR-101-3p	0.132	0.51035	24	0.08372
let7f-5p	-0.129	0.52030	25	0.08721
let7a-5p	-0.121	0.54659	26	0.09070
miR-26a-5p	-0.108	0.59244	27	0.09419
miR-27b-3p	-0.106	0.59989	28	0.09767
miR-143-3p	-0.102	0.61166	29	0.10116
miR-30a-5p	0.108	0.61703	30	0.10465
miR-106b-3p	-0.104	0.63019	31	0.10814
miR-148a-3p	0.089	0.65859	32	0.11163
miR-10b-5p	0.083	0.68572	33	0.11512
miR-30e-3p	-0.078	0.71743	34	0.11860
miR-181a-5p	0.070	0.72964	35	0.12209
miR-30c-5p	-0.048	0.81357	36	0.12558
miR-532-5p	-0.047	0.81781	37	0.12907
miR-127-3p	0.051	0.82948	38	0.13256
miR-451a	0.026	0.89594	39	0.13605
miR-744-5p	-0.024	0.91287	40	0.13953
miR-21-5p	0.007	0.97210	41	0.14302
miR-320a	-0.007	0.97331	42	0.14651
miR-486-5p	0.007	0.97331	43	0.15000

Table S6. Full-length version of Table 3: correlations of CIR-miRNAs with free Hb levels in non-infective SIRS. MiRNAs significantly correlating with disease severity (as by SOFA) are highlighted in bold black (SIRS) or red (sepsis).

Micro-RNA species§	Hb Spearman correlation (ρ)*	Correlation significance p#	Benjamini Hochberg (BH) rank	BH critical value (FDR 5%)
miR-21-5p	-0.6308	5.78E-06	1	0.00116
miR-10b-5p	-0.5297693	2.59E-04	2	0.00233
miR-320a	-0.5080983	0.000504483	3	0.00349
miR-486-5p	0.5080983	0.000504483	4	0.00465
miR-375	-0.5562014	0.000521672	5	0.00581
miR-451a	0.5024351	0.000596273	6	0.00698
miR-30e-3p	-0.4792892	0.001521716	7	0.00814
miR-30a-5p	-0.4631706	0.001761638	8	0.00930
miR-92b-3p	-0.4986164	0.001966918	9	0.01047
miR-22-3p	-0.4430098	0.002929346	10	0.01163
miR-122-5p	-0.4416506	0.00302822	11	0.01279
miR-28-3p	-0.4402159	0.003135754	12	0.01395
miR-146a-5p	-0.4328161	0.00374532	13	0.01512
miR-192-5p	-0.4219428	0.004828522	14	0.01628
miR-101-3p	-0.4151471	0.005636129	15	0.01744

miR-30d-5p	-0.4115382	0.006110867	16	0.01860
miR-27b-3p	-0.4023106	0.007485402	17	0.01977
let7f-5p	-0.3962699	0.008523216	18	0.02093
miR-151a-3p	-0.3890965	0.009914354	19	0.02209
miR-107	-0.3855476	0.01067175	20	0.02326
miR-26a-5p	-0.380262	0.01189136	21	0.02442
miR-744-5p	-0.3832098	0.01224583	22	0.02558
miR-378a-3p	-0.3773172	0.01262106	23	0.02674
let7a-5p	-0.3755805	0.01306903	24	0.02791
miR-23a-3p	-0.3574584	0.0186085	25	0.02907
miR-130a-3p	-0.3562502	0.01903926	26	0.03023
miR-423-5p	-0.3510401	0.02099429	27	0.03140
miR-10a-5p	-0.3413912	0.02892798	28	0.03256
miR-103a-3p	-0.3304262	0.03045712	29	0.03372
miR-148a-3p	-0.3062634	0.04578071	30	0.03488
miR-143-3p	-0.2963718	0.05363466	31	0.03605
miR-941	-0.3071781	0.08205511	32	0.03721
miR-106b-3p	-0.2697505	0.09676793	33	0.03837
miR-182-5p	-0.2561087	0.1060366	34	0.03953
miR-30c-5p	-0.2478952	0.1089817	35	0.04070
miR-423-3p	-0.206894	0.1831324	36	0.04186
miR-181a-5p	-0.1957942	0.2082848	37	0.04302
miR-191-5p	-0.1871107	0.2295738	38	0.04419
let7b-5p	-0.1788802	0.2510852	39	0.04535
miR-127-3p	-0.1535263	0.3574384	40	0.04651
miR-532-5p	-0.1378109	0.3902074	41	0.04767
let7i-5p	-0.07029863	0.6541891	42	0.04884
miR-223-3p	0.04115226	0.7933117	43	0.05000

Table S7. Full-length version of Table 4: correlations of CIR-miRNAs with Prdx-1 levels in non-infective SIRS. MiRNAs significantly correlating with disease severity (as by SOFA) are highlighted in bold black (SIRS) or red (sepsis).

Micro-RNA species§	Prdx1 Spearman correlation (ρ)*	Correlation significance p#	Benjamini Hochberg (BH) rank	BH critical value (FDR 5%)
miR-192-5p	0.590909	3.02E-05	1	0.00116
miR-30a-5p	0.548626	1.39E-04	2	0.00233
miR-22-3p	0.536243	2.10E-04	3	0.00349
miR-122-5p	0.505436	0.000545917	4	0.00465
miR-148a-3p	0.485956	0.00095437	5	0.00581
miR-378a-3p	0.485503	0.000966472	6	0.00698
miR-532-5p	0.465157	0.002181351	7	0.00814
miR-320a	0.453186	0.002274844	8	0.00930
miR-486-5p	-0.45319	0.002274844	9	0.01047

miR-21-5p	0.437632	0.003337895	10	0.01163
miR-101-3p	0.413168	0.005892309	11	0.01279
miR-423-5p	0.340985	0.02524517	12	0.01395
miR-30d-5p	0.318571	0.03733837	13	0.01512
miR-375	0.342017	0.04432343	14	0.01628
miR-10b-5p	0.266687	0.08386032	15	0.01744
miR-106b-3p	-0.26761	0.09954696	16	0.01860
miR-451a	-0.24524	0.1129453	17	0.01977
miR-941	0.278409	0.116678	18	0.02093
miR-143-3p	0.230746	0.1365694	19	0.02209
miR-127-3p	0.216763	0.1911489	20	0.02326
miR-146a-5p	0.200997	0.1962112	21	0.02442
miR-30e-3p	0.198781	0.2127893	22	0.02558
miR-10a-5p	0.198258	0.2140192	23	0.02674
miR-28-3p	0.191634	0.2183055	24	0.02791
miR-23a-3p	0.173815	0.2649776	25	0.02907
let7f-5p	-0.16626	0.2866147	26	0.03023
miR-130a-3p	0.165358	0.2892862	27	0.03140
miR-27b-3p	0.155542	0.3192592	28	0.03256
let7i-5p	-0.1264	0.4192846	29	0.03372
let7a-5p	-0.09619	0.5394614	30	0.03488
miR-744-5p	-0.08387	0.597466	31	0.03605
miR-103a-3p	-0.07581	0.6289866	32	0.03721
miR-92b-3p	-0.07362	0.6696049	33	0.03837
let7b-5p	-0.06312	0.6875891	34	0.03953
miR-182-5p	0.055401	0.7308226	35	0.04070
miR-191-5p	-0.05044	0.7480572	36	0.04186
miR-30c-5p	-0.03972	0.8003754	37	0.04302
miR-107	-0.03186	0.8392622	38	0.04419
miR-223-3p	-0.02764	0.8603651	39	0.04535
miR-26a-5p	-0.02718	0.8626321	40	0.04651
miR-423-3p	-0.02235	0.8868777	41	0.04767
miR-151a-3p	0.012534	0.9364187	42	0.04884
miR-181a-5p	0.00453	0.9769984	43	0.05000

[§]Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

*Blue and violet-shadowed cells indicate miRNAs that returned positive and negative correlations ($\rho \geq 0.2$ or $\rho \leq -0.2$), respectively.

[#]Brown and red-shadowed cells indicate miRNAs that returned significant correlations ($p \leq 0.05$) and additionally passed the correction for multiple comparisons, respectively.

Table S8. Medication at the time of admission in the study cohort. The data of medication targeting inflammation (steroids, NSAIDs and other immune-suppressants) used by patients at the time of admission is shown for non-infective SIRS and Sepsis patients. The third column (significance) shows whether the distribution differed between the two groups.

	SIRS	Sepsis	Significance
Steroids	4/43 (9.3%)	5/29 (17%)	ns
NSAIDs	3/43 (7.0%)	2/29 (6.9%)	ns
Immuno-suppressants	6/43 (14%)	1/29 (3.4%)	ns
Total with anti-inflammatory drug treatment at admission	9/43 (21%)	6/29 (21%)	ns

Supplementary Materials and Methods

Patients and healthy donors.

The patient population used in this study was previously described in detail (1) (2). Briefly, patients comprised unselected adult admissions to a mixed medical / surgical intensive / high-dependency care unit (ICU/HDU) at an English acute hospital (Brighton and Sussex University Hospitals NHS Trust). Patients were categorized as having non-infective (n=44) or infective (n=29) SIRS, following standard criteria(1). For each patient, we gathered data describing demographics, reason for admission to ICU/HDU, severity of illness (by SOFA and APACHE II scores, in the first 24 hours), comorbidities, focus of infection (for sepsis patients), and routine clinical blood test results. We defined distinct levels of non-infective SIRS severity: severe (SOFA≥6) and non-severe (SOFA≤3); patients with intermediate SOFA scores of 4-5 were excluded. Only patients with abdominal sepsis were included in this study. Blood samples were collected within <6 hours from admission and time of sample collection did not affect levels of CIR-miRNAs(2). We gathered information about any medication affecting inflammation that SIRS/sepsis patients were taking at the time of admission in ICU/HDU. A minority (21%) of patients had received previous treatment with anti-inflammatory drugs, including steroids, non-steroid anti-inflammatory drugs (NSAIDs) and other immuno-suppressants (Table S8). The number of patients taking anti-inflammatory drug medication was not significantly different in the non-infective SIRS and sepsis cohorts (Table S8). Multiple regression models designed to assess the variation in blood levels of miR-378a-3p, miR-30a-5p, miR-30d-5p and miR192-5p dependent on the severity of SIRS showed no significant contribution of anti-inflammatory drug medication at the time of admission (Supplementary Fig. 8). Healthy donors were recruited at Brighton and Sussex Medical School (BSMS) in the phlebotomy facility of the University of Sussex (Falmer, Brighton, UK). Generally healthy donors (aged 22-63; 50% males) according to criteria outlined in the National Blood Service guidelines were enrolled,

excluding pregnant women and individuals with: diabetes, asthma, past or present cancer, a known infection (e.g., HIV); blood disorders (e.g., anemia), fever, any chemotherapy, antibiotics and/or any other treatment/vaccination or long-term medication.

Haemoglobin analysis in plasma samples.

RBC lysis can bias miRNA content in plasma(3, 4). The concentration of free hemoglobin ([Hb]) was independently measured in patient plasma by the Harboe spectrophotometric method(5, 6) and hemolytic samples(7) with [Hb]>0.6g/L(8) were excluded (n=2). In parallel, we scored hemolysis in qPCR miRNA arrays as miR23a/miR451a ratio and excluded one sample that scored >7(2, 9) from further analysis.

Human samples and T cell cultures.

Fresh human PBMCs were derived from heathy donor blood after isolation by centrifugation over Ficoll-Hypaque density gradient as previously described(10). Stimulated and unstimulated cultures were seeded under identical conditions (i.e., same input; same cell concentration; exactly the same volumes). First, cells were washed in sterile PBS (ThermoFisherScientific) and counted with 0.1% Trypan Blue cell-viability exclusion dye (Sigma). Thereafter, for each condition, $20x10^6$ viable PBMCs were resuspended in 10 ml ($2x10^6$ cells/ml) of complete media: RPMI containing 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all from ThermoFisherScientific) and 10% of exosome-depleted, heat-deactivated fetal calf serum (FCS), to minimize the contamination with bovine blood-derived miRNAs in our analyses (System Biosciences). PBMCs were then seeded in 24 well-plates in replicate wells and stimulated with the bacterial superantigen (SAg), streptococcal pyrogenic exotoxin K/L (SPE-K/L, purified from *Escherichia coli* transfected with a spe-K/L-expressing vector(11), a gift from Prof Thomas Proft - University of Auckland, New Zealand) as described before(10), in parallel to unstimulated control cultures. After 5 days, cell cultures were harvested and viability of the cells was tested with Trypan-blue (0.1%) dye exclusion (Supplementary Fig. 7). Equal volumes of culture supernatants were then recovered, frozen at -80°C and total RNA was then extracted as described below.

RNA extraction and microRNA real-time qPCR array.

Total plasma and supernatant RNA was extracted using the miRCURY™ RNA isolation - biofluids kit (Exiqon, Denmark). After thawing on ice, an RNA spike-in template mixture was added to the samples. Eight ml of supernatant per sample was mixed with 2.4 ml of Lysis solution BF containing 16.67 µg/mL of MS2 bacteriophage RNA (UniSp6), prior to proceeding to the purification as by manufacturer instructions. 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 for 3 min at room temperature, before adding 20 µL Protein Precipitation solution BF. Samples were vortexed again, incubated for 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, total RNA was eluted in RNase-free H_2O by centrifugation (11000g) and stored at -80°C.

RNA (2 µl) was reverse transcribed (RT) 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, Polyadenylation and cDNA synthesis method (Exiqon). For plasma samples derived from patients, each microRNA was assayed by qPCR (microRNA Ready-to-Use PCR, Pick-&-Mix using ExiLENT SYBR® Green master mix) in 2

independent technical repeats including negative controls (no-template from the RT reaction) using a LightCycler® 480 Real-Time PCR System (Roche). In each experimental group, ≥8 biological replicates were included. Superantigen stimulation experiments were performed as 10 biological replicates. 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 crossing point (Cp) values less than the negative control and Cp<37 were accepted. Excluding spike-ins and no template controls, we analyzed 44 CIR-miRNAs in Q-PCR arrays from sepsis/SIRS patients. Although out of these, most miRNAs (33 and 29 respectively in SIRS and sepsis patients, including normalizer miRNAs) were detectable in all individuals, some miRNAs were not expressed in all individuals (i.e., Cp>37), affecting n in correlation analyses as indicated in individual figure legends. Further, miR-103b assay did not return acceptable Cp values in any donor and was excluded, leading to final 43 CIR-miRNA species analyzed. The most significantly affected miRNAs (miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-5p) were expressed in (89-100%) of sepsis/SIRS individuals. The stability values of candidate normalizers were assessed using the 'NormFinder' software(12). Any qPCR data was normalized to the average Cp of internal normalizers (miR-320a and miR-485-5p (2)), detected in all plasma samples as described previously (2) or the Cp of normalizer spike-in ((UniSp6), in the case of culture supernatants (delta Cp, dCp=normalizer Cp–assay Cp) as indicated in individual experiments. Hence relative to the normalizer/s, dCp values that become more positive correspond to an increase in the abundancy of specific miRNA species.

Supplementary Material References

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