



# 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

## 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)  $\alpha$ , 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 TNF $\alpha$ , IL-1, and IL-6 have been reported to occur more often in sepsis than SIRS (1, 20–22). Anti-inflammatory mediators (e.g., TGF $\beta$  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  $\geq 6$ ) and non-severe (SOFA  $\leq 3$ ); 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,  $20 \times 10^6$  viable PBMCs were resuspended in 10 ml ( $2 \times 10^6$  cells/ml) of complete media: RPMI containing 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine (all from ThermoFisherScientific) and 10% of exosome-depleted, heat-inactivated 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  $-80^{\circ}\text{C}$  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  $\mu\text{g}/\text{ml}$  of MS2 bacteriophage RNA (UniSp6), prior to RNA purification, as specified by the manufacturer's instructions. Extracted total RNA was stored in a  $-80^{\circ}\text{C}$  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 ( $\geq 8$  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  $\text{Cp} < 37$  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); ( $\Delta\text{Cp}$ ,  $\text{dCp} = \text{normalizer Cp} - \text{assay Cp}$ ). 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 ( $\rho$ ) 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  $\sim 1/6$  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  $\text{dCp}$  values that increase with the abundance of specific miRNAs. Severity of disease, according to the SOFA score, was then correlated with  $\text{dCp}$  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 <sup>a</sup>	SOFA Spearman correlation ( $\rho$ ) <sup>b</sup>	Correlation significance $p$ <sup>c</sup>	Benjamini–Hochberg (BH) rank	BH critical value (FDR 15%)
<b>miR-378a-3p</b>	0.491	0.00084	1*	0.00349
<b>miR-30a-5p</b>	0.433	0.00370	2*	0.00698
<b>miR-30d-5p</b>	0.412	0.00609	3*	0.01047
<b>miR-192-5p</b>	0.378	0.01253	4*	0.01395
<b>miR-122-5p</b>	0.359	0.01799	5	0.01744
<b>miR-101-3p</b>	0.351	0.02092	6	0.02093
<b>miR-21-5p</b>	0.336	0.02769	7	0.02442
<b>miR-148a-3p</b>	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
<b>miR-22-3p</b>	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.

<sup>a</sup>Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

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

<sup>c</sup>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 <sup>a</sup>	SOFA Spearman correlation ( $\rho$ ) <sup>b</sup>	Correlation significance $p^c$	Benjamini-Hochberg (BH) rank	BH critical value (FDR 15%)
<b>miR-22-3p</b>	0.447	0.01942	1	0.00349
<b>miR-191-5p</b>	-0.432	0.02436	2	0.00698
<b>miR-375</b>	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
<b>miR-378a-3p</b>	0.282	0.16305	7	0.02442
let7b-5p	-0.269	0.17521	8	0.02791
<b>miR-122-5p</b>	0.262	0.19539	9	0.03140
let7i-5p	-0.252	0.20556	10	0.03488
<b>miR-192-5p</b>	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.

<sup>a</sup>Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

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

<sup>c</sup>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 <sup>a</sup>	Hb Spearman correlation ( $\rho$ ) <sup>b</sup>	Correlation significance $p^c$	Benjamini-Hochberg (BH) rank	BH critical value (FDR 5%)
<b>miR-21-5p</b>	-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
<b>miR-375</b>	-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
<b>miR-30a-5p</b>	-0.4631706	0.001761638	8	0.00930
miR-92b-3p	-0.4986164	0.001966918	9	0.01047
<b>miR-22-3p</b>	-0.4430098	0.002929346	10	0.01163
<b>miR-122-5p</b>	-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
<b>miR-192-5p</b>	-0.4219428	0.004828522	14	0.01628
<b>miR-101-3p</b>	-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.

<sup>a</sup>Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

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

<sup>c</sup>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 <sup>a</sup>	Prdx1 Spearman correlation ( $\rho$ ) <sup>b</sup>	Correlation significance $p^c$	Benjamini-Hochberg (BH) rank	BH critical value (FDR 5%)
<b>miR-192-5p</b>	0.590909	3.02E-05	1	0.00116
<b>miR-30a-5p</b>	0.548626	1.39E-04	2	0.00233
<b>miR-22-3p</b>	0.536243	2.10E-04	3	0.00349
<b>miR-122-5p</b>	0.505436	0.000545917	4	0.00465
<b>miR-148a-3p</b>	0.485956	0.00095437	5	0.00581
<b>miR-378a-3p</b>	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
<b>miR-21-5p</b>	0.437632	0.003337895	10	0.01163
<b>miR-101-3p</b>	0.413168	0.005892309	11	0.01279
miR-423-5p	0.340985	0.02524517	12	0.01395
<b>miR-30d-5p</b>	0.318571	0.03733837	13	0.01512
<b>miR-375</b>	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.

<sup>a</sup>Green-shadowed cells indicate miRNAs that passed the BH correction for multiple comparisons.

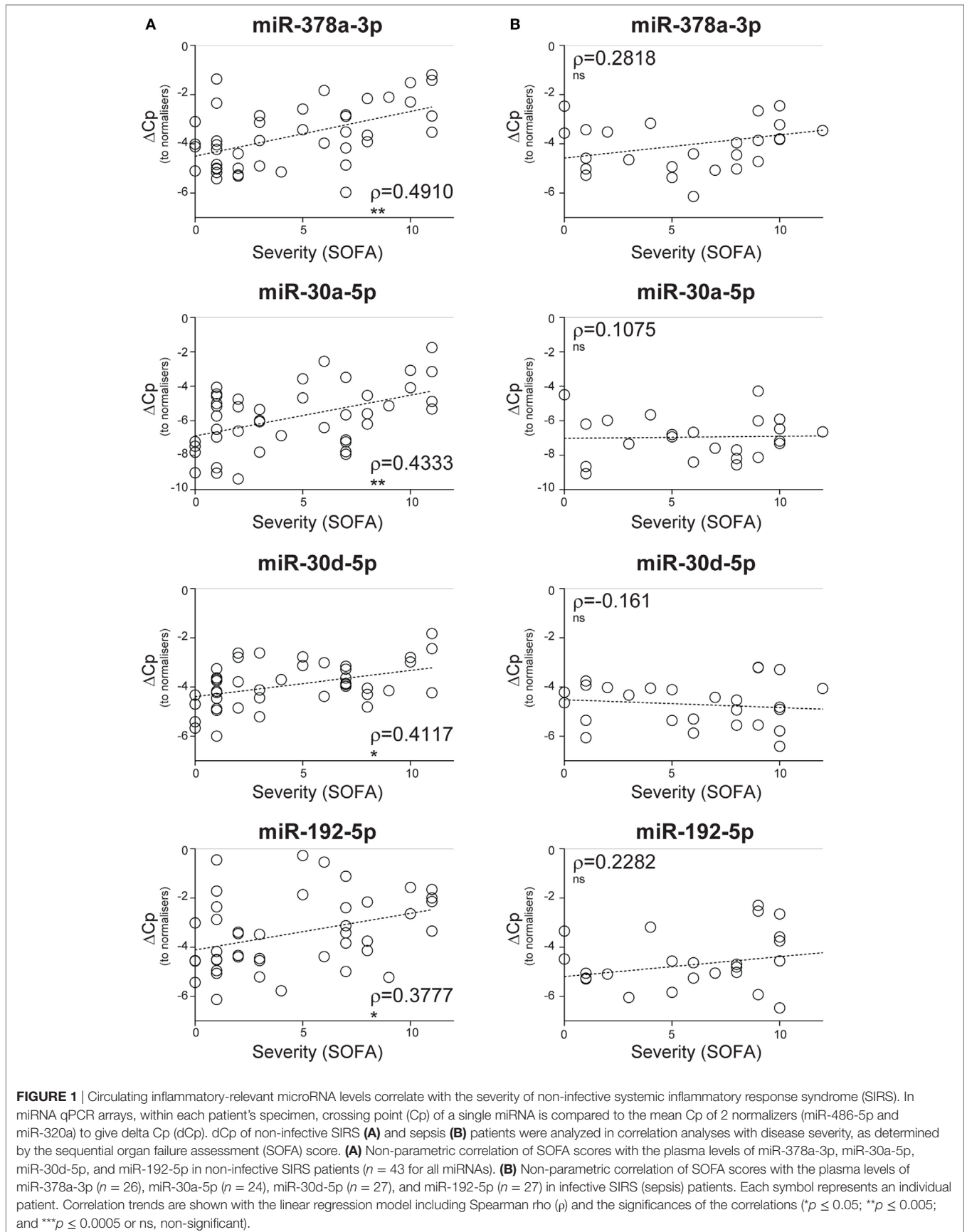
<sup>b</sup>Blue- and violet-shadowed cells indicate miRNAs that returned positive and negative correlations ( $\rho \geq 0.2$  or  $\rho \leq -0.2$ ), respectively.

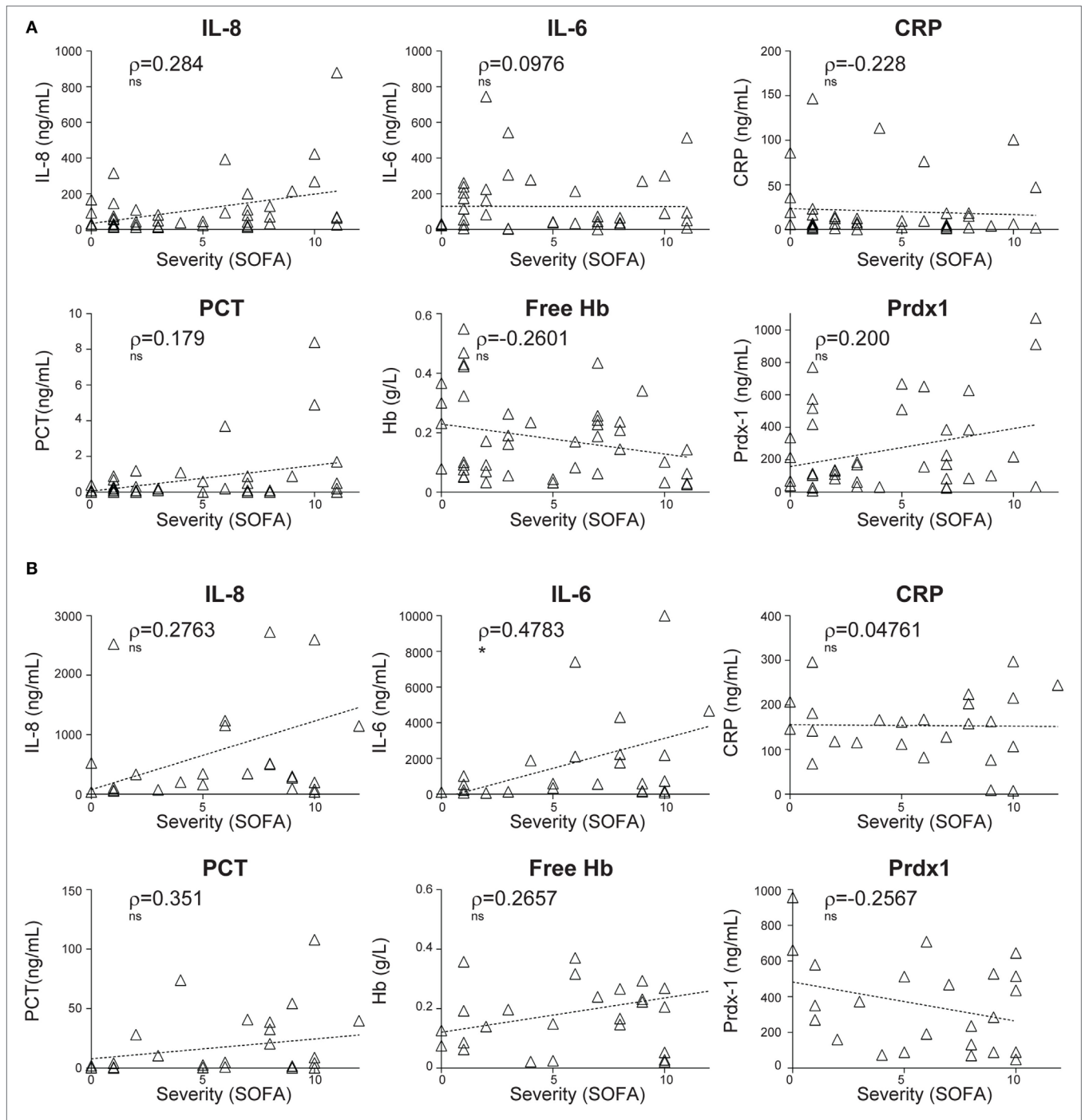
<sup>c</sup>Brown- and red-shadowed cells indicate miRNAs that returned significant correlations ( $p \leq 0.05$ ) and additionally passed the correction for multiple comparisons, respectively.

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 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 ( $\rho$ ) 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.

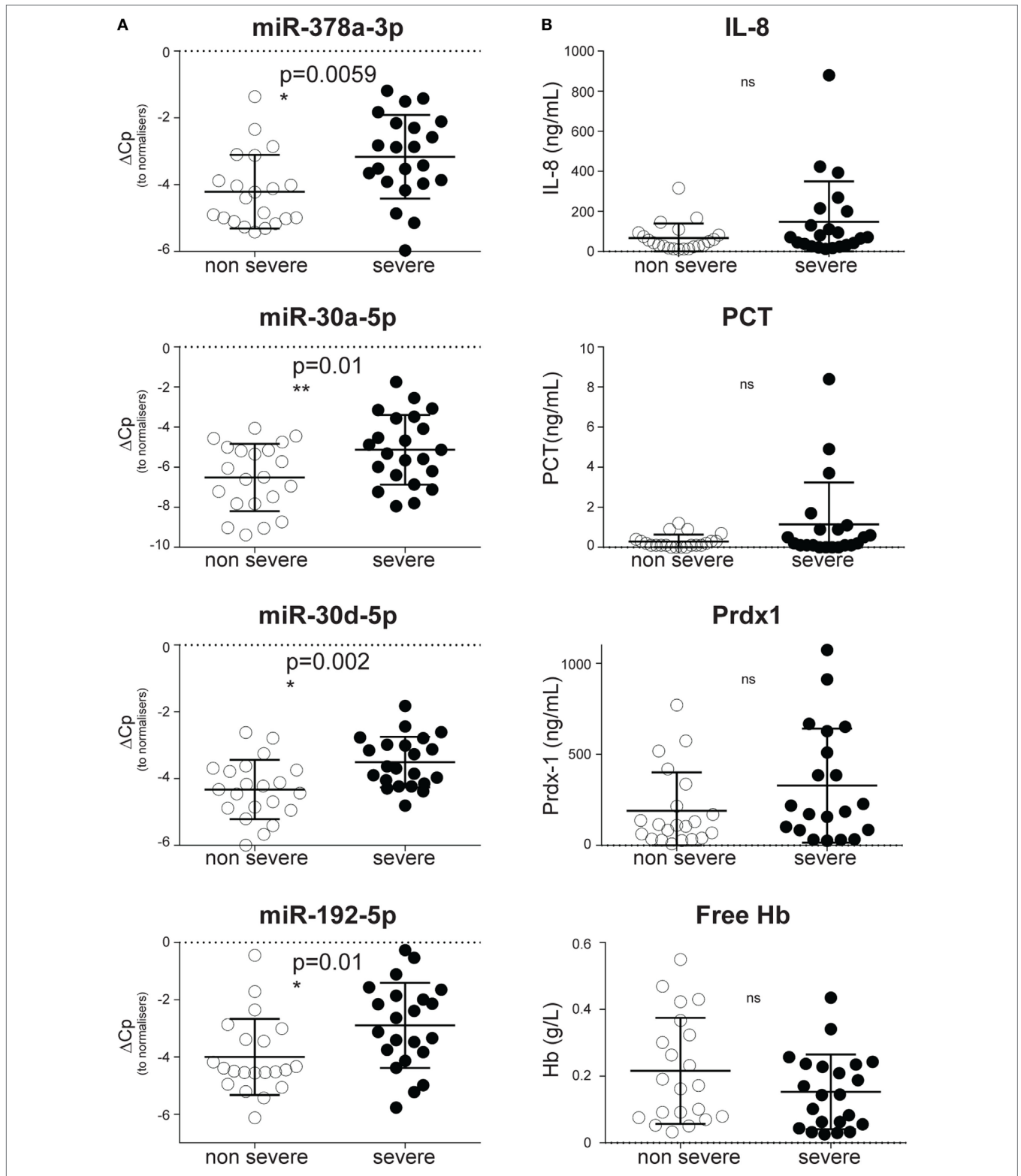
## Stimulated Immune Cells Produce CIR-miRNAs Affected by Severity of SIRS

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

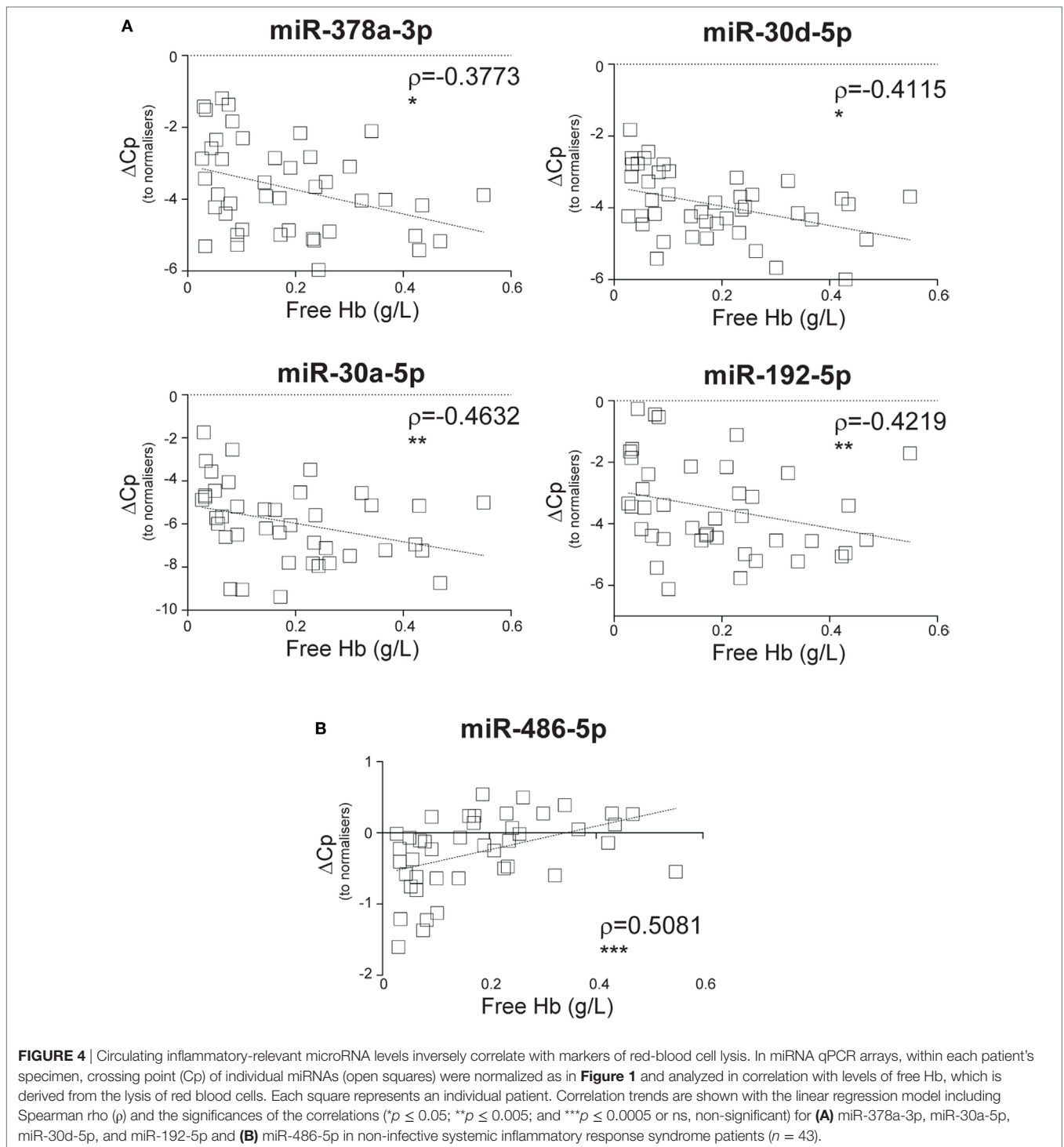
We report here that, during non-infective SIRS, the blood levels of CIR-miRNAs increase in parallel with the severity of inflammation. Levels of CIR-miRNAs do not correlate with those of free Hb, indicating that they do not derive from RBCs during SIRS. Instead, CIR-miRNAs positively correlate with levels 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.

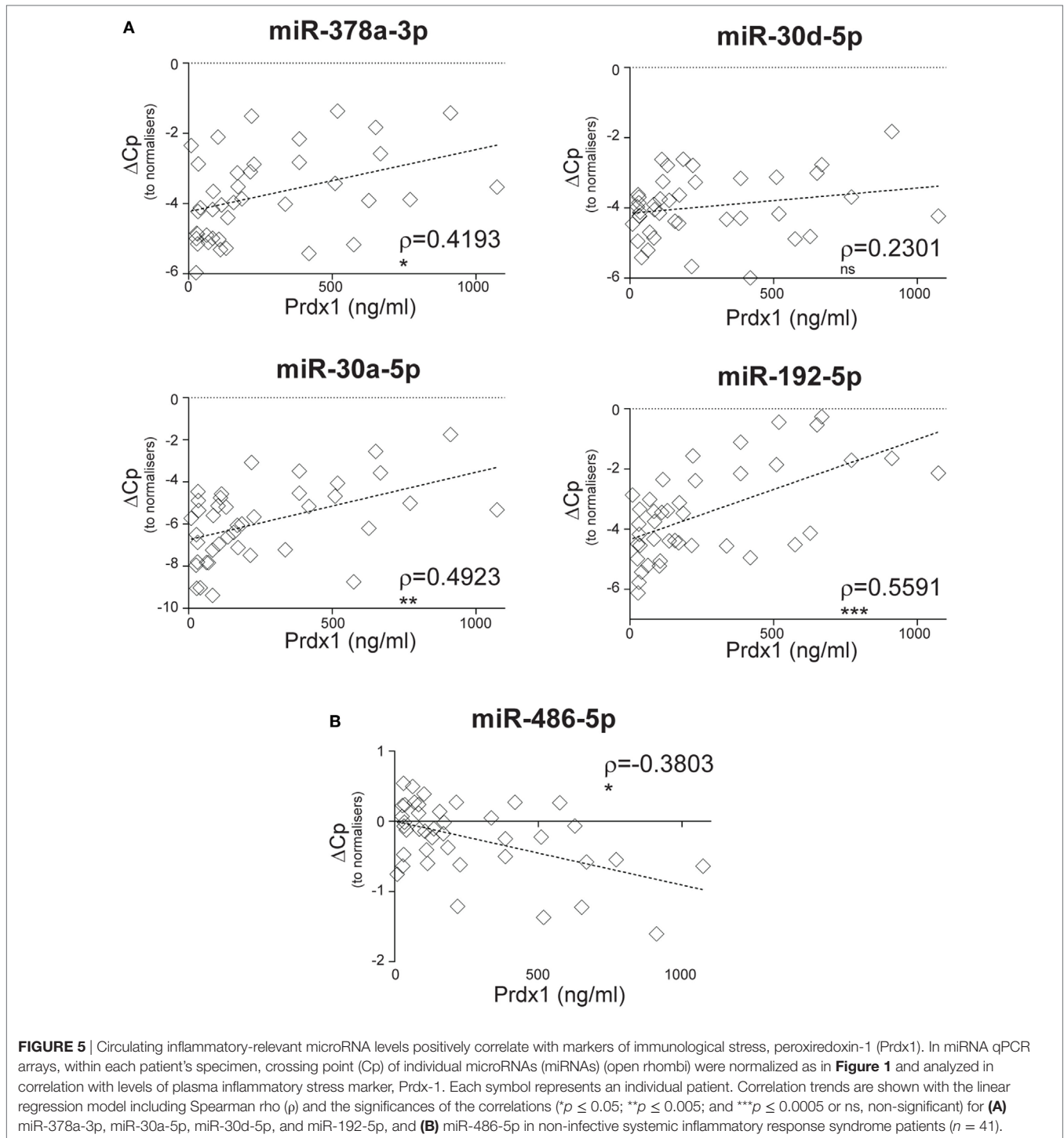




from whether patients were taking anti-inflammatory drugs (at the time of admission) (Figure S8 and Table S8 in Supplementary Material).

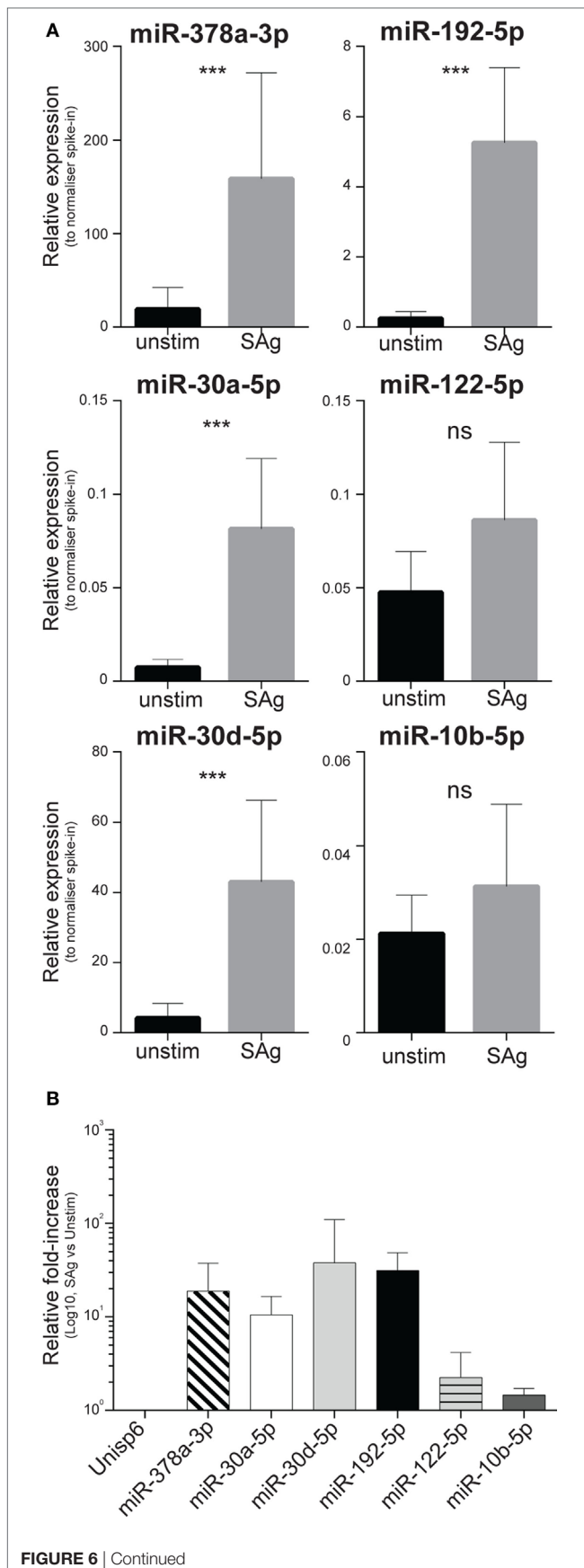
CIR-miRNAs may act as regulators of inflammation (35) by targeting the 3'UTR of mRNAs encoding pro-inflammatory cytokines/mediators (58–61). Many miRNAs are involved in

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



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 sequester miRNAs in sepsis, but not in other trauma including SIRS (74). Although miR-30a was recently associated with



**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  $2 \times 10^6$  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<sub>2</sub>O<sub>2</sub> 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.

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## 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.

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