Serum microRNAs as Biomarkers of Sepsis and Resuscitation

SUPPLEMENTARY MATERIALS

INTRODUCTION

Commonly used biomarkers for the diagnosis of sepsis and to monitor the response to therapy are C-reactive protein (CRP), procalcitonin (PCT) and interleukin (IL)-6. CRP is an acute phase protein produced in the liver upon IL-6 stimulation. It is related to the severity of infection and is sensitive to the response to therapy (32). However, it is not specific for infection as is elevated in other inflammatory conditions without sepsis, such as burns (33). PCT is a prohormone of calcitonin synthesized and secreted by neuroendocrine cells in the thyroid gland, and from other tissues under conditions of infection. PCT is more sensitive and specific than CRP for the diagnosis of bacterial infections, and is useful to guide antibiotic therapy in septic patients (34). IL-6 is released after stimulation of the innate immune cells and activates the synthesis of acute phase proteins. It is useful as a prognostic biomarker of sepsis but it is elevated nonspecifically in other conditions such as trauma or surgery (35), therefore lacking specificity for sepsis. In this context, many different molecules have been proposed as biomarkers for the diagnosis of sepsis (36).

Micro RNAs (miRNAs) are critically involved in the innate and adaptive immunity in various conditions including atherosclerosis, immunological disorders, diabetes, and bacterial infection (37). The role of miRNAs in sepsis is supported by experimental studies showing (i) that certain miRNAs are mediators of the effects observed after a septic challenge; or (ii) that key genes in the pathophysiology of sepsis are regulated by certain miRNAs. For instance, lipopolysaccharide (LPS) and tumor necrosis factor (TNF)-α upregulate miR-155 expression in macrophages and liver, and treatment with TNF-α induces a more severe disease in miR-155 transgenic mice (38). Reciprocally, miRNAs regulate the expression of several messenger RNAs encoding for components of the Toll-like receptor (TLR) signaling which mediates the response to LPS. For instance, miR-146a regulates TLR4 expression in the human monocytic cell line THP-1 (39).

METHODS

Animal preparation and monitoring

We followed the current European and National legislation on the use of laboratory animals (Principles of Laboratory Animal Care, 2010/63/UE and Real Decreto 53/2013 BOE-08/02).

We used stored blood and renal tissue samples obtained in a previous study on the circulatory effects of shock resuscitation. Intact pigs (Sus scrofa domesticus), purchased from an authorized breeder (Agropardal de Almendros S.L., Spain), aged 2.5-3 months old and weighing between 25 and 30 Kg were enrolled in the study. Each pig was housed individually after a one-week acclimation period at our animal care facility (registration number ES280650001166), with a 12h:12h light:dark cycle and room temperature between 20-24 °C. They were fed once daily (127 SAFE, standard diet for pigs, Panlab, Cornella, Spain) and water was provided ad libitum. All animals were considered healthy based on physical examination by the facility's veterinarian.

miRNA isolation from serum

Total RNA was isolated from 200 µl human serum using miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, 200 µl serum was mixed vigorously for 15 sec with 1 mL QIAzol Lysis reagent (Qiagen, Hilden, Germany) followed by incubation at room temperature for 10 min. 3.5 µl of miRNeasy Serum/Plasma Spike-In Control (1.6x108 copies/uL of C. elegans miR-39) and a 1.2 µL of carrier MS2 RNA (0.8 µg/µL; Roche, Mannheim, Germany) were added to the serum before starting the isolation procedure for sample normalization. miRNAs were eluted in 15 µl of RNase-free water and stored at -80°C until further analysis. To assess the quality and quantity of RNA, samples were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Tech, DE, USA). All RNA samples showed A260/280 ratios between 1.9 and 2.1.

miRNA cDNA Preparation

Total RNA including miRNA from each sample was reverse transcribed using the miScript® II Reverse Transcription Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. Briefly, a master mix of 3 µL of miScript HiSpec Buffer, 1,5 µL of miScript Nucleics mix and 1.5 µl of miScript Reverse Transcriptase Mix per tube was prepared and distributed to 0.2 mL PCR tubes (Applied Biosystems, Foster City, CA, USA) on ice. 4 µl of DNase/RNase free water was added to 5 µl of RNA to bring the final volume to 15 µL. Samples were incubated at 37°C for 60 minutes and at 95°C for 5 minutes. The reaction mixture was placed on ice and was diluted 10-fold with DNase/RNase free water before amplification. cDNA was stored at −80°C until required.

RT-qPCR

The miScript Primer Assays (Ss_miR-155-5p, Ss_miR-146a-5p, Ss_miR-150-5p, Ss_miR-21a-5p, Ss_miR-34a-5p, Ss_miR-486a-5p, Ss_miR-133a-3p and Syn-cel-miR-39) were diluted according to the manufacturer's protocol. Quantification of these miRNAs was performed by using miScript PCR System (Qiagen, Hilden, Germany). Briefly,1.5 µL of diluted sample was used for quantitative real-time PCR in a total volume of 15 µL using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) and miRNA specific primers on a qPCR machine (Applied Biosystems 7500 Fast Real-Time PCR System, Applied Biosystems, Life Technologies Corporation, Carlsbald, CA, USA). All real-time PCR reactions were performed in triplicate. Syn-cel-miR-39 was used for normalization. A relative fold change in expression of the target gene transcript was determined using the comparative cycle threshold method (2DDCT) using the SDS 1.4.1 software packages.

DISCUSSION

Functional roles of the miRNAs studied

miR-21a-5p is implicated in numerous biological processes (40). RAW246.7 cells from BCG infected mouse treated with miR-21a mimic showed decreased expression of TLR4 and MyD88 and decreased cell viability, whereas TNF-α, IL-6 and IL-10 levels, apoptosis and necrosis were increased, suggesting that miR-21a can negatively modulate the TLR4/MyD88 signaling pathway (15). In human studies, high levels were found in 33 patients with late sepsis compared to 18 healthy controls (41). However, miR-21a expression was not different in 70 patients with sepsis and 30 with SIRS (42). Given those results, and the lack of differences among the various groups in our study, whether miR-21a levels are sensitive to the sampling time and to the state of resuscitation is unknown.

miR-34a-5p is involved in tumorigenesis (43). Recent studies have implicated miR-34a in the sepsis-induced inflammatory response. In silico analysis revealed that miR-34a impacts the NF-κB signalosome with miR-34a binding sites in 14 key members of the NF-κB signaling pathway. Overexpression of miR-34a in CD4+ and CD8+ T cells led to a significant decrease of NFKBIA (as the most downstream cytoplasmic NF-κB) member, and reduced T cell killing capacity (16). LPS-treated HUVEC s howed attenuation of LPS-induced effects on TNF-α and NF-κB after treatment with miR-34a lentivirus (17). Our finding of elevated serum expression of miR-34a-5p in sepsis is in line with the results of those in vitro studies and supports its role as a potential biomarker of sepsis.

miR-133a-3p has been associated with SIRS, the β1-adrenergic receptor transduction cascade, and anti-apoptotic and anti-fibrotic effects in mice subject to transaortic constriction (18). As to the role of miR-133 in sepsis, Tacke et al (44) showed an increase of circulating miR-133a and miR-133b in Gram-positive and cecal ligation and puncture induced sepsis in mice. The same group reported in 138 septic patients, 85 critically ill patients without sepsis and 76 healthy controls a higher serum concentration of miR-133a in the septic group (44). In addition, they observed a correlation between these levels and severity and mortality. Unlike this study (44), we have not found a relationship between sepsis and an increase in gene expression of miR-133a-3p. Our results do not allow us to conclude as to miR-133 expression in sepsis, given the below discussed limitations of the present study.

miR-146a-5p is associated with anti-inflammatory effects. It provides a negative feedback after initiation of TLR and cytokine signaling by down-regulation of IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 protein levels (19). miR-146a serum expression has been shown to be down regulated in 50 patients with sepsis as compared to 30 patients with systemic inflammatory response (SIRS) and 20 healthy controls (28). A subsequent study comparing patients with non-septic SIRS and patients with sepsis (29), found higher gene expression in patients without sepsis. Finally, comparing 40 patients with septic shock, 29 with sepsis and 24 healthy controls, no differences were found in the expression of miR-146a (30). Given those previous results, our findings of high levels in the LoR group and normal levels in the HiR group, allow for the speculation that miR-146a-5p could be a marker of the intensity of resuscitation, being its levels high only if resuscitation is insufficient.

miR-150-5p inhibits cell proliferation, blocks the cell cycle and promotes cell apoptosis in pancreatic cancer cells (45); regulates hematopoiesis (46); and participates in the control B and T cell differentiation (20). miR-150-5p expression has been measured in humans with sepsis in different studies (30, 47-50), reporting generally down regulation of its expression. Reduced let-7a and miR-150-5p levels in peripheral leukocytes were measured in patients with gram-negative urological sepsis (50). In a study comparing 138 patients with sepsis and 76 healthy controls, miR-150 serum concentrations were only slightly reduced in critically ill patients compared to healthy controls (49). Levels did not significantly differ in critically ill patients with or without sepsis, but correlated with hepatic or renal dysfunction and mortality (49). Only Puskarich et al (30) reported up-regulation of miR-150 serum expression in patients with sepsis. This discrepancy could be explained by differences in the sampling time. In the cited studies (47-50) serum sampling was from 6 hours to 7 days after admission, whereas in the study by Puskarich et al (30) sampling was within the first 5 hours of ICU admission. Our results of a trend towards increased levels of miR-150-5p in sepsis, and attenuation of that change in the HiR as compared to the LoR group, are in line with the results of Puskarich et al (30) and allow the speculation on the role of this miRNA as a biomarker of resuscitation.

miR-155-5p is an oncomiR and targets include regulatory proteins for myelopoiesis and leukemogenesis, inflammation and known tumor suppressors (51). It post-transcriptionally

regulates innate immune signaling pathways (i.e., after TLR stimulation) (21). miR-155 expression was up regulated in 60 patients with sepsis compared to 30 healthy controls, and correlated with disease severity and mortality (31). In our model, in line with Liu et al (31), we found a strong trend toward increased expression of miR-155-5p in sepsis. Also, expression tended to be lower in the HiR versus the LoR groups. As differences did not reach statistical significance, we cannot conclude as to the role of miR-155-5p as a biomarker of sepsis and resuscitation.

miR-486a-5p has a tumor-suppressive role. Reduced miR-486a–5p expression is a frequent molecular event in human cancers (52). miR-486a-5p also induces antiapoptotic and antioxidant effects by targeting PTEN y FOXO1 (22). In a study using genome-wide miRNA profiling by microarray in peripheral blood leukocytes, comparing 24 patients with sepsis and 32 healthy controls, it was found that miR-486 (as well as miR-150, miR-182 and miR-342-5p) expression profile was higher in sepsis (47). In another study comparing survivors and non survivors of sepsis, miR-486a–5p was not differentially expressed (53). Measuring expression in serum, we did not find differences in miR-486a-5p expression among the different groups.

Table S1. Effect of sepsis and resuscitation on hemodynamic variables.

		Baseline	Absolute change versus baseline				
	Groups	t=0 h	t=1 h	t=2 h	t=3 h	t=4 h	t=5 h
Sample size	Control	15	15	15	15	15	15
	Sepsis 4 mL/Kg/h	8	8	7	3	3	3
	Sepsis 10 mL/Kg/h	8	8	8	8	5	5
	Sepsis 17 mL/Kg/h	8	8	8	8	8	7
HR (bpm)	Control	109 (86 - 144)	1 (-9 - 28)	-6 (-28 - 18)	-8 (-25 - 24)	-13 (-24 - 20)	-11 (-31 - 19)
	Sepsis 4 mL/Kg/h	87 (70 - 105)	24 (-7 - 74)	45 (0 - 72)	4 (2 - NA)	19 (6 - NA)	19 (-2 - NA)
	Sepsis 10 mL/Kg/h	87 (80 - 99)	12 (-7 - 46)	23 (10 - 53)	23 (-8 - 53)	25 (-8 - 83)	14 (-15 - 51)
	Sepsis 17 mL/Kg/h	101 (75 - 109)	34 (-15 - 59)	29 (-11 - 57)	41 (14 - 62)	37 (9 - 51)	80 (-6 - 105) *
	p value	0.152	0.549	0.067	0.104	0.042	0.047
MAP (mm Hg)	Control	97 (93 - 104)	6 (-2 - 15)	-2 (-8 - 15)	-7 (-11 - 8)	-10 (-14 - -2)	-12 (-21 - 2)
	Sepsis 4 mL/kg/h	98 (91 - 103)	-21 (-48 - -10) *	-26 (-39 - -15) *	-27 (-39 - NA) *	-22 (-47 - NA)	-36(-54 - NA)
	Sepsis 10 mL/Kg/h	100 (91 - 113)	-16 (-29 - 9)	-11 (-39 - -3) *	-33 (-59 - -14)	-33 (-50 - -14)	-33 (-56 - 9)
	Sepsis 17 mL/Kg/h	102 (100 - 126)	-12 (-18 - -9) *	-32 (-50 - -26) *	-25 (-44 - -21) *	-35 (-38 - -24) *	-28 (-36 - -12)
	p value	0.265	0.003	<0.001	<0.001	0.002	0.115
RAP (mm Hg)	Control	11.0 (9.0 - 15.0)	0.0 (-1.0 - 1.0)	1.0 (-1.0 - 1.0)	1.0 (0.0 - 1.0)	-1.0 (-3.0 - 1.0)	-1.0 (-2.0 - 1.0)
	Sepsis 4 mL/Kg/h	13.5 (11.0 - 16.8)	-4.5 (-6.5 - 0.5)	-2.0 (-6.0 - 0.0)	-6.0 (-7.0 - NA)	-6.0 (-6.0 - NA) *	-5.0 (-10.0 - NA)
	Sepsis 10 mL/Kg/h	10.5 ((8.5 - 15.5)	3.0 (-0.2 - 5.8) †	3.5 (-1.0 - 6.5)	3.0 (-1.3 - 10.2)	4.0 (-0.5 - 9.0) †	5.0 (1.0 - 7.5)
	Sepsis 17 mL/Kg/h	12.5 (10.2 - 15.0)	0.5 (0.0 - 1.0)	1.5 (1.0 - 3.5)	2.0 (0.2 - 3.5)	1.0 (-0.7 - 4.5)	2.0 (1.0 - 3.0)
	p value	0.392	0.007	0.027	0.093	0.010	0.005
MPAP (mm Hg)	Control	25 (22 - 32)	0 (-3 - 2)	-1 (-4 - 7)	-1 (-7 - 5)	-2 (-7 - 4)	-2 (-7 - 1)
	Sepsis 4 mL/Kg/h	24 (22 - 29)	15 (11 - 17) *	24 (17 - 28) *	18 (18 - NA) *	21 (19 - NA)	22 (22 - NA)
	Sepsis 10 mL/Kg/h	22 (19 - 24)	14 (8 - 17) *	26 (11 - 35) *	23 (21 - 29) *	20 (15 - 28) *	16 (11 - 27) *
	Sepsis 17 mL/Kg/h	27 (21 - 28)	13 (10 - 18) *	18 (13 - 22) *	21 (15 - 26) *	22 (12 - 29) *	19 (9 - 31)
	p value	0.200	<0.001	<0.001	0.001	0.004	0.006
PAOP (mm Hg)	Control	13.0 (12.0 - 15.0)	1.0 (1.0 - 2.0)	0.0 (-1.0 - 1.0)	0.0 (-1.0 - 1.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 1.0)
	Sepsis 4 mL/Kg/h	15.5 (13.2 - 16.0)	0.5 (-3.0 - 4.3)	-1.0 (-2.0 - 5.0)	0.0 (-1.0 - NA)	0.0 (0.0 - NA)	0.0 (0.0 - NA)
	Sepsis 10 mL/Kg/h	12.5 (12.0 - 14.0)	0.5 (0.0 - 3.2)	2.5 (1.2 - 6.0)	2.0 (0.5 - 6.0)	1.0 (1.0 - 4.5) *	2.0 (-0.5 - 5.5)
	Sepsis 17 mL/Kg/h	15.5 (10.5 - 19.0)	1.5 (0.0 - 4.5)	1.0 (-0.7 - 3.7)	2.5 (0.2 - 3.0)	4.0 (0.7 - 4.0)	4.0 (-2 - 6.0)
	p value	0.236	0.804	0.180	0.332	0.016	0.337
SVR (AU)	Control	650 (507 - 722)	106 (19 - 195)	104 (-6 - 208)	105 (-60 - 355)	192 (-34 - 288)	121 (39 - 604)
	Sepsis 4 mL/Kg/h	659 (571 - 760)	-75 (-114 - 167)	2 (-168 - 106)	168 (-280 - NA)	55 (-92 - NA)	66 (-37 - NA)
	Sepsis 10 mL/Kg/h	664 (529 - 806)	-66(-226 - 139)	-54 (-236 - 135)	-45 (-313 - 193)	-257(-310 - 430)	-105 (-128 - 353)
	Sepsis 17 mL/Kg/h	561 (499 - 768	-76 (-231 - 128)	-140 (-391 - -41) *	-117 (-356 - 167)	-32 (-210 - 105)	99 (-17 - 244)
	p value	0.850	0.257	0.031	0.334	0.254	0.487
PVR (AU)	Control	97 (75 - 121))	-12 (-28 - 26)	2 (-28 - 23)	-6 (-30 - 59)	14 (-9 - 76)	8 (-4 - 132)
	Sepsis 4 mL/Kg/h	74 (61 - 82)	177 (75- 267) *	270 (198 - 427) *	284 (143 - NA)	256 (190 - NA)	312 (309 - NA)
	Sepsis 10 mL/Kg/h	69 (45 - 78)	80 (59 - 135) *	247 (117 - 308) *	301 (188 - 362) *	282 (90 - 392)	240 (98 - 378)
	Sepsis 17 mL/Kg/h	68 (59 - 91)	81 (54 - 128) *	134 (60 - 181)* †	141 (72 - 231) *	231 (74 - 293) *	218 (121 - 307)
	p value	0.063	<0.001	<0.001	<0.001	0.006	0.008
Q_{TOT} (mL/kg/min)	Control	126 (107 - 165)	-12 (-33 - 6)	-24 (-35 - 6)	-33 (-67 - 8)	- 32 (-58 - -18)	-39 (-71 - -20)
	Sepsis 4 mL/Kg/h	121 (107 - 162)	-31 (-54 - -19)	-29 (-100 - 10)	-55 (-59 - NA)	-38 (-62 - NA)	-50 (-69 - NA)
	Sepsis 10 mL/Kg/h	135 (107 - 153)	-2 (-10 - 9)	-23 (-37 - -12)	-57 (-71 - -28)	-67 (-128 - 27)	-66 (-130 - 4))
	Sepsis 17 mL/Kg/h	162 (128 - 192)	10 (-60 - 26)	-23 (-62 - -2)	-33 (-85 - -6)	-61 (-92 - -6)	-70 (-91 - -27)
	p value	0.411	0.193	0.792	0.755	0.718	0.746
SV (mL/kg/min/beat)	Control	1.34 (0.79 - 1.58)	-0.05 (-0.29 - 0.10)	-0.16 (-0.21 - 0.10)	-0.14 (-0.27 - -0.03)	-0.21 (-0.53 - 0.03)	-0.25 (-0.53 - 0.08)
	Sepsis 4 mL/Kg/h	1.60 (1.42 - 1.78)	-0.40 (-0.97 - -0.18)	-0.71 (-1.26 - -0.25)	-0.71 (-1.27 - NA)	-0.56 (-1,31 - NA)	-0.95 (-1.32 - NA)
	Sepsis 10 mL/Kg/h	1.53 (1.24 - 1.76)	-0.17 (-0.53 - 0.13)	-0.54 (-0.83 - -0.30) *	-0.81 (-1.05 - -0.38) *	-0.42 (-1.74 - 0.03)	-0.45 (-1.65 - -0.05)
	Sepsis 17 mL/Kg/h	1.74 (1.49 - 2.04)	-0.43 (-0.49 - -0.29) *	-0.49 (-0.62 - -0.26) *	-0.79 (-1.00 - -0.44) *	-0.83 (-1.01 - -0.69) *	-0.86 (-1.29 - -0.85) *
	p value	0.173	0.024	0.005	0.012	0.006	0.015

Control, non septic animals. Sepsis groups were resuscitated with 4 mL/Kg/h. 10 mL/Kg/h or 17 mL/Kg/h saline 0.9%. HR, heart rate. MAP, mean arterial pressure. RAP, right atrial pressure. MPAP, mean pulmonary arterial pressure. PAOP, pulmonary artery occlusion pressure. SVR, systemic vascular resistance. PVR, pulmonary vascular resistance. Q_{TOT}, systemic blood flow. VS, stroke volume. p values indicate the overall significance for between group differences (Kruskal Wallis test). * $p < 0.05$ versus control. † $p < 0.05$ versus sepsis 4 mL/Kg/h group (Mann Whitney U test with Holm´s correction for multiple comparisons). There were no significant differences between the sepsis 10 mL/kg/h and the sepsis 17 mL/kg/h groups in any of the parameters compared. Values are medians and 25th and 75th percentiles.

Table S2. Effect of sepsis and resuscitation on biochemical variables.

		Baseline	Absolute change versus baseline		
	Groups	t=0 h	t=1 h	t=3 h	t=5 h
Sample size	Control	15	15	15	15
	Sepsis 4 mL/Kg/h	8	8	3	3
	Sepsis 10 mL/Kg/h	8	8	8	5
	Sepsis 17 mL/Kg/h	8	8	8	7
Creatinine (mg/dL) [a]	Control	0.90 (0.80 - 1.00)	0.00 (0.00 - 0.10)	0.00 (-0.10 - 0.00)	0.00 (-0.10 - 0.01)
	Sepsis 4 mL/Kg/h	1.00 (0.75 - 1.10)	0.20 (0.06 - 0.38)	0.40 (0.10 - NA)	0.50 (0.10 - NA) *
	Sepsis 10 mL/kg/h	0.95 (0.83 - 1.10)	0.00 (-0.10 - 0.18)	-0.09 (-0.35 - 0.28)	0.00 (-0.20 - 0.70)
	Sepsis 17 mL/Kg/h	0.90 (0.80 - 0.98)	0.05 (-0.90 - 0.10)	0.05 (-0.30 - 0.10)	0.20 (0.00- 0.50)
	p value	0.380	0.182	0.107	0.042
Arterial lactate (mmol/L) [b]	Control	0.9 (0.7 - 1.4)	0.0 (0.0 - 0.0)	0.0 (-0.5 - 0.2)	-0.2 (-0.5 - 0.2)
	Sepsis 4 mL/Kg/h	1.0 (0.8 - 1.1)	0.7 (0.0 - 1.7)	0.8 (-0.5 - NA)	0.4 (-0.2 - NA)
	Sepsis 10 mL/kg/h	1.2 (0.9 - 1.5)	0.0 (-0.1 - 1.4)	1.4 (0.1 - 1.6)	3.2 (-0.5 - 3.6) *
	Sepsis 17 mL/Kg/h	1.1 (1.0 - 1.5)	0.0 (0.0 - 0.4)	0.4 (-0.4 - 1.3)	0.1 (-0.1 - 1.1)
	p value	0.500	0.050	0.036	0.009
Arterial pH [b]	Control	7.410 (7.410 - 7.530)	0.000 (0.000 - 0.000)	0.020 (-0.010 - 0.070)	0.030 (-0.030 - 0.100)
	Sepsis 4 mL/Kg/h	7.480 (7.450 - 7.498)	0.000 (-0.140 - 0.000)	-0.180 (-0.300 - NA)	-0.260 (-0.300 - NA)
	Sepsis 10 mL/kg/h	7.460 (7.440 - 7.503)	-0.005 (-0.058 - 0.000)	-0.070 (-0.120 - -0.023) *	-0.150 (-0.215 - -0.100) *
	Sepsis 17 mL/Kg/h	7.410 (7.335 - 7.510)	0.000 (-0.063 - 0.000)	-0.010 (-0.095 - 0.018)	-0.070 (-0.190 - -0.010) *
	p value	0.315	0.125	0.026	0.001
PaCO2 (mm Hg) [b]	Control	37.0 (34.0 - 45.0)	-3.0 (-6.0 - 2.0)	-4.0 (-9.0 - 5.0)	-5.0 (-8-0 - 4.0)
	Sepsis 4 mL/Kg/h	36.0 (33.0 - 38.0)	1.0 (-4.0 - 3.5)	-4.0 (-7.0 - NA)	-1.0 (-5.0 - NA)
	Sepsis 10 mL/kg/h	40.0 (33.0 - 46.0)	-2.0 (-3.0 - 1.3)	2.5 (-4.3 - 3.8)	2.0 (1.5 - 7.5)
	Sepsis 17 mL/Kg/h	38.0 (34.0 - 43.0)	-0.5 (-2.0 - 2.5)	2.5 (-0.3 - 3.0)	4.0 (-1.0 - 6.0)
	p value	0.489	0.330	0.466	0.193
PaO2 (mm Hg) [b]	Control	252 (206 - 320)	0 (-5 - 0)	-30 (-50- -2)	-15 (-69 - 12)
	Sepsis 4 mL/Kg/h	341 (226 - 365)	-102 (-246 - -42)	-101 (-160 - NA) *	-111 (-166 - NA)
	Sepsis 10 mL/kg/h	289 (225 - 337)	-18 (-88 - 0)	-118 (-165 - -93) *	-136 (-196 - -54)
	Sepsis 17 mL/Kg/h	262 (223 - 334)	0 (-130 - 0)	-73 (-255 - 42)	-26 (-219 - 8)
	p value	0.588	0.062	0.009	0.212

Legends as in Table S1. a, serum concentration. b, measurements form an arterial blood sample.

Table S3. Sample size for cytokine and miRNA determinations in the different groups.

	Control	Sepsis LoR	Sepsis HiR
Cytokines			
IL-1β (serum)	6	5	10
TNFα (serum)	6	5	9
IL-6 (serum)	6	6	9
IL-1β (renal cortex)	6	6	8
IL-1β (renal medulla)	6	6	10
miRNAs			
miR-155-5p	6	4	5
miR-146a-5p	6	4	5
miR-150-5p	6	3	5
miR-21a-5p	6	3	3
miR-34a-5p	6	3	4
miR-133a-3p	5	3	5
miR-486a-5p	6	3	5

Table S4. Effect of sepsis and resuscitation on cytokine concentration.

Cytokines (pg/mL)	Control (n=6)	Sepsis LoR (n=4)	Sepsis HiR (n=5)	p value ‡
IL-1β (serum)	0	1179.4(492.6 to 2046.6) *	439.7(194.1 to 1223.2) *	0.001
TNFα (serum)	6.8 (5.8-12.9)	374.8 (328.5-1535.7) *	152.8 (120.2-161.6) * †	0.001
IL-6 (serum)	20.5 (8.06-25.1)	150.7 (96.6-284.9) *	69.06 (50.7-117.4) *	0.001
IL-1β (renal cortex)	366.5 (268.5-589.2)	939.7 (778.3-1008.8)	801.5 (723.4-952.9) *	0.007
IL-1β (renal medulla)	148.2 (30.4-361.1)	945.2 (623.2-1208.1) *	589.1 (431.01-869.4) *	0.015

Legends as in Table S3.

Table S5. Effect of sepsis and resuscitation on serum miRNA expression.

miRNA	Control	Sepsis LoR	Sepsis HiR	p value ‡
miR-155-5p	0.06 (0.02 - 1.6)	21.7 (9.8 - 27.7)	4.4 (0.04 - 10.2)	0.051
miR-146a-5p	0.01 (0.006 - 0.05)	13.80 (6.80 - 23.80) *	0.60 (0.10 - 0.70) †	0.010
miR-150-5p	0.17 (0.04 - 0.50)	3.80 (1.30 - 105.10)	0.70 (0.08 - 1.04)	0.056
miR-21a-5p	0.20 (0.01 - 0.50)	1.40 (1.09 - 65.50)	0.4 (0.2 - 6.20)	0.098
miR-34a-5p	0.7 (0.1 - 0.46)	2.05 (1.7 - 4.1)	2.40 (1.80 - 2.80)	0.016
miR-486a-5p	0.50 (0.10 - 2.40)	0.50 (0.30 - 0.80)	1.10 (0.40 - 3.30)	0.877
miR-133a-3p	2.10 (1.01 - 4.60)	1.50 (0.10 - 2.80)	4.4 (0.9 - 11.9)	0.891

Legends as in Table S3.‡ Kruskal Wallis test. (*) $p<0.05$ versus control. (†) $p<0.05$ versus sepsis-LoR (Mann Whitney U test with Holm´s correction for multiple comparisons). Values are medians and 25th and 75th percentiles. Units are counts per million copies.

Table S6. MiRNAs differentially expressed in control versus septic animals: miRNA146-5p and miRNA34a-5p.

miR-146a-5p	**p value (uncorrected) ***	**p value (corrected) †**
Control - Sepsis LoR	0.019	0.048
Control - Sepsis HiR	0.082	0.082
Sepsis LoR - Sepsis HiR	0.016	0.048
miR-34a-5p		
Control - Sepsis LoR	0.024	0.072
Control - Sepsis HiR	0.024	0.072
Sepsis LoR - Sepsis HiR	1.000	1.000

Legends as in Table S3. * Mann Whitney U test. † Mann Whitney U test with Holm´s correction for multiple comparisons.

Table S7. Pathways targeted by both miRNA34a-5p and miR-146a-5p (KEGG enrichment analysis) (adjusted p<0.01).					
Pathway	**Total**	**Expected**	**Hits**	**Pval**	**Adjusted p value**
Toxoplasmosis	93	783	24	3.77e-7	3,77E-05
Pancreatic cancer	69	581	18	0.00000947	3,866667E-04
Toll-like receptor signaling pathway	97	817	22	0.0000116	3,866667E-04
Pathways in cancer	310	261	48	0.0000159	3,975E-04
Prostate cancer	87	732	20	0.0000236	4,72E-04
Chronic myeloid leukemia	73	615	17	0.0000815	1,358333E-03
HTLV-I infection	199	168	33	0.0000956	1,365714E-03
Colorectal cancer	49	413	13	0.000139	1,7375E-03
Endometrial cancer	44	37	12	0.000188	2,088889E-03
Melanoma	68	572	15	0.000402	4,02E-03
Influenza A	107	901	20	0.000488	4,125E-03
TGF-beta signaling pathway	84	707	17	0.000495	4,125E-03
MAPK signaling pathway	265	223	38	0.000635	4,884615E-03
Acute myeloid leukemia	57	48	13	0.000693	4,95E-03
Glioma	65	547	14	0.000805	5,16875E-03
Small cell lung cancer	80	674	16	0.000827	5,16875E-03
Non-small cell lung cancer	52	438	12	0.000991	5,829412E-03
Focal adhesion	200	168	30	0.00114	6,052632E-03
T cell receptor signaling pathway	98	825	18	0.00115	6,052632E-03
Apoptosis	83	699	16	0.00125	6,142857E-03
Epstein-Barr virus infection	91	766	17	0.00129	6,142857E-03
Cell cycle	124	104	21	0.00137	6,227273E-03
Measles	102	859	18	0.00184	8E+00
Bladder cancer	29	244	8	0.00207	8,625E-03

Table S8. Proteins in our network participating in the Toll-like receptor and in the toxoplasmosis pathways. In red, proteins common to both pathways.	
Toll-like receptor pathway	**Toxoplasmosis pathway**
AKT2	CASP8
BIRC3	CD40
CASP8	CD86
CD40	CXCL10
CD40LG	CXCL8
CYCS	FOS
GNAI2	IFNB1
HSPA1A	IL6
HSPA2	IRAK1
IRAK1	IRF3
ITGA6	IRF7
LAMB3	JUN
LAMC2	MAP2K2
LAMC3	MAP2K7
MAP3K7	MAP3K7
MAPK3	MAPK3
NFKB1	NFKB1
PIK3CA	PIK3CA
PIK3CG	PIK3CG
PIK3R2	PIK3R2
SOCS1	STAT1
STAT1	TAB2
TAB2	
TNFRSF1A	

Table S9. Enrichment analysis (KEGG) of proteins in the Toll-like receptor pathway (STRING) (adjusted p value<0.0000001).					
#term ID	term description	observed gene count	background gene count	strength	false discovery rate
hsa04620	Toll-like receptor signaling pathway	21	102	226	3,24E-42
hsa05167	Kaposi's sarcoma-associated herpesvirus infection	17	183	192	1,41E-26
hsa05161	Hepatitis B	14	142	194	3,61E-21
hsa05164	Influenza A	14	168	187	2,53E-20
hsa04668	TNF signaling pathway	12	108	199	3,06E-18
hsa05142	Chagas disease (American trypanosomiasis)	11	101	199	2,26E-16
hsa04621	NOD-like receptor signaling pathway	12	166	181	2,92E-16
hsa04380	Osteoclast differentiation	11	124	19	1,43E-15
hsa05162	Measles	11	133	187	2,64E-15
hsa04657	IL-17 signaling pathway	10	92	199	8,98E-15
hsa05168	Herpes simplex infection	11	181	173	5,51E-14
hsa04660	T cell receptor signaling pathway	9	99	191	2,05E-12
hsa05145	Toxoplasmosis	9	109	187	4,31E-12
hsa05169	Epstein-Barr virus infection	10	194	166	7,82E-12
hsa04622	RIG-I-like receptor signaling pathway	8	70	201	1,4E-11
hsa05140	Leishmaniasis	8	70	201	1,4E-11
hsa05160	Hepatitis C	9	131	179	1,59E-11
hsa05133	Pertussis	8	74	198	1,77E-11
hsa04933	AGE-RAGE signaling pathway in diabetic complications	8	98	186	1,39E-10
hsa04722	Neurotrophin signaling pathway	8	116	179	4,78E-10
hsa04926	Relaxin signaling pathway	8	130	174	1,09E-09
hsa04917	Prolactin signaling pathway	7	69	196	1,16E-09

hsa04210	Apoptosis	8	135	172	1,33E-09
hsa04662	B cell receptor signaling pathway	7	71	194	1,33E-09
hsa05200	Pathways in cancer	11	515	128	1,6E-09
hsa04010	MAPK signaling pathway	9	293	144	1,1E-08
hsa05203	Viral carcinogenesis	8	183	159	1,17E-08
hsa05165	Human papillomavirus infection	9	317	14	2,02E-08
hsa04623	Cytosolic DNA-sensing pathway	6	62	193	5,4E-08
hsa05418	Fluid shear stress and atherosclerosis	7	133	167	6,68E-08

Table S10. Enrichment analysis (Reactome) of proteins in the Toll-like receptor pathway (STRING) (adjusted p value<0.001).					
#term ID	term description	observed gene count	background gene count	strength	false discovery rate
HSA-1280215	Cytokine Signaling in Immune system	5	328	113	2,6E-04
HSA-1059683	Interleukin-6 signaling	2	11	221	4,2E-04
HSA-109582	Hemostasis	5	591	88	9,7E-04
HSA-1168372	Downstream signaling events of B Cell Receptor (BCR)	2	22	191	9,7E-04
HSA-1257604	PIP3 activates AKT signaling	3	118	135	9,7E-04

Table S11. Enrichment analysis (Gene-Ontology Biological Process) of proteins in the Toll-like receptor pathway (STRING) (adjusted p value<0.0000001).					
#term ID	term description	observed gene count	background gene count	strength	false discovery rate
GO:0002684	positive regulation of immune system process	19	882	128	7,37E-18
GO:0034097	response to cytokine	19	1035	121	7,28E-17
GO:0019221	cytokine-mediated signaling pathway	16	655	134	1,04E-14
GO:0050776	regulation of immune response	17	873	124	1,04E-14
GO:0009967	positive regulation of signal transduction	19	1493	105	2,26E-14
GO:0048584	positive regulation of response to stimulus	20	2054	94	1,01E-13
GO:0002764	immune response-regulating signaling pathway	13	365	15	2,99E-13
GO:0071310	cellular response to organic substance	20	2219	9	3,76E-13
GO:0010033	response to organic substance	21	2815	82	5,9E-13
GO:1902533	positive regulation of intracellular signal transduction	16	959	117	8,49E-13
GO:0051173	positive regulation of nitrogen compound metabolic process	21	2946	8	1,21E-12
GO:0050778	positive regulation of immune response	14	589	133	1,58E-12
GO:0009966	regulation of signal transduction	21	3033	79	1,96E-12
GO:0031325	positive regulation of cellular metabolic process	21	3060	79	2,22E-12
GO:0010604	positive regulation of macromolecule metabolic process	21	3081	78	2,43E-12
GO:0048583	regulation of response to stimulus	22	3882	7	3,1E-12
GO:1902531	regulation of intracellular signal transduction	18	1764	96	6,88E-12
GO:0032103	positive regulation of response to external stimulus	13	499	136	6,99E-12
GO:0007166	cell surface receptor signaling pathway	19	2198	89	7,22E-12
GO:0051707	response to other organism	16	1173	108	1,01E-11
GO:0035556	intracellular signal transduction	17	1528	10	1,58E-11
GO:0002376	immune system process	19	2370	85	2,28E-11
GO:0038095	Fc-epsilon receptor signaling pathway	8	64	205	3,47E-11
GO:0032496	response to lipopolysaccharide	11	298	152	3,75E-11
GO:0007165	signal transduction	22	4738	62	1,37E-10
GO:1901701	cellular response to oxygen-containing compound	14	896	114	2,02E-10
GO:0006950	response to stress	20	3267	74	2,15E-10

GO:0048522	positive regulation of cellular process	22	4898	6	2,61E-10
GO:0031349	positive regulation of defense response	11	365	143	2,76E-10
GO:0032101	regulation of response to external stimulus	14	955	112	4,32E-10
GO:0051704	multi-organism process	18	2514	8	1,56E-09
GO:0010557	positive regulation of macromolecule biosynthetic process	16	1758	91	3,02E-09
GO:0045935	positive regulation of nucleobase-containing compound metabolic process	16	1770	91	3,28E-09
GO:1901700	response to oxygen-containing compound	15	1427	97	3,36E-09
GO:0043900	regulation of multi-organism process	12	653	121	3,37E-09
GO:0045893	positive regulation of transcription, DNA-templated	15	1435	97	3,49E-09
GO:0051246	regulation of protein metabolic process	18	2668	78	3,72E-09
GO:0002757	immune response-activating signal transduction	10	332	143	3,85E-09
GO:0031347	regulation of defense response	12	676	12	4,58E-09
GO:0031328	positive regulation of cellular biosynthetic process	16	1846	89	5,36E-09
GO:0051239	regulation of multicellular organismal process	18	2788	76	7,04E-09
GO:0002753	cytoplasmic pattern recognition receptor signaling pathway	6	32	222	7,06E-09
GO:0006952	defense response	14	1234	10	9,02E-09
GO:0071222	cellular response to lipopolysaccharide	8	146	169	9,41E-09
GO:0001819	positive regulation of cytokine production	10	390	136	1,49E-08
GO:0043902	positive regulation of multi-organism process	10	394	135	1,57E-08
GO:0080134	regulation of response to stress	14	1299	98	1,59E-08
GO:0002768	immune response-regulating cell surface receptor signaling pathway	9	266	148	1,86E-08
GO:0002833	positive regulation of response to biotic stimulus	9	277	146	2,61E-08
GO:0001932	regulation of protein phosphorylation	14	1370	96	3,07E-08
GO:0002831	regulation of response to biotic stimulus	10	426	132	3,07E-08
GO:0001817	regulation of cytokine production	11	615	12	3,63E-08
GO:0014070	response to organic cyclic compound	12	873	109	6,07E-08

Table S12. Enrichment analysis (KEGG) of proteins in the toxoplasmosis pathway (STRING) (adjusted p value<0.0000001).					
#term ID	term description	observed gene count	background gene count	strength	false disco-very rate
hsa05145	Toxoplasmosis	22	109	222	1,01E-42
hsa04620	Toll-like receptor signaling pathway	11	102	194	2,78E-15
hsa05222	Small cell lung cancer	10	92	195	9,51E-14
hsa04668	TNF signaling pathway	10	108	188	3,23E-13
hsa05200	Pathways in cancer	14	515	135	4,7E-13
hsa04380	Osteoclast differentiation	10	124	182	7,97E-13
hsa05162	Measles	10	133	179	1,33E-12
hsa05165	Human papillomavirus infection	12	317	149	1,93E-12
hsa05142	Chagas disease (American trypanosomiasis)	9	101	186	8,93E-12
hsa05164	Influenza A	10	168	169	8,93E-12
hsa05167	Kaposi's sarcoma-associated herpesvirus infection	10	183	165	1,79E-11
hsa05169	Epstein-Barr virus infection	10	194	162	2,87E-11
hsa04210	Apoptosis	9	135	174	7,4E-11
hsa04064	NF-kappa B signaling pathway	8	93	185	3,07E-10
hsa04510	Focal adhesion	9	197	157	1,67E-09
hsa04917	Prolactin signaling pathway	7	69	192	3,39E-09
hsa01524	Platinum drug resistance	7	70	191	3,51E-09
hsa04151	PI3K-Akt signaling pathway	10	348	137	5,28E-09
hsa05161	Hepatitis B	8	142	166	5,67E-09
hsa05152	Tuberculosis	8	172	158	2,34E-08
hsa04660	T cell receptor signaling pathway	7	99	176	2,82E-08
hsa04010	MAPK signaling pathway	9	293	14	3,52E-08
hsa04071	Sphingolipid signaling pathway	7	116	169	7,4E-08

Table S13. Enrichment analysis (Reactome) of proteins in the toxoplasmosis pathway (STRING) (adjusted p value<0.001).					
#term ID	term description	observed gene count	background gene count	strength	false discovery rate
HSA-109582	Hemostasis	6	591	92	7,9E-04

Table S14. Enrichment analysis (Gene-Ontology Biological Process) of proteins in the toxoplasmosis pathway (STRING) (adjusted p value<0.0000001).					
#term ID	term description	observed gene count	background gene count	strength	false discovery rate
GO:0035556	intracellular signal transduction	18	1528	98	1,99E-10
GO:0050790	regulation of catalytic activity	19	2249	84	2,57E-09
GO:0070887	cellular response to chemical stimulus	20	2672	79	2,57E-09
GO:0071310	cellular response to organic substance	19	2219	84	2,57E-09
GO:0010033	response to organic substance	20	2815	76	3,29E-09
GO:0032268	regulation of cellular protein metabolic process	19	2486	79	5,33E-09
GO:0043085	positive regulation of catalytic activity	16	1381	98	5,33E-09
GO:0044093	positive regulation of molecular function	17	1713	91	5,33E-09
GO:0050776	regulation of immune response	14	873	112	5,33E-09
GO:0051173	positive regulation of nitrogen compound metabolic process	20	2946	74	5,33E-09
GO:0031325	positive regulation of cellular metabolic process	20	3060	73	7,47E-09
GO:0010604	positive regulation of macromolecule metabolic process	20	3081	72	7,81E-09
GO:0032270	positive regulation of cellular protein metabolic process	16	1496	94	8,37E-09
GO:1902533	positive regulation of intracellular signal transduction	14	959	108	8,37E-09
GO:0019221	cytokine-mediated signaling pathway	12	655	117	4,15E-08
GO:0002684	positive regulation of immune system process	13	882	108	5,2E-08
GO:0031347	regulation of defense response	12	676	116	5,36E-08

GO:0031399	regulation of protein modification process	16	1747	87	5,51E-08
GO:1902531	regulation of intracellular signal transduction	16	1764	87	6,09E-08
GO:0042221	response to chemical	21	4153	62	6,2E-08
GO:0042325	regulation of phosphorylation	15	1465	92	7,21E-08
GO:0048522	positive regulation of cellular process	22	4898	56	7,21E-08
GO:0007166	cell surface receptor signaling pathway	17	2198	8	8,46E-08
GO:0009967	positive regulation of signal transduction	15	1493	91	8,46E-08
GO:0042981	regulation of apoptotic process	15	1501	91	8,73E-08

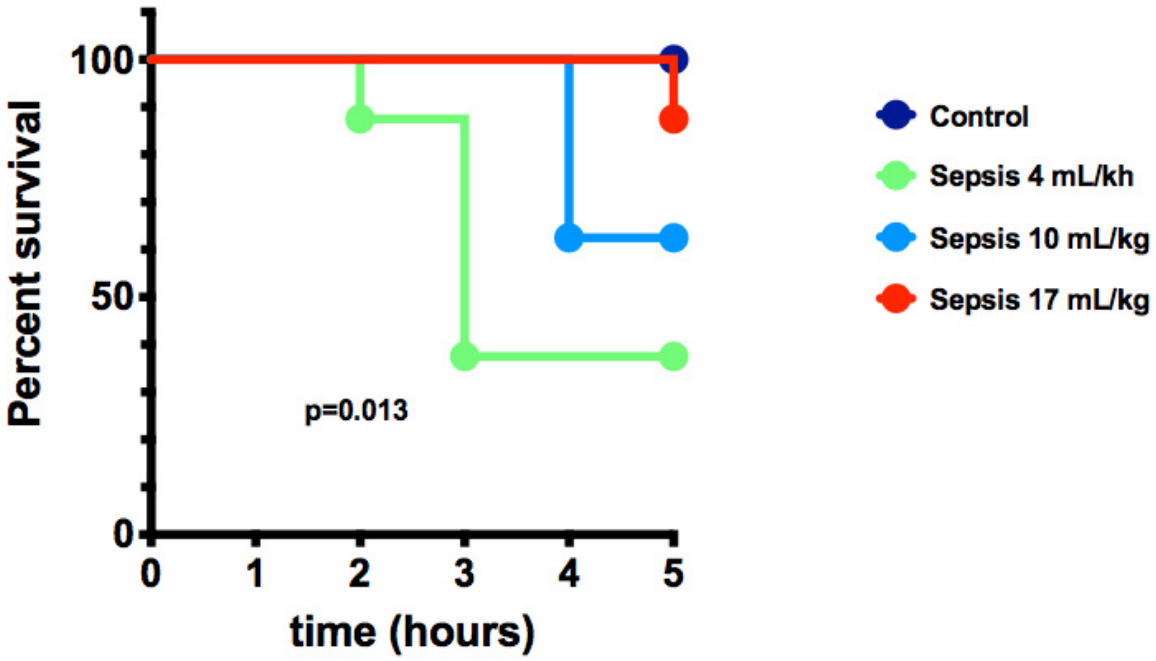

Figure S1. Mortality of the different experimental groups (control, n=15; sepsis 4 ml/kg/h, n=8; sepsis 10 ml/kg/h, sepsis 17 ml/kg/h).

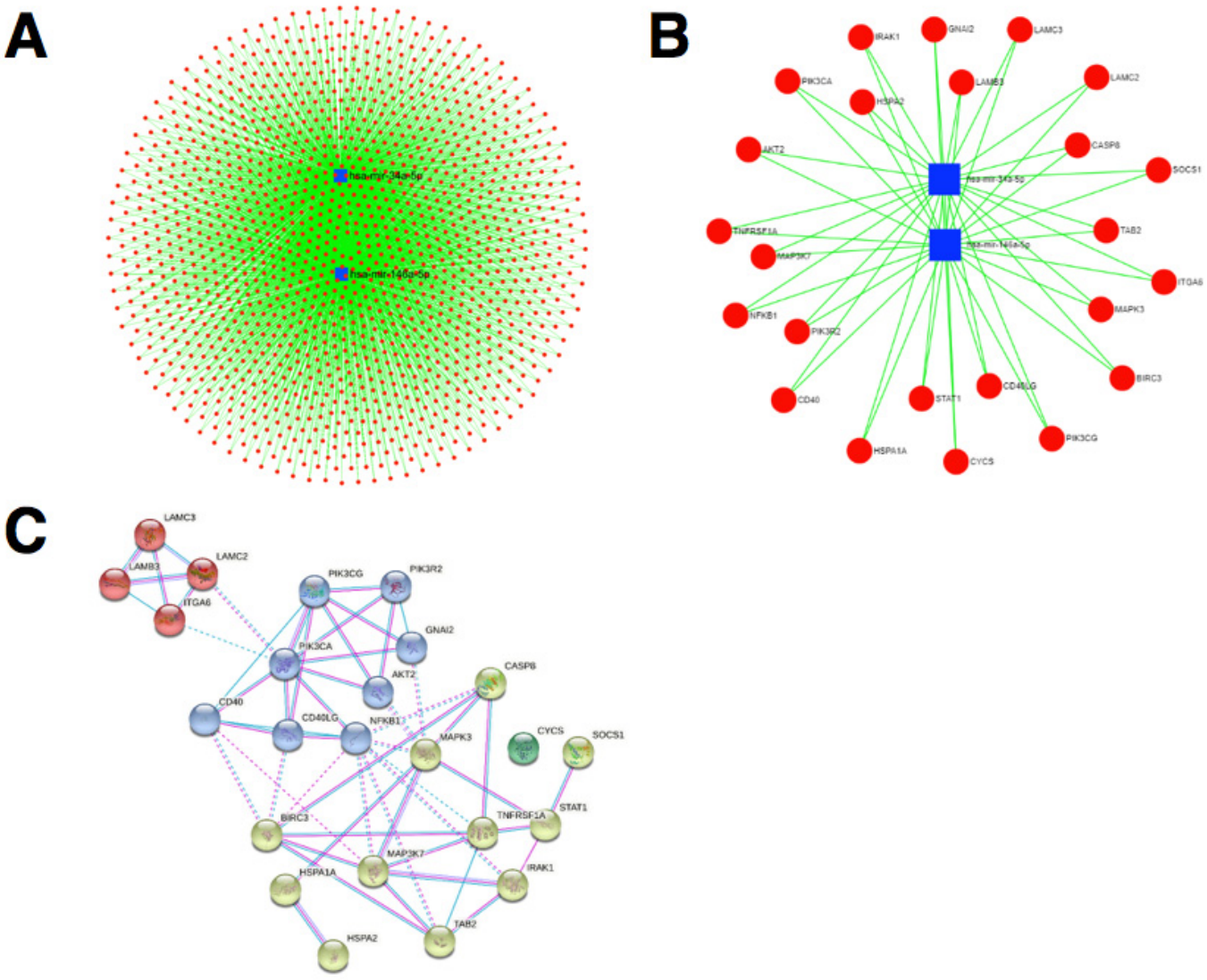

Figure S2. **Panel A**: Genes targeted by both miRNAs of interest (miRNA146a-5p and miRNA34a-5p) (miRNet); 1386 genes were identified as regulated by both miRNAs. **Panel B**: Toxoplasmosis as the most highly significant pathway regulated by the miRNAs of interest (KEGG enrichment analysis), with 24 hits (adjusted p value = 3.77×10^{-5}) of which 10 were common with the Toll-like receptor signaling pathway. **Panel C**: Protein-protein interaction of the 24 proteins (STRING, confidence 0.9, in 4 clusters). PPI enrichment p-value: < 1.0×10^{-16}. Purple line, experimental evidence. Light blue line, database evidence. Dotted lines, interactions appearing only after clustering.