MicroRNA Fingerprints Identify miR-150 as a Plasma Prognostic Marker in Patients with Sepsis

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Abstract

Background: The physiopathology of sepsis continues to be poorly understood, and despite recent advances in its management, sepsis is still a life-threatening condition with a poor outcome. If new diagnostic markers related to sepsis pathogenesis will be identified, new specific therapies might be developed and mortality reduced. Small regulatory non-coding RNAs, microRNAs (miRNAs), were recently linked to various diseases; the aim of our prospective study was to identify miRNAs that can differentiate patients with early-stage sepsis from healthy controls and to determine if miRNA levels correlate with the severity assessed by the Sequential Organ Failure Assessment (SOFA) score.

Methodology/Principal Findings: By using genome-wide miRNA profiling by microarray in peripheral blood leukocytes, we found that miR-150, miR-182, miR-342-5p, and miR-486 expression profiles differentiated sepsis patients from healthy controls. We also proved by quantitative reverse transcription-polymerase chain reaction that miR-150 levels were significantly reduced in plasma samples of sepsis patients and correlated with the level of disease severity measured by the SOFA score, but were independent of the white blood counts (WBC). We found that plasma levels of tumor necrosis factor alpha, interleukin-10, and interleukin-18, all genes with sequence complementarity to miR-150, were negatively correlated with the plasma levels of this miRNA. Furthermore, we identified that the plasma levels ratio for miR-150/interleukin-18 can be used for assessing the severity of the sepsis.

Conclusions/Significance: We propose that miR-150 levels in both leukocytes and plasma correlate with the aggressiveness of sepsis and can be used as a marker of early sepsis. Furthermore, we envision miR-150 restoration as a future therapeutic option in sepsis patients.

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Introduction

Sepsis represents a serious medical condition characterized by a whole-body inflammatory state called systemic inflammatory response syndrome, which is caused by a suspected or proven severe infection [1–3]. Severe sepsis occurs when sepsis leads to dysfunction of at least one organ or system due to hypoperfusion or hypotension, while septic shock is associated with refractory arterial hypotension despite aggressive fluid resuscitation and with multiple organ dysfunction syndrome. The criteria for diagnosis of sepsis and severity were established by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus in 1992 and remain valid [2]. Despite recent advances in management, sepsis is still a life-threatening condition with a poor outcome and is the major cause of death among critically ill patients in intensive care units (ICUs) [3]. The physiopathology of sepsis continues to be poorly understood and, consequently, only a few specific therapies are available to treat this condition. Several scoring systems are used, including the sequential organ failure assessment (SOFA) score, but these systems evaluate clinical parameters related to the associated multiple organ dysfunction syndrome and do not include any factor involved in pathogenesis of the sepsis itself. Intrabdominal sepsis after abdominal surgery is responsible for approximately 13% of all ICU admissions [4,5]. Studies have suggested that clearance of intraperitoneal sepsis may be beneficial when patients develop signs of intraabdominal sepsis.
and 7 after admission); for one patient, we collected a sample only remaining 7 sepsis patients and 20 controls. For seven of the sepsis patients and controls were white Caucasians (according to medical infection or known medical condition at the time of the study. All study. ''Healthy'' was defined as the absence of any type of infections (n = 9) (Table 1). Peripheral blood leukocytes were obtained from 5 ml blood, and total RNA was purified using Trizol (Invitrogen, Carlsbad, CA). RNA quality was assessed by Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA), and only RNA with an RNA integrity number (a measure of RNA quality) higher than 7 was used for the expression-profiling study. Total RNA in plasma was isolated using Total RNA purification Kit (Norgen Biotek Corporation, Ontario, Canada) according to the manufacturer’s instructions.

Ethics Statement
For de-identification, the samples were codified S (for sepsis) and C (for controls) followed by a codified number to protect the privacy of individuals during all the further molecular study. All participants gave informed written consent to participate in this study and the samples were processed under approval of the Fundeni Hospital Ethics Committee.

Genome-wide Human miRNA Expression Detection
Sixteen RNA samples from leukocyte (8 control, 8 sepsis) were hybridized on a human miRNA microarray (G4470A, Agilent Technologies). This microarray consisted of 60-mer DNA probes for 470 human miRNAs, sourced from the Sanger miRBase public database (Release 9.1). One-color miRNA expression was performed according to the manufacturer’s procedure. Briefly, total RNA was obtained from samples by using the Trizol reagent (Invitrogen). Labeled miRNAs were obtained from 500 ng of total RNA through the ligation of a 5'-cytidine bisphosphate-Cy3 (pCp-Cy3, Agilent Technologies) group at the 3'-end of each miRNA. To enhance the T4 RNA-ligase (Promega, Madison, WI) efficiency, we had previously treated total RNA with alkaline phosphatase (Amersham, Piscataway, NJ) at 37°C for 30 min. Labeled miRNAs were purified on chromatography columns (Micro Biospin 6, Biorad Laboratories, Hercules, CA) and then hybridized on a microarray. Hybridizations were performed at 55°C for 17 h in a rotating oven. Images at 5-μm resolution were generated by a scanner (Agilent Technologies), and the Feature Extraction 9.5 software (Agilent Technologies) was used to obtain the microarray raw data.

Microarray Data Analysis
Microarray results were independently analyzed in two distinct ways by MF in Ferrara, Italy and by SR in Houston, TX, respectively. First, using GeneSpring GX software version 7.3 (Agilent Technologies), we preprocessed data files with the plug-in for the Agilent Feature Extraction software results. Data transformation was applied to set all negative raw values at 5.0, followed by on-chip and on-gene median normalization. We filtered data for low gene expression so that only probes expressed (flagged as “present”) in at least one sample were kept; probes that did not change between samples, i.e., identified as having an expression value across all samples between median±1.5, were removed. Next, samples were grouped according to their status and then compared. Differentially expressed genes were selected as having a 2-fold expression difference between their geometrical mean in the two groups of interest (sepsis and control) and a statistically significant p-value (p<0.05) by analysis of variance statistics. Independently, a second type of data analysis was performed by using extracted fluorescence intensity values (Agilent Feature Extraction) from all 16 hybridizations. The data were imported into Biometric Research Branch (BRB) array tool version 3.7.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) for subsequent microarray analysis. MiRNAs with less than 20% expression...
qRT-PCR for miRNA Expression

MiRNA levels were detected by qRT-PCR using the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Experiments were performed in triplicate wells. To normalize the expression levels of target genes, we used U6B small nuclear RNA for the experiment performed with RNAs from leukocytes, while miR-192 was used for plasma RNA normalization. The relative expression of each miRNA was calculated as the ratio of the value from sepsis to the value from controls, producing a fold-change value. Data for sepsis/control samples were compared using the two-sided Student t-test (p<0.05). MiRNA Target Prediction

We used two independent and complementary ways to predict miRNA targets. First, we used miRGen at http://www.diana.pcbi.upenn.edu/miRGen.html, which contains animal miRNA targets according to combinations of the widely used target-prediction programs miRanda, TargetScanS, and PicTar and experimentally supported targets from TarBase. Second, we used RNA22 at http://cbcsrv.watson.ibm.com/rna22.html, a pattern-based method for identifying miRNA-target sites and their corresponding RNA/RNA complexes, by using not only the 3' untranslated region of the mRNA but also the full messenger sequence. We used the Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov) to identify the pathway distribution of predicted targets. These pathways were presented according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). This is a database of biological systems, consisting of the genetic building blocks of genes and proteins; the identified pathways are composed by molecular interactions and reaction networks for metabolism, various cellular processes, and human diseases.

Enzyme-Linked Immunosorbent Assay (ELISA)

Due to the availability of material, the levels of human tumor necrosis factor alpha (TNF-alpha, 12 control and 11 sepsis samples), human interleukin-10 (IL-10, 11 control and 20 sepsis samples), and human interleukin-18 (IL-18, 22 control and 18 sepsis samples) were measured by ELISA in undiluted plasma samples. Commercially available ELISA kits for TNF-alpha and IL-18 (R & D Systems, Minneapolis, MN) and IL-10 (BD Biosciences, San Jose, CA) were used according to the manufacturers' instructions. Results were read at an optical density of 450 nm using a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA). Measurements were performed in
duplicate, and \( p \)-values were computed using the two-sided Student t-test (\( p<0.05 \)).

### Statistical Analysis

The t-test on microarray data identified miRNAs that were differentially expressed between sepsis and control patients (\( p<0.01 \), FDR<0.09). We also identified miRNAs and cytokines that were differentially expressed in sepsis and control subjects for qRT-PCR data using t-test (\( p<0.05 \)). Matlab 6.5 (www.mathworks.com) analysis was used for the Pearson’s correlation. All statistical tests were two-sided, and statistical significance was defined as \( p<0.05 \).

### Results

**MiRNA Genome-Wide Profiling in Peripheral Blood Leukocytes Differentiate Sepsis Patients from Healthy Controls**

To determine if miRNA patterns in patients with sepsis are significantly different from those in healthy controls, we first used microarray to compare the expression of 470 human miRNAs in leukocytes from eight patients at day 1 (first day in ICU) with expression profiles from eight healthy controls (Figure 1). As array data analyses could be performed by several methods that could reciprocally confirm each other, independent investigators performed this essential step using two different tools—the BBR array and GeneSpring GX software, respectively (see Materials and Methods). We found that a set of 17 miRNAs correctly differentiated 100% of the samples belonging to the two groups (sepsis and control), meaning that expression of these miRNAs was a good classifier for each category (Figure 1A). In both types of analyses, we identified four miRNAs—miR-150, miR-182, miR-342-5p and miR-486—whose expression levels differed by at least a factor of 2 between sepsis and healthy samples (\( p<0.01 \); FDR<0.08); miR-486 and miR-182 were overexpressed, while miR-150 and miR-342-5p were downregulated in sepsis patients. Of note, miR-153 and miR-125b, the former upregulated, while the latter down-regulated by LPS stimulation of Raw 264.7 mouse macrophages [13], were not statistically significant dysregulated in the analyzed set of patients.

For independent confirmation, we performed a distinct type of quantification, qRT-PCR amplification for the active miRNA and its converse expression profiles from eight healthy controls (\( p<0.01 \) and \( p<0.001 \), respectively) whose expression levels differed by at least a factor of 2 between sepsis and healthy samples (\( p<0.01 \); FDR<0.08); miR-486 and miR-182 were overexpressed, while miR-150 and miR-342-5p were downregulated in sepsis patients. Of note, miR-153 and miR-125b, the former upregulated, while the latter down-regulated by LPS stimulation of Raw 264.7 mouse macrophages [13], were not statistically significant dysregulated in the analyzed set of patients.

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**MiR-150 Levels in Plasma Are Significantly Reduced in Sepsis Patients Compared with Controls and Correlate with the Level of Sepsis Severity**

Recently, miRNA expression in plasma was detected by using qRT-PCR techniques [14]. We focused for the present study on miR-150, as it has the highest levels of dysregulation in sepsis samples versus controls (Figure 1B). Therefore, we investigated the expression of miR-150 in plasma by performing qRT-PCR on 24 sepsis (including 16 at day 1 and 8 at day 7) and 32 control samples. We initially used U6 and U6B for the selection of the normalizer gene, but since these RNAs were degraded in plasma we could not obtain reproducible results in triplicate experiments. Thereafter, we analyzed two miRNAs (miR-192 and let-7a) that, according to the array data, had no variance in expression between sepsis samples and controls. The best reproducible results were obtained with miR-192; therefore, we used the expression of this miRNA as a reference value (Figure 2A). We found that miR-150 was significantly down regulated in plasma from sepsis patients compared with controls at both days 1 and 7 and at highly statistically significant values (\( p = 0.001 \) and \( p = 0.005 \), respectively) (Figure 2A). Therefore, plasma levels of miR-150 (expressed as ratio with miR-192) reproduced the variations in leukocytes, and represent a reliable indicator of early sepsis. Importantly, we did not find any correlation between the plasma levels of miR-150 and the number of leukocytes (expressed as white blood count, WBC) in sepsis patients (Figure S1), meaning that this downregulation is not just a biomarkers for the amount of circulating leukocytes. MiR-192 and miR-342-5p, differentially expressed in leukocytes were studied also in plasma; the trend of variation was as in leukocytes for miR-342-5p and opposite for miR-192, and both of them were not significantly differentially expressed, probably due to the small number of sepsis samples with detectable expression (4 for miR-192 and 3 for miR-342-5p).

In studying the expression of miR-150 in plasma of the sepsis patients (qRT-PCR data), we noticed a correlation between the fold-difference value and SOFA score and/or associated severity of sepsis (grades labeled as sepsis, severe sepsis, and septic shock). Interestingly, by considering an 8.5-fold cutoff value for the expression ratio miR-150/miR-192 (meaning at least three amplification cycles’ difference for the ACT between miR-150 and miR-192) at day 1 (Figure 2B), we found that the 6 patients with higher ratios (15.07 to 97.24) had significantly lower SOFA scores than the 10 patients with lower ratios (0.44 to 8.20) (2.83+/−2.64 versus 7.80+/−5.41 mean ratio ± SD, respectively; \( p=0.028 \)) (Table 1). All three patients with septic shock had levels of miR-150 lower than 8.5. Furthermore the miR-150/miR-192 relative expression was negatively correlated with the SOFA score (Figure 2C) and this ratio decreased from the sepsis to severe sepsis to sepsis shock patients (Figure S2). These data are in agreement with our initial observation that sepsis patients have lower levels of miR-150 than healthy controls; in fact, the patient with the highest miR-150/miR-192 ratio (97.24) had the lowest SOFA score at both days 1 and 7. Further strengthening this conclusion, we did not find any significant differences in miR-150 expression between day 1 and day 7 (43.98+/−29.35 versus 28.08+/−25.32; \( p \) not significant), as the SOFA score was quite similar for this limited set of patients (day 1+/−SD = 3.28+/−2.69 versus 1.86+/−2.61 for day 7; \( p \) not significant). Also, six of seven patients with measurements on both day 1 and day 7 had miR-150/miR-192 ratios concordant with their SOFA scores (Table 1). Therefore, consistently lower levels of miR-150 were found in both leukocyte and plasma samples from sepsis patients than in healthy controls, and lower expression was associated with poor clinical condition as indicated by the SOFA score.

**MiR-150 Expression Profile Is Correlated with Expression of Immune System Genes**

As the next step, we tried to understand if the variations in miR-150 expression were only bystanders for other unknown causal effects or if they were linked to the pathogenesis of this disease. One way to do this was to identify correlations in clinical samples between levels of miR-150 expression and that of important...
protein-coding genes involved in the pathogenesis of sepsis. Dysregulation of miRNA levels would be anticipated to affect the translation of multiple protein-coding genes. Therefore, first we performed target prediction for miR-150 by using miRGen and found that among the predicted targets, at least 20 genes were functionally related to immune system processes; among these was IL-18, which is reportedly increased in patients with sepsis [15–17]. Using the Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov) to identify overrepresented pathways, we found that predicted miR-150 targets were significantly (p<0.05) clustered in a few KEGG pathways, and the five most overrepresented pathways were all
miR-150 as Marker in Sepsis

In the present prospective study we interrogated miRNA expression in a set of sepsis patients and found, by performing genome-wide profiling by microarray in leukocytes followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on plasma samples that miR-150 is significantly downregulated in sepsis patients and that the plasma levels ratio for miR-150/interleukin-18 can be used for assessing the severity of the sepsis.

Discussion

In the present prospective study we interrogated miRNA expression in a set of sepsis patients and found, by performing genome-wide profiling by microarray in leukocytes followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on plasma samples that miR-150 is significantly downregulated in sepsis patients and that the plasma levels ratio for miR-150/interleukin-18 can be used for assessing the severity of the sepsis.
of three miRNAs — miR-150, miR-182, and miR-342-5p — are at least twice as dysregulated in leukocytes from patients than in those from healthy controls. Supporting these findings is a recent in vivo profile of the human leukocyte miRNA response to endotoxemia in which leukocyte RNA was isolated from venous blood samples obtained from three healthy male volunteers before and 4 h after LPS infusion and profiled for miRNA expression [27]. Five miRNAs consistently responded to LPS infusion, four of which were downregulated (miR-146b, miR-150, miR-342, and let-7g) and one of which was upregulated (miR-143) [27]. Also, miR-150 was found to control c-Myb expression in vivo in a dose-dependent manner over a narrow range of miRNA and c-Myb concentrations, and this dramatically affected lymphocyte development and response [28]. These data further strengthen the functional significance of miR-150 downregulation in sepsis patients. Furthermore, abnormal processing of miR-21 transcript was recently reported in ICU patients suffering from sepsis-induced multiple organ failure [29], expanding the spectrum of miRNA alterations in sepsis.

Second, the recent identification of miRNAs in serum and plasma from healthy individuals and individuals with pathologic conditions, such as cancer, opens up the possibility of exploring miRNAs as biomarkers of disease [30]. To our knowledge, this is the first report of miRNA measurement in plasma from sepsis patients. We found not only that miR-150 levels are significantly different in patients and healthy controls, but also that the levels of miR-150 correlate with SOFA scores (but not with WBC). SOFA is a scoring system used to track a patient’s status while in the ICU; it is based on six different scores, one each for the respiratory, cardiovascular, hepatic, coagulation, renal, and neurological systems [30]. We identified a ratio between the quantitative RT-PCR expression of miR-150 and a nonvariable control miR-192 that can be used to assess the severity of sepsis based on its correlation with the SOFA score. Additional candidates for large studies could be miR-182 and miR-342-5p that we found differentially expressed in sepsis versus control leukocytes.

Finally, we revealed a new potential pathogenetic mechanism explaining some of the immune system dysfunctions in sepsis patients. The malfunction of regulatory mechanisms during sepsis can result in a loss of control of inflammation, eventually leading to profound immunosuppression and host damage [31–33]. Our study points to a miRNA regulation of pro- and anti-inflammatory genes involved in sepsis. We found that the expression levels of miR-150 correlated with those of main immune response genes, such as TNF-alpha, IL-10, and IL-18. Furthermore, the putative spectrum of targets of miR-150 is highly enriched in genes involved in immune system functions. Therefore, in addition to miR-155 [34] and miR-125 [35], miR-150 could be one of the main regulatory miRNAs of immune function, and our study unraveled the clinical significance of the miR-150 expression correlation with cytokine expression in patients with sepsis.

In conclusion, although the functions of most human miRNAs have yet to be discovered, miRNAs have emerged as key regulators of gene expression. The present data support the hypothesis that miRNAs are main regulators of the immune system, and abnormal expression has been found and can be used as a diagnostic and prognostic marker in immune disease. Sepsis is the newest addition to the long list of disease states proved by studies in patients to be linked to abnormal miRNA expression. One of the important regulators is miR-150, and this is significantly abnormally expressed in both leukocytes and plasma from sepsis patients. Our study is the first to identify a specific miRNA profile and to interrogate about the clinical significance of miRNA variations in sepsis patients. Due to the limited number of cases originating in the same center and to the bias toward intra-abdominal causes of sepsis, larger multi-institutional studies with higher numbers of patients will establish the final prognostic significance of our initial findings.

Supporting Information

Figure S1 White blood count (WBC) and miR-150/miR-192 relative expression plot. No correlation was found in 23 sepsis samples (the WBC was missing for one sample) after standardization of miR-150 relative expression values and WBC values meaning that miR-150/miR-192 ratio is not just a biomarker for presence or absence of circulating leukocytes in sepsis. The standard values were derived by subtracting the mean of the relative expressions for miR-150 and miR-192 and mean of the WBC, respectively from each individual relative expression value and WBC value, respectively, and then dividing the difference by the standard deviation, calculated for each one of the data series. Found at: doi:10.1371/journal.pone.0007405.s001 (2.61 MB TIF)

Figure S2 miR-150/miR-192 relative expression correlates with sepsis grade. The mean +/- standard deviation of miR-150/miR-192 fold difference related to sepsis grade (labeled as sepsis, SP, severe sepsis, SSP, and septic shock, SoP) is reported. As expected, miR-150 relative expression is higher in low sepsis grade samples
Figure 3. Negative Correlation Between Plasma Levels of miR-150 and Cytokines. (A) The complementary sequence between miR-150 and the mRNAs of TNF-alpha, IL-10, and IL-18 is shown. Complementary sequences are reported, as well as the relative folding energy between miR-150 and mRNAs by using RNA22. (B) ELISA determination for plasma markers of sepsis and correlation with miR-150 expression in plasma are depicted in the graphs. The left panels show IL-10, IL-18 and TNF-alpha measurements by ELISA in plasma, mean Ct +/- standard deviation have been reported. The right panels show the negative correlation between miR-150 and IL-10, IL-18 and TNF-alpha, respectively. Values, on a patient by patient basis, have been reported for each cytokine studied. doi:10.1371/journal.pone.0007405.g003
### Table 3. Cytokine measurements from Septic Shock cases and Controls.

| Cytokine | Control Subjects | Patients with Sepsis | P value |
|----------|------------------|----------------------|---------|
| IL-10    | 6.55, 21 < DL    | 33.39 (5.79–112.1)   | 6E-04   |
| IL-18    | 162.05 (15.23–532.60) | 672.08 (107.55–2201.15) | 6.8E-04 |
| TNF-alpha| 24.69 (11.05–31.85) | 34.51 (26.01–44.16)  | 0.026   |

Definition of abbreviations: < DL = less than the detectable limit of 4 pg/ml for TNF-alpha or 5 pg/ml for IL-10 and IL-18. Displayed are the plasma cytokine determinations quantified by chemiluminescence in picograms per millimeter (median/s–range). The actual number of analyzed patients and controls are as in Materials and Methods. doi:10.1371/journal.pone.0007405.t003

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### Author Contributions

Conceived and designed the experiments: CV GC. Performed the experiments: ST. Contributed reagents/materials/analysis tools: ST. Analysis and interpretation of data: CV GC. Wrote the paper: CV GC.

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