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REVIEW

Novel Transcriptional Biomarkers for Diagnosis and Prognosis of Sepsis

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ABSTRACT

Sepsis is a lethal disease that has a complex pathophysiology including a dysregulated inflammatory response, endothelial injury, microvascular thrombosis, vasoplegia and myocardial depression leading to multiorgan failure. Although there are advancements in the management of the disease, incidence and mortality rates are still high even in the developed countries. Prompt recognition of sepsis, early initiation of antibiotics, source control, optimal fluid and vasopressor therapy are of utmost importance. Currently, there is no gold standard biomarker that can allow clinicians to diagnose and prognosticate sepsis and to monitor the response to treatment in a precise, accurate and time efficient way. Current single-protein and multi-protein biomarkers have certain caveats and only partially helpful to diagnose and manage sepsis. Transcriptomics is a widely used approach for biomarker research, especially in sepsis. Technologies that apply to this area are easy, affordable and time efficient; further, the developments in next generation sequencing makes transcriptomics even more applicable. Even though the term transcriptomics includes all RNAs, microRNAs (miRNAs), which are short noncoding RNAs are especially under the spotlight for the search of sepsis biomarkers and some of them have already been validated to be specific. Specifically their high abundancy in circulation and their stability for long periods make them strong candidates for further research. Identification of new biomarkers can help enlightening the unknown sides of sepsis, which might lead to new therapeutic advancements in the management of the disease. Also the search for reliable biomarkers gives hope to clinicians and patients for a better management for this highly mortal and devastating condition.

Key words: sepsis, prognosis, diagnosis, transcriptomics, biomarker, miRNA

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Introduction

Sepsis is a lethal disease with a complex pathophysiology that is yet to be understood. Number of severe sepsis cases has increased by 71%, while the hospital costs has increased by 57% in the US between the years 2003 and 2007, making sepsis the most expensive disease condition for hospitalization [1]. Although there are advancements in the management of the disease, incidence and mortality rates are still high even in developed countries [2].

Sepsis is a threat to patients with chronic diseases, severe trauma or burns; who are immunocompromised or receiving immunosuppressive therapy; who are malnourished and debilitated, although people of any age without any chronic condition, neonates or those who undergo a surgical operation might also be affected [3]. The management of sepsis requires a multidisciplinary, high quality and personalized care starting from the initial venue where the patient enters the hospital.

Sepsis arises from the host response to infection; its pathophysiology is complex and yet to be understood. Basically, the infection causes a pro-inflammatory response and results in the excessive release of mediators, causing a cytokine storm and the Systemic inflammatory response syndrome (SIRS). Recent evidence suggest that pro- and anti-inflammatory pathways are activated in a parallel fashion to result in a dysregulated, generalized immune response along with the dominance of the proinflammatory response for the first few days of sepsis [4]. In time the pro-inflammatory response overrules the

anti-inflammatory response and as a result immunoparalysis occurs; inflammatory mediators overwhelm the immune system and paralyze it [5]. It is thought that pro-inflammatory response is responsible for tissue damage and organ failure, while the suppression of anti-inflammatory response increases the susceptibility to secondary infections [6].

In 1992, American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference defined 'sepsis' as systemic inflammatory response syndrome (SIRS) with the addition of infection, 'severe sepsis' as sepsis associated with organ dysfunction, hypoperfusion and hypotension and 'septic shock' as sepsis with arterial hypotension despite adequate fluid resuscitation [7]. Another consensus established in 2001 expanded the list of manifestations that can be associated with sepsis [8]. However, there are still several areas of uncertainty to define sepsis in the continuum to sepsis shock [9], which hinders the efforts to establish the standards of diagnosis, treatment and stratification for clinical research.

Prompt recognition of sepsis, early initiation of antibiotics, source control, optimal fluid and vasopressor therapy are of utmost importance. Although advances have been seen in the diagnosis of sepsis and septic shock in recent years such as the utilization of laboratory tests such as lactate and procalcitonin (PCT) and focused ultrasonography, caveats do still exist such as the normotensive shock with isolated hyperlactatemia or cardiogenic shock in a patient with infection [10]. Furthermore, it is sometimes impossible to point out the focus or even the presence of infection when the signs and symptoms of infection are subtle or masked. Making things more complicated, myocardial dysfunction develops in more than half of the severe sepsis and septic shock patients [11]. That's why clinicians need culture-independent, rapid and reliable methods –biomarkers- to recognize occult presentations or to differentiate complex cases as infection vs. sterile inflammation, septic shock vs. cardiogenic shock [12].

A *biomarker*, a term abbreviated from *biological marker*, is usually described as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [13]. Several chemical, physical or biological parameters can be considered as biomarkers, if they are measurable and repeatable. Four classes of biomarkers have been specified; diagnostic biomarkers, screening biomarkers, monitoring biomarkers and risk stratification biomarkers [14].

Biomarkers that could differentiate true bacterial infections and sepsis from other non-infectious and non-bacterial causes have received attention with a goal of early diagnosis and optimization of antibiotic therapy. Among these, C-reactive protein (CRP) and procalcitonin (PCT) have been the two most widely studied. CRP can assess the presence of inflammation but not specifically sepsis and it also increases in some non-infectious conditions. In addition to this, Monneret et al. showed that CRP levels in the plasma increase with a 24 hours delay when compared to PCT [15]. Serum PCT levels above a certain threshold is a marker of bacterial infection and tissue damage, however it might give false positive results if there is a trauma or tissue injury [16]. Moreover, it lacks specificity and its concentration increases with up to a 48 hour lag following the onset of infection, which precludes its use as a biomarker to guide sepsis diagnosis and initiation of antibiotic treatment [17].

Early diagnosis of sepsis is very critical for the timely and efficient use of treatment modalities, however there are no reliable, specific biomarkers that can guide the diagnosis of sepsis at the moment. In addition to this, determining prognosis and stratification of the disease is extremely important for the management of sepsis, and this has not been achieved yet either by any of the known biomarkers. Hence novel, satisfactory, specific and sensitive candidate biomarkers should be discovered to develop biological guidance in the management of sepsis syndromes.

With the completion of the Human Genome Project, there has been an increase in the number of identified genes responsible for diseases. Moreover, it was realized that studying the genetic material and its products as a whole provided exciting evidence to unanswered questions. This wider approach, currently called 'omics', has become more important with the developments in high-throughput methods that could obtain large amounts of data in a short amount of time. Knowing that many diseases are polygenic, including sepsis, it is obvious that approaching diseases through 'omics' would provide an insight to their unknown pathophysiology. Indeed; *genomics*, *transcriptomics*, *proteomics* and *metabolomics* have been widely used in many studies to investigate sepsis as well as to find candidate biomarkers for its diagnosis and prognosis.

Table 1. Genes whose expressions are determined as potential diagnostic, stratification and prognostic biomarkers.

f	BIOMARKER	FUNCTION
DIAGNOSTIC	TLR2/MyD88 AK/STAT3 – dependent signaling pathway	Early upregulation in sepsis [19]
	TLR2, TLR4, TLR5	Differential expression up to 48 hours before the onset of sepsis [24]
STRATIFICATION	IL2, IL7, IL23, IFNγ, TNFα	Downregulated in severe sepsis [21]
	IL10	Upregulated in severe sepsis [21]
	IL18R1	Neutrophil migration and activation, biomarker for several systemic inflammatory conditions [27]
	IL1R2	Suggested as a marker for sepsis, high circulating levels are seen in critically ill patients [27]
	MMP9	Downregulated in sepsis, correlating with multiple organ dysfunction [27]
	TNFRSF9	Downregulated by TNFα, inducing inflammatory responses [27]
	CCL22	Shown that it plays a role in neutrophil chemotaxis in a sepsis model [27]
	TLR2	Overexpressed in septic shock [27]
PROGNOSTIC	BCL-2 rhAPC	Overexpressed in sepsis survivors [25], [26]
	VPS9D1	Overexpressed in sepsis survivors [22]
	TNFAIP6, FCN1, CXCL10, GBP1, CXCL5, PID1	Potential biomarkers for INFγ treatment, management of septic shock [29]

This review focuses on transcriptomics because of its ability to tie genomics to proteomics, and suggests miRNAs as one of the most promising transcriptomic approach for the search of biomarkers for sepsis.

An omics approach to sepsis biomarkers: Transcriptomics

Every cell type has the same genetic code, however the transcriptional pattern is not the same. Function of a cell is determined by gene expression and this differs from cell to cell; in other words, every tissue has its own gene expression pattern. *Transcriptome* is the set of all RNA transcripts (both coding and non-coding) in a cell under a specific condition while *transcriptomics* is the study of these transcripts, focusing on the gene expression at the RNA level [18]. Transcriptomics employs high throughput methods such as microarray and RNA sequencing. Transcriptomic profiling makes it possible to monitor gene expression patterns in a genome wide manner. Analyzing the differences between healthy and disease states, points out the genes that are over- or under-expressed and helps to explain the pathophysiological background of the diseases. Since transcription patterns vary with regard to different diseases, the alteration in expression of specific genes can be valued as a marker for sepsis. By using a transcriptomic approach, novel candidate biomarkers that can help the diagnosis, prognosis and stratification of sepsis have been detected (Table 1).

Rudiger et al. studied the early transcriptomic changes in myocardium in the fluid resuscitated long-term (3-day) rodent model of sepsis through network based gene expression analysis. The cardiac genome-wide profiling showed that there was an early up-regulation of TLR2/MyD88 and JAK/STAT3-dependent signaling inflammatory pathway with sepsis [19]. Thus, detection of this up-regulation can be a potential marker for the early detection of sepsis, which is critical for its management.

In sepsis, there is a release of excessive pro-inflammatory mediators, referred to as "cytokine storm", which results in a devastating inflammation for the host [20]. In a research by Grealy et al., cytokine gene expression signatures in peripheral blood mononuclear cells (PBMC) were analyzed. As a result, it was found that the gene expression of interleukin-2 (IL2), interleukin-7 (IL7), interleukin-23 (IL23), interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNFα) were lower in severe sepsis patients when compared to patients with infection. In addition, interleukin-10 (IL10) was found to have greater expression in patients with severe sepsis [21]. The expression

Table 2. Potential and candidate miRNA biomarkers for prognosis and diagnosis of sepsis.

	miRNA	FUNCTION
DIAGNOSTIC	miR-96, miR-101, miR-122, miR-182, miR-185, miR-141, miR-143, miR-181a, miR-29a, miR-1184	Immune regulatory miRNAs that show significant expression changes between neonatal sepsis patients and healthy individuals [39]
	miR-150, miR-182, miR-342-5p, miR-486	Shows different expression levels between sepsis patients and healthy individuals [40]
PROGNOSTIC	miR-15a, miR-16, miR-122, miR-193*, miR-483-5p	Expressed differentially between sepsis survivors and non-survivors [37]
	miR-150	Candidate prognostic biomarker for sepsis [41]
	miR-574-5p	Candidate prognostic biomarker for sepsis, correlating with non-survival [42]
	miR-297	Candidate prognostic biomarker for sepsis, correlating with survival [42]
	miR-133a	Potential biomarker for mortality prediction in sepsis patients [43]
	miR-223	Higher in patients with sepsis more than healthy patients [44]
	miR-499-5p, miR-122, miR-193b*	Lower in patients with sepsis more than healthy patients [44]
	miR-15a/16	Upregulated in sepsis patients, downregulating the TLR4 and IRAK-1 [45]
	miR-27	Upregulated in sepsis, candidate biomarker for the prognosis of sepsis [46]
	miR-200a, miR-200b, miR-200c	Regulates TLR4 signaling and NF-κB activation [47]
	miR-149	High levels results in reduced inflammation, potential biomarker for prognosis [49]

levels of these cytokines can be used as markers to differentiate between infection and sepsis syndromes.

One of the challenges in sepsis diagnosis is to differentiate it from SIRS, which may be incited by many other insults aside from infection. In a research that employed RNA sequencing, 338 genes, which are mostly related to immune activation pathways, differed between SIRS and sepsis. Those genes were mostly upregulated in sepsis patients, hence they have the potential to be used as novel diagnostic biomarkers for sepsis. In addition to this result, it was found that vacuolar protein sorting domain-containing gene 1 (VPS9D1) mRNA variants had functional effects on the sepsis outcome [22]. Sutherland et al. enrolled sepsis patients, post-surgical patients and healthy individuals in order to test a panel of gene expression biomarkers to see if they could distinguish between sepsis patients and surgical patients. As a result of this study, a panel of 42 gene expression markers that were selected *a priori* was found promising in order to differenciate between SIRS and sepsis patients [23]. In another study that focused on differentiating between sterile SIRS and early sepsis, it was shown that toll-like receptor (TLR) pathway, mitogen-activated protein kinase (MAPK) signaling pathway, cytokine receptors, signal transduction through IL-1R pathway, T-cell differentiation pathways, apoptosis pathway, regulation of eIF-4E and p70 S6 kinase pathway have differentially expressed genes that can provide that distinction [24]. When these results are taken into consideration, it is obvious that a transcriptomic approach can provide various options for sepsis diagnosis, especially for stratification.

Prognosis of sepsis is equally important as diagnosing it, since it affects the triage and the clinical decision making pathways. Previously mentioned *VPS9D1* gene was also found to be overexpressed in sepsis survivors. Thus, it was shown that *VPS9D1* has a potential to be used as a prognostic biomarker to predict the outcome of a disease [22]. In addition, it was shown that the overexpression of B-cell lymphoma 2 (BCL-2) [25] as well as recombinant human activated protein C (rhAPC) [26] improves survival in sepsis patients by preventing sepsis-induced apoptosis.

It was demonstrated that a neutrophil reporter assay coupled with whole transcriptome readout can determine the severity of sepsis. In this study, three different cell types in plasma (polymorphonuclear cells (PMNs), peripheral blood mononuclear cells (PBMCs), and monocyte-derived dendritic cells (MoDCs) were the focus. A set of 30 transcripts showed high accuracy to identify the severity

of sepsis; further, a functional network for the septic severity biomarker signature has been created containing those transcripts. Among the genes that were determined, interleukin 18 receptor 1(IL18R1), interleukin 1 receptor, type II (IL1R2), toll-like receptor 2 (TLR2), matrix metallopeptidase 9 (MMP9), tumor necrosis factor receptor super family 9 (TNFRSF9), chemokine motif ligand 22 (CCL22) are found [27]. However, blood cell population has high heterogeneity, hence the cell types that are excluded might also have important expression information that needs to be taken into consideration [28].

Transcriptomics can also be used for developing biomarkers for patients to distinguish if they can or cannot recieve a specific treatment. In a study by Allantaz-Frager et al., biomarkers have been established in order to identify sepsis patients that are eligible for immunotherapy, more specifically interferon-gamma (IFNγ), by employing the microarray technology. It was shown that six transcripts, tumor necrosis factor-alpha induced protein 9 (TNFAIP6), ficolin-1 (FCN1), C-X-C motif chemokine 10 (CXCL10), guanylate binding protein 1 (GBP1), C-X-C motif chemokine 5 (CXCL5) and phosphotyrosine interaction domain containing 1(PID1) are potential biomarkers for the identification of sepsis patients that can recieve the IFNγ treatment [29].

As evident from this literature review, transcriptomic approach can give way to the discovery of novel biomarkers in the diagnosis, prognosis or stratification of sepsis. One of the biggest challenges in transcriptomic approach for biomarker development is that mRNAs are prone to degradation easily; hence they are quite hard to isolate and to work with. Although this remains a problem, current research on microRNAs, small single strand non-coding RNAs, tries to eliminate this inconvenience.

Novel transcriptomic biomarkers for sepsis: miRNAs

Discovered by Lee et al in 1993 [30], microRNAs are short noncoding RNAs with the length of ~22 nucleotides. The main mechanism of the regulatory effect of miRNA is suppression of translation of mRNA [31] miRNA usually targets the 3'UTR (untranslated region) (and sometimes 5'UTR (untranslated region) or coding regions) of mRNA and complements partially or completely in order to suppress it [32]. The suppression is done by degradation (removal of cap (decapping) or shortening of poly A tail (deadenylation)) or by cleavage [32]. They usually participate in the early stages of gene expression, hence they have the potential to be used in early diagnosis [33] miRNAs contain 'seed sequences' which are found in the 5' UTR of miRNAs, and these sequences complement with the mRNA sequences. From miRNA to miRNA, these sequences differ only with one or a few bases, hence more than one miRNA can silence an mRNA.

One of the biggest advantages of miRNA is that it is highly stable even after long storage periods of serum and plasma samples and is not easily degraded by RNases. Incorporation into RNA-induced silencing complex (RISC) as well as being contained in vesicles called exosomes provides protection for miRNAs. Furthermore, in humans more than 3000 miRNAs are known [31] and until now, no tissue has been identified that lacks miRNAs [32] miRNAs are usually tissue specific, and in pathological conditions the miRNA expression levels can be altered due to changes in the transcriptional and posttranscriptional regulation of miRNA expression [34].

miRNAs are highly conserved among organisms and are quite abundant in blood. However, since miRNAs are conserved among organisms, bacterial or viral miRNAs may affect the host mRNAs, affecting the infectivity, rather than the host miRNAs [35]. Furthermore, the fact that some miRNAs target multiple mRNAs, expression level change of miRNAs might not always give the accurate result. One of the major limitations of miRNA is that there are no control miRNAs, i.e. housekeeping miRNAs, which can provide a satisfying comparison between healthy and sick. In order to eliminate this problem, the control population must be kept high [36]. For the detection of miRNAs, hybridization based techniques (especially microarray) as well as reverse transcriptase PCR (RT-PCR) and cloning based techniques are used. With the next generation sequencing era, RNA sequencing has become the focus for miRNA researches.

There are numerous studies on miRNAs and their roles as biomarkers, especially on sepsis. Various miRNAs have been identified and selected as promising candidate biomarkers for sepsis. A research by Wang et al. employed Solexa sequencing as an approach for determining serum miRNAs in order to predict mortality in sepsis patients. Six serum miRNAs, miR-223, miR-15a, miR-16, miR-122, miR-193* and miR-483-5p, were expressed differentially between sepsis survivors and non-survivors [37]. Another study supports the possibility of miR-223 as a biomarker for sepsis as well as suggesting miR-146a

as a candidate [38]. Chen et al. also applied a broad approach to show the changes in miRNA expression during neonatal sepsis and demonstrated that there were 10 immune regulatory miRNAs which had significant expressional changes between neonatal sepsis patients and healthy controls. Moreover, they pointed out how those 10 miRNAs (miR-96, miR-101, miR-122, miR-182, miR-185, miR-141, miR-143, miR-181a, miR-29a and miR-1184) are affiliated with immune associated genes [39]. Any one of those miRNAs can be seen as potential biomarkers for the diagnosis of neonatal and adult sepsis.

Vasilescu et al. identified four miRNAs (miR-150, miR-182, miR-342-5p and miR-486) with different expression levels between sepsis patients and healthy controls. It was shown that miR-150 levels were decreased in both leukocytes and plasma, correlated with the severity of the sepsis and they proposed that miR-150 is not only a promising biomarker, but also might be a target for therapeutic intervention [40]. However, Roderburg et al. showed that there is a minor, statistically insignificant difference in miR-150 levels between sepsis patients and healthy individuals, hence stated that it could not be used as a diagnostic biomarker. Nevertheless, they found that there is a correlation between low miR-150 levels and organ dysfunction and mortality. Thus, they suggested that miR-150 can be used as a prognostic biomarker for sepsis [41]. Another study determined miR-574-5p as a candidate prognostic biomarker, which correlates with the non-survival in sepsis patients, while miR-297 was correlating with survival from this disease [42]. Study of Tacke et al. showed that the miR-133a levels in circulation was higher in sepsis patients and suggested that its expression was upregulated in sepsis. In addition to this, they suggested that miR-133a can be used as a biomarker to predict the mortality in sepsis patients [43]. Wang et al., pointed out four novel miRNA biomarkers that can identify sepsis; miR-223 is significantly higher in patients with mild sepsis, severe sepsis and septic shock than healthy patients while miR-499-5p, miR-122, and miR-193b* was significantly lower. Furthermore, it was shown that mir-499-5p can distinguish between the three different forms of sepsis [44]. In a study that focused on neonatal sepsis patients, it was shown that miR-15a/16 was upregulated when compared to healthy subjects. It was demonstrated that miR-15a/16 inhibits lipopolysaccharide induced inflammatory pathway; furthermore it down regulates the toll-like receptor 4 (TLR4) and interleukin-1 receptor associated kinase 1 (IRAK-1) [37]. Since miR-15a/16 plays such a pivotal role in inflammatory response, it can be evaluated as a potential biomarker for early diagnosis. In addition to this, another study showed that both miR-15a and miR-16 are able to distinguish between sepsis patients and SIRS patients, hence they can be used for differential diagnosis as well [45].

NF-κB activated pathway is one of the inflammatory pathways that sepsis employs. It is also one of the pathways that regulate inflammatory responses as well as cell apoptosis. Wang et al. proposed that miR-27a is up-regulated in sepsis, and knocking down of miR-27a in mice revealed that the expression levels of TNF-α and IL-6 are down-regulated, which was maintained by reducing the phosphorylation level of NF-κB [46]. Therefore, miR-27 is one of the powerful candidate biomarkers in order to identify prognosis of sepsis patients. Additionally, Wendlandt et al. showed that miR-200a, miR-200b, miR-200c can regulate TLR4 signaling and NF-κB activation [47]. Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein that is important for the signaling cascade of most TLRs and interleukin-1 [48]. Xu et al. provided information about miR-149 that it can regulate MyD88 protein levels negatively [49]. Similar to miR-27, miR-149 can be used as a prognostic biomarker, since high levels of miR-149 will result in reduced inflammation biomarkers.

Even though the research on miRNAs as potential biomarkers is stimulating, there are contradictory results as well. For instance, recently Benz et al. provided information about miR-223 levels indicating it could not predict sepsis or its outcome [50]. Nevertheless, miRNAs are promising candidates as novel sepsis biomarkers.

In addition to sepsis patients, various animal models have been used for investigating the levels of circulating miRNA expression. Blood samples obtained from mice with experimental sepsis induced by cecal ligation and puncture was analyzed using an miRNA array. Upregulation of the circulating miR-16, miR-17, miR-20a, miR-20b, miR-26a, miR-26b, miR-106a, miR-106b, miR-195, and miR-451 were detected [51]. Among them, miR-16 was detected as a prognostic biomarker and it also showed differential expression between sepsis survivor and non-survivor patients [37].

In another study, downregulation of MyD88/TAK1/IKKβ/IκB-α/NF-κB pathway and decreased miR-150, miR-223, and miR-297 expression levels

were analyzed in the rat model of septic shock. As a result, vasodilatory and inflammatory mediator production was detected [52]. All of the three miRNAs mentioned above were also detected as prognostic biomarkers in sepsis patients.

Conclusion

Sepsis is a lethal disease that has a complex pathophysiology including a dysregulated inflammatory response, endothelial injury, microvascular thrombosis, vasoplegia and myocardial depression leading to multiorgan failure. Currently, there is no gold standard biomarker that can allow clinicians to diagnose and prognosticate sepsis and to monitor the response to treatment in a precise, accurate and time efficient way. Current single-protein and multi-protein biomarkers are only helpful and more research has to be done in order to discover candidate biomarkers for sepsis.

Transcriptomics is a widely used approach for biomarker research, especially in sepsis. Technologies that apply to this area are easy, affordable and time efficient; further, the developments in next generation sequencing makes transcriptomics even more applicable. Even though the term transcriptomics includes all RNAs, miRNAs are especially under the spotlight for the search of sepsis biomarkers and some of them have already been validated to be specific. Specifically their high abundancy in circulation and their stability for long periods make them strong candidates for further research. Identification of new biomarkers can help enlightening the unknown sides of sepsis, which might lead to new therapeutic advancements to the management of the disease. Also the search for reliable biomarkers gives hope to clinicians and patients for a better management for this highly mortal and devastating condition.

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