

Rethinking Sepsis: New Insights from Gene Expression Profiling Studies

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Introduction

Critically ill patients encompass an enormously heterogeneous population and, as such, therapeutic interventions, including drug therapy, can produce multiple outcomes in different patient subgroups. For example, researchers not only look for an 'average effect' of a drug on a typical patient, but also seek to understand individual variability. The presence of variability impacts significantly on the success of clinical trials and failure to identify this variability can result in the clinical trial being under-powered to detect a treatment effect. For clinicians, failure to recognize variability can result in unintended toxicity or excessive harm in certain patients. Hence, understanding variability is critically important in both research and clinical practice.

Nowhere is the relevance of patient variability more evident than in sepsis. Over the last two decades, numerous clinical trials have been conducted, all producing mixed results. It has been commonly observed that various patient populations responded differently to the same drug, ranging from marginal beneficial effect in some subgroups, to nil effect or increased toxicity in others.

Investigators have attempted to address the heterogeneity issue by stratifying patients into groups who have different baseline mortality risk. Theoretically, identifying those patients who are most likely to respond to treatment will ensure maximal benefits and minimal harms. In the case of recombinant activated protein C (drotrecogin alfa (activated)), such subgroups have been identified [1, 2]. For many other drugs, no particular subgroups were found, although investigators have long suspected patient heterogeneity was the reason for failure in these trials [3].

There is now an increased recognition that our failure to give the right treatment to the right patient reflects our current limitations in identifying and measuring heterogeneity in critically ill patients [4, 5]. In this chapter, we will redefine heterogeneity in sepsis patients using a simple conceptual model. We then review findings from recent studies that provide new insights into the sources of heterogeneity in these patients.

How to Identify and Measure Heterogeneity

Current methods to define patient heterogeneity in sepsis are grossly inadequate. Traditional criteria such as age, clinical settings or disease severity are commonly used to enlist patients into clinical trials. However, these are crude measures of the inherent heterogeneity of a very complex syndrome in a diverse patient population.

Although simple physiological parameters (e.g., systemic inflammatory response syndrome [SIRS] criteria), organ level indices (e.g., circulatory failure), or a combination of both (e.g., APACHE score) have been proved to be helpful in epidemiological studies, they are too non-specific as criteria to stratify patients in clinical trials. With the exception of recombinant activated protein C and anti-tumor necrosis factor (TNF) therapy, attempts to select patients based on disease severity or baseline mortality risk have consistently failed, as evidenced from analyses of clinical trials on anti-coagulant therapy, anti-inflammatory drugs, or low-dose corticosteroids [11, 12]. Investigators can also measure a vast array of physiological parameters and serum cytokines in sepsis patients. However, we do not know how these measurements relate to the observed heterogeneity, nor do we know how they can be used to predict a patient’s possible response to a new drug. Consequently, there is currently no agreed upon method to identify and measure heterogeneity in sepsis patients.

Sources of Heterogeneity in Sepsis Patients

The sources of heterogeneity are multiple and manifest at different levels. Study and patient level variables (e.g., trial design, disease severity) are easy to discern, as this information is readily available from published reports of clinical trials. Our current understanding of heterogeneity derives mainly from these variables [8]. While the data from these variables is useful, they represent only the tip of an iceberg (Fig. 1).

The iceberg model provides a qualitative overview of the sources of heterogeneity. The complexity of the data increases progressively downwards in this model (Fig. 1). Data on organ and cellular level variables demonstrate a diverse range of complex behavior exhibited by different organs (e.g., liver vs. kidney) [9] and different cells

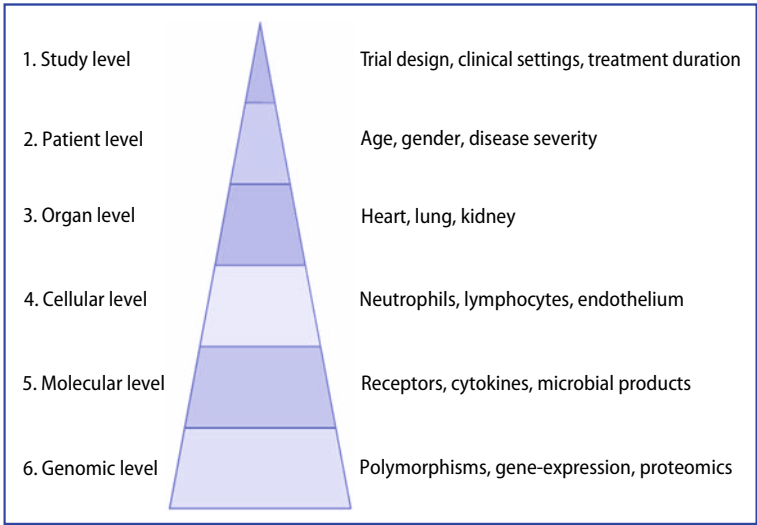


Fig. 1. The iceberg model to conceptualize the sources of heterogeneity in sepsis trials. Variables on the upper levels of the model are easier to discern and study. Complexity of the data increases towards the lower levels, with most variables yet to be discovered or understood at the genomic level.

(e.g., leukocytes vs. endothelium) [10]. Data from molecular level studies are even more complex, with over 50 mediators found to be involved at multiple points during the host response to sepsis [11].

The highest level of complexity, however, lies at the genomic level. Here, a vast myriad of data is accessible to only a handful of researchers in a few highly specialized research institutions. Yet, these data are potentially the richest source of information and may help us identify and measure the observed clinical heterogeneity in sepsis patients. Here, we will highlight some important findings from this rapidly expanding area of research.

New Insights from Gene-expression Studies

The field of genomic science includes the study of genetic polymorphism, proteomics, and gene-expression profiling (see [Table 1](#) for more details). The emerging fields of proteomics and genetic polymorphism have been reviewed elsewhere [12, 13]. This chapter will focus on insights obtained from studies of gene-expression profiling, a field with the most promising potential to assist us understand the sources of heterogeneity in sepsis.

Over the last 5 years, we have undertaken a large scale, systematic interrogation of the host response in sepsis at a transcriptional level [14–16]. The microarray technique is a powerful tool that allows us to sift through a massive amount of the

Table 1. Glossary

Proteomics: A new technology that involves large-scale study of protein composition and function. Typically, it involves cataloguing all the expressed proteins in a particular cell or tissue type, using techniques such as two-dimensional gel electrophoresis or mass spectrometry. A single mass spectrometry experiment can identify over 2,000 proteins.

Gene-expression profiling: A high-throughput technology that measures the activity of thousands of genes at once, to create a global picture of cellular function. Cells respond changes in their environment by making messenger RNA (i.e., gene-expression), which in turn encodes for various proteins that carry out the appropriate cellular function. A single experiment can measure an entire genome simultaneously, in some cases over 25,000 genes. This technology therefore provides a more global picture than proteomics.

Polymorphism: A common biological phenomenon in which phenotype variations arise due to difference in DNA sequence among individuals. The most frequent type of polymorphism is the single-nucleotide polymorphism (SNP), which can be a substitution, a deletion, or an insertion of a single nucleotide. It is thought to be one of the causes of the individual variability in the susceptibility to infectious disease.

Microarray: The commonest platform used in gene-expression profiling experiments. It is a two-dimensional grid of DNA genes or gene fragment spots, usually arranged on a glass slide or silicone wafer. A typical microarray contains 10,000–200,000 microscopic probes. The probes on the microarray are either a short oligonucleotide or a cDNA. Probe-target hybridization is usually detected and quantified by fluorescence-based detection. This allows the determination of relative abundance of nucleic acid sequences in the sample.

Network Analysis: An analysis method that seeks to study the relationships and interactions between various parts of a cell signaling system (metabolic pathways, organelles, cells, and organisms) and to integrate this information to understand how biological systems function.

genetic information contained within the human genome (see [Table 1](#) for more details). We examined the gene-expression profiles of 164 critically ill patients admitted to the intensive care unit (ICU) of a university-affiliated teaching hospital. The patient cohort consists of a full range of sepsis syndromes (from sepsis to septic shock) in a wide variety of clinical settings (medical, surgical and obstetric). Our findings reveal some interesting insights with regard to transcriptional heterogeneity.

Limitation of Current Risk Stratification Methods

Our data shows that there is no difference in the host response between sepsis, severe sepsis, and septic shock at a transcriptional level. Classification of septic patients into sepsis, severe sepsis, and septic shock is one of the commonest ways to stratify patient into different risk groups and is supported by a large amount of data from epidemiological studies [17]. However, the use of these criteria has failed to identify treatment-responsive groups in most clinical trials. Fundamental questions have therefore been raised on the effectiveness of such criteria to define the complex range of heterogeneity found in septic patients [18]. Our data provide the first genomic evidence that the grouping of patients based on such criteria is too limited to represent the full spectrum of heterogeneity in sepsis patients. A more precise definition of the subgroups in sepsis is needed, perhaps by using not just simple clinical variables (e.g., heart rate or creatine values), but also more sophisticated methods such as genomic studies.

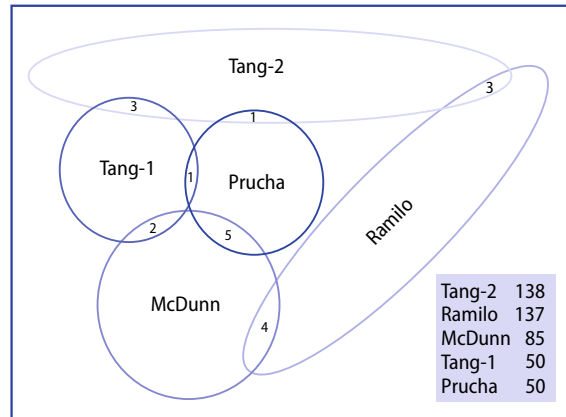
Host Response in Sepsis is More Complex Than Previously Thought

Investigators have delivered a huge amount of molecular information on sepsis at an unprecedented level of complexity. This is well demonstrated by a seminal study by Calvano et al., in which volunteers were given endotoxin and their gene-expression profiles measured [19]. A total of 3,714 genes were found to have their expression intensity altered by the endotoxin challenge. This is an impressively large number because it represents over 14 % of the protein-coding genes. When these genes were followed at 2, 4, 6, 9 and 24 hours, more complex changes were exhibited. In addition, the authors performed network analysis (see [Table 1](#) for more details) and reported a further discovery of a vast interconnecting network of cellular activities. For example, by honing in on just one gene alone (i.e., nuclear factor kappa B), the authors unveiled a total of 619 interactions between 150 genes. With over three thousand genes showing simultaneous changes, the number of potential interactions is immeasurable. The immense complexity of these data provides an exciting opportunity to develop a potentially more powerful method to classify sepsis patients into clinically relevant and molecularly precise subgroups.

There Is Strong Evidence of Heterogeneity at the Genomic Level

Patient subgroups can be identified using gene-expression studies, but first there is a need to explore all the genomic variability within any defined population of sepsis patients. To this end, we recently undertook a review of all the gene-expression studies that had identified genomic markers of sepsis [14–16, 20–22]. We performed a pair-wise comparison of all the signature genes between each study. Our analysis of these studies reveals two important findings ([Fig. 2](#)).

Fig. 2. List of putative genes in sepsis. Total number of genes in each data set is shown in the insert. References for included studies: Tang-2 [14], Ramilo [20], McDunn [21], Tang-1 [15], Prucha [22]



First, there are over 400 signature genes that were identified as putative genomic markers of human sepsis. The vast majority of these genes have never been studied in the past. These data sets contain valuable sources of information and could potentially lead to the discovery of novel pathways which may help researchers explain heterogeneity. In fact, many of these genes are subsequently identified to be those involved in nucleosome assembly, signal transduction, transcription/translation regulation, and control of protein complex assembly.

The second finding is that there is minimal overlapping in the lists of signature genes among studies. The genomic markers of sepsis seem to vary from one patient population to another. This finding persists even after the analysis is restricted to studies that had comparable study design, disease spectrum, or clinical settings. This finding indicates that the spectrum of heterogeneity at a transcriptional level is large. It is likely that the current studies revealed only a very small portion of this enormous variability.

Genomic Heterogeneity

To explore genomic heterogeneity further in the context of biological pathways, we recently conducted network analysis (see [Table 1](#) for more details) using our microarray datasets. We examined all the relevant biological pathways implicated in sepsis, including those involved in immunity and inflammatory responses. We then compared our findings to other gene-expression studies that used similar analysis techniques [19, 23, 24]. These analyses addressed two important questions: 1) what give rise to genomic heterogeneity; 2) where are the main sources of variability?

What Gives Rise To Genomic Heterogeneity?

Our analyses suggest that the vast connectivity of the molecular signaling system gives rise to heterogeneity. Traditionally, cellular function is conceptualized as individual components working in a linear fashion to produce a series of predictable biological effect. However, network analysis data suggest a far more complex picture. It shows that cellular functions are governed by vast gene regulatory networks. While there are main hubs in these networks, there are also extensive collateral sub-

networks that will provide alternative routes. This is akin to the airline network, where travelers can arrive at the same destination via complex re-routing or the use of alternative airlines. Consequently, there is a large amount of redundancy in its signaling system. To complicate matters further, there are conflicting feedback loops acting on each hub. For any given biological signal, multiple outcomes are possible depending on a variety of factors, such as the temporal pattern of each feedback loop and summation of individual stimuli. Cellular function is therefore a result of an integrative process. Such a system gives rise to a huge potential for variability and, hence, heterogeneity.

Main Sources of Genomic Variability

Given the enormity and the complexity of the data, investigators need to hone into the main sources of the variability. Our data, along with those from other studies [19, 23, 24], allow us to narrow our focus down to four gene regulatory networks (Fig. 3).

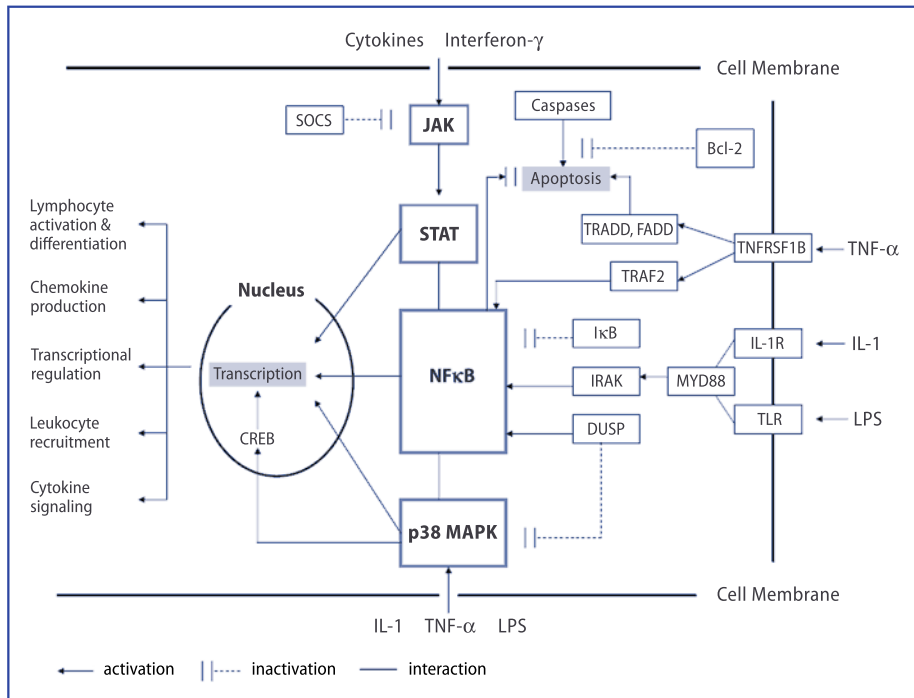


Fig. 3. Four gene regulatory networks (JAK, STAT, NF-κB and p38 MAPK). BCL2: B-cell CLL/lymphoma 2; CREB: cAMP responsive element binding protein; DUSP: dual specificity phosphatase; FADD: Fas-associated death domain protein; IFNGR: interferon gamma receptor; IκB: inhibitor of kappa-B; IL-R: interleukin-1 receptor; IRAK: interleukin-1 receptor-associated kinase; JAK: Janus kinase; LPS: lipopolysaccharide; MAPK: mitogen activated protein kinase; MyD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor kappa-B; SOCS: suppressor of cytokine signaling; STAT: transducer and activator of transcription protein; TLR: Toll-like receptor; TNF: tumor necrosis factor; TNFRSF1B: TNF receptor superfamily, member 1B; TRADD: TNF receptor-associated death domain protein; TRAF2: TNF receptor-associated factor-2.

All four networks have been implicated in the immune response to sepsis. Although other molecular networks have also been implicated, these four networks are the most consistent findings reported by the gene-expression studies we surveyed. While each network has been extensively studied in the past, the gene-expression studies provide a global overview of all these networks and their individual components. They reflect a growing body of data that will help researchers explain heterogeneity. However, these data represent only a glimpse of the vast genomic landscape. We still do not fully understand the complex inter-relationship between these networks and the dynamic interaction between components of each pathway. More in-depth studies focusing on gene regulatory networks are therefore needed in the future.

Further Questions on an Existing Sepsis Model

Our analysis of the gene-expression studies also revealed two unexpected findings with regard to the role of the immune response. First, there is a noticeable absence of the activation of pro-inflammatory genes. According to the currently accepted model of sepsis, the host response is a biphasic process in which an initial hyper-inflammatory phase is followed by a later, anti-inflammatory phase that manifests as functional immune suppression [25]. This has not been supported by data from the gene-expression studies we surveyed, where investigators rarely reported the activation of well-known inflammatory genes, such as TNF, interleukin (IL)-1, IL-2, IL-6, or IL-10. Second, the gene-expression studies suggest that immune suppression is present in both the early and late phases of sepsis. Again, this is in contrast to the established model of sepsis where immune suppression is thought to occur later. In fact, it is now well established that the simplistic strategy of treating early sepsis as a pro-inflammatory phenomenon has been proven ineffective.

Put together, these data suggest that the established model of sepsis is too simplistic to account for the wide range of immune abnormalities observed in sepsis patients. The insight that both hyper-immune and hypo-immune status can occur early in sepsis further reinforces our notion that the pathogenesis of sepsis is much more complex and heterogeneous than we previously thought.

There are important therapeutic implications of the above findings. First, sepsis has long been defined as a pro-inflammatory or hypo-inflammatory syndrome. Such a dichotomization ignores the complexity of sepsis and leads to simplistic strategies such as neutralizing elevated cytokines or replacing a compound when its serum level is low. Second, septic patients have been treated as an immunologically homogeneous group. However, there are likely to be many heterogeneous immune phenotypes. Giving drugs without sufficient information about the patient's underlying immunological status can result in benefit in some phenotypes but harm in others.

With an increased recognition of immunological heterogeneity, some authors are now advocating that the immune status needs to be accurately assessed before patients are recruited into clinical trials [26]. However, currently available biomarker assays capture only a fraction of all known immune aberrations in sepsis. For example, serum measurements of inflammatory cytokines (e.g. IL-1, IL-6, or TNF- α) are widely used. But a far greater number of molecules have been observed to be abnormally elevated in septic patients. Functional testing of immune cells (e.g., cell proliferation or human-leukocyte antigen [HLA]-DR expression) has also been used, but it measures only a few pathways and hence provides only a partial view of the over-

all immunological status. Here, we propose that a gene-expression profiling technique is better suited to assess global immune dysfunction in sepsis.

Functional Mapping of Sepsis Genome to Monitor Immune Function

Gene-expression profiling can be used to characterize the immunological status of septic patients on a genome-wide scale. This is because there are advantages of this technique over conventional biomarker assays. First, gene-expression profiling can handle much larger volumes of data, often measuring thousands of genes simultaneously. This capability is unmatched by conventional assays. Second, many cellular dysfunctions are often unmeasurable by normal assays, because their expression is downregulated or their expressed proteins are below the dynamic range of detection. These dysfunctions can be easily detected by gene-expression profiling.

We undertook gene-expression analysis of thirty-five critically ill patients (sepsis = 25, control = 10). Circulating mononuclear cells were used because these cells play a major role in the immune response in sepsis. We then compared the gene-expression profile of the sepsis and control patients. The analysis was performed on over 130 biological pathways, including those known to be involved in immunological functions. Some of the important findings are presented in [Table 2](#).

Table 2. Biological pathways implicated in sepsis

BioCarta Pathway	Pathway description	Number of genes	p-value
1 h_crebPathway	Transcription factor CREB and its extracellular signals	9	1e-05
2 h_egfr_smrtePathway	MAPK inactivation of SMRT co-repressor	8	1e-05
3 h_hcmvPathway	Human cytomegalovirus and MAPK pathways	8	1e-05
4 h_hdacPathway	Control of skeletal myogenesis by HDAC & calcium/calmodulin-dependent kinase (CaMK)	12	1e-05
5 h_mapkPathway	MAPK signaling pathway	28	1e-05
6 h_p38mapkPathway	p38 MAPK signaling pathway	23	1e-05
7 h_tollPathway	Toll-like receptor pathway	13	1e-05
8 h_dspPathway	Regulation of MAPK pathways through dual specificity phosphatases	6	9.11e-05
9 h_SARSPathway	SARS coronavirus protease	7	9.63e-05
10 h_stressPathway	TNF/stress related signaling	7	9.67e-05
11 h_tall1Pathway	TACI and BCMA stimulation of B cell immune responses.	7	9.84e-05
12 h_fMLPPathway	fMLP induced chemokine gene expression in HMC-1 cells	11	0.0001511
13 h_biopeptidesPathway	Bioactive peptide induced signaling pathway	13	0.0001688
14 h_41bbPathway	The 4–1BB-dependent immune response	7	0.000176

Table 2. (cont.)

BioCarta Pathway	Pathway description	Number of genes	p-value
15 h_pyk2Pathway	Links between Pyk2 and MAPKs	9	0.0003106
16 h_nfataPathway	NFAT and hypertrophy of the heart (Transcription in the broken heart)	12	0.0003726
17 h_eif4Pathway	Regulation of eIF4e and p70 S6 kinase	15	0.0006778
18 h_Ccr5Pathway	Pertussis toxin-insensitive CCR5 signaling in macrophage	9	0.001729
19 h_keratinocytePathway	Keratinocyte differentiation	15	0.0021693
20 h_arenf2Pathway	Oxidative stress induced gene expression Via Nrf2	11	0.0022934
21 h_GATA3pathway	GATA3 participate in activating the Th2 cytokine gene expression	10	0.0026139
22 h_ranklPathway	Bone remodeling	5	0.0040675
23 h_IL12Pathway	IL12 and Stat4 dependent signaling pathway in Th1 development	17	0.0041499
24 h_ifnaPathway	IFN alpha signaling pathway	8	0.0043606
25 h_egfPathway	EGF signaling pathway	8	0.0179649
26 h_gleevecpathway	Inhibition of cellular proliferation by gleevec	7	0.0537086
27 h_ifngPathway	IFN gamma signaling pathway	6	0.0820155
28 h_tcraPathway	Lck and Fyn tyrosine kinases in initiation of TCR activation	23	0.1777127
29 h_asbcellPathway	Antigen dependent B cell activation	9	0.2645085
30 h_bbcellPathway	Bystander B cell activation	9	0.2645085

As expected, well known pathways such as Toll-like receptor (TLR) or TNF signaling are confirmed to be involved in sepsis. However, our analysis also discovered a large number of pathways, many of which have not been studied previously with regard to their involvement in sepsis. This analysis demonstrates that it is feasible to assay immunological dysfunction on a global scale and to yield highly valuable biological information regarding the roles of both established and unknown pathways. Based on the data above, we hypothesize that a comprehensive architecture of the gene regulatory network of immune response in sepsis can be constructed using gene-expression data. Such a database should include transcriptional information on: 1) all functional pathways; 2) all possible interactions between genes and molecules; 3) how the system functions as a whole in response to perturbations (e.g., to trauma, ischemia, or infectious stimuli); 4) mathematical modeling which will help investigators predict the existence of hidden interactions or feedback loops.

Conclusion

Based on the review above, we would argue for a greater appreciation of the complexity of the immune status in sepsis. Current models of sepsis are limited in their ability to account for the huge range of heterogeneity in sepsis patients. New data show that immunological dysfunction gives rise to much of the observed variability. We, therefore, propose that functional mapping of immunological aberrations by gene-expression studies holds the key to the understanding, measuring, and monitoring of heterogeneity in sepsis patients. Such a database will allow future researchers to better understand the variability of drug response. In the long term, it will help clinicians design drug treatment based on individual variability; this is the ultimate goal of individualized medicine.

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