Genetics and genomics in pediatric septic shock

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Objectives: **Pediatric septic shock continues to be an important public health problem. Several investigative groups have applied genetic and genomic approaches as a means of identifying novel pathways and therapeutic targets, discovery of sepsis-related biomarkers, and identification of septic shock subclasses. This review will highlight studies in pediatric sepsis with a focus on gene association studies and genome-wide expression profiling.**

Data Sources: **A summary of published literature involving gene association and expression profiling studies specifically involving pediatric sepsis and septic shock.**

Summary: **Several polymorphisms of genes broadly involved in inflammation, immunity, and coagulation have been linked with susceptibility to sepsis, or outcome of sepsis in children. Many of these studies involve meningococcemia, and the strongest association involves a functional polymorphism of the plasminogen activator inhibitor-1 promoter region and meningococcal sepsis. Expression profiling studies in pediatric septic shock have identified zinc supplementation and inhibition of matrix metalloproteinase-8 activity as potential, novel therapeutic approaches in sepsis. Studies focused on discovery of sepsis-related biomarkers have identified interleukin-8 as a robust outcome biomarker in pediatric septic shock. Additional studies have demonstrated the feasibility and clinical relevance of gene expression-based subclassification of pediatric septic shock.**

Conclusions: **Pediatric sepsis and septic shock are increasingly being studied by genetic and genomic approaches and the accumulating data hold the promise of enhancing our future approach to this ongoing clinical problem. (Crit Care Med 2012; 40: 1618–1626)**

Key Words: children; expression profiling; genomics; polymorphism; sepsis

Pediatric septic shock remains a major public health problem despite the development of effective antibiotics, vaccines, intensive care unit-based support modalities, and standardized treatment guidelines (1–5). The recognition of septic shock as a persistent challenge in the pediatric intensive care unit has led several investigators to study this syndrome using genetic and genomic approaches (6–8). This review will focus on the two areas of genomics most widely applied thus far to the field of pediatric septic shock: *gene association studies* and *genome-wide expression profiling*. The concluding section will briefly speculate on the potential link between epigenetics and long-term outcomes.

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Gene Association Studies

Death from infection is reported to have a stronger heritable component than death from cancer or cardiovascular disease (9). While this observation did not identify the causative genetic alterations and involves a relatively noncontemporary patient cohort, the study nonetheless provides compelling evidence that genetics play an important role in both susceptibility and response to infection. The existence of a singular "sepsis gene" is not biologically plausible. More plausible is the existence of genetic variations within multiple candidate genes that affect how the host responds to an infectious challenge.

The majority of gene association studies involving pediatric sepsis have focused on polymorphisms: the regular occurrence (>1%) of two or more alleles at a particular chromosome location. The most frequent type of polymorphism is called a single nucleotide polymorphism (SNP): a substitution, deletion, or insertion of a single nucleotide occurring in approximately one per 1000 base pairs of human DNA. SNPs can result in an altered protein, a change in the amount of normal protein expression, or no discernable change in protein function.

Many SNPs fall into the latter category because they occur in noncoding regions, or they are synonymous SNPs that do not lead to an amino acid change. These SNPs may be nonetheless worthy of study because they may be coinherited along with causal variants through linkage disequilibrium, which refers to the nonrandom association of alleles at two or more chromosome locations. Related to the concept of linkage disequilibrium is that of haplotype, which refers to a set of multiple SNPs on a single chromosome that are typically coinherited. These haplotype "blocks" can be identified by haplotype tag SNPs, and the International HapMap project is developing a haplotype map of the entire human genome as means to more effectively conduct genetic association studies (10).

A selected group of gene association studies in pediatric sepsis will be discussed below. Several reviews exist on the topic involving both adults and children, which also discuss methodological issues and limitations (6, 11–15). Notably, there are rigorous criteria dictating the quality of an ideal gene association study (16–20). Unfortunately, many gene association studies in critical care medicine do not meet this level of rigor (19).

Plasminogen Activator Inhibitor-1 (PAI-1). PAI-1 is the principal inhibitor of tissue plasminogen activator and urokinase (21, 22). As such, PAI-1 can be viewed as a procoagulant factor in that it

inhibits fibrinolysis. The PAI-1 promoter contains an insertion/deletion polymorphism at –675 base pairs: 5G/4G (23). *In vitro*, the 4G allele produces six times the amount of PAI-1 mRNA compared to the 5G allele, and individuals homozygous for the 4G allele produce greater amounts of PAI-1 compared to individuals homozygous for the 5G allele (23).

Microvascular thrombosis is a common pathologic component of sepsis, particularly in meningococcemia (24-27). Accordingly, the PAI-1 4G/5G polymorphism has been a focus of investigation in meningococcal sepsis. Hermans et al (28) first demonstrated that children with meningococcemia and the 4G/4G genotype produce higher concentrations of systemic PAI-1 and have worse outcomes compared to children with the 4G/5G or 5G/5G genotype. This link between the 4G/4G genotype and severity of meningococcal disease has been independently corroborated by several investigators (29-31), and a recent meta-analysis provides further confirmation that the 4G/4G allele is associated with mortality (32).

The studies linking the PAI-1 4G/5G insertion/deletion polymorphism and meningococcal sepsis provide relevant examples of well-conducted gene association studies (16–20). The association between the gene and the disease has a high level of biological plausibility. The allele (4G) affects the gene product in a physiologically meaningful manner. Cases are clearly defined and represent a spectrum of disease severity. The initial study has been independently replicated. Thus, the impact of the 4G allele on outcome in meningococcal disease is perhaps the most well-founded association between genetic variation and outcome in pediatric septic shock. However, this "genomic knowledge" has yet to be unambiguously translated to the bedside in the form of a novel therapy. This unfortunate circumstance reflects the complexities of coagulation balance in septic shock, as demonstrated by the inability of activated protein-C therapy to improve outcome in a heterogeneous cohort of children with septic shock (33).

Toll-Like Receptors (TLRs) and Related Signaling Molecules. TLRs are a family of pathogen-recognition receptors that provide a major mechanism for cells of the innate immune system to recognize and respond to pathogens (34). TLR4 is responsible for recognizing lipopolysaccharide from Gram-negative bacteria, while TLR2 is responsible for recognizing cell wall components of Gram-positive bacteria (lipoteichoic acid and peptidoglycan).

The coding region of the human TLR2 gene contains a nonsynonymous SNP leading to a substitution of arginine for glutamine at amino acid 753 (Arg753Gln). Lorenz et al (35) first reported this polymorphism and that the Arg753Gln polymorphism renders TLR2 less responsive to components of Gram-positive bacteria. Lorenz et al (35) also detected this polymorphism in two of 91 patients with septic shock, both of whom had *staphylococcal* infections. Subsequent studies, however, have not been able to confirm a strong association between the Arg753Gln allele and severity of Gram-positive infection in adults (36, 37). Studies in pediatric populations indicate an association between the Arg753Gln polymorphism and risk of recurrent infection (38), urinary tract infection (39), premature birth (40), and acute rheumatic fever (41). Other TLR2 SNPs have been described that may warrant further investigation (42, 43).

TLR4 mutations exist in mice that lead to abnormal responses to endotoxin and increase susceptibility to Gram-negative infections (44–47). The human TLR4 gene contains two mutations (Asp299Gly and Thr399Ile) that lead to hyporesponsiveness when human volunteers are challenged with inhaled endotoxin (48, 49). Conversely, a study involving peripheral blood mononuclear cells from children, showed no differential response to endotoxin or respiratory syncytial in association with these two mutations (50). Nonetheless, studies comparing adults with septic shock and healthy blood donor controls revealed the TLR4 Asp299Gly allele exclusively in the patients with septic shock, and also found that patients with the Asp299Gly/Thr399Ile alleles had a higher prevalence of Gram-negative infections (51, 52). In one report involving children with meningococcal disease, a heterozygous Asp299Gly genotype was associated with increased mortality (53), while two other reports have not been able corroborate this association (54, 55). Smirnova et al (56) have reported no link between "common" TLR4 variants and meningococcal disease, but have provided evidence that "rare" TLR4 coding variants are substantially overrepresented in patients with meningococcal disease. Finally, TLR4 polymorphisms have been linked with susceptibility to malaria in children (57).

Despite the biological importance of TLRs (a focus of the most recent Nobel Prize in Medicine), an absolute and unambiguous link between TLR genetic variants and human septic shock remains relatively elusive. Accordingly, investigators have recently focused on adapter proteins constituting the downstream signaling apparatus of TLRs. Polymorphisms of one such adapter protein, Mal (aka TIRAP), have been linked to invasive pneumococcal disease (58), an evolution-related increased resistance to infection (59), increased risk of infection in critically ill adults (60), and susceptibility to invasive *Haemophilus influenzae* infection in immunized children (61). Given the existence of several other adapter proteins that contribute to TLR signaling (34), it would be expected that several other gene association studies, focused on these adapter protein genes, are forthcoming.

Tumor Necrosis Factor-α. Tumor necrosis factor-α (TNFα) is recognized as a primary mediator in the pathophysiology of sepsis and septic shock (62-64), and has well-described polymorphisms (65). A substitution polymorphism of the TNFα promoter region involves a guanine (TNF1 allele) or an adenine (TNF2 allele) at –308 base pairs (66), and the TNF2 allele correlates with increased production of TNFα (67-69). The TNF2 allele has been associated with increased susceptibility to septic shock and mortality from septic shock in adults (70). However, this association has not been consistently observed (71, 72), and a recent meta-analysis involving 25 selected articles concluded that the TNF2 allele is associated with the development of sepsis, but not with sepsis mortality (73). In children with meningococcemia, Nadel et al (74) reported an association between the TNF2 allele and illness severity, whereas Read et al (75) reported an association between the TNF2 allele and susceptibility to meningococcemia in a mixed population of adults and children. A small study involving children with heterogeneous sepsis etiologies suggested that the TNF2 allele is more common in patients with septic shock compared to normal controls, but could not detect an association between the TNF2 allele and mortality.

A related polymorphism involves lymphotoxin-α, a member of the TNF superfamily (TNF-α) (74). The first intron of the lymphotoxin-α gene contains a restriction length polymorphism: the TNFB1 and TNFB2 alleles. Adults with septic shock, and homozygous for the TNFB2 allele, are characterized by higher systemic levels of TNFα and a higher mortality rate

(76). In bacteremic children, the TNFB2 allele was also demonstrated to be associated with higher systemic levels of TNFα and higher mortality (77).

Summary and Perspective. Several other polymorphisms have been studied in pediatric sepsis, and a selected group is summarized in a Table 1. Presently, no gene association study has directly impacted care in the pediatric intensive care unit. Nonetheless, the concept of genetics influencing pediatric sepsis remains valid. To translate this concept to the bedside, large-scale collaborations will need to be developed, positive association studies will need to be validated, and the field should consider focusing on functional polymorphisms for which there potentially exist reasonable therapeutic options.

Expression Profiling Studies

Expression profiling involves the use of microarray technology to simultaneously measure mRNA abundance of thousands of transcripts from biological specimens (78, 79). The approach is said to be "discovery oriented" in that no *a priori* assumptions are made regarding the relevance of any particular genes to the biological process of interest. This relatively unbiased, whole-genome approach is also referred to as "transcriptomics," and is generally hypothesis generating, rather than hypothesis driven (Fig. 1). Several whole-genome expression profiling studies have been conducted in human volunteers challenged with endotoxin and adults with sepsis (80-91), and excellent reviews have detailed the technical aspects, caveats, and limitations of expression profiling (78, 79). This section will review the analogous studies involving children, with a focus on leveraging expression data for the discovery of novel pathways and therapeutic targets, biomarker discovery, and gene expression-based subclassification.

Table 1. Selected gene-association studies in pediatric sepsis and septic shock

Reference	Gene/Polymorphism	Main Findings
Read et al [143], Brouwer et al [32]	Polymorphisms of IL-1B (–511) and IL-1RN (+2018).	IL-1B (–511) allele associated with increased survival in meningococcemia. Combination of the IL-1B (–511) and IL-1RN (+2018) alleles associated with decreased survival.
Endler et al [144], Brouwer et al [32]	Multiple polymorphisms for the IL-1 locus.	The IL-1RN (+2018) polymorphism was associated with risk of meningococcal disease and with its outcome.
Michalek et al [145]	IL-6 polymorphisms (G-174C and G-572C).	Both polymorphisms could be predictors of risk of development and/or predictors of sepsis severity.
Lehrnbecher et al [146]	IL-6 G-174C polymorphism	Population of children with acute myeloid leukemia. G allele associated with risk of infection with Gram-negative bacteria.
Artifoni et al [147]	IL-8 –251 A>T polymorphism	A allele associated with pyelonephritis.
Binder et al [148]	Polymorphisms of the protein C promoter: C-1654T and A-1641G	Carriers of the CG allele had an increased risk of developing meningococcal sepsis.
Multiple [149-153]	Fc gamma receptor polymorphisms	Increased risk of meningococcal disease and increased illness severity.
Hibberd et al [154]	MBL polymorphisms	Increased susceptibility to meningococcal disease.
Summerfield et al [155]	MBL polymorphisms	Increased susceptibility to severe infections.
Koch et al [156]	MBL polymorphisms	Increased risk of acute respiratory infections in children 6 to 17 months of age.
Michalek et al [157]	Bactericidal permeability increasing protein polymorphisms	Increased risk of Gram-negative sepsis and increased risk of death.
El Saleeby et al [158]	Surfactant protein A2 polymorphisms	Increased illness severity in infants with respiratory syncytial virus infection.
Dahmer et al [159]	Surfactant protein B polymorphisms	Increased severity of acute lung injury after community acquired pneumonia in African-American children.
Agbeko et al [160]	Functional polymorphisms of the complement activation cascade.	Homozygosity for the complement factor H Y402H polymorphism carries a decreased risk of sepsis.
Haralambous et al [161]	Complement factor H polymorphisms	Increased risk of invasive meningococcal disease, in association with increased serum factor H levels and reduced bactericidal activity against meningococcus.
Davila et al [162]		
Harding et al [163]	Insertion/deletion polymorphism of angiotensin converting enzyme.	DD genotype associated with increased illness severity in meningococcal disease.
Cogulu et al [164]	Angiotensin-converting enzyme insertion/deletion polymorphism.	DD genotype associated with decreased risk of sepsis.
Tekin et al [165]	Nucleotide-binding oligomerization domain-containing protein 2 receptor (pathogen recognition receptor) polymorphism.	Gene variants of the Nucleotide-binding oligomerization domain-containing protein 2 receptor associated with increased risk of sepsis and increased illness severity.
Khor et al [166]	Cytokine-inducible Src homology 2 domain protein polymorphisms: a suppressor of cytokine signaling.	Cytokine-inducible Src homology 2 domain protein variants are associated with increased risk of various types of infections in a mixed population of adults and children. Over 8,000 individuals sampled.

IL, interleukin; IL-1RN, IL-1 receptor antagonist; MBL, mannose binding lectin.

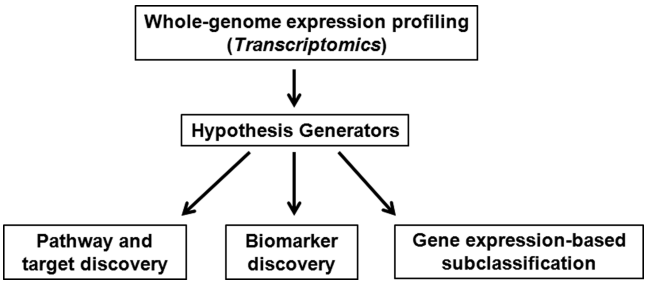

Figure 1. Schematic illustrating the discovery-oriented and hypothesis-generating approach of whole-genome expression profiling. The potential deliverables of expression profiling data include the discovery of novel pathways and therapeutic targets, biomarker discovery, and expression-based subclassification of patients with septic shock.

Discovery of Novel Pathways and Therapeutic Targets. The ability to interrogate the entire genome provides an opportunity to discover previously unrecognized targets and pathways relevant to sepsis biology. For example, Pathan et al (92) have taken this approach to address the phenomenon of myocardial dysfunction in meningococcal sepsis. Using a combination of expression profiling and *in vitro* approaches, these investigators identified interleukin (IL)-6 as a major contributor to myocardial depression in meningococcal sepsis.

Multiple expression profiling studies in children with septic shock have documented early and persistent repression of gene programs directly related to, or dependent on, zinc homeostasis, as well as low serum zinc concentrations (93–97). Since normal zinc homeostasis is critical for normal immune function (98), these observations raise the possibility of zinc supplementation as a potential therapeutic strategy for sepsis (99–101). Independent of the pediatric studies, Knoell et al (102, 103) demonstrated that zinc supplementation is a beneficial strategy in experimental sepsis. Additional studies by Knoell et al (104) corroborated decreased plasma zinc concentrations in patients with sepsis, and a correlation between low plasma zinc concentrations and higher illness severity. These same investigators have also reported that plasma zinc concentrations correlate inversely with monocyte expression of the zinc transporter gene SLC39A8 (104, 105), and expression profiling studies in children with septic shock have corroborated high level SLC39A8 expression in nonsurvivors relative to survivors (97).

Despite this interesting convergence of independent data sources, the safety and efficacy of zinc supplementation in clinical sepsis remains to be directly demonstrated. The pediatric critical illness stress-induced immune suppression (CRISIS) trial tested the efficacy of enteral zinc supplementation, along with selenium, glutamine, and metoclopramide, as a means of preventing nosocomial infection or sepsis in critically ill children (106). This trial was terminated early for futility (http://clinicaltrials.gov; NCT00395161). Potential confounders in this trial included the testing of multiple agents, thus making it difficult to assess the effect of any single agent (107, 108), and decreased bioavailability of enteral zinc (100). Consequently, there is an active Phase 1 trial involving intravenous zinc supplementation in critically ill children (NCT01062009).

In multiple studies involving children with septic shock, metalloproteinase-8 (MMP-8) has consistently been the highest expressed gene in patients with septic shock, relative to normal controls (93-97, 109, 110). MMP-8 is also more highly expressed in patients with septic shock compared to patients with sepsis, and in septic shock nonsurvivors compared to septic shock survivors (111). While MMP-8 is best known as a neutrophil-derived protease that cleaves extracellular matrix collagen, MMP-8 has other cellular sources and nonextracellular matrix substrates (112). The discovery of high level MMP-8 expression in clinical septic shock has led to the formal study of MMP-8 in experimental sepsis. These studies demonstrated that pharmacologic inhibition of MMP-8, or genetic ablation of MMP-8, confers a significant survival advantage in a murine model of sepsis (111). Collectively, these studies identify MMP-8 as a novel, candidate therapeutic target in sepsis, and this assertion is particularly intriguing given the existence of drugs to inhibit MMP-8 activity in the clinical setting (113).

Triggering receptor expressed on myeloid cells-1 is critical for amplification of the inflammatory response to pathogen challenge and there is interest in blockade of the triggering receptor expressed on myeloid cells-1 pathway in sepsis (114). A recent gene expression profiling study in pediatric septic shock compared four distinct developmental age groups (110). A primary finding of this study was that children in the "neonate" group (0 to 28 days of age) had widespread repression of genes corresponding to the triggering receptor expressed on myeloid cells-1 signaling pathway compared to older children. The observation that the triggering receptor expressed on myeloid cells-1 pathway may not be particularly active in neonates with sepsis illustrates how some candidate therapeutic strategies may not have a biological basis across all developmental age groups.

Biomarker Discovery. The diagnostic approach to the febrile child without an obvious source of infection, and distinguishing viral from bacterial infection, remain important challenges in clinical pediatrics (115). Ramilo et al (116) have applied gene expression profiling to differentiate bacterial vs. viral infection in hospitalized febrile children. Specifically, they have reported expression signatures that can distinguish Influenza A infection from bacterial infection, and *Escherichia coli* infection from *Staphylococcus aureus* infection. In a conceptually analogous study, Allantaz et al (117) reported a gene expression signature that differentiates children with systemic onset juvenile idiopathic arthritis (e.g., "sterile inflammation") from children with acute bacterial or viral systemic infections. These data provide a foundation to better address an important problem in clinical pediatrics.

Another area of interest for sepsis-related biomarker discovery involves outcome biomarkers (118–120). Expression profiling experiments in children with septic shock identified IL-8 as a differentially regulated gene between survivors and nonsurvivors, and this observation was validated by serum IL-8 protein measurements (97). A subsequent study tested the ability of serum IL-8 levels, measured within 24 hrs of admission to the pediatric intensive care unit, to predict survival/nonsurvival in pediatric septic shock (121). Using separate derivation and validation cohorts, this study demonstrated that serum IL-8 measurements could predict a 95% probability of survival with standard care. Interestingly, IL-8 was not able to predict survival with this degree of robustness in a cohort of adults with septic shock (122). It has been proposed

that serum IL-8 levels can be used to exclude children from future interventional clinical trials as a means of improving the risk-to-benefit ratio of a given therapy (121). Using a similar approach, Nowak et al (123) identified chemokine ligand (C-C motif) ligand 4 as an outcome biomarker in pediatric septic shock, but this observation remains to be validated.

Currently, there is an ongoing effort to derive and validate a multibiomarker sepsis outcome risk model in pediatric septic shock. The foundation of this effort is the unbiased selection of a panel of candidate outcome biomarkers using microarray data from a large cohort of children with septic shock (118, 124).

Gene Expression-Based Septic Shock Subclasses. Viewing septic shock as a heterogeneous syndrome implies the existence of "disease subclasses," analogous to the oncology field (119, 120). Recent studies reported pediatric septic shock subclasses based exclusively on genome-wide expression profiles. In the initial study, three subclasses of children with septic shock (subclasses "A," "B," and "C") were identified using a computer algorithm (unsupervised hierarchical clustering) that groups patients based on statistically similar patterns of gene expression, with no *a priori* knowledge of the clinical phenotype (96). *Post-hoc* analysis of the clinical subclass phenotypes revealed that subclass A patients had a significantly higher level of illness severity, including mortality.

Recognizing that standard genomic data outputs are not clinically intuitive, a subsequent study explored the feasibility of bringing expression-based subclassification closer to the bedside. The subclass-defining gene expression patterns were distilled to a 100 gene-expression signature and depicted using visually intuitive gene expression mosaics (125–127). Clinicians, without any bioinformatics training, were able to reliably allocate patients to the correct subclasses with a high degree of sensitivity and specificity. In a follow-up study, the 100 gene-expression signature and the expression mosaics were used to classify a separate validation cohort, and again, the subclass A patients were characterized by higher illness severity (128). Thus, gene expression-based subclassification of pediatric septic shock is feasible and clinically relevant. The assertion of clinical relevance is further substantiated given that the 100 class-defining genes correspond to adaptive immunity, glucocorticoid receptor signaling, and peroxisome proliferator activated receptor-α signaling (128).

Epigenetics

Epigenetics refers to heritable changes in gene expression that are not related to direct DNA sequence changes (129). The epigenetic mechanisms dictating increased or decreased gene expression include chemical modifications of DNA and posttranslational modifications of histones. A key concept of epigenetics is that the epigenetic modifications can be "inherited" (i.e., passed on to daughter cells) and can therefore lead to long-lasting effects on gene expression.

Immunity- and inflammation-related genes are subject to epigenetic regulation (130–137), and experimental data indicate that sepsis induces epigenetic changes in dendritic cells and lymphocytes, rendering the host immune deficient for a long period after the initial sepsis challenge (138–140). In children with septic shock, there is evidence of differential expression of genes involved in epigenetic regulation, in parallel with suppression of adaptive immunity genes (109).

Patients that recover from critical illness, sepsis in particular, are at increased risk of death for several years after discharge (5, 141, 142). Czaja et al (5) recently studied over 7,000 pediatric severe sepsis cases. Almost one half of the patients that were discharged after the initial admission were readmitted at least once, at a median of 3 months after discharge. Respiratory infection was the most common indication for readmission, and >30% of these readmissions were in children without comorbidities. An additional 6.5% of patients died during these readmissions. While the cause of these late deaths and the high rate of readmission are likely to be multifactorial, it is tempting to speculate on a potential role for epigenetic mechanisms involving the immune system.

CONCLUSIONS

Genetic/genomic approaches to pediatric septic shock have proliferated over the last decade. While novel information has been derived from these studies, it must be kept in mind that none of these data have been directly translated to the bedside of the critically ill child, yet. Meeting the lofty goal of clinical translation will require multi-investigator collaborations and further rigorous studies with an emphasis on independent validation. The potential deliverables of clinical translation include robust and clinically effective patient stratification strategies, and novel therapies, which will enhance, rather than replace, our current clinical protocols and guidelines.

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