

2. Price OJ, Ansley L, Menzies-Gow A, Cullinan P, Hull JH. Airway dysfunction in elite athletes—an occupational lung disease? *Allergy* 2013;68:1343–1352.
3. Dryden DM, Spooner CH, Stickland MK, Vandermeer B, Tjosvold L, Bialy L, Wong K, Rowe BH. Exercise-induced bronchoconstriction and asthma. *Evid Rep Technol Assess (Full Rep)* 2010;189:1–154.
4. Anderson SD, Argyros GJ, Magnussen H, Holzer K. Provocation by eucapnic voluntary hyperpnoea to identify exercise induced bronchoconstriction. *Br J Sports Med* 2001;35:344–347.
5. Hurwitz KM, Argyros GJ, Roach JM, Eliasson AH, Phillips YY. Interpretation of eucapnic voluntary hyperventilation in the diagnosis of asthma. *Chest* 1995;108:1240–1245.
6. Argyros GJ, Roach JM, Hurwitz KM, Eliasson AH, Phillips YY. Eucapnic voluntary hyperventilation as a bronchoprovocation technique: development of a standardized dosing schedule in asthmatics. *Chest* 1996;109:1520–1524.
7. Helenius IJ, Tikkanen HO, Haahtela T. Occurrence of exercise induced bronchospasm in elite runners: dependence on atopy and exposure to cold air and pollen. *Br J Sports Med* 1998;32:125–129.
8. Price OJ, Ansley L, Hull JH. Diagnosing exercise-induced bronchoconstriction with eucapnic voluntary hyperpnea: is one test enough? *J Allergy Clin Immunol Pract* 2015;3:243–249.
9. Nielsen EW, Hull JH, Backer V. High prevalence of exercise-induced laryngeal obstruction in athletes. *Med Sci Sports Exerc* 2013;45:2030–2035.

Copyright © 2016 by the American Thoracic Society

Nasopharyngeal Microbiome in Respiratory Syncytial Virus Resembles Profile Associated with Increased Childhood Asthma Risk

To the Editor:

Early-life acute respiratory infection (ARI) with respiratory syncytial virus (RSV) has been strongly associated with the development of childhood asthma (1), but the pathways underlying this association are poorly understood. We hypothesized that RSV alters the infant nasopharyngeal microbiome in a way that may help explain how RSV contributes to asthma development (1, 2). As a first step to address this question, we characterized the nasopharyngeal microbiome of healthy and RSV-infected infants using bacterial 16S ribosomal RNA (rRNA) gene sequencing.

We compared the nasopharyngeal microbiome of 33 infants assessed during a well-child visit with that of 99 infants assessed during confirmed RSV ARI. These 132 infants were enrolled as part of a large (N = 1,952) prospective cohort of previously healthy infants recruited near birth and with routine respiratory illness surveillance during their first winter season (3). Eligible infants were born between June and December, so they were on average ≤ 6 months of age during their first winter viral season. Thus, an RSV infection during this time likely represents their first exposure to this virus. The Institutional Review Board of Vanderbilt University approved this study, and parents provided informed consent.

Supported by National Institutes of Health grants U19AI095227, K24AI77930, U54RR24975, and U19AI110819 and contract number HHSN272200900007C.

Author Contributions: C.R.-S., J.D.C., E.K.L., M.L.M., L.J.A., and T.V.H. contributed to the study design, data collection, and manuscript writing. M.H.S., A.T., K.E.N., and S.R.D. contributed to the sample processing, data analysis, and manuscript writing.

The nasopharyngeal microbiome was sampled using a dry filter paper in healthy infants and a nasal wash in infants with RSV, as previously described (3, 4). The diagnosis of RSV was made by reverse transcriptase–polymerase chain reaction. We have also previously reported the methods used to characterize the nasopharyngeal microbiome (4). In brief, after cell lysis and extraction of the microbial genomic DNA from the samples, the V1–V3 region of 16S rRNA was amplified using 27F/534R primers and pyrosequencing was performed on a 454 sequencer. A mothur-based automated annotation pipeline, YAP, was used to perform initial processing of the 16S rRNA datasets (5). Operational taxonomic units (OTUs) were clustered at 97% sequence identity. After quality filtering, 1,231,758 reads were retained (range, 424–20,987 reads per sample). For statistical analyses, only samples with sequence counts of more than 2,000 sequences per sample were retained, and these were rarified to 2,047 sequences. Statistical analyses were done with MGSAT, using R (6).

The median age for healthy infants and for those with RSV was 5 (interquartile range, 2–9) and 22 (interquartile range, 13–27) weeks, respectively ($P = 0.001$). There were no other significant differences between groups in sex, race or ethnicity, gestational age, birth weight, mode of delivery, exposure to antibiotics *in utero* or after birth, breastfeeding, maternal smoking, maternal asthma, or type of insurance (data not shown).

The most abundant genera in samples collected among healthy infants included *Streptococcus*, *Corynebacterium*, *Staphylococcus*, and *Dolosigranulum*, whereas the most abundant genera during acute RSV infection were *Streptococcus*, *Moraxella*, *Corynebacterium*, and *Haemophilus* (Figure 1). Other than *Dolosigranulum* (base mean = 456.7; $\log_2$ fold change = -0.9321 ; q -value = 0.36), the abundance of these dominant genera was significantly different between healthy infants and those with RSV (Figure 2). *Staphylococcus* (base mean = 265.1; $\log_2$ fold change = -2.48 ; q -value = 2.14×10^{-3}) and *Corynebacterium* (base mean = 1,694; $\log_2$ fold change = -2.31 ; q -value = 1.39×10^{-3}) abundance was higher in healthy infants. In contrast, *Haemophilus* (base mean = 1,384; $\log_2$ fold change = 8.83; q -value = 1.96×10^{-27}), *Moraxella* (base mean = 2,075; $\log_2$ fold change = 4.77; q -value = 4.55×10^{-7}), and *Streptococcus* (base mean = 2,991; $\log_2$ fold change = 2.04; q -value = 7.15×10^{-5}) abundances were higher during RSV infection.

The taxonomic composition of the nasopharyngeal microbiome also differed between health and RSV at both the genus ($P < 0.001$; $R^2 = 0.08$) and the OTU ($P < 0.001$; $R^2 = 0.05$) level when using the Bray-Curtis dissimilarity index. In addition, there was a significant decrease in richness at both the genus and OTU levels during RSV, using the abundance-based estimates Chao1, observed species counts, and inverted Shannon index Hill number N_1 when applying a linear model ($P < 0.05$ for all estimates).

To control for the potential confounding effect of age, we further compared a subset of healthy infants ($n = 21$) who overlapped in age with children with acute RSV infection ($n = 37$) by restricting the analysis to infants between 3 and 20 weeks of age. The median age for these healthy infants and for those with RSV was 9 (interquartile range, 6–14) and 10 (interquartile range, 8–13) weeks, respectively ($P = 0.7032$). In this exploratory analysis, we found similar results to our main analysis in the larger dataset with regard to the differences in genus abundance and richness. The nasal microbiome significantly differed by the Bray-Curtis index at both the genus and OTU levels, and richness remained significantly lower

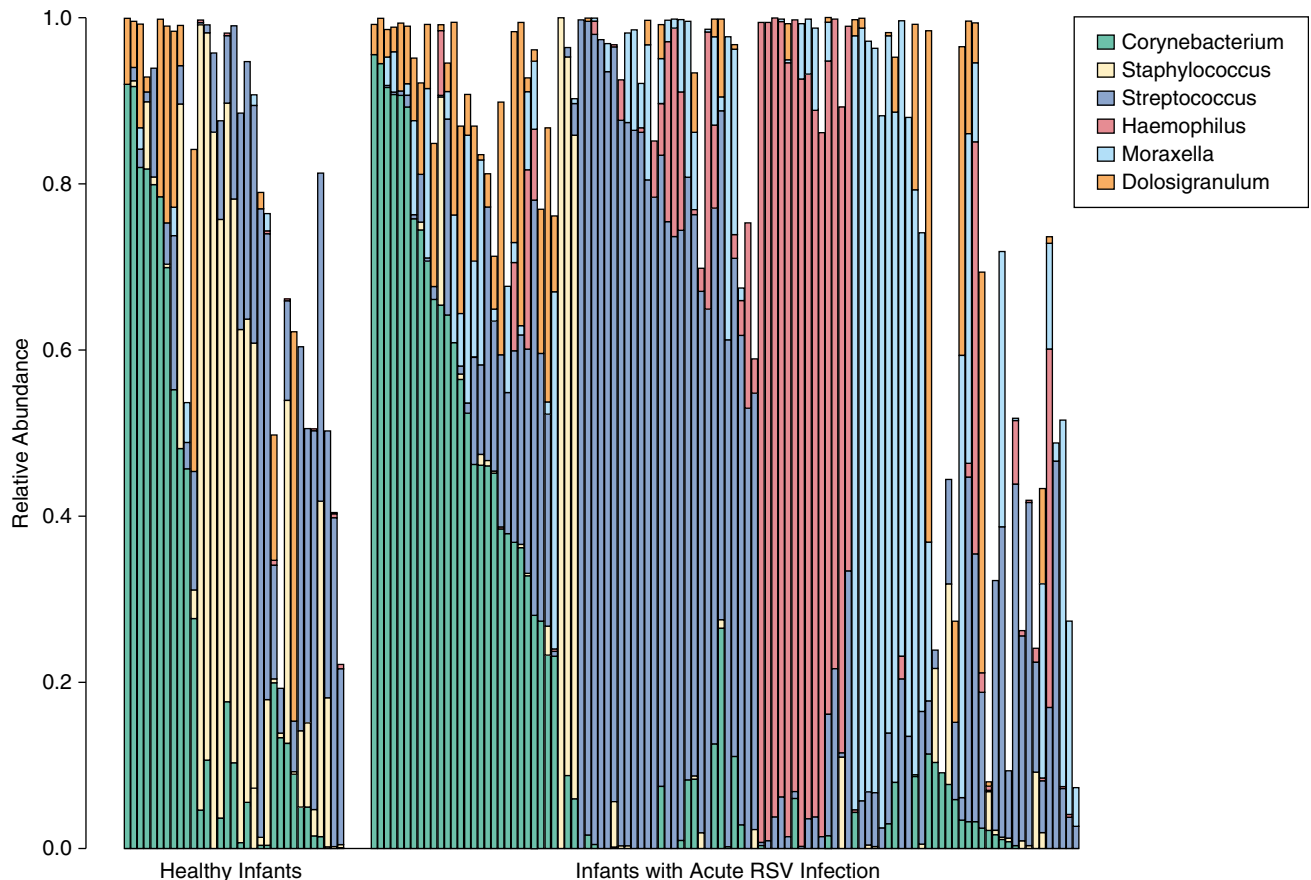


Figure 1. Nasopharyngeal microbiome composition of healthy infants (*left*) and infants during acute respiratory syncytial virus infection (RSV; *right*). Stacked bar graph showing genus-level taxonomic composition for each individual nasopharyngeal sample, expressed as a proportion of reads of infants in each group. All samples are shown in this figure, regardless of sequence count. The relative abundance of the six most prevalent genera is shown; all other genera are not shown in this figure. Within each grouping, samples are sorted by predominant genera within that sample. Infants with acute RSV infection had a nasopharyngeal microbiome dominated by *Streptococcus*, *Haemophilus*, or *Moraxella*, whereas *Haemophilus* and *Moraxella* were observed at very low frequencies in healthy infants.

during acute RSV infection ($P < 0.05$ for all estimates). Furthermore, *Moraxella*, *Haemophilus*, and *Streptococcus* remained higher during acute RSV infection, whereas *Staphylococcus* and *Corynebacterium* remained lower during this illness (data not shown). These findings suggest that the nasopharyngeal microbiome changes we observed are not a result of the age difference between groups.

This study adds to the small but increasing literature on the association of early-life ARIs and the nasopharyngeal microbiome in children. To our knowledge, this is the first study to directly compare the nasal microbiome of healthy infants with the nasal microbiome during acute RSV infection in early life, using bacterial 16S rRNA gene sequencing. Prior studies using conventional culture techniques have also shown an increase in *Haemophilus*, *Moraxella*, and/or *Streptococcus* in the nasopharynx of infants with RSV when compared with healthy controls (7, 8). More recently, another study using 16S rRNA profiling in infants at high risk for atopy also found that these bacteria were more frequent in infants with ARIs, even after adjusting for viral detection (9). In another study using 16S rRNA sequencing, *Moraxella*-dominated profiles in infancy were associated with a lower rate of parental report of ARIs; however, most of these were upper ARIs, and viral detection

was not done (10), which may explain the discrepant findings. Furthermore, there were several *Moraxella* OTUs noted in this study, and RSV could have distinct effects on different species within this genus. Taken together, these studies provide evidence for physiologic linkage between respiratory viruses and compositional shifts in the nasopharyngeal microbiome.

Interestingly, the predominant genera found in infants with RSV have been previously associated with an increased risk for childhood asthma (2). Thus, RSV may in part increase the risk for asthma through alterations in the composition of the nasopharyngeal microbiome.

One limitation to our findings is that the methods to collect the nasal samples were different between the two groups. We have recently shown that nasal dry filter papers, similar to nasal washes, can capture a wide variety of expected nasopharyngeal taxa (4); however, it is possible that certain bacterial genera could be under- or overrepresented by one of these methods when compared with the other. In addition, the region sampled by nasal filters is likely to be more proximal than that sampled by nasal washes. Thus, our findings merit replication in larger longitudinal cohorts. It is also possible that the microbial changes

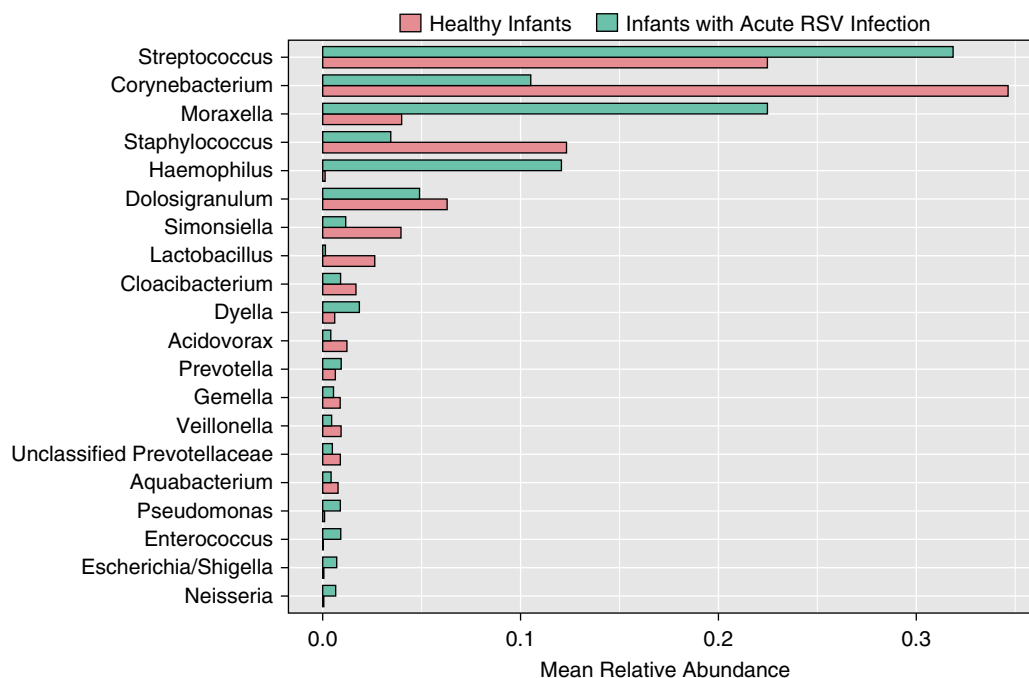


Figure 2. Average relative abundance of genera in nasopharyngeal samples of healthy infants and infants with acute respiratory syncytial virus (RSV) infection. Within each sample, counts were normalized to simple proportions. The relative abundance of the 20 most prevalent genera is shown; all other genera are not shown in this figure.

associated with acute RSV infection are not exclusive to this respiratory virus but are a result of the local effects of viral ARIs in general; however, limited evidence suggests that compositional shifts in the airway microbiome during childhood ARIs are virus specific (11). In spite of these limitations, our study lays the groundwork for future studies to examine the interactions among RSV infection, the infant's nasopharyngeal microbiome, and how RSV may contribute to the development of childhood asthma. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Christian Rosas-Salazar, M.D., M.P.H.*
Vanderbilt University School of Medicine
Nashville, Tennessee

Meghan H. Shilts, M.H.S., M.S.*
Andrey Tovchigrechko, Ph.D.
J. Craig Venter Institute
Rockville, Maryland

James D. Chappell, M.D., Ph.D.
Emma K. Larkin, Ph.D.
Vanderbilt University School of Medicine
Nashville, Tennessee

Karen E. Nelson, Ph.D.
J. Craig Venter Institute
Rockville, Maryland

Martin L. Moore, Ph.D.
Larry J. Anderson, M.D.
Emory University School of Medicine
Atlanta, Georgia

and
Children's Healthcare of Atlanta
Atlanta, Georgia

Suman R. Das, Ph.D.
J. Craig Venter Institute
Rockville, Maryland

Tina V. Hartert, M.D., M.P.H.
Vanderbilt University School of Medicine
Nashville, Tennessee

ORCID ID: 0000-0001-7470-1166 (T.V.H.).

*These authors contributed equally to this letter.

References

1. Feldman AS, He Y, Moore ML, Hershenson MB, Hartert TV. Toward primary prevention of asthma. Reviewing the evidence for early-life respiratory viral infections as modifiable risk factors to prevent childhood asthma. *Am J Respir Crit Care Med* 2015;191:34–44.
2. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, Brasholt M, Heltberg A, Vissing NH, Thorsen SV, *et al.* Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;357:1487–1495.
3. Larkin EK, Gebretsadik T, Moore ML, Anderson LJ, Dupont WD, Chappell JD, Minton PA, Peebles RS Jr, Moore PE, Valet RS, *et al.*; INSPIRE Study. Objectives, design and enrollment results from the infant susceptibility to pulmonary infections and asthma following RSV exposure study (INSPIRE). *BMC Pulm Med* 2015;15:45.
4. Shilts MH, Rosas-Salazar C, Tovchigrechko A, Larkin EK, Torralba M, Akopov A, Halpin R, Peebles RS, Moore ML, Anderson LJ, *et al.* Minimally invasive sampling method identifies differences in

- taxonomic richness of nasal microbiomes in young infants associated with mode of delivery. *Microb Ecol* 2016;71:233–242.
5. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75:7537–7541.
 6. Tovchigrechko A. MGSAT semi-automated differential abundance analysis of omics datasets. San Francisco: Atlassian [updated 2015 Jul 9; accessed 2015 Feb 20]. Available from: <https://bitbucket.org/andreyto/mgsat>
 7. Suárez-Arrabal MC, Mella C, Lopez SM, Brown NV, Hall MW, Hammond S, Shiels W, Groner J, Marcon M, Ramilo O, *et al.* Nasopharyngeal bacterial burden and antibiotics: influence on inflammatory markers and disease severity in infants with respiratory syncytial virus bronchiolitis. *J Infect* 2015;71:458–469.
 8. van den Bergh MR, Biesbroek G, Rossen JW, de Steenhuijsen P, Biesbroek WA, Bosch AA, van Gils EJ, Wang X, Boonacker CW, Veenhoven RH, Bruin JP, *et al.* Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. *PLoS One* 2012;7:e47711.
 9. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, Holt BJ, Hales BJ, Walker ML, Hollams E, *et al.* The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 2015;17:704–715.
 10. Biesbroek G, Tsvitvadze E, Sanders EA, Montijn R, Veenhoven RH, Keijser BJ, Bogaert D. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am J Respir Crit Care Med* 2014;190:1283–1292.
 11. Hyde ER, Petrosino JF, Piedra PA, Camargo CA Jr, Espinola JA, Mansbach JM. Nasopharyngeal Proteobacteria are associated with viral etiology and acute wheezing in children with severe bronchiolitis. *J Allergy Clin Immunol* 2014;133:1220–1222.

Copyright © 2016 by the American Thoracic Society

Tempering Sepsis-induced Hyperbilirubinemia: Implications for Short- and Long-Term Outcomes

To the Editor:

We read with enthusiasm the recent study by Nesseler and colleagues regarding liver dysfunction associated with long-term mortality in septic shock (1).

Using a cohort from the PROWESS-SHOCK (Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis and Septic Shock) study, the authors identified a strong relationship between the occurrence or worsening of liver dysfunction in septic shock as measured by a Sequential Organ Failure Assessment (SOFA) liver score greater than 0 and mortality (1). We agree with the authors' premise that a low SOFA liver injury score (hypothesizing a low-grade injury) can be associated with major effects. We would like to emphasize implications for short-term outcomes, but we would also encourage caution in linking sepsis-induced hyperbilirubinemia to poor long-term outcomes.

Short-term outcomes related to hyperbilirubinemia seem clear and are supported by additional studies (2–4). In fact, sepsis-induced hyperbilirubinemia is most commonly due to cholestasis, implicating a cytokine- and lipopolysaccharide-mediated decrease in basolateral and canalicular transport (5). Zhai and colleagues demonstrated that an admission bilirubin of greater than 2 mg/dl was independently associated with the development of acute

respiratory distress syndrome (5). In patients in the surgical intensive care unit, Field and colleagues described a threefold risk for infection with elevated bilirubin (2). Our group demonstrated that a serum bilirubin greater than 1 mg/dl in severe sepsis was associated with an increased risk for mortality (odds ratio, 3.85; 95% confidence interval [CI], 1.21–12.2), increased length of stay in the intensive care unit (odds ratio, 1.55; 95% CI, 1.20–2.03), increased duration of mechanical ventilation (2.39-fold; 95% CI, 1.38–4.14), and increased duration of vasopressor use (1.2-fold; 95% CI, 1.04–1.33) (4). Implications for short-term management include early sepsis recognition, early resuscitation, and early eradication of infectious foci to mitigate downstream organ injury (6).

Implications for long-term outcome are not as clear and may be related to methodologic problems. First, even though SOFA liver scores were evaluated daily from baseline to Days 7, 14, and 28, the authors do not provide delta SOFA liver score (or bilirubin levels) at 28, 90, or 180 days. Temporal changes in SOFA score and/or bilirubin could provide support to associate biologic plausibility (e.g., induction of chronic liver dysfunction) for the increased mortality risk. Second, a composite severity of illness score, such as total SOFA and/or Acute Physiology and Chronic Health Evaluation II, was not reported. The independent association of hyperbilirubinemia and mortality should adjust for severity of illness; otherwise, bilirubin may simply be a covariate. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Jayshil Patel, M.D.
Amit Taneja, M.D.
Department of Medicine
Medical College of Wisconsin
Milwaukee, Wisconsin

References

1. Nesseler N, Launey Y, Aninat C, White J, Corlu A, Pieper K, Mallédant Y, Seguin P. Liver dysfunction is associated with long-term mortality in septic shock. *Am J Respir Crit Care Med* 2016;193:335–337.
2. Field E, Horst HM, Rubinfeld IS, Copeland CF, Waheed U, Jordan J, Barry A, Brandt MM. Hyperbilirubinemia: a risk factor for infection in the surgical intensive care unit. *Am J Surg* 2008;195:304–306. [Discussion, pp. 306–307].
3. Patel JJ, Taneja A, Niccum D, Kumar G, Jacobs E, Nanchal R. The association of serum bilirubin levels on the outcomes of severe sepsis. *J Intensive Care Med* 2015;30:23–29.
4. Chand N, Sanyal AJ. Sepsis-induced cholestasis. *Hepatology* 2007;45:230–241.
5. Zhai R, Sheu CC, Su L, Gong MN, Tejera P, Chen F, Wang Z, Convery MP, Thompson BT, Christiani DC. Serum bilirubin levels on ICU admission are associated with ARDS development and mortality in sepsis. *Thorax* 2009;64:784–790.
6. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky JE, Sprung CL, Douglas IS, Jaeschke R, *et al.*; Surviving Sepsis Campaign Guidelines Committee including The Pediatric Subgroup. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock, 2012. *Intensive Care Med* 2013;39:165–228.

Copyright © 2016 by the American Thoracic Society

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.