

such as hypoventilation and hypereosinophilic syndrome is surprising, and we propose that these findings may indicate novel functions for these gene products relevant to the pathogenesis of lung disease in smokers, a concept that requires further study.

Our study has limitations. Subsequent functional investigation is needed to determine how these genes may contribute to the pathogenesis of complex, smoking-related acquired lung disease. This is especially true for genes whereby loss-of-function mutations are implicated in the lung disorder but were up-regulated in healthy smokers in this study. In addition, only a subset of samples were confirmed by RNA sequencing. Bearing these limitations in mind, the data support the concept that genes implicated in rare monogenic lung diseases may have roles in the pathogenesis of smoking-related lung diseases and that smoking-induced changes in expression of these genes could be early molecular events in the development of diverse smoking-related clinical phenotypes. ■

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Ann E. Tilley, M.D.\*  
Michelle R. Staudt, Ph.D.\*  
Jacqueline Salit, M.S.  
Benjamin Van de Graaf, M.B.A.  
Yael Strulovici-Barel, M.S.  
Robert J. Kaner, M.D.  
Thomas Vincent, Ph.D.  
Francisco Agosto-Perez, M.S.  
Weill Cornell Medical College  
New York, New York

Jason G. Mezey, Ph.D.  
Weill Cornell Medical College  
New York, New York  
and  
Cornell University  
Ithaca, New York

Benjamin A. Raby, M.D., M.P.H.  
Harvard Medical School  
Boston, Massachusetts

Ronald G. Crystal, M.D.  
Weill Cornell Medical College  
New York, New York

\*These authors contributed equally to this study.

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## Key Role of $\alpha$ -Toxin in Fatal Pneumonia Caused by *Staphylococcus aureus* Sequence Type 398

To the Editor:

Pneumonia is a widespread and severe disease and a leading cause of health care-associated infections. *Staphylococcus aureus* is the most important pathogen in all types of pneumonia (1). *S. aureus* sequence type (ST) 398 is a clone that is traditionally associated with animal farming (2). Both methicillin-sensitive (MSSA) and methicillin-resistant ST398 are predominant in livestock, but only ST398 MSSA has been reported to spread among humans (3). These potentially human-adapted ST398 MSSA strains have primarily been associated with skin and bloodstream infections (4). Notably, there are virtually no data on the role of virulence factors in isolates of the ST398 lineage achieved by advanced molecular investigation.

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Author Contributions: M.F.B., A.J.Y., A.E.V., J.M., H.-S.J., and G.Y.C.C. performed experiments; M.T.S. and C.F.R. obtained patient samples; C.M.C.B.F. and R.S.C. collected clinical data; M.F.B., H.-S.J., G.Y.C.C., and M.O. analyzed data; M.F.B., M.L.R.S.C., and M.O. conceived the study and supervised experiments; and M.O. wrote the paper.

We performed a prospective study at the hospital of Botucatu Medical School, Brazil, from November 2011 to August 2013, that included all adult patients at the intensive care unit under mechanical ventilation ( $n = 270$ ). *S. aureus* was isolated from 47 patients, and 27 of those patients developed pneumonia. Methicillin resistance occurred in 22 isolates (47%) and was more common in hospital-associated pneumonia (7 of 9; 78%) than in community-associated pneumonia (1 of 7; 14%) or only colonizing (without progression to respiratory infection, 6 of 18; 33%) isolates.

Multilocus sequence typing revealed that a considerable number of isolates (five; 11%) belonged to the ST398 lineage. Intriguingly, the rate of pneumonia cases was highest among the ST398 lineage (80%), as was the fatality rate resulting from pneumonia (three of four pneumonia cases; 75%). Three (27%) of 11 fatal pneumonia cases in the reported time frame were a result of ST398 isolates. All ST398 isolates were methicillin-sensitive, positive for the *chp* and *scn* genes found to be associated with human-adapted ST398 MSSA (3), and belonged to *spa* type t1451. Brief case reports of the three ST398-associated fatal pneumonia cases are shown in Table 1. Notably, there was at least one case of fatal ST398 pneumonia, for which the patient did not report animal contact, indicative of nosocomial acquisition of the infecting strain.

We found no significant correlation between the presence of key virulence genes and pneumonia or fatality. Prompted by our recent findings that the severity of staphylococcal skin infection is influenced to a considerable degree by gene expression levels (5), we hypothesized that this may also be the case for ST398 pneumonia.

**Table 1.** Brief Case Reports of Fatal Pneumonia Cases Caused by ST398 Isolates

Patient	Isolate	Brief Case Report
39-yr-old man	A16	The patient presented at the intensive care unit for head trauma and pulmonary contusion. He developed skin and soft tissue infection, which was treated with cefepime and clindamycin. He died from pneumonia that developed within 48 h of admission. This patient was a rural worker.
61-yr-old man	A19	The patient presented at the intensive care unit for chronic renal failure with acute worsening and necessity of dialysis. He was treated with amoxicillin and clavulanic acid because of sinusitis for 3 d after admission. Afterward, the treatment was changed to imipenem and vancomycin because the patient developed ventilator-associated pneumonia. The patient died from ventilator-associated pneumonia. He reported no animal contact.
76-yr-old man	A52	The patient presented to the intensive care unit for urinary tract infection with septic shock. He received imipenem, polymyxin E, and linezolid. He died from hospital-associated pneumonia that was not associated with mechanical ventilation. He reported contact with pigs and poultry.

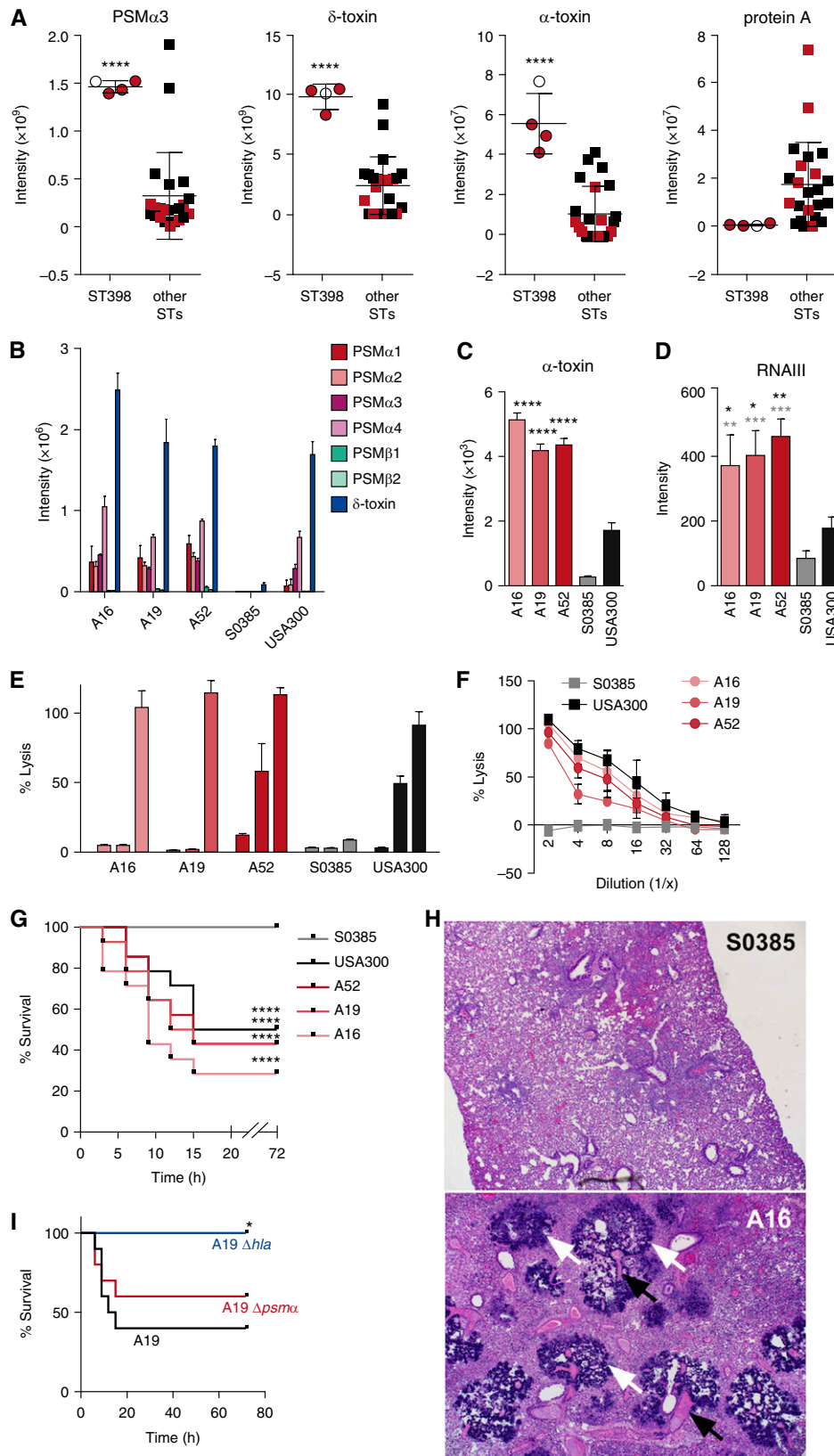
Definition of abbreviation: ST = sequence type.

We focused on the major factors shown to affect staphylococcal lung infection:  $\alpha$ -toxin, phenol-soluble modulins (PSM) peptides, and protein A (6–8). The genes encoding Pantone-Valentine leukocidin were not present in our ST398 isolates. When we compared all pneumonia isolates, the ST398 isolates showed significantly higher *in vitro* expression levels of the most cytolytic PSM, PSM $\alpha$ 3,  $\delta$ -toxin (a readout for functionality of the accessory gene regulator *Agr*), and  $\alpha$ -toxin, than non-ST398 isolates (Figure 1A). Differences in other PSMs were similar (data not shown). Furthermore, PSM and  $\alpha$ -toxin levels were similar to or exceeded those detected in the highly virulent community-associated methicillin-resistant *S. aureus* clone USA300, which is known to strongly express PSMs and  $\alpha$ -toxin and whose virulence potential relies on those cytolytins (6, 9) (Figures 1B and 1C). Notably, ST398 PSM and  $\alpha$ -toxin levels strongly exceeded those detected in the standard ST398 strain S0385, a Dutch livestock-associated methicillin-resistant *S. aureus* isolate from a case of human endocarditis (10). In contrast, production of the negatively *Agr*-regulated protein A was significantly lower in the ST398 isolates compared with the other isolates (Figure 1A). Thus, our data were indicative of a highly functional *Agr* system in the ST398 isolates, which we confirmed by quantitative real-time polymerase chain reaction of RNAPIII (Figure 1D). Furthermore, among isolates that caused fatal pneumonia, high production of PSM $\alpha$  and  $\alpha$ -toxin only occurred in ST398 strains, indicating there is a specific role for PSM $\alpha$  peptides and  $\alpha$ -toxin in the extraordinary virulence of ST398 isolates. The ST398 isolates showed pronounced capacities to lyse human erythrocytes and neutrophils (Figures 1E and 1F), demonstrating that high cytolytin expression levels translate to strong cytolytic capacities.

In a mouse pneumonia model (Figure 1G), the fatality rate in mice infected with the three ST398 strains was high, at least equaling that caused by strain USA300, and significantly higher than that caused by strain S0385. Accordingly, histological analysis demonstrated extensive pathology in the lungs of mice infected with the ST398 or USA300 strains, which all showed signs of severe and multifocal necrotizing pneumonia, with acute inflammation, necrotizing vasculitis, thrombosis, and large numbers of bacterial colonies, to an extent that was indistinguishable between groups (shown in Figure 1H for strain A16). Mice infected with strain S0385, in contrast, showed only signs of moderate peribronchiolar pneumonia and no bacterial colonies (Figure 1H). Thus, the murine pneumonia model well reflected the clinical results and confirmed the high virulence of the ST398 isolates.

Then, to analyze the effect of PSM $\alpha$  and  $\alpha$ -toxin, we produced isogenic gene deletion mutants in the *psm $\alpha$*  operon and *hla* gene in isolate A19. Both *psm $\alpha$*  and *hla* mutants caused a lower number of deaths than the wild-type A19 isolate (Figure 1I). However, only the *hla* mutant survival rates were significantly different from those of the wild-type strain, with no animals infected by the A19 *hla* mutant succumbing to the infection. These results attribute a central function to  $\alpha$ -toxin and high  $\alpha$ -toxin expression in the pathogenesis of fatal pneumonia by ST398 *S. aureus*.

In conclusion, our study identifies ST398 MSSA as a dangerous and highly virulent emerging source of fatal pneumonia. Furthermore, although our results emphasize strain dependence, they are in accordance with the notion of a generally crucial role of  $\alpha$ -toxin in lung infection. Finally, our findings call for surveillance measures analyzing not only antibiotic resistance but



**Figure 1.** Molecular investigation of fatal pneumonia resulting from sequence type (ST) 398. (A) *In vitro* production levels of phenol-soluble modulins (PSM)  $\alpha$ 3 and  $\delta$ -toxin (by high-performance liquid chromatography/mass spectrometry) and  $\alpha$ -toxin and protein A (by Western blot densitometry) of all

also virulence potential of *S. aureus* as an important factor that contributes to infection outcome. ■

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Mariana F. Bonesso, Ph.D.  
National Institutes of Health  
Bethesda, Maryland

and  
Universidade Estadual Paulista  
Botucatu, Brazil

Anthony J. Yeh, B.Sc.  
Amer E. Villaruz, Ph.D.  
Hwang-Soo Joo, Ph.D.  
Joshua McCausland, B.Sc.  
National Institutes of Health  
Bethesda, Maryland

Carlos M. C. B. Fortaleza, M.D.  
Ricardo S. Cavalcante, M.D.  
Moises T. Sobrinho, M.D.  
Carlos F. Ronchi, M.D.  
Universidade Estadual Paulista  
Botucatu, Brazil

Gordon Y. C. Cheung, Ph.D.  
National Institutes of Health  
Bethesda, Maryland

Maria L. R. S. Cunha, M.D., Ph.D.  
Universidade Estadual Paulista  
Botucatu, Brazil

Michael Otto, Ph.D.  
National Institutes of Health  
Bethesda, Maryland

ORCID ID: 0000-0002-2222-4115 (M.O.).

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## Written Asthma Action Plans: The Devil's in the Details

To the Editor:

The report by Sheares and colleagues found no benefit from written action plans provided to patients of subspecialist physicians (1). However, examination of the data presented in that manuscript indicates control of asthma was suboptimal, whether or not the written action plan was provided. Specifically, an average of 4 symptomatic days in the previous

**Figure 1.** (Continued). pneumonia isolates (here and all other experiments: grown to stationary growth phase in tryptic soy broth). Values corresponding to fatal cases are shown in red. \*\*\*\* $P < 0.0001$  (unpaired  $t$  test). (B and C) Comparison of *in vitro* production levels of PSMs (B) and  $\alpha$ -toxin (C) to those of USA300 (the USA300 isolate SF8300 was used for all analyses in the present study) and the ST398 endocarditis isolate S0385. \*\*\*\* $P < 0.0001$  (vs. USA300 and S0385, one-way analysis of variance [ANOVA]). (D) RNAlII expression levels by quantitative real-time polymerase chain reaction. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (one-way ANOVA; black asterisks, vs. USA300; gray asterisks, vs. S0385). (E) Neutrophil lysis. Culture filtrates were measured for their capacity to lyse human neutrophils by release of lactate dehydrogenase. The three bars for each strain represent decreasing dilutions: 1:10; 1:5; and 1:2. (F) Lysis of human erythrocytes. Culture filtrates were used at the given dilutions. (G–I) Mouse pneumonia model. Mice were infected intranasally with  $1 \times 10^8$  colony-forming units, and disease development was monitored for a total of 72 hours. (G) Comparison of ST398 pneumonia isolates with USA300 and S0385.  $n = 25$  mice per group. \*\*\*\* $P < 0.0001$  (log-rank test, vs. data obtained with S0385). (H) Histology. Microscopic images of lung tissue; magnification 40 $\times$ . White arrows point to bacterial colonies, black arrows to necrotic vessels. An image of lung tissue of a mouse infected with strain A16 is shown; pathology in the lungs of mice infected with other ST398 strains and USA300 were similar. (I) Comparison of ST398 A19 isolate wild-type versus isogenic *hla* and *psmA* deletion strains.  $n = 10$  mice per group. \* $P < 0.05$  (log-rank test; vs. data obtained with wild-type strain A19). (A–F) Error bars show  $\pm$ SD.