PATHOLOGY

Human genetic and metabolite variation reveals that methylthioadenosine is a prognostic biomarker and an inflammatory regulator in sepsis

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Sepsis is a deleterious inflammatory response to infection with high mortality. Reliable sepsis biomarkers could improve diagnosis, prognosis, and treatment. Integration of human genetics, patient metabolite and cytokine measurements, and testing in a mouse model demonstrate that the methionine salvage pathway is a regulator of sepsis that can accurately predict prognosis in patients. Pathway-based genome-wide association analysis of nontyphoidal *Salmonella* bacteremia showed a strong enrichment for single-nucleotide polymorphisms near the components of the methionine salvage pathway. Measurement of the pathway's substrate, methylthioadenosine (MTA), in two cohorts of sepsis patients demonstrated increased plasma MTA in nonsurvivors. Plasma MTA was correlated with levels of inflammatory cytokines, indicating that elevated MTA marks a subset of patients with excessive inflammation. A machine-learning model combining MTA and other variables yielded approximately 80% accuracy (area under the curve) in predicting death. Furthermore, mice infected with *Salmonella* had prolonged survival when MTA was administered before infection, suggesting that manipulating MTA levels could regulate the severity of the inflammatory response. Our results demonstrate how combining genetic data, biomolecule measurements, and animal models can shape our understanding of disease and lead to new biomarkers for patient stratification and potential therapeutic targeting.

INTRODUCTION

The 20th century witnessed a remarkable decrease in infectious disease deaths. Although a great deal of the decline was due to improved sanitation (*1*), early antibiotics, resuscitation, and supportive hospital care have also played major roles, particularly in improving outcomes in bacteremia and sepsis (*2*, *3*). With sepsis mortality rates up to 30% even in advanced care settings (*4*, *5*), there has long been hope that this therapeutic arsenal could be complemented by host-directed sepsis therapies. However, failures in more than 100 clinical trials aimed at modulating the immune response in sepsis have demonstrated that a better understanding of host biology and differences in clinical factors is necessary (*6*). Heterogeneous host responses to infection, some on opposite ends of an inflammatory spectrum (*7*), have made progress difficult. It is unclear whether these failed clinical trials can be more accurately attributed to a failure of the treatments or to a failure in the diagnostic criteria. Most recently, the Sepsis Definitions Task Force has updated definitions for sepsis and septic shock, underscoring the importance of sepsis criteria for clinical trials of molecular therapies (*8*–*10*). Notably, the new definitions remain broad in their criteria, encompassing a wide variety of patients with varying etiology and pathophysiology. Therefore, an important goal toward personalized treatment is to improve stratification of patients for clinical trials by developing better biomarkers and integrating information on clinical course, site of infection, cellular dysfunction, and other factors (*11*). Metabolite markers are particularly attractive for this goal because they serve to integrate multiple inputs (transcriptional, translational, and environmental) into an active biomolecule that can have large effects on physiology. On the other hand, genetic markers of susceptibility have the advantage of not changing during the course of disease, making the direction of causation for true genetic associations unambiguous. Therefore, an improved understanding of human genetic differences that contribute to regulation of metabolite levels could powerfully couple the larger effect sizes of metabolites to the causality of genetic variants for prioritizing and designing interventions.

One component of the host response that has received significant interest in characterizing and possibly treating sepsis is the activation of inflammatory caspases (*12*, *13*). A variety of bacterial patterns and danger signals can trigger the formation of molecular scaffolds called inflammasomes that serve to activate the inflammatory caspases (caspase-1, caspase-4, and caspase-5 in humans and caspase-1 and caspase-11 in

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mice) (*14*–*16*). The two best characterized effects of this activation are secretion of cleaved/activated forms of interleukin-1β (IL-1β) and IL-18 and induction of a proinflammatory form of cell death known as pyroptosis. In addition to releasing proinflammatory cellular components, pyroptosis likely also serves as a means of depriving some bacteria of their intracellular niche (*17*). Targeting some of these downstream effects of inflammatory caspases has previously been examined for sepsis therapy. In clinical trials, IL-1 receptor antagonist (IL-1ra) showed a small but significant improvement in 28-day survival time for a select group of patients presenting with severe sepsis without shock (*18*), but a subsequent multicenter trial could not recapitulate these results (*19*). A synonymous coding variant in IL-1ra that leads to increased plasma levels of the protein was associated with improved survival from septic shock, further suggesting that modulation of the inflammatory response by IL-1ra may benefit select patients (*20*). In regard to the second major function of caspase activation, inhibition of cell death has shown benefit in some animal sepsis models (*21*, *22*), but the relevance of pyroptosis during sepsis in humans remains poorly understood.

Here, we have developed a hypothesis from the discovery of naturally occurring genetic variation modulating pyroptosis (*23*, *24*) and tested it in multiple patient data sets. Specifically, we previously determined that a single-nucleotide polymorphism (SNP) associated with the expression level of the *APIP* gene was associated with both *Salmonella*-induced pyroptosis in vitro and bacteremia and risk of death in patients with the systemic inflammatory response syndrome (SIRS) (*25*). Because *APIP* encodes a methionine salvage enzyme and regulates the levels of the substrate of the pathway, methylthioadenosine (MTA) (*25*–*27*), one hypothesis is that MTA could serve as a rheostat for inflammatory response that could modulate susceptibility and severity of sepsis (*28*). Although other studies have demonstrated an anti-inflammatory effect of MTA in lipopolysaccharide (LPS) endotoxemia (*29*) and in chemically induced colitis (*30*), our previous work suggests a proinflammatory activity during infection because exogenous MTA increased *Salmonella*-induced pyroptosis in cultured cells (*25*). Thus, there is controversy surrounding the role of MTA in inflammation. Furthermore, how MTA levels change during the course of infection and its possible value as a biomarker in sepsis have never before been examined.

In this study, we have confirmed that *APIP* is associated with bacteremia using a nontyphoidal *Salmonella* (NTS) bacteremia data set from Kenya (*31*). We also have found a clear enrichment of bacteremia-associated SNPs near components of the methionine salvage pathway, implicating, for the first time, the entire methionine salvage pathway as a regulator of bacteremia and sepsis. We then used samples from two prospective clinical studies to examine the relationships among *APIP*, MTA levels, and sepsis outcome. We discovered that elevated plasma MTA levels near the time of suspected infection are associated with death in sepsis and that measurement of this single metabolite provides prognostic ability comparable to the frequently used APACHE II (Acute Physiology and Chronic Health Evaluation II) score (*32*); however, unlike APACHE II, elevated MTA levels are specific to infection. A mouse model of *Salmonella* infection mirrored the elevated plasma levels of MTA observed in humans. Furthermore, we found that tissue MTA levels in mice were depleted during infection, lending insight into the directionality of the MTA response. Finally, administration of exogenous MTA to mice before infection prolonged survival and suppressed levels of proinflammatory cytokines. Our study shows that the methionine salvage pathway is an important metabolic regulator of sepsis and that MTA could be used as a powerful prognostic biomarker with potential therapeutic benefit in sepsis.

RESULTS

SNPs in the methionine salvage pathway are associated with NTS bacteremia

We previously showed that natural genetic variation affecting *APIP* gene expression could regulate the susceptibility of cells to undergo a form of proinflammatory cell death called pyroptosis, triggered by *Salmonella* and other infections. This effect on pyroptosis was mediated by the role of APIP in the methionine salvage pathway. In patients from Seattle hospitals meeting the physiologic criteria for SIRS, the allele associated with reduced expression of *APIP* was associated with reduced odds of culture-verified sepsis and reduced odds of death (*25*). However, it remained unclear whether association with the lower-expressed, proinflammatory *APIP* allele was specific to infection or a more general indicator of a severe inflammatory response from both infectious and noninfectious causes. To further explore the relationship with infection in human cohorts, we examined genome-wide association study (GWAS) data performed in 180 Kenyan children with blood culture–positive (bacteremia) NTS infection and 2677 controls from a birth cohort of the same population (*31*) to determine whether SNPs in/near *APIP* demonstrated an association with this specific diagnosis. All studies used in this study are summarized in Table 1.

We observed an association signal near the *APIP* gene with NTS bacteremia (Fig. 1A). The strongest association was seen with the noncoding SNP rs2433162 [$P = 4.8 \times 10^{-7}$; odds ratio (OR) = 0.43, dominant model], but there was also an association, albeit weaker, with rs514182 ($P = 0.03$; OR = 0.71, dominant model). The rs514182 SNP is the same SNP associated with reduced expression levels of *APIP* and increased pyroptosis in the cell culture model (*25*). The directionality of effect seen in the current study was consistent with the findings of our previous patient studies: The proinflammatory allele of *APIP* is associated with reduced odds of bacteremia and death in SIRS patients in Seattle (*25*) and with reduced odds of NTS bacteremia in Kenya.

The association of the *APIP* SNPs with NTS bacteremia confirms the role of this gene in the regulation of bacteremia and sepsis. However, these results do not show whether this effect is mediated through the role of APIP in the methionine salvage pathway. APIP has been reported to have distinct roles in two cell death pathways, pyroptosis and apoptosis. We previously have shown (*25*) and others have confirmed (*26*) that, although the enzymatic methionine salvage activity is required for regulation of pyroptosis, regulation of apoptosis is due to a different domain of the protein.

If methionine salvage is the mechanism by which APIP regulates sepsis, then we would predict that other genetic variants near or in genes encoding enzymes in the methionine salvage pathway might also be associated with NTS bacteremia. Pathway analysis using INRICH (*33*) showed that the only pathway showing significant enrichment in the NTS bacteremia GWAS was "metabolism of polyamines" (empirical $P = 2.0 \times 10^{-5}$, $P = 0.01$ after multiple-test correction). Three of the individual GWAS signals identified by this pathway-based analysis are from components of the methionine salvage pathway (half of the six enzymes that make up the pathway), while a fourth is part of the polyamine synthesis pathway, which results in MTA production (Fig. 1, B and C). The findings strongly implicate the methionine salvage pathway in defense against NTS infection. Although each SNP contributes a small fraction to overall genetic risk for bacteremia, we hypothesized that the metabolite that feeds into the pathway (MTA) could be playing a central regulatory role, larger than the role of the individual SNPs (Fig. 1D). Therefore, we characterized the role of MTA in sepsis patients.

MTA is associated with sepsis death

To determine whether there was an association between MTA levels and sepsis outcomes, we used plasma samples that had previously undergone metabolomics profiling as part of the Community Acquired Pneumonia and Sepsis Outcome Diagnostics (CAPSOD) study (*34*–*36*). Briefly, 1152 patients with suspected community-acquired infection and who met SIRS criteria were enrolled in the emergency rooms of three U.S. hospitals, and extensive phenotypic and laboratory data were collected. Some were later adjudicated as having noninfectious SIRS, allowing grouping based on etiology (noninfectious SIRS versus sepsis) and by primary outcome (survival versus death). A 121-subject subset with sepsis previously underwent metabolomics profiling (Table 2) (*34*). Our subsequent evaluation of the MTA levels in these CAPSOD samples demonstrated an association between relative MTA levels and sepsis outcome (Fig. 2A, $P = 0.002$). Sepsis survivors had significantly lower levels of MTA compared to the sepsis nonsurvivors.

Although we had hypothesized that an association would be present based on the genetic data, what was unexpected was the direction of effect. Because the low–*APIP* expression allele (presumably resulting in higher steady-state MTA) is associated with a decreased odds of bacteremia and death in SIRS patients (*25*) and decreased odds of NTS bacteremia, we had anticipated that higher MTA would also be protective against infection. The fact that higher plasma MTA was associated with death prompted us to explore two possible explanations: (i) that there might be temporal variation in MTA levels during infection (the CAPSOD study has no information regarding when the suspected infection occurred relative to the measurement of MTA) or (ii) that changes in extracellular (plasma) MTA levels measured on patient samples might not reflect intracellular changes important for response to infection.

Response to infection may require differential regulation of inflammation during the course of infection. For example, a proinflammatory response may be beneficial early during infection, allowing a stronger bactericidal effect, whereas reduction of the inflammatory response later in infection may be beneficial to prevent tissue injury. To test for replication of the association of MTA level with sepsis outcome and to determine how this association changed during the progression of sepsis, we used patient samples collected as part of a prospective study on hospitalized patients (Table 2). This study was originally designed to evaluate the risk of health care–associated infections (HAIs) with a focus on ventilator-associated pneumonia (VAP) (HAI-VAP study).

Patients admitted to the hospital for noninfectious reasons (for example, after surgery, trauma, stroke, or cardiac arrest) who were at risk for HAI by virtue of intubation were enrolled into the study and monitored for the development of infection, and blood samples were obtained serially. Patients who had multiple time points and onset of HAI during the observation and sampling period were selected for MTA analysis. This study design not only allowed validation of the MTA association with sepsis outcome found in the CAPSOD cohort

Table 1. Summary of patient populations used in this work. ICU, intensive care unit; UNC, University of North Carolina.

Study	Patient population	Cases	Controls	Measurements	Major finding regarding methionine salvage	First reference on patient cohort
Seattle SIRS	Adult ICU patients in Harborview Medical Center (Seattle, WA) meeting at least three of four SIRS criteria	(i) SIRS nonsurvivors (n = 128)	(i) SIRS survivors (n = 776)	Genotyping for *APIP*	*APIP* genotype associated with SIRS death (*25*)	(*25*)
		(ii) Culture-positive sepsis (n = 149)	(ii) Culture-negative SIRS (n = 755)	Genotyping for *APIP*	*APIP* genotype associated with culture-verified sepsis (*25*)	
Kenyan bacteremia	Children in the Kilifi District Hospital (Kilifi, Kenya)	NTS bacteremia (n = 180)	Birth cohort population controls (n = 2677)	Genotyping on Affymetrix array 6.0 for GWAS and pathway enrichment	*APIP* genotype associated with NTS bacteremia (this study)	(*31*)
					Enrichment of SNPs in the methionine salvage pathway with NTS bacteremia (this study)	
CAPSOD	Emergency room patients in the Henry Ford Hospital (Detroit, MI), the Duke University Medical Center (Durham, NC), and the Durham Veteran Affairs Medical Center (Durham, NC) meeting at least two of four SIRS criteria and with suspected infection	Sepsis nonsurvivors (n = 31)	Sepsis survivors (n = 90)	Metabolomics including MTA measurement	MTA is associated with death in sepsis patients (this study)	(*34*)
HAI-VAP	Adult patients in the Duke University Medical Center (Durham, NC), the Durham VA Medical Center (Durham, NC), and the UNC Medical Center (Chapel Hill, NC) at high risk of health care–associated infection	Sepsis nonsurvivors (n = 37 samples; n = 16 patients)	Sepsis survivors (n = 68 samples; n = 20 patients)	MTA measurement	MTA is associated with death in sepsis patients (this study)	This study

but also let us examine the temporal relationship of MTA both before and after sepsis onset.

In the HAI-VAP study, elevated MTA was associated with sepsis nonsurvival, and the association was strongest soon after the time of suspected infection (Fig. 2B; $P = 0.03$ at 0 to 1 day and $P = 0.01$ at 2 to 3 days after apparent onset of infection). These associations remained significant after controlling for age and sex ($P = 0.03$ at 0 to 1 day after suspected infection). At later time points, the association was no longer observed (Fig. 2B; $P = 0.6$ at 4 to 8 days after suspected infection). We were unable to draw conclusions about the prognostic value of MTA levels before infection because of an insufficient number of samples collected before infection for patients in the nonsurvivor group. The association was specific only to the sepsis patients; no association was found between MTA levels and death in the uninfected control patients (Fig. 2B). Furthermore, no difference in MTA levels was observed in the VAP samples comparing overall sepsis versus noninfectious SIRS (fig. S1).

In the same mass spectrometry assay used for measuring MTA (see Materials and Methods), we also measured related metabolites [methionine, *S*-adenosyl homocysteine (SAH), and *S*-adenosyl methionine (SAM)], several amino acids (Leu, Ile, Phe, and Arg), and the polyamine spermine. As might be expected on the basis of their

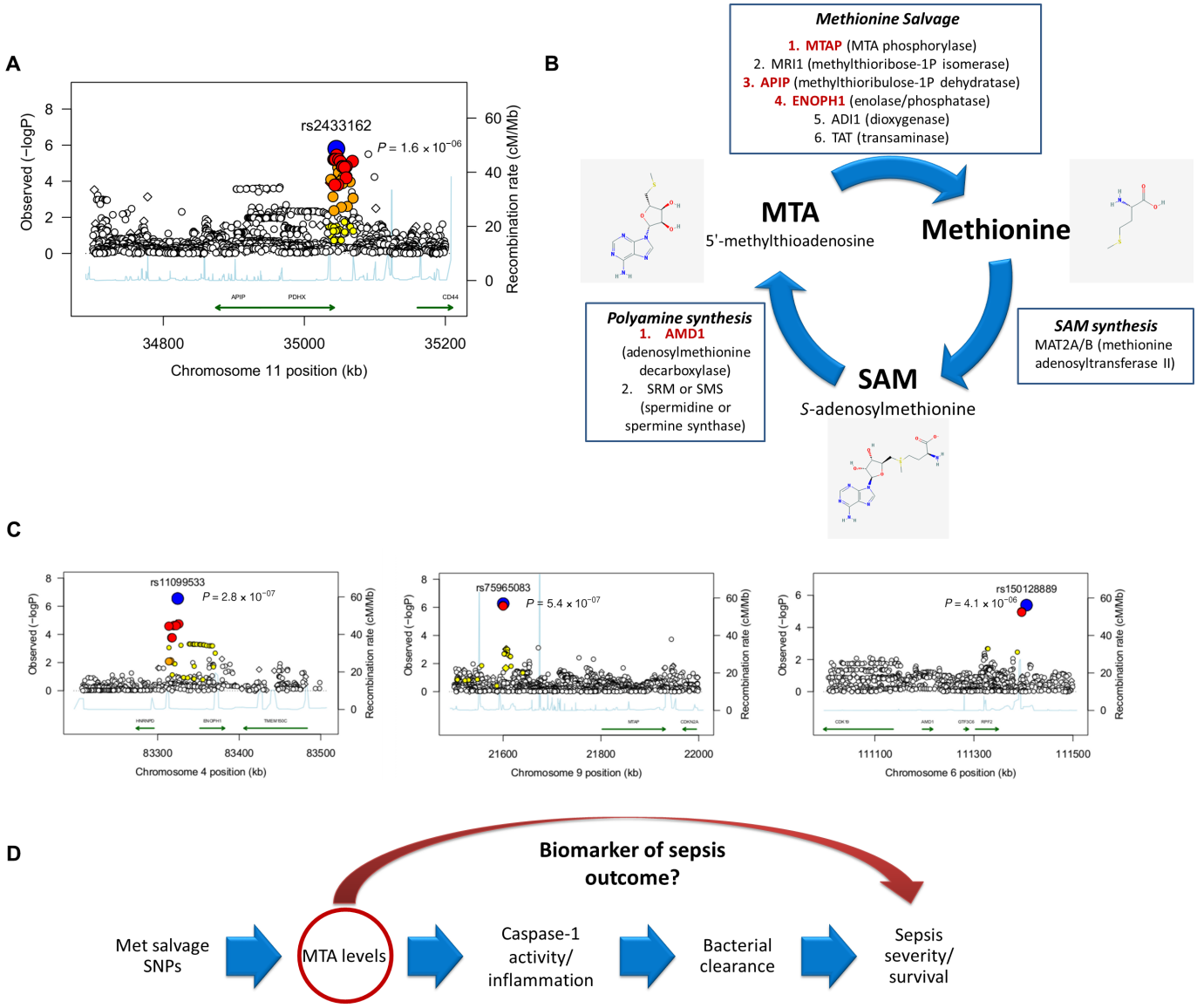

Fig. 1. SNPs near methionine salvage genes are associated with NTS bacteremia. (**A**) Association plot of NTS bacteremia susceptibility in the *APIP* region. Imputed SNPs are plotted as circles, and directly genotyped SNPs are plotted as diamonds. SNPs are colored according to strength of linkage disequilibrium (r^2) to rs2433162. Association statistics are calculated with a dominant model (the most likely model of association at rs2433162). (**B**) Methionine metabolism schematic with genes listed for each enzymatic step and structures for three major metabolites. The four genes implicated with pathway enrichment analysis for NTS bacteremia GWAS are in red boldface. (**C**) Association plots of the three regions (in addition to *APIP*) associated with NTS bacteremia and involved in methionine salvage: *ENOPH1*, *MTAP*, and *AMD1*. Imputed SNPs are plotted as circles, and directly genotyped SNPs are plotted as diamonds. SNPs are colored according to strength of linkage disequilibrium (r^2) to the lead SNP. Association statistics are calculated with the model of association observed for the lead SNP in each region (ENOPH1, recessive; MTAP, recessive; AMD1, dominant). (**D**) Model for how the SNPs near methionine salvage genes could affect sepsis. MTA may integrate genetic variation and other inputs to serve as a sepsis biomarker.

metabolic relationships (see Fig. 1B), hierarchical clustering of metabolites by patient profiles suggested coregulation of MTA, SAH, and SAM (Fig. 2C). Although SAH and SAM approached significance ($P = 0.05$ and $P = 0.06$, respectively), none of the other metabolites examined showed a statistically significant association with sepsis survival (Table 3). Thus, we have demonstrated that high MTA levels in plasma are associated with increased mortality in sepsis patients in two independent cohorts and that the association is strongest within 3 days of suspected infection.

Lethal NTS infection of mice decreased MTA levels in tissues but increased levels in plasma

Measurement of MTA levels in the HAI-VAP study confirmed the association between MTA levels and sepsis death seen in the CAPSOD samples and demonstrated the timing of this response. However, the increased temporal resolution of the HAI-VAP cohort did not reveal why the direction of effect was opposite of what was predicted from the *APIP* genetic association. Therefore, we turned to a mouse *Salmonella* infection model to determine how tissue levels of MTA differed in comparison to plasma levels.

Mice underwent intraperitoneal infection with *Salmonella* Typhimurium at a dosage (1×10^6) that usually results in death in 1 to 4 days (Fig. 3A). Plasma MTA levels spiked at 6 hours after infection but then returned largely to normal values by 24 hours (Fig. 3B). This is consistent with the high levels of plasma MTA seen near the time of infection in the sepsis nonsurvival cohort of the HAI-VAP study. However, MTA levels in the spleen showed the opposite relationship at 6 hours. MTA levels were markedly decreased and then showed a trend toward returning to normal by 24 hours (Fig. 3, C and D). A similar decline was noted in the liver, although the decrease was still apparent at 24 hours (Fig. 3D). Therefore, although plasma MTA levels are increased in severe bacterial infection, there is actually a drop in tissue MTA levels. These opposite changes in tissue and plasma MTA levels provide one plausible explanation for why a higher mortality could be associated with both higher plasma levels of MTA in the CAPSOD and HAI-VAP cohorts and the *APIP* SNP allele predicted to result in lower intracellular MTA levels in the Seattle SIRS cohort (*25*); the reduction in tissues might be the source for the increase in plasma during infection. Future studies of human sepsis tissues or peripheral blood mononuclear cells will be necessary to test the model that a reduction of MTA in tissues is accompanied by a rise in plasma levels of MTA early in the temporal course of infection and associated with worse outcome in sepsis.

MTA is a prognostic biomarker for death in sepsis

The strong association between MTA and sepsis survival prompted us to evaluate the utility of MTA as a prognostic biomarker. We found that, in the CAPSOD cohort, MTA had moderate prognostic power [receiver operating characteristic (ROC) curve with area under the curve (AUC), 0.69; 95% confidence interval (CI), 0.59 to 0.80; Fig. 4A]. However, we suspected that the power might be improved if the time relative to suspected infection were known. In the HAI-VAP study, plasma MTA levels at the time of suspected infection and soon after had improved predictive power [ROC curves with AUC, 0.74 (95% CI, 0.55 to 0.93) for 0 to 1 day, 0.79 (95% CI, 0.61 to 0.98) for 2 to 3 days, and 0.60 (95% CI,

Table 2. Clinical variables and demographics for VAP and CAPSOD patient cohorts. Data are presented as means ± SD. NI-SIRS, noninfectious SIRS; B/W/O, black, white, other; BMI, body mass index.

Clinical variable	CAPSOD sepsis metabolomics cohort		VAP cohort			
	Sepsis survivors	Sepsis nonsurvivors	NI-SIRS survivors	NI-SIRS nonsurvivors	Sepsis survivors	Sepsis nonsurvivors
No. of samples			40	12	68	37
No. of patients	90	31	20	6	20	16
Age (years)	56.4 ± 19.2	68.8 ± 16.7	55 ± 17.3	61.5 ± 20.2	52 ± 21.5	58 ± 16.7
Gender (% male)	58.9	54.8	100.0	83.3	82.4	67.6
Race (B/W/O)	56/28/6	23/7/1	6/13/1	2/4/0	8/10*/1	2/14/0
APACHE II	15.0 ± 7.1	22.8 ± 7.8	17 ± 9.6	18.5 ± 8.8	17 ± 4.9	22 ± 10.2
BMI	—	—	32 ± 10.8	25 ± 4.0	27.5 ± 5.1	29 ± 5.2
Tube feeds (%)	—	—	30.0	50.0	91.2	91.9
Comorbidities						
Diabetes (%)	34.4	41.9	35.0	33.3	5.9	51.4
End-stage liver disease (%)	4.4	19.4	0	0	0	16.2
Chronic kidney disease (%)	22.2	22.6	20.0	16.7	12.8	16.2
Cancer (%)	6.7	22.6	10.0	0.0	8.8	8.1
Immunosuppression (%)	5.6	6.5	10.0	0.0	5.9	8.1
Current smoker (%)	28.9	25.8	30.0	16.7	10.0	18.8

*Race data missing for one individual in the sepsis survivor group.

0.21 to 0.98) for >3 days; Fig. 4B]. Levels of MTA in HAI-VAP had prognostic power comparable to the commonly used APACHE II score, calculated from a dozen physiological parameters, measured at the same time points [AUC, 0.76 (95% CI, 0.58 to 0.94) for 0 to 1 day and 0.84 (95% CI, 0.68 to 1) for 2 to 3 days; Fig. 4C and fig. S2]. With a decision boundary for classifying likely survivors versus nonsurvivors of 0.15 in the 0- to 1-day interval, MTA has a sensitivity of 0.75 and a specificity of 0.77. Because the metabolites in the HAI-VAP study were measured by a different laboratory on a completely different set of sepsis patients compared to the CAPSOD samples, this represents a highly stringent form of biomarker replication. Our data demonstrate that measurement of a single metabolite, MTA, especially within 3 days of the onset of suspected infection, can serve as a useful predictor of sepsis outcome and that its accuracy was similar to that of APACHE II in the HAI-VAP cohort.

Although MTA appears to be a good biomarker in isolation, we tested whether the utility of MTA in predicting sepsis outcome could be increased through the incorporation of clinical parameters that would be available in a hospital setting. The relative importance of MTA along with several other parameters was evaluated using a support

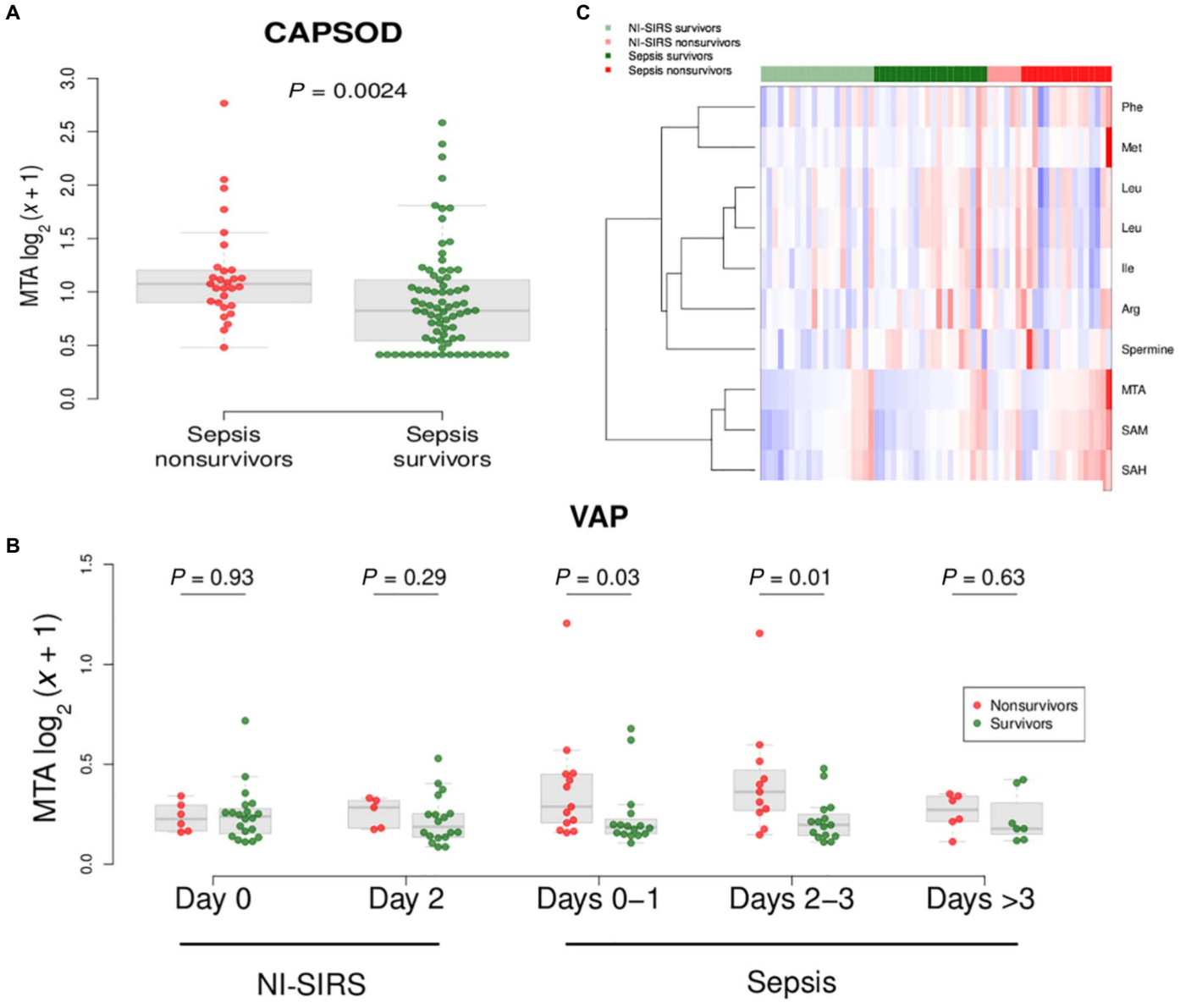

Fig. 2. High plasma MTA levels are associated with sepsis death in ICU patients. (**A**) CAPSOD data show significantly higher MTA levels in sepsis nonsurvivors compared to sepsis survivors. (**B**) Noninfectious SIRS individuals in the VAP cohort show no association between MTA and death at two time points. Day 0 is the time of the first blood draw; day 2 denotes that blood is drawn 2 days later. Early time points during sepsis for the HAI-VAP cohort (days 0 to 1 and days 2 to 3 of suspected infection) confirm significantly lower MTA in sepsis survivors. Later time points (days >3) show no significant difference between sepsis survivors and sepsis nonsurvivors. (**C**) Hierarchical clustering of metabolites (rows) with samples grouped by outcome (columns). MTA is clustered with two other metabolites in the methionine salvage pathway, SAM and SAH (refer to the diagram in Fig. 1D). Peak areas were used for spermine, SAH, and SAM due to lack of internal standards, whereas all other metabolites were scaled to internal standards. The metabolites are transformed into $\log_2$ values and then normalized to *z* scores. All *P* values were calculated by a two-tailed Mann-Whitney test.

Table 3. Summary of metabolite levels (at 0 to 1 day after suspected onset of infection) and association with death in sepsis and in NI-SIRS patients. All calculations were based on $\log_2(x + 1)$ transformations of either ratios of metabolite to internal standards or metabolite peak areas (for SAM, SAH, and spermine). *P* values were calculated by two-tailed Wilcoxon rank-sum test.

Metabolite	VAP sepsis			VAP NI-SIRS		
	Sepsis survivors (mean ± SD)	Sepsis nonsurvivors (mean ± SD)	*P*	NI-SIRS survivors (mean ± SD)	NI-SIRS nonsurvivors (mean ± SD)	*P*
MTA	0.24 ± 0.17	0.38 ± 0.28	0.03	0.25 ± 0.14	0.24 ± 0.07	0.93
SAM	12.72 ± 0.90	13.50 ± 1.16	0.06	12.94 ± 0.97	13.00 ± 0.78	1
SAH	11.28 ± 1.09	12.10 ± 1.27	0.05	11.43 ± 1.25	11.81 ± 0.94	0.46
Methionine	0.22 ± 0.07	0.37 ± 0.45	0.75	0.24 ± 0.10	0.20 ± 0.12	0.46
Phe	0.81 ± 0.11	0.82 ± 0.19	0.78	0.78 ± 0.16	0.76 ± 0.17	0.93
Ile	0.54 ± 0.20	0.53 ± 0.30	0.85	0.54 ± 0.24	0.51 ± 0.26	0.42
Leu	0.74 ± 0.22	0.71 ± 0.33	0.56	0.71 ± 0.24	0.70 ± 0.33	0.74
Arg	0.63 ± 0.19	0.60 ± 0.29	0.40	0.58 ± 0.25	0.63 ± 0.23	0.79
Spermine	14.26 ± 1.10	14.19 ± 1.95	0.31	13.43 ± 1.17	13.34 ± 0.69	0.93

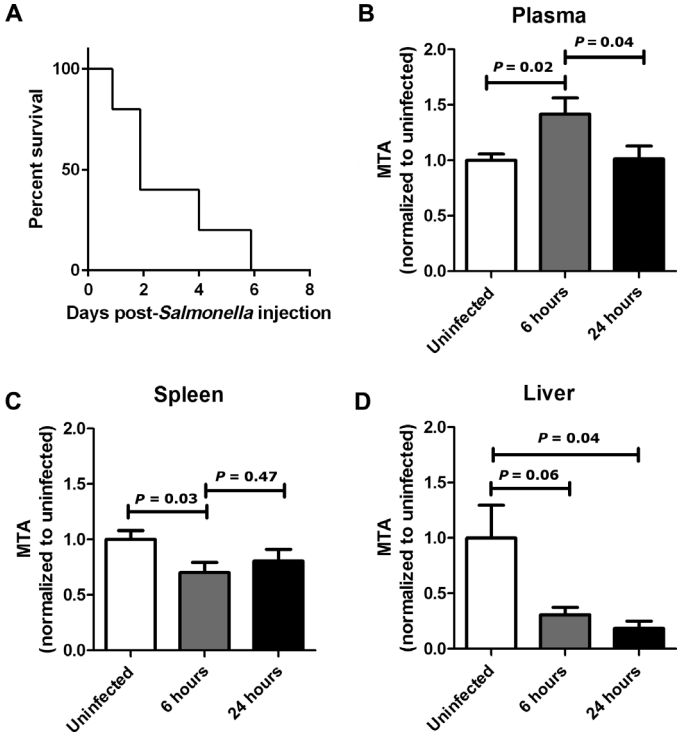

Fig. 3. Mouse NTS infection transiently increases MTA levels in plasma but decreases levels in the spleen. (**A**) Survival curve for mice injected with 1×10^6 *S.* Typhimurium by the intraperitoneal route. Most of the mice die by 2 days. (**B**) Mouse plasma MTA levels for uninfected mice and mice at 6 and 24 hours after infection. (**C**) Mouse spleen MTA levels for uninfected mice and mice at 6 and 24 hours after infection. (**D**) Mouse liver MTA levels for uninfected mice and mice at 6 and 24 hours after infection. For each time point, means ± SEM are from 10 mice, except for 4 mice for liver, and *P* values are from *t* test.

vector machine implemented in the R caret package (*37*). We found that, in the CAPSOD cohort, MTA was the variable of second highest importance (Fig. 4D). If variable selection was instead performed on a larger set of 10 clinical variables using the HAI-VAP study at the 0- to 1-day time interval, MTA was the variable of highest importance (Fig. 4D). A model incorporating APACHE II, MTA, and age developed using the CAPSOD data showed robust cross-validation with the CAPSOD cohort (AUC, 0.81; median, 0.83; 95% CI, 0.59 to 0.96) (Fig. 4E). This model developed from the CAPSOD data performed well on the independent HAI-VAP data set for days 0 to 1 (AUC, 0.77; 95% CI, 0.75 to 0.78) (Fig. 4E). Similar results were observed at the 2- to 3-day time interval (AUC, 0.82; 95% CI, 0.79 to 0.84; Fig. 4E). Thus, MTA alone had similar prognostic ability compared to a model incorporating MTA with clinical parameters.

MTA levels are correlated with high levels of proinflammatory cytokines

On the basis of our previous studies indicating that exogenous MTA could increase inflammation, as measured by pyroptosis in cultured cells (*25*), we hypothesized that the prognostic ability of MTA could be indicative of a harmful proinflammatory state. To test this possibility, we determined whether MTA levels were correlated with levels of proinflammatory cytokines. Early proinflammatory cytokines [IL-1β and tumor necrosis factor–α (TNFα) (*38*)] were either largely undetected in our patient samples (IL-1β) or weakly correlated with MTA (TNFα; Fig. 5A). However, both IL-6 and IL-8 showed significant positive correlations with MTA levels in the sepsis patients but not in the noninfectious SIRS patients (Fig. 5, B and C). Furthermore, measurement of IL-6 and IL-8 each had significant prognostic power at this early time point (0 to 1 day) used for cytokine measurement (for IL-6: AUC, 0.85; 95% CI, 0.62 to 1.0; for IL-8: AUC, 0.81; 95% CI, 0.55 to 1.0; fig. S3). As shown above, sepsis patients with elevated plasma MTA have a worse prognosis, and these cytokine data indicate that the elevated MTA levels may identify a subset of sepsis patients with a hyperinflammatory state.

Exogenous MTA prolongs survival in lethal NTS infection of mice

Connecting the methionine salvage pathway to sepsis through association of both genetic differences in the pathway and the metabolite they regulate led us to hypothesize that MTA might not only serve as a biomarker but could also modulate the course of infection. We determined whether survival of mice would be affected by administering exogenous MTA in the NTS challenge model. Mice were injected with MTA either 30 min before infection or 6 hours after infection. MTA administered before infection prolonged survival ($P = 0.01$; Fig. 6A). This is consistent with elevated MTA before infection having a protective effect against NTS bacteremia, and we hypothesize that beneficially elevated MTA could occur either through exogenous administration or through a genetic difference that reduces expression of *APIP*. In contrast, when administered 6 hours after infection (concomitant with the natural peak in plasma MTA; see Fig. 3), no protective effect was seen ($P = 0.45$; Fig. 6B).

To examine the mechanism of prolonged survival with early administration of MTA, we measured levels of proinflammatory cytokines (TNFα and IL-6). TNFα and IL-6 were both induced in plasma relative to uninfected mice, but the level of induction was suppressed in the MTA-pretreated mice ($P = 0.01$ for TNFα and $P = 0.03$ for IL-6; Fig. 6C). This difference is not secondary to differences in bacterial load because bacterial colony-forming units (CFUs) were similar ($P = 0.90$; fig. S4). Although the patient data demonstrate that elevated plasma MTA during infection is a biomarker of a hyperinflammatory state, exogenous MTA suppresses inflammatory cytokine levels during NTS infection in mice.

DISCUSSION

As of 2010, 170 biomarkers had been evaluated for use in sepsis (*39*), including gene expression, proteins, and metabolites, but only a handful have been adopted for clinical use. It is increasingly popular to take a completely agnostic approach in identification of biomarkers and use machine learning to deconvolute omics data sets into biomarker panels for the particular application (*40*). The approach demonstrated here integrates genotype, cytokine, and metabolite data with testing in an animal model to reveal a robust biomarker that, at least in mice, can be added exogenously to affect the course of infection. Genotype markers are unique in that they are unaffected during the course of the disease (except in the context of cancer), and therefore, the direction of causation from genotype to disease is unambiguous. However, as the last decade of GWAS has demonstrated, effect sizes of most associated common genetic variants are small (*41*). Biomarkers based on gene expression have increased in popularity, and some are in clinical use, particularly in oncology (*42*, *43*). However, the exquisite regulation of gene expression means that it can serve a causal role in disease, but dysfunctional expression can also be a consequence of disease. Other molecular

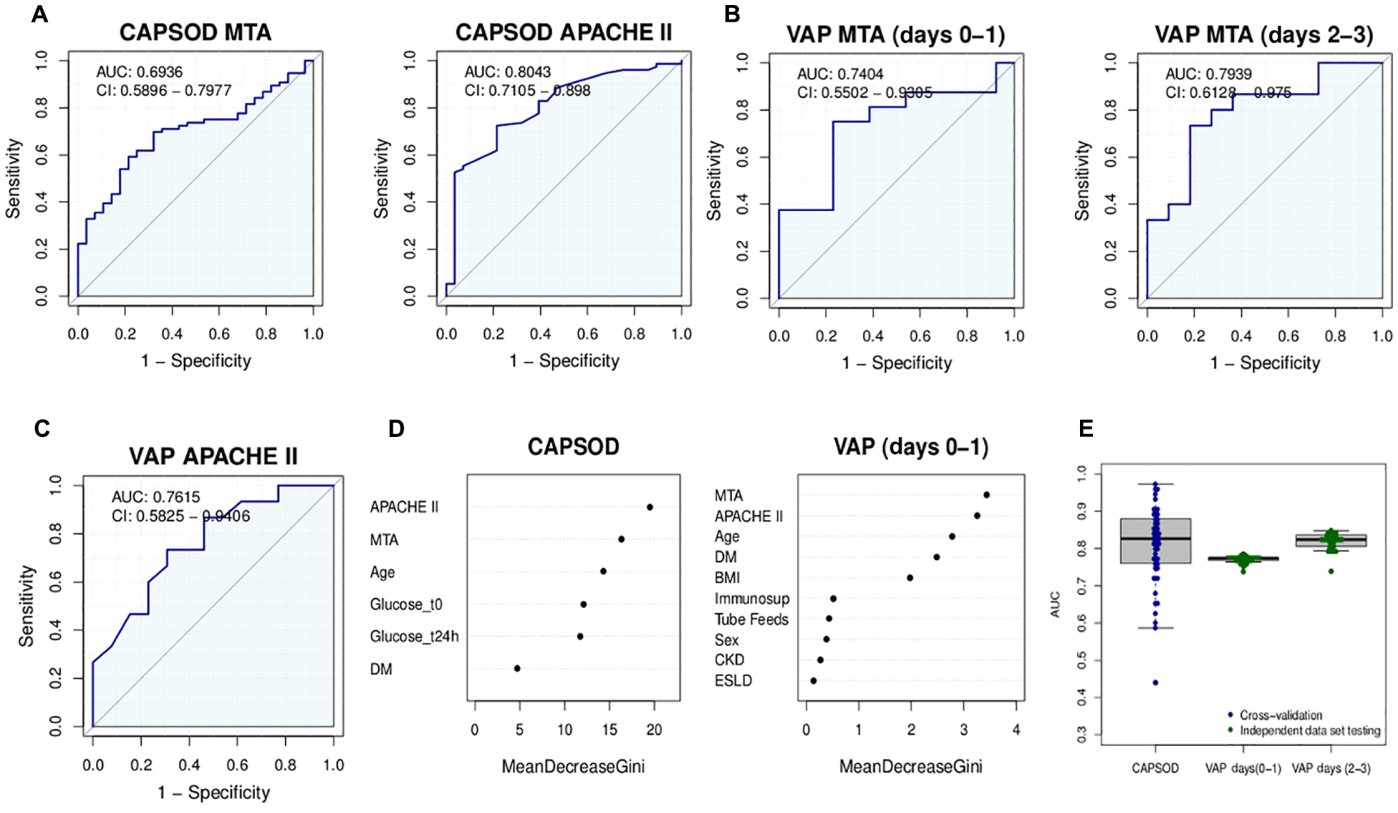

Fig. 4. Evaluation of MTA as a biomarker for sepsis survival. (**A**) ROC curves for MTA and APACHE II from the CAPSOD cohort. (**B**) ROC curves for MTA from the HAI-VAP cohort at days 0 to 1 and days 2 to 3. (**C**) ROC curve for APACHE II from HAI-VAP at enrollment. (**D**) Ranked variable importance for the CAPSOD cohort and the HAI-VAP cohort at days 0 to 1 for prognosis of death. MTA ranks as the second most important variable among all six clinical parameters in the CAPSOD cohort and the most important among 10 clinical parameters in the HAI-VAP cohort. (**E**) Distribution of AUC values for cross-validation with CAPSOD or with the independent HAI-VAP data set using an SVM approach. The SVM model was constructed using the CAPSOD cohort with the three most important variables (APACHE II, MTA, and age). Testing of the model was carried out using CAPSOD for cross-validation and independent testing with two time points of the HAI-VAP cohort at days 0 to 1 and days 2 to 3 (50 iterations were run for each data set).

biomarkers (metabolites and cytokine levels) also serve as the dynamic effectors of the physiological process being studied. Our work demonstrates that combining measurement of biomolecules with genetic association studies allows for the identification of biomarkers that are not only symptoms but also likely regulators of disease physiology.

Here, we started with a genetic biomarker of bacteremia and death in a SIRS cohort and validated the SNP association and implicated the entire methionine salvage pathway in a separate NTS bacteremia data set. We then evaluated the metabolite regulated by this pathway in two different patient cohorts. Therefore, both the genetic association and the metabolite association with sepsis outcome have now been observed in two independent patient populations and under a variety of sepsis conditions.

Closer examination of the details of these studies also demonstrates the advantages and limitations of genetic and metabolite biomarkers. Our initial discovery that the SNP in *APIP* (rs514182) was associated with bacteremia came from a study that used a cohort of nearly a thousand subjects and revealed an OR of 2.0 (*25*). The OR in the NTS data set of nearly 3000 subjects was quite similar [OR, 2.3 (for rs2433162) and 1.4 (for rs514182)]. In contrast, the OR for sepsis death using MTA is 7.18 from the HAI-VAP study of 62 patients, indicating that the odds of death are increased 7.18-fold when MTA concentration is doubled. Although ORs for genotypes are not entirely comparable to the OR of a continuous variable, the significant association from such a vastly smaller group of patients underscores the robustness and large effect of the MTA metabolite biomarker. Our work demonstrates the advantage in combining genetic association studies for causal inference with metabolite association studies of larger effect size and possibly greater clinical utility.

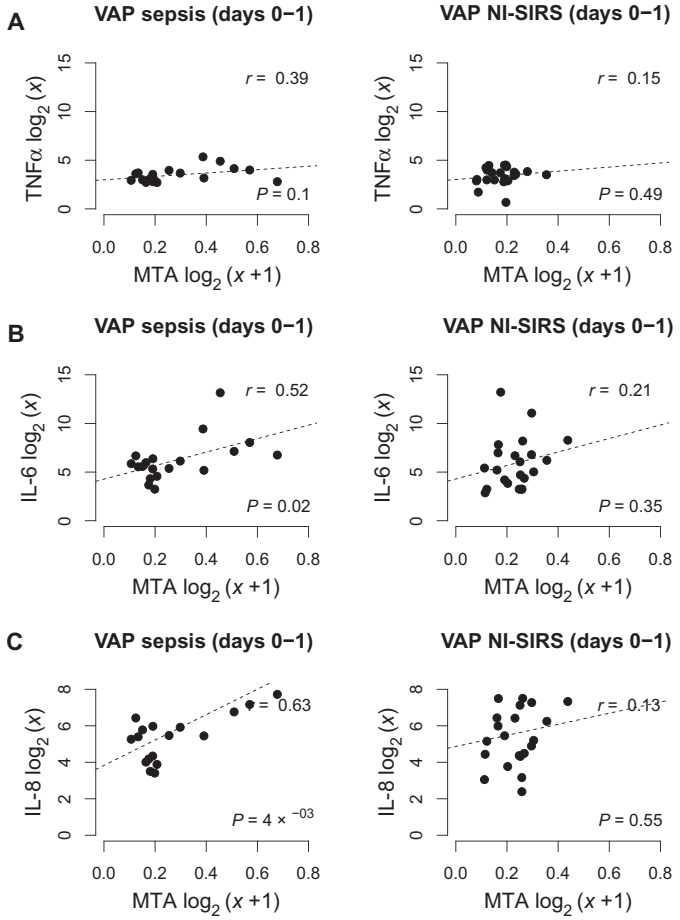

Fig. 5. Correlation of MTA and plasma levels of proinflammatory cytokines in the HAI-VAP cohort. (**A**) MTA is not correlated with plasma levels of TNFα for both VAP sepsis and NI-SIRS patients at days 0 to 1. (**B**) MTA is significantly correlated with plasma levels of IL-6 in HAI-VAP sepsis patients at days 0 to 1 but not in NI-SIRS patients. (**C**) MTA is significantly correlated with plasma levels of IL-8 in HAI-VAP sepsis patients at days 0 to 1 but not in NI-SIRS patients.

MTA could be a valuable biomarker not only for prognosis but also in determining whether patients could benefit from therapies that either enhance or suppress the immune system. An emerging view is that sepsis immunomodulatory therapies have failed in clinical trials because different subpopulations benefit from either increasing or suppressing inflammation (*44*). Specifically, sepsis patients early in their course might benefit from therapies that reduce the action of proinflammatory cytokines. Conversely, patients later in the course of their infection often have an immunosuppressed state that might benefit from therapies that enhance immune function. The timing of elevated plasma MTA (and decreased tissue MTA) suggests that it represents an acute response to bacterial infection. Our data support the idea that elevated MTA levels, which are prognostic of later death, are indicative of an excessive immune response. IL-6 and IL-8 levels are correlated with MTA levels in sepsis patients, indicating that MTA levels could be a marker of "cytokine storm." However, MTA administered before infection suppresses inflammatory cytokine production in mice and prolongs survival. This suppression of inflammation was surprising given our previous studies demonstrating increased pyroptosis with MTA in lymphoblastoid cell lines and macrophages, but it is consistent with studies that have demonstrated that MTA protects against LPS endotoxemia (*29*) and can reprogram macrophages to have reduced TNFα production (*45*). Together, the human and mouse data suggest that MTA could serve not only as a prognostic biomarker for identification of patients in need of more aggressive care but also as a predictive one for identifying which patients would benefit from immunosuppressive therapies, including MTA itself. Notably, MTA remains elevated longer (at least up to 3 days in our data) compared to the cytokines themselves, which have a much more transient increase and may start declining rapidly within the first 24 hours of infection (*46*). The more prolonged elevation of MTA may enhance its clinical utility.

An additional level of complexity in understanding the utility and biological effects of MTA comes from variation in intracellular versus extracellular levels. We propose that steady-state intracellular levels of MTA are regulated by genetic variation in the methionine salvage and polyamine synthesis pathways. With acute infection, there is an increase in plasma MTA with a decrease seen in tissues. This decrease in intracellular MTA is likely not mediated by an increased flux through the pathway because the product of the pathway, methionine, was not observed to increase as MTA decreased; rather, we observed a decrease of methionine to 56% of uninfected levels in the spleens of mice at 6 hours after infection with *Salmonella*. We speculate that high levels of MTA in patients might be released from cells as part of the host response to suppress and resolve inflammation. One possible model reconciling our genetic, metabolite, and mouse data shows that high intracellular levels before infection (due to *APIP* genotype) are protective, possibly because the cells are more susceptible and poised to undergo pyroptosis. However, if the infection is not effectively resolved, the patient's course worsens, leading to further release of MTA (possibly through pyroptosis, another form of cell death, or an unknown mechanism) that is indicative

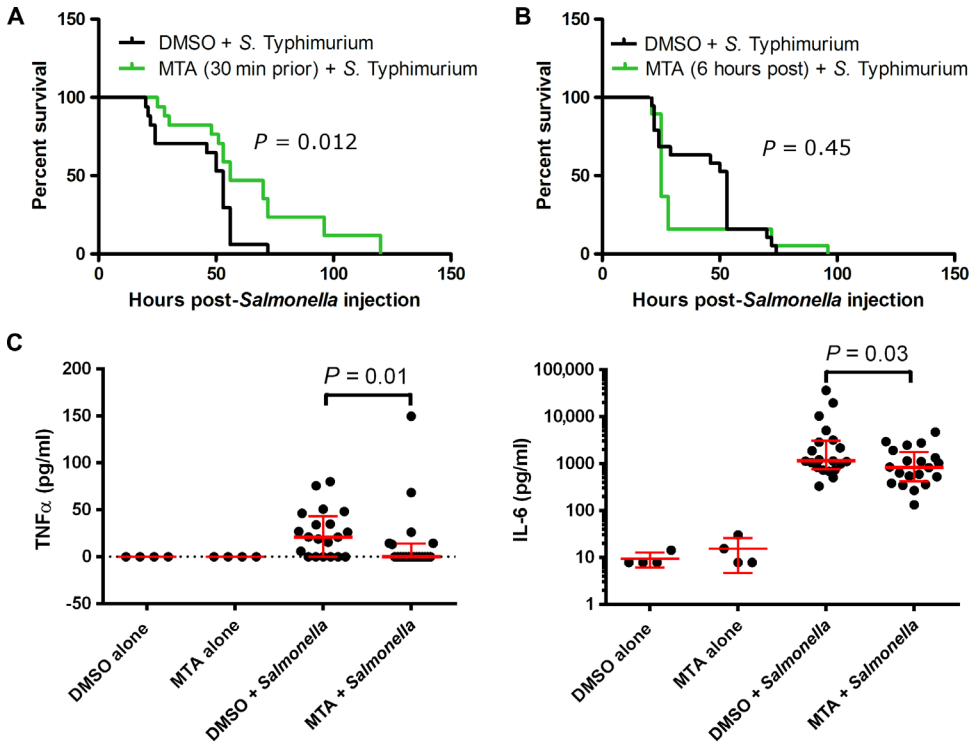

Fig. 6. Exogenous MTA prolongs survival and suppresses proinflammatory cytokines. (**A**) MTA injection 30 min before *S.* Typhimurium infection (1×10^6 CFUs by intraperitoneal injection) prolongs survival. Data were compared by log-rank test ($P = 0.012$; hazard ratio, 2.1) with $n = 17$ in each group, combined from three different experiments that each showed the same trend. (**B**) MTA injection 6 hours after *S.* Typhimurium infection does not prolong survival. Data were compared by log-rank test ($P = 0.45$; hazard ratio, 0.82) with $n = 18$ in each group combined from three different experiments. (**C**) *Salmonella*-induced cytokine levels are decreased with MTA before treatment. MTA was administered 30 min before *S.* Typhimurium injection. Plasma was collected at 3 hours after infection and measured by enzyme-linked immunosorbent assay (ELISA). $n = 20$ for each infected condition measured over three experiments and $n = 4$ for uninfected controls. Median and interquartile range are shown in red. For TNFα, values were compared by the Mann-Whitney test [$P = 0.01$; median of 21.1 pg/ml for dimethyl sulfoxide (DMSO) + *Salmonella* and median below limit of detection for MTA + *Salmonella*; means of 25.3 pg/ml for DMSO + *Salmonella* and 14.3 pg/ml for MTA + *Salmonella*]. For IL-6, log transformation resulted in normal distributions that were compared by unpaired *t* test ($P = 0.03$; medians of 1134 pg/ml for DMSO + *Salmonella* and 831 pg/ml for MTA + *Salmonella*; means of 4604 pg/ml for DMSO + *Salmonella* and 1242 pg/ml for MTA + *Salmonella*).

of excessive inflammation in the setting of uncontrolled infection. Our study demonstrates that MTA timing, localization, and regulation are complex, and MTA levels not only are regulated by genetic differences but also change dynamically during the course of infection.

Although the mouse data demonstrate an inverse relationship of MTA levels in tissues and plasma, future studies are required to verify that this relationship holds in humans and is associated with sepsis outcome. Furthermore, whereas the experiments with exogenous MTA demonstrate that lethal NTS infection and cytokine levels in mice can be modulated by MTA, whether endogenous MTA definitively plays this role during infection and the mechanism of its immune modulation will require genetic loss-of-function experiments in mice (likely in a cell type–specific manner) with more detailed phenotyping.

Future studies aimed at moving MTA into the clinic will need to incorporate accurate absolute quantification of MTA in a clinical laboratory platform that is more common than liquid chromatography–mass spectrometry. In addition, it will be crucial to further examine the utility, dosing, pharmacodynamics, and mechanism of modulating MTA levels to suppress inflammation in animal models and, if results are promising, to validate in patients. The goal is to ultimately help the more than 1 million patients hospitalized with sepsis in the United States every year (*47*) and the millions more throughout the world.

MATERIALS AND METHODS

GWAS populations

Ethical approval for the study was obtained from the Kenya Medical Research Institute National Scientific Steering and Research Committees and the Oxford Tropical Research Ethics Committee. Following explanation of the study, written informed consent was obtained from the parent or guardian of each child included in the study. Children (under 13 years of age) presenting with bacteremia to Kilifi District Hospital, Kenya, between 1 August 1998 and 30 October 2010 were recruited to the Wellcome Trust Case Control Consortium 2 (WTCCC2) study of bacteremia, as described elsewhere (*48*). Briefly, blood samples for bacterial culture (BACTEC 9050, Becton Dickinson) were taken from every child admitted to the hospital during the study period, with the exception of elective surgical admissions and children admitted following minor accidents. Control samples were collected as part of a birth cohort study from consecutive births between 1 May 2006 and 30 April 2008, among the same population as the case samples in the Kilifi district.

GWAS analysis

Genome-wide genotyping (Affymetrix SNP 6.0 chip) was performed for samples from 218 cases of NTS bacteremia and 3000 controls. Following whole-genome imputation and quality control (*31*), 180 cases

of NTS bacteremia and 2677 control samples were included in the analysis. Genome-wide association analysis was performed under additive and genotypic models of association with SNPTEST2 (*49*), with the first four principal components of genome-wide genotyping data included in the models to account for the underlying population structure. SNPs with minor allele frequencies >0.05 (additive model; >0.1, genotypic model), with imputation info scores >0.8, and without evidence for departure from Hardy-Weinberg equilibrium ($P < 1 \times 10^{-10}$) were included in the analysis (7,950,619 SNPs, additive model; 4,669,669 SNPs, genotypic model). Genotype data for Kenyan GWAS samples will be made available via the European Genome-phenome Archive (EGAS00001001756).

Pathway enrichment analysis of NTS GWAS

To test for the enrichment of biologically related genes at NTS-associated loci, we used INRICH (*33*). We defined NTS-associated regions in PLINK (*50*), as nonoverlapping genomic regions containing an NTS-associated SNP ($P < 1 \times 10^{-5}$ in either an additive or a genotypic model), bounded in the 3′ and 5′ directions by the extent of linkage disequilibrium ($r^2 > 0.2$) to the most-associated SNP. We identified 73 independent NTS-associated genomic regions, which we tested for enrichment in 1320 gene sets in the curated canonical pathway gene set in GSEA (Gene Set Enrichment Analysis) (*51*). Empirical *P* values for enrichment were calculated with 100,000 replicates, and multiple-testing correction of *P* values was performed with 10,000 bootstraps.

Metabolite study populations and clinical data collection

For the CAPSOD cohort, informed consent was obtained, and 1152 patients who met at least two SIRS criteria and had suspected infection were enrolled prospectively from emergency departments at three urban, tertiary care centers (*29*). Demographic and clinical data as well as venous plasma and whole blood were collected at time of enrollment and 24 hours later. Clinical adjudication for clear presence of infection and the etiologic infectious agent was performed by a board-certified clinician. Survival at 28 days was determined.

The HAI-VAP study was approved by the Institutional Review Board of the Duke University Medical Center, the Durham Veterans Affairs Medical Center, and UNC Health Care. This prospective observational cohort study targeted patients who were at high risk of developing VAP by virtue of recent intubation and an expected duration of ventilation of greater than 48 hours. Enrollment occurred from the emergency department, intensive care units, step-down units, and other appropriate locations within the Duke University Health System. The study was also conducted at the Durham Veterans Affairs Medical Center and UNC Health Care. Clinical information and biological samples were collected daily through day 6. If no VAP or other HAI occurred, sample collection ended at day 6, and this patient served as an uninfected control. However, if an HAI occurred within this window, postinfection samples were collected every other day for an additional week. Survival status was determined at day 30. An adjudication committee of physicians used all prospectively collected clinical data to categorize patients into specific HAI groups, incorporating clinical, microbiological, and radiographic data.

Patients were grouped as sepsis survivors, sepsis nonsurvivors, noninfection survivors, and noninfection nonsurvivors. APACHE II scores were calculated for time of enrollment. Plasma samples were used for metabolite profiling, and whole blood was used for transcriptome sequencing, as described below. For the study presented here, 62 patients were included, and 157 samples from those patients were subjected to further measurement and analysis.

Metabolite profiling

Semiquantitative metabolomics analysis for the CAPSOD samples was previously described (*34*). The metabolite profiling of the HAI-VAP samples was performed by the Duke Proteomics and Metabolomics Shared Resource. A method was developed for the measurement of 10 metabolites (leucine, isoleucine, spermidine, methionine, phenylalanine, spermine, arginine, MTA, SAM, and SAH) in human plasma samples. A total of 157 human plasma samples were block-randomized before sample submission based on outcomes to reduce any batch effects. After thawing, all samples were vortexed briefly and centrifuged at 1700 rpm for 10 min. Then, 100 µl of each sample was pipetted directly into a 96-well, 1-ml Eppendorf plate. For each sample well, 900 µl of methanol/0.1% formic acid containing internal standards was added. The plate was capped and allowed to mix at 650 rpm at 25°C for 30 min. The plate was then centrifuged for 10 min at 3000 rpm. The supernatant (400 µl) from each well was transferred to another 96-well plate, and the extracts were dried under a gentle stream of nitrogen. Samples were reconstituted in 32 µl of 50:49:1 acetonitrile/water/trifluoroacetic acid (TFA) and mixed at 650 rpm for 5 min, and then 128 µl of 1% TFA in water was added. Quality control was monitored by a pooled sample injected several times before the start of the run queue, once after every 20 sample runs, and once at the end to measure the performance of the assay during sample analysis.

Liquid chromatography separation of these 10 polar metabolites was performed on a Waters Acquity UPLC (Milford) using a Waters Acquity 2.1-mm × 10-mm, 1.7-µm BEH C18 column, followed by introduction into a Xevo TQ-S mass spectrometer (Waters) using electrospray ionization operating in multiple reaction monitoring (MRM) mode. MRM transitions (compound-specific precursor to product ion transitions) for each analyte and internal standard were collected over the appropriate retention time. The total run time per injection was 17.5 min. The data were imported into Skyline software (https://skyline.ms/project/home/software/Skyline/begin.view) for peak integration and exported into Excel for ratio calculation of analyte to internal standard, where applicable. Note that because all spermidine data were below the limit of quantitation in all plasma samples, there are no data present for this analyte.

The mouse plasma samples were prepared in the same manner as the human plasma samples, except for the fact that formic acid was left out of the methanol extraction after it was determined to more efficiently extract MTA. For the mouse spleens, phosphate-buffered saline (PBS) was added to each at 1 ml per 100 mg of wet weight. Samples were then probe-sonicated at power level 3 for three bursts of 5 s each, cooling on ice between bursts. Bradford assay was performed on each solubilized tissue extract using 10-fold diluted material. Samples corresponding to 575 µg of total protein from each tissue were taken out and normalized to 100 µl in total with PBS in a 96-well plate. Samples were then processed as above with 900 µl of methanol.

Mouse infections

Mouse studies were approved by the Duke Institutional Animal Care and Use Committee and adhere to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (NIH). *S.* Typhimurium (14028s + p67GFP) were grown overnight in LB media + ampicillin. Bacteria were diluted 1:33 and grown for an additional 2 hours and 40 min. Bacteria were washed and resuspended in PBS. Mice (age/sex-matched in each experiment; 9 to 14 weeks old) were injected with 1×10^6 bacteria by the intraperitoneal route. Following 6 or 24 hours, mice were euthanized, and blood collected by cardiac puncture

and spleens were harvested. Blood and spleens from uninfected control mice were harvested at 0 hours. Plasma from blood samples was collected using Microtainer plasma separator tubes with lithium heparin (Becton Dickinson) and stored at −80°C until ready for analysis. Spleens were weighed and stored at −80°C until analysis. For survival curves, mice were assessed using a morbidity scale twice daily. MTA injections were given intraperitoneally (96 μg/kg body weight, dissolved in DMSO) either 30 min before or 6 hours after *Salmonella* infection. TNFα and IL-6 levels were measured by ELISA according to the manufacturer's instructions (R&D Systems).

Statistical analysis

All samples were binned into four time points according to days relative to onset of infection. The four time points used in this study are the following: before onset of infection, days 0 to 1 of infection, days 2 to 3 after infection, and days 4 to 9 after infection. For some time points, MTA levels were measured for multiple times from the same patient, and they were averaged for the subsequent analyses.

Before the statistical analyses, metabolite ratios (metabolite peak area over the internal standard) were rescaled via the $\log_2(x + 1)$ transformation. Wilcoxon rank-sum test was used to detect the significance of differential levels among groups (sepsis versus noninfectious SIRS and survivors versus nonsurvivors) at different time points, as well as the correlation of outcome and clinical demographic parameters. Kruskal-Wallis test was performed for samples at different time points and among groups. Ward's hierarchical clustering of Pearson product-moment correlations was used to group different metabolites and patients.

To evaluate the sensitivity and specificity of MTA as a biomarker for sepsis risk, we used a machine learning method, the Support Vector Machine (SVM), implemented in the R caret package version 6.0-58 using default parameters (*37*). Briefly, the SVM algorithm was used to calculate the importance of all variables and rank them. The top three variables were then selected to classify the samples into sepsis survivors and nonsurvivors. The CAPSOD study was used as the discovery panel and to construct the optimized prediction model, and then the HAI-VAP study served as an independent data set to test the model. Eighty percent of the CAPSOD data set served as the training data set for model building, and the remaining 20% served as the testing data set. Cross-validation was applied on both the CAPSOD data and the independent HAI-VAP data set to evaluate the accuracy and specificity of prediction performance for 50 iterations. ROC curves and AUC values were calculated using the R pROC package version 1.8 (*52*). All statistical analyses and modeling were performed with the R program (*53*).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/3/e1602096/DC1

fig. S1. MTA levels in noninfectious SIRS versus sepsis patients in the HAI-VAP study.

fig. S2. ROC curves for APACHE II from HAI-VAP (on days 2 to 3).

fig. S3. ROC curves for IL-6 and IL-8 from HAI-VAP (on days 0 to 1).

fig. S4. *Salmonella* spleen CFUs are not altered by MTA at the time of cytokine measurement (3 hours).

Consortia author list

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