ORIGINAL ARTICLE

Plasma sRAGE Acts as a Genetically Regulated Causal Intermediate in Sepsis-associated Acute Respiratory Distress Syndrome

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Abstract

Rationale: Acute respiratory distress syndrome (ARDS) lacks known causal biomarkers. Plasma concentrations of sRAGE (soluble receptor for advanced glycation end products) strongly associate with ARDS risk. However, whether plasma sRAGE contributes causally to ARDS remains unknown.

Objectives: Evaluate plasma sRAGE as a causal intermediate in ARDS by Mendelian randomization (MR), a statistical method to infer causality using observational data.

Methods: We measured early plasma sRAGE in two critically ill populations with sepsis. The cohorts were whole-genome genotyped and phenotyped for ARDS. To select validated genetic instruments for MR, we regressed plasma sRAGE on genome-wide genotypes in both cohorts. The causal effect of plasma sRAGE on ARDS was inferred using the top variants with significant associations in both populations ($P < 0.01$, $R^2 > 0.02$). We applied the inverse

variance-weighted method to obtain consistent estimates of the causal effect of plasma sRAGE on ARDS risk.

Measurements and Main Results: There were 393 European and 266 African ancestry patients in the first cohort and 843 European ancestry patients in the second cohort. Plasma sRAGE was strongly associated with ARDS risk in both populations (odds ratio, 1.86; 95% confidence interval [1.54–2.25]; 2.56 [2.14–3.06] per log increase). Using genetic instruments common to both populations, plasma sRAGE had a consistent causal effect on ARDS risk with a β estimate of 0.50 (95% confidence interval [0.09–0.91] per log increase).

Conclusions: Plasma sRAGE is genetically regulated during sepsis, and MR analysis indicates that increased plasma sRAGE leads to increased ARDS risk, suggesting plasma sRAGE acts as a causal intermediate in sepsis-related ARDS.

Keywords: respiratory distress syndrome; adult; Mendelian randomization analysis

(Received in original form October 28, 2018; accepted in final form September 5, 2019)

*J.D.C. is Associate Editor of *AJRCCM*. His participation complies with American Thoracic Society requirements for recusal from review and decisions for authored works.

This work was funded by NIH grants HL137915 (N.J.M.), HL137006 (N.J.M.), HL140026 (C.S.C.), HL051856 (M.A.M.), HL115354 (J.D.C.), HL101779 (M.M.W.), HL060710 (D.C.C.), HL103836 (L.B.W.), and the American Thoracic Society Foundation (N.J.M.). T.K.J. reports institutional training grant funding from the NIH (HL007891). C.S.C., M.A.M., J.D.C., and N.J.M. also report funding from GlaxoSmithKline.

Author Contributions: T.K.J. and N.J.M. had access to all data and take responsibility for the integrity of the work. T.K.J. and N.J.M. conceived of and designed the study. D.C.C., M.M.W., L.B.W., C.S.C., M.A.M., J.D.C., and N.J.M. obtained funding. T.K.J., J.P.R., B.J.A., M.G.S.S., F.W., T.G.D., T.R.R., J.A., C.A.G.I., D.C.C., C.M., M.M.W., C.S.C., M.A.M., and N.J.M. acquired data. T.K.J., R.F., V.E.K., J.P.R., B.J.A., M.G.S.S., F.W., L.B.W., J.D.C., and N.J.M. analyzed and interpreted the data. T.K.J. and N.J.M. drafted the manuscript. All authors made significant contributions to the final manuscript and approve its submission.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 201, Iss 1, pp 47–56, Jan 1, 2020

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Originally Published in Press as DOI: 10.1164/rccm.201810-2033OC on September 5, 2019

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Although multiple plasma biomarkers have demonstrated a significant association with acute respiratory distress syndrome (ARDS) risk, few have been subjected to causal inference analyses. A causal biomarker would have a scientific basis for targeting in clinical trials of preventive and/or therapeutic interventions for ARDS and might act as a response indicator. sRAGE (soluble receptor for advanced glycation end products) is an important ARDS biomarker that warrants testing for causal inference.

What This Study Adds to the Field:

Employing an instrumental variable analysis technique, we 1) report that genetically predicted plasma sRAGE levels associate with sepsis-related ARDS risk and 2) infer that plasma sRAGE may act as a causal intermediate in ARDS development. These findings were identified using two independent cohorts of patients with sepsis. These findings represent an early application of Mendelian randomization methodology to plasma biomarkers in ARDS.

Acute respiratory distress syndrome (ARDS) complicates up to 10% of ICU admissions (1). It is a heterogeneous syndrome that includes an inflammatory pattern of lung injury, loss of alveolar-capillary barrier integrity, alveolar fluid accumulation, and profound hypoxemia (2–5). Despite decades of research, there is a paucity of directed pharmacological therapies for ARDS prevention and treatment (6–8). Although multiple biomarkers have been shown to associate with ARDS (9), the causality of these biomarkers in ARDS risk remains unknown. Identification of a causal biomarker with strong biological plausibility may allow future therapeutic innovation in ARDS (8). A causal biomarker may also facilitate the identification of subgroups for precision therapies and improve preclinical trial efficacy through the validation of an intermediate phenotype prior to proceeding with a clinical trial (10). However, it is not

feasible to randomize patients to a low or high plasma biomarker level to assess the effect of the biomarker on ARDS directly.

Mendelian randomization (MR) is a methodology to assess causality in observational studies that is particularly well suited to infer the potential causal contribution of measured biomarkers (11, 12). A type of instrumental variable analysis, MR uses genetic variation to consider patients effectively randomized to a high or low expressing genotype by genetic recombination at gametogenesis in accordance with Mendel's law of independent assortment (11, 13–15). MR controls for threats to internal validity common to the study of ARDS biomarkers, such as reverse causality, measurement error, spuriousness, simultaneity, and confounding variables (16, 17). If the genetically predicted proportion of the biomarker, which is randomly assigned by parental allele assortment, retains an association with the outcome, then the measured biomarker is inferred to have a causal effect on the outcome (18).

sRAGE (soluble receptor for advanced glycation end products) is a strong candidate for a causal intermediate in ARDS. Plasma sRAGE is an isoform of membrane-bound RAGE (receptor for advanced glycation end products), a multiligand transmembrane receptor constitutively expressed in the lung on alveolar type I cells (19), which binds multiple advanced glycation end products and damage-associated molecular patterns (20, 21). This binding initiates a signaling cascade that potentiates the inflammatory response (22). In a murine model of hyperoxia lung injury, RAGE-null mice lived 3 days longer than their age-matched wild-type counterparts (23). The RAGE-null mice also demonstrated less airway cellular infiltration, less protein permeability, and less alveolar damage on immunohistochemistry (23). In an acid lung injury model, plasma and BAL levels of sRAGE associated with the severity of lung injury in a dose-response fashion (20, 24). In humans, plasma sRAGE is a top-performing biomarker for ARDS risk and mortality (7, 9, 25), and elevated plasma sRAGE levels are associated with impaired alveolar epithelial fluid clearance, a putative functional readout for epithelial function (26–28). The levels of plasma sRAGE are also significantly higher in patients with ARDS/acute lung injury as compared

with patients with hydrostatic pulmonary edema (20).

Despite the proposed mechanistic links between plasma sRAGE and ARDS, it remains unclear whether plasma sRAGE represents an epiphenomenon of lung injury or a causal contributor to risk of ARDS during sepsis. We hypothesized that the biology represented by plasma sRAGE associates with ARDS risk even when accounting for unmeasured confounding using MR for causal inference. In an MR analysis of critically ill patients with sepsis from two independent cohorts, we examined whether genetically predicted plasma sRAGE is associated with ARDS risk to evaluate the biomarker as a causal contributor. We propose that if plasma sRAGE acts as a causal contributor to ARDS risk, then strategies to modify plasma sRAGE or the pathways that generate it may warrant testing as novel targets for ARDS prevention or treatment. Some of the results of these studies have been previously reported in the form of an abstract (29, 30).

Methods

Study Design and Clinical Variables

We analyzed all patients with available DNA and early plasma prospectively enrolled in the MESSI (Molecular Epidemiology of Sepsis in the ICU) cohort at the University of Pennsylvania from September 2008 to February 2015 (31). Eligible patients were admitted to the medical ICU with sepsis-associated organ dysfunction in accordance with Sepsis-2 criteria as previously described (32). Major exclusion criteria included an alternative etiology for organ dysfunction, a decision to pursue comfort measures, or prior enrollment into the cohort. We abstracted clinical parameters from the electronic medical record. All chest-imaging studies were evaluated as previously described (31, 33). ARDS was defined by Berlin criteria requiring that chest radiograph and oxygenation criteria be fulfilled on the same calendar day that the patient was invasively ventilated (3). Mortality was determined at 30 days from admission. The Institutional Review Board of the University of Pennsylvania approved the MESSI cohort study. A second population, the iSPAAR (Identification of SNPs Predisposing to Altered Acute Lung Injury Risk) consortium study, was used to conduct an sRAGE protein quantitative

trait loci analysis (pQTL) and confirm the validity of the genetic instruments applied to test causal inference. The iSPAAR cohort consisted of genotyped subjects of European ancestry with an ARDS risk factor or enrolled as an ARDS case into a clinical trial (34). We limited the analysis to iSPAAR subjects with plasma sRAGE measured and with sepsis or pneumonia as their ARDS risk factor. This population has been previously described (10).

Plasma Analysis and Genotyping

In the MESSI cohort, we used ELISAs optimized for human plasma on samples collected at emergency department presentation for emergency department admitted patients or ICU admission (Day 0) for floor-admitted patients to measure plasma sRAGE (R&D Systems). In iSPAAR, we also used the ELISA kits to quantify plasma sRAGE levels within 48 hours of presentation. For MESSI, the genomic DNA was extracted from whole blood using the QIAamp DNA Mini kit (Qiagen) and assayed with the Affymetrix Axiom TxArray v.1, a genome-wide platform comprising approximately 780,000 polymorphisms (see SUPPLEMENTAL METHODS section in the online supplement). Genome-wide genotyping for the iSPAAR subjects was performed using the Illumina 660 W platform (35). To discover shared SNP-sRAGE loci, we undertook imputation using the Michigan Imputation Server (36). After postimputation quality control and sample filtering, there were 843 iSPAAR subjects included as the secondary population (SUPPLEMENTAL METHODS, online supplement).

Statistical Analysis

Genetic ancestry was determined by using all markers on the array and clustering with HapMap populations as previously described (10). As prespecified in our analytical approach, we conducted primary analyses separately in genetically determined European ancestry (EA) and African ancestry (AA) subjects to mitigate population stratification using principle components analysis and to test the consistency of effect in distinct ancestral populations within the MESSI population. MESSI subjects from other ancestries were excluded due to low enrollment ($n = 119$). All iSPAAR subjects were of European ancestry. In both populations, plasma sRAGE levels were log-transformed for normality.

Mendelian Randomization Procedure

The study was designed as a single-sample MR in MESSI with a second sample from iSPAAR used to mitigate reliance on weak or irreproducible genetic instruments (37). The MR analyses proceeded with the following steps:

Association of plasma RAGE levels with ARDS. We used logistic regression to evaluate the univariate and multivariate associations between plasma sRAGE and potential confounders with ARDS in the MESSI and iSPAAR populations.

Association of SNPs and plasma sRAGE in MESSI and iSPAAR. We conducted separate ancestry-specific genome-wide pQTL analyses on log-transformed plasma sRAGE using logistic regression for each common SNP based on minor allele frequency > 0.02 and assuming an additive genetic model (10).

Determination of genetic instruments. Top SNPs from the pQTL in both iSPAAR and MESSI with $P < 0.01$ were selected as candidate instruments. The SNPs with a relatively strong association with plasma sRAGE ($R^2 > 2\%$ and F

Figure 1. Mendelian randomization (MR) can be applied to assess the association between plasma sRAGE (soluble receptor for advanced glycation end products) and acute respiratory distress syndrome (ARDS) for a causal effect. The genetically determined portion of plasma sRAGE is considered the least vulnerable to unobserved confounding, spuriousness, and reverse causality (22). In this study, genetic variants were used as instrumental variables to evaluate the effects of plasma sRAGE on ARDS. (A) In the first stage of the analysis, measured plasma sRAGE levels were regressed on genome-wide genotypes to generate a protein quantitative trait locus, a set of SNPs that determine plasma sRAGE levels. This protein quantitative trait locus can be quantitatively leveraged to generate genetically determined plasma sRAGE (1). For causal inference, plasma sRAGE was considered to have a statistically significant causal effect if the association between the genetically determined plasma sRAGE values remained associated with ARDS risk after adjustment for confounders and the nongenetic residual (2). (B) This MR framework relies on underlying assumptions: 1) there is an association between SNPs and plasma sRAGE (e.g., plasma sRAGE is genetically predictable), 2) there is no direct association between plasma sRAGE-determining SNPs and ARDS that excludes plasma sRAGE, and 3) the plasma sRAGE-determining SNPs are not associated with the confounders. The MR method appears in solid lines, and assumptions appear in dashed lines in the figure. APACHE = Acute Physiology and Chronic Health Evaluation; pQTL = protein quantitative trait locus analysis.

statistic > 6) in MESSI were chosen as the final genetic instruments to reduce weak instrument bias. The selection of ancestry-specific genetic instruments was performed separately for the MESSI EA and AA populations. We used ancestry-specific populations for the SNP-sRAGE association in MESSI because inflammation-evoked plasma expression has been shown to be ancestry specific (38); however, we could not identify a replication population of African ancestry with ARDS phenotype and plasma sRAGE measurement. Therefore, we used the iSPAAR population for SNP selection in both MESSI ancestral populations. We optimized the number of genetic instruments by confirming that the instruments did not violate an assumption of homogeneity (e.g., heterogeneity test P value < 0.05) prior to proceeding with subsequent causal analyses (39).

Assessment of a statistically consistent causal effect. The approach of using multiple genetic instruments from different gene regions has been validated as a suitable method for polygenic or complex traits (40). For each selected SNP instrument, we enacted the limited information maximum likelihood method to estimate the causal effect of sRAGE levels on ARDS after controlling for confounding (Figure 1). We used the conventional inverse variable-weighted (IVW) method to obtain a consistent causal effect across all genetic instruments. To assess the accuracy of our causal inference, we applied MR-Egger regression to test for bias from pleiotropy and to provide a complementary approach for estimating the causal effect. We generated MR-Egger plots to evaluate for heterogeneity visually (41–43). We assessed for a putative functional relationship between the top sRAGE-determining exonic variant identified in the EA subjects with *AGER* by generating a coexpression network based on Gene Expression Omnibus using GeneMANIA with default coexpression settings (44).

Results

The MESSI population and enrollment is described in Table 1. Between September 2008 and February 2015, 9,265 intensive care patients were screened for inclusion in MESSI, 2,163 had sepsis, and 1,263 were enrolled (Figure E1 in the online

supplement). Of the enrolled patients, 659 had DNA and plasma sRAGE measured. Reasons for lack of DNA included inadequate DNA quantity (e.g., most commonly observed in patients with leukopenia), poor DNA quality, or failure for the DNA sample to be collected. The primary reason for a lack of plasma protein measurement was that the sample was not obtained within the 24-hour period immediately following ICU admission, particularly for subjects transferred from another facility, or sample depletion. ARDS developed in 259 (39%) patients in the MESSI cohort, and patients who developed ARDS had nearly double the mortality of those who did not (Table 1). Plasma sRAGE was associated with ARDS adjusting for Acute Physiology and Chronic Health Evaluation III score and pulmonary source of infection in each of the ancestries tested (Table 2).

Our primary single-sample MR analysis investigated plasma sRAGE as a potential causal intermediate in ARDS risk within the MESSI cohort (Figure 1). Plasma sRAGE demonstrated trans-regulation (45), with SNPs outside the *AGER* locus – the gene encoding RAGE – most strongly associating with early-evoked levels of the biomarker (Tables 3 and 4). Heterogeneity

of the causal effect was statistically minimized with 13 EA and 35 AA genetic instruments on IVW testing. A test for heterogeneity of the limited information maximum likelihood method effect yielded nonsignificant heterogeneity in both the MESSI EA-iSPAAR group ($P = 0.75$) and the MESSI AA-iSPAAR group ($P = 0.45$) using the top sRAGE-determining SNPs. Therefore, we selected the top 13 SNPs that shared a common pQTL association in the MESSI EA-iSPAAR group and the top 35 SNPs in the MESSI AA-iSPAAR group as genetic instruments. Genetically determined sRAGE was associated with ARDS in both populations, with β estimates for the effect of 0.50 [95% confidence interval, 0.09–0.91] per log increase in sRAGE in MESSI EA-iSPAAR group and 0.81 [95% confidence interval, 0.53–1.10] per log increase in AA on IVW analysis. There was a consistent causal effect of genetically predicted sRAGE on ARDS risk across the distinct genetic instruments evaluated in each ancestry (Figure 2). Among the set of 13 SNP genetic instruments from the MESSI EA-iSPAAR analysis, 1 SNP was exonic, rs2452600 in the gene *PDLIM5* which encodes for PDLIM5, an Enigma homolog protein. A GeneMANIA-created coexpression network

Table 1. MESSI Population with Genotype and Plasma sRAGE Measured

	ARDS (n = 261)	Non-ARDS (n = 411)	P Value
Age, yr	60 (51–69)	62 (53–71)	0.18
Sex, F	107 (41.0%)	177 (43.2%)	0.58
European ancestry	157 (60.2%)	228 (55.6%)	0.101
African ancestry	94 (36.0%)	167 (40.7%)	0.101
Asian ancestry	7 (2.69%)	7 (1.70%)	0.101
Genetically admixed	3 (1.15%)	8 (1.95%)	0.101
APACHE III	85 (67–111)	69 (53–88)	< 0.001
Pulmonary sepsis	164 (62.8%)	148 (36.1%)	< 0.001
Comorbidities			
Immunocompromised	88 (33.7%)	119 (29.0%)	0.38
Malignancy	69 (26.4%)	84 (20.4%)	0.12
Cirrhosis, decompensated	28 (10.7%)	35 (8.5%)	0.41
Diabetes	53 (20.3%)	92 (22.4%)	0.54
sRAGE, ng/ml	1,932 (960–4,267)	1,028 (592–1,855)	< 0.001
Mortality, 30 d	174 (66.7%)	145 (35.4%)	< 0.001

Definition of abbreviations: APACHE = Acute Physiology and Chronic Health Evaluation; ARDS = acute respiratory distress syndrome; MESSI = Molecular Epidemiology of Sepsis in the ICU; sRAGE = soluble receptor for advanced glycation end products. Subjects with ARDS were more likely to present with pulmonary sepsis and had a higher APACHE III score compared with patients with sepsis without ARDS. A high proportion of patients with ARDS carried an underlying immunocompromised state. Subjects with ARDS had higher ICU Day 0 plasma sRAGE and higher mortality than non-ARDS controls. Continuous data are summarized as mean (SD) or median (interquartile range) and compared between ARDS and non-ARDS by the Wilcoxon rank-sum test; and categorical variables are summarized as frequency (%) and compared by a chi-square test.

Table 2. Higher Concentrations of Plasma sRAGE Were Associated with Increased Odds of Acute Respiratory Distress Syndrome in MESSI and iSPAAR

	European Ancestry		African Ancestry	
	Adjusted OR (95% CI)	P Value	Adjusted OR (95% CI)	P Value
MESSI				
Measured log sRAGE	1.73 (1.35–2.21)	<0.001	2.05 (1.50–2.83)	<0.001
iSPAAR				
Measured log sRAGE	2.56 (2.14–3.06)	<0.001	—	—

Definition of abbreviations: ALI = acute lung injury; CI = confidence interval; iSPAAR = Identification of SNPs Predisposing to Altered ALI Risk; MESSI = Molecular Epidemiology of Sepsis in the ICU; OR = odds ratio; sRAGE = soluble receptor for advanced glycation end products.

Among 393 subjects with European ancestry and 266 subjects with African ancestry in the MESSI population, there was a significant positive association between higher plasma concentrations of sRAGE and increased odds of acute respiratory distress syndrome. The logistic regression analyses in MESSI were adjusted for pulmonary source of infection and Acute Physiology and Chronic Health Evaluation III score, which possessed independent associations with acute respiratory distress syndrome on univariate analyses. The same association was observed within the 843 iSPAAR subjects.

(Figure 3) depicts potential relationships between *AGER* and *PDLIM5* (44).

Discussion

Our causal inference analyses suggest that plasma sRAGE may be a potential causal intermediate—conferring risk for sepsis-associated ARDS. Using an approach to MR with two validated methodologies and

multiple genetic instruments replicated in two independent cohorts (13, 16, 46), there was a consistent causal effect of plasma sRAGE on ARDS development. We found that plasma sRAGE is genetically regulated during sepsis, and we also learned that levels of plasma sRAGE early in the course of sepsis were subject to trans-regulation in both MESSI and iSPAAR. These results suggest that the primary genetic determinants of plasma sRAGE in

sepsis-associated ARDS are not within *AGER*, which encodes plasma sRAGE, but rather in distant genes (45). Demonstrating the significance of genetic studies in diverse populations (47), we found that the genetic determinants of early-evoked plasma sRAGE differed by genetic ancestry but that common pQTLs were detectable. Although our results suggest that reducing plasma sRAGE may have preventive or therapeutic ARDS potential, the complex regulation of plasma sRAGE (22, 48–51) indicates that further clarity on which processes control plasma sRAGE elevation may be necessary to design a reasonable intervention strategy.

Existing studies strongly support the notion that endogenous plasma sRAGE plays a mechanistic role in ARDS pathophysiology in acid and hyperoxia models of lung injury (20, 23, 24). Human studies similarly noted that plasma levels of sRAGE are elevated in patients with ARDS and positively associated with mortality in patients with sepsis (9, 52). Reports are conflicting on whether exogenous plasma sRAGE potentiates or attenuates lung injury (53). In a murine model of lung infection, the administration of exogenous sRAGE worsened bacterial burden and neutrophilic infiltration, suggesting a direct pathogenic role (51). In contrast, treatment

Table 3. Top SNPs Associated with Early-Evoked Plasma sRAGE Concentrations on Protein Quantitative Trait Locus Analysis in European Ancestry Subjects from MESSI and iSPAAR

CHR	SNP	MAF	MESSI SNP-sRAGE			MESSI SNP-ARDS P Value	Gene	Function
			β	P Value	R^2			
3	rs4681329	0.36	−0.19	7.66×10^{-3}	0.04	0.62	<i>PLSCR5</i> , <i>LINC0200</i>	Intergenic
4	rs2452600	0.24	0.20	7.10×10^{-3}	0.03	0.20	<i>PDLIM5</i>	Exonic
6	rs72897292	0.09	−0.30	9.14×10^{-3}	0.03	0.14	<i>MEI4</i> , <i>IRAK1BP1</i>	Intergenic
5	rs77879804	0.06	0.41	6.31×10^{-3}	0.03	0.88	<i>LINC01411</i> , <i>MSX2</i>	Intergenic
5	rs72759624	0.09	0.37	7.80×10^{-3}	0.03	0.46	<i>PDZD2</i>	Intronic
12	rs6488383	0.14	0.18	6.31×10^{-3}	0.03	0.59	<i>CD163</i> , <i>APOBEC1</i>	Intergenic
16	rs170364	0.27	0.23	4.55×10^{-3}	0.03	0.57	<i>CX3CL1</i>	Intronic
6	rs17076148	0.09	0.33	9.27×10^{-3}	0.03	0.27	<i>ADGB</i>	Intronic
12	rs66656935	0.33	−0.29	8.66×10^{-5}	0.02	0.41	<i>LINC02370</i> , <i>LINC02414</i>	Intergenic
4	rs6831515	0.23	0.31	1.42×10^{-3}	0.02	0.58	<i>DCHS2</i>	Intronic
9	rs62573374	0.15	0.29	1.30×10^{-3}	0.02	0.33	<i>GNA14-AS1</i>	Intronic ncRNA
5	rs72780184	0.13	−0.34	8.76×10^{-3}	0.02	0.061	<i>LINC01950</i> , <i>EFNA5</i>	Intergenic
1	rs6679878	0.37	0.29	3.71×10^{-4}	0.02	0.60	<i>MTOR</i>	Intronic

Definition of abbreviations: ALI = acute lung injury; ARDS = acute respiratory distress syndrome; CHR = chromosome; iSPAAR = Identification of SNPs Predisposing to Altered ALI Risk; MAF = minor allele frequency; MESSI = Molecular Epidemiology of Sepsis in the ICU; ncRNA = non-coding RNA; sRAGE = soluble receptor for advanced glycation end products.

Note: β represents the expected change in plasma sRAGE per each additional risk-conferring allele in an additive genetic model. Mendelian randomization P values were considered statistically significant at $\alpha = 0.05$. Gene annotations were derived from Annovar. For intergenic SNPs, the nearest annotated genes are listed.

The table depicts results for 393 European ancestry subjects from MESSI and 843 European ancestry subjects from iSPAAR. A 13-SNP set of genetic instruments was used to test causal inference. The results reveal that early-evoked plasma sRAGE demonstrates trans-regulatory elements, indicating that genes outside *AGER*, which encodes sRAGE and is located on chromosome 6, govern its expression.

Table 4. Top SNPs Associated with Early-Evoked Plasma sRAGE Concentrations on Protein Quantitative Trait Locus Analysis in African Ancestry Subjects from MESSI and European Ancestry Subjects from iSPAAR

CHR	SNP	MAF	MESSI SNP-sRAGE			MESSI SNP-ARDS P Value	Gene	Function
			β	P Value	R^2			
2	rs4671615	0.35	−0.29	5.47×10^{-3}	0.04	0.56	<i>SERTAD2, LINC01800</i>	Intergenic
15	rs8039831	0.23	−0.34	4.60×10^{-3}	0.04	0.059	<i>PWRN4, PWRN2</i>	Intergenic
12	rs75606969	0.02	−1.13	6.00×10^{-3}	0.04	0.99	<i>RASSF3</i>	UTR3
2	rs1128965	0.44	0.25	5.44×10^{-3}	0.04	0.87	<i>CAVIN2</i>	UTR3
11	rs6592724	0.31	0.28	3.10×10^{-3}	0.04	0.13	<i>PAK1, LOC646029</i>	Intergenic
8	rs1608995	0.39	0.35	2.34×10^{-4}	0.04	0.05	<i>MSR1, FGF20</i>	Intergenic
18	rs1365253	0.24	−0.37	4.32×10^{-3}	0.03	0.88	<i>LINC01922, FAM69C</i>	Intergenic
12	rs10859579	0.34	0.32	4.65×10^{-3}	0.03	0.07	<i>CRADD</i>	Intronic
6	rs6457116	0.05	0.46	4.34×10^{-3}	0.03	0.24	<i>HLA-A, HCG9</i>	Intergenic
1	rs12404037	0.11	−0.38	7.11×10^{-3}	0.03	0.04	<i>SMYD3</i>	Intronic
9	rs7862786	0.07	0.44	8.13×10^{-4}	0.03	0.72	<i>UNQ6494, LOC101927847</i>	Intergenic
8	rs2980699	0.49	−0.24	4.93×10^{-3}	0.03	0.77	<i>MCPH1-AS1</i>	ncRNA
2	rs62152427	0.15	0.52	5.99×10^{-3}	0.03	0.73	<i>TSN, LINC01826</i>	Intergenic
16	rs10514543	0.38	−0.28	4.33×10^{-3}	0.03	0.13	<i>MPHOSPH6, CDH13</i>	Intergenic
9	rs10816581	0.33	0.26	6.56×10^{-3}	0.03	0.03	<i>KLF4, ACTL7B</i>	Intergenic
5	rs10462994	0.40	0.27	2.70×10^{-3}	0.03	0.01	<i>DOCK2</i>	Intronic
12	rs76306506	0.06	−0.95	8.44×10^{-3}	0.03	0.57	<i>DCD, MUCL1</i>	Intergenic
7	rs3808330	0.23	0.25	7.49×10^{-3}	0.03	0.27	<i>EN2</i>	UTR3
2	rs17772356	0.17	0.29	8.78×10^{-3}	0.03	0.78	<i>ARHGAP15</i>	Intronic
8	rs4371989	0.41	0.28	1.53×10^{-3}	0.03	0.57	<i>CPA6, PREX2</i>	Intergenic
4	rs17045076	0.06	−0.41	6.74×10^{-3}	0.03	0.63	<i>ANK2</i>	Intronic
3	rs115515578	0.02	−1.83	1.55×10^{-3}	0.03	0.99	<i>TMEM158, LARS2</i>	Intergenic
11	rs2897997	0.12	−0.38	2.23×10^{-3}	0.03	0.82	<i>DYNC2H1, MIR4693</i>	Intergenic
3	rs77744581	0.10	−0.61	2.43×10^{-3}	0.03	0.36	<i>TP63</i>	Intronic
7	rs2710995	0.46	−0.27	1.90×10^{-3}	0.03	0.17	<i>OSBPL3</i>	Intronic
6	rs78860635	0.14	−0.28	4.50×10^{-3}	0.03	0.08	<i>TBX18, LOC101928820</i>	Intergenic
2	rs2167461	0.09	−0.32	5.56×10^{-3}	0.03	0.82	<i>LOC285000, NCK2</i>	Intergenic
13	rs79104916	0.03	0.89	3.73×10^{-3}	0.03	0.29	<i>MIR3169, PCDH20</i>	Intergenic
1	rs913178	0.32	−0.23	9.65×10^{-3}	0.03	0.07	<i>KIAA1324</i>	Intronic
8	rs16905009	0.03	0.71	8.43×10^{-3}	0.03	0.98	<i>LOC101927822</i>	ncRNA
8	rs11785751	0.33	0.28	1.00×10^{-3}	0.02	0.01	<i>MSR1, FGF20</i>	Intergenic
13	rs73175166	0.11	−0.44	5.69×10^{-3}	0.02	0.41	<i>LHFP</i>	Intronic
2	rs10200410	0.38	0.26	9.42×10^{-3}	0.02	0.05	<i>IL1RL2, IL1RL1</i>	Intergenic
17	rs9906785	0.26	−0.30	5.47×10^{-3}	0.05	0.56	<i>SERTAD2, LINC01800</i>	Intergenic
17	rs4890093	0.27	−0.35	4.60×10^{-3}	0.05	0.06	<i>PWRN4, PWRN2</i>	Intergenic

Definition of abbreviations: ALI = acute lung injury; ARDS = acute respiratory distress syndrome; CHR = chromosome; iSPAAR = Identification of SNPs Predisposing to Altered ALI Risk; MAF = minor allele frequency; MESSI = Molecular Epidemiology of Sepsis in the ICU; ncRNA = non-coding RNA; sRAGE = soluble receptor for advanced glycation end products; UTR = untranslated region.

Note: β represents the expected change in plasma sRAGE per each additional risk-conferring allele in an additive genetic model. Mendelian randomization P values were considered statistically significant at $\alpha = 0.05$. Gene annotations were derived from Annovar. For intergenic SNPs, the nearest annotated genes are listed.

The table depicts results for 266 African ancestry subjects from MESSI and 843 European ancestry subjects from the iSPAAR study. A suitable replication population composed of African ancestry subjects with plasma sRAGE and acute respiratory distress syndrome ascertained could not be identified. A 35-SNP set of genetic instruments was used for Mendelian randomization. A relatively large SNP set (compared with that used in Table 3) was required owing to the underlying differences in population substructure in the cross-ancestry comparison. As in Table 3, the results reveal that early-evoked plasma sRAGE demonstrates trans-regulatory elements, indicating that genes outside *AGER*, which encodes sRAGE and is located on chromosome 6, govern its expression.

with recombinant sRAGE in an LPS model of lung injury reduced neutrophil infiltration, lung permeability, and production of inflammatory cytokines (50). In a model of delayed-type hypersensitivity, mice-administered recombinant sRAGE or anti-RAGE antibody also demonstrated decreased inflammation following antigen exposure (48). Further, plasma sRAGE, to our knowledge, is the only biomarker that has been correlated with a functional

readout of epithelial barrier function, alveolar fluid clearance (27, 28).

Our study contributes to the current understanding of sRAGE by providing further support for the critical role of endogenous plasma sRAGE within the RAGE axis as a potential driver of lung injury. Offering additional support for a potential hyperinflammatory role of plasma sRAGE, our causal inference analyses suggest that plasma sRAGE or the pathways

that act to generate sRAGE may be actual contributors to ARDS pathophysiology. However, MR can only estimate a causal effect based on the parameters entered into the model. What is quantified as plasma sRAGE represents the totality of a variety of processes including metalloproteinase activity, alternative splicing of RAGE mRNA, increased circulating advanced glycation end products (AGE) or other damage-associated molecular patterns, and

Figure 2. Plasma sRAGE (soluble receptor for advanced glycation end products) demonstrated a consistent causal effect on acute respiratory distress syndrome (ARDS) risk using a genetic instrument derived from replication in two independent cohorts. (A and B) Mendelian randomization–Egger analysis was separately applied in MESSI (Molecular Epidemiology of Sepsis in the ICU)–European ancestry and iSPAAR (Identification of SNPs Predisposing to Altered ALI Risk) (A) and MESSI–African ancestry and iSPAAR (B) groups. The horizontal axis represents the genetic effect estimate (β) of a single SNP on plasma sRAGE levels. The vertical axis represents the genetic effect estimate (β) of a single SNP on ARDS. The dots represent single SNPs. The horizontal bars denote the 95% confidence interval for the protein quantitative trait locus effect, whereas the vertical bars denote the 95% confidence interval for the genome-wide association study effect of the SNP on ARDS. A consistent causal effect is observed if the slope of the line of best fit is constant, nonzero, and approximates the origin. The Mendelian randomization–Egger regression plot demonstrated a consistent, causal effect for the association of plasma sRAGE on ARDS in each ancestry.

a response to epithelial cell injury. It remains possible that one of these pathways may have been responsible for the observed causal effect of plasma sRAGE on ARDS risk. Further mechanistic research into the AGE–RAGE axis will be necessary to illuminate the precise cause of the plasma sRAGE–ARDS causal effect identified in this study.

Critical gaps in our knowledge of the AGE–RAGE axis during sepsis-associated ARDS remain. In lung epithelial cells, AGE–RAGE signaling depends not only on the role of RAGE and its isoforms, but also on the activity of RAGE ligands (54). Adding additional complexities, our genetic analyses indicate that plasma sRAGE has trans-regulatory factors outside *AGER* that govern early-evoked sRAGE expression during sepsis. Functional genomic studies to better understand the trans-regulation of sRAGE may help to disentangle which elements are most critical (45). Prior studies have demonstrated that the genetic regulation of plasma protein expression differs in an inflammatory state (38).

Although additional work is necessary to understand the sepsis-evoked regulation of plasma sRAGE, our genetic analyses identified rs2452600 located in *PDLIM5* as the top exonic SNP determining plasma sRAGE levels that shared its association with ARDS in both MESSI and iSPAAR. *PDLIM5* encodes PDLIM5, an Enigma homolog protein from the Enigma subfamily of PDZ-LIM proteins (55, 56). *PDLIM5* has been implicated in protein complex assembly and basement membrane cellular targeting (56). We used GeneMANIA to assess for a functional relationship between *PDLIM5* and *AGER* based on Gene Expression Omnibus data. The coexpression network demonstrated links in protein expression between *PDLIM5* and three well-established RAGE ligands, S100P, S100A12, and high-mobility group box 1 protein (Figure 3). We also confirmed basal expression of both *AGER* and *PDLIM5* in the lung using the Gene-Tissue Expression (GTEx) database (57). These findings are hypothesis-generating and will need to be explored in future studies of the RAGE axis. Ultimately, a rigorous knowledge of the AGE–RAGE axis requires an integrative systems biology approach including simultaneous testing of RAGE isoforms and RAGE ligands in cellular and circulating compartments during stress conditions. This approach

Figure 3. *PDLIM5* shares a coexpression network with *AGER* (blue circles). On protein quantitative trait locus testing, we identified trans-regulation within the top sRAGE (soluble receptor for advanced glycation end products)-determining SNPs that govern evoked expression levels. The top exonic sRAGE-determining SNP (rs2452600) is located within *PDLIM5*. *PDLIM5* encodes the Enigma homolog protein, which regulates protein–protein interactions and the activity of ID2, a transcription factor. We generated a coexpression network based on Gene Expression Omnibus using GeneMANIA to determine if there are coexpression links between *AGER*, which encodes RAGE, and *PDLIM5* observed in prior studies. We found that *PDLIM5* shares indirect coexpression links with S100A12, S100P, and HMGB1 (high-mobility group box 1 protein) (green circles), which are known RAGE ligands.

may allow for the identification of the best target within the pathway for ARDS therapeutics.

This study has limitations. For our causal inference analyses, we relied on Mendelian randomization, a robust and validated method that enhances causal inference compared with conventional studies of circulating biomarkers (11, 58). However, our study relies on single-sample MR which differs from a two-sample approach in which the genetic instrument determination and causal effect estimation occur in distinct samples (37, 59). Single-sample MR can be associated with a greater potential for weak instruments to generate spurious and confounded effect estimates, underestimation of true causal effects, and reduced power (60). Despite the potential for underestimation of true causal effects and reduced power, we have observed a statistically significant causal effect estimate for plasma sRAGE as a risk factor for ARDS using single-sample MR methodology. In addition, two-sample MR approaches may be more vulnerable to bias if the two samples used for causal inference are not

representative of the same reference population with respect to age, race, and other confounders (60, 61). Acknowledging that genetic studies on evoked levels of plasma sRAGE during critical illness remain limited precluding a two-sample MR approach currently, we have attempted to mitigate weak instrument bias by using a multivariable (e.g., multi-SNP) approach, selecting genetic instruments that have an association with early-evoked plasma sRAGE levels in two independent cohorts, and assessing statistically for instrument strength using stringent *F* statistics and *R*² values (39, 41, 46).

Although we have examined the data for violations of the three main assumptions of MR, it is plausible that our primary results may have been subject to weak instrument bias, pleiotropy, and/or canalization (62). To mitigate concerns regarding unidentified violations of MR assumptions, we also applied sensitivity analyses to test for the heterogeneity of the causal effects of the SNPs and used the MR-Egger regression as a complementary approach for estimating causal effects (43). We also

applied MR-Egger regression to evaluate for bias from pleiotropy. We recognize that our instrument developed in the MESSI AA-iSPAAR population was less parsimonious than the one developed with the MESSI EA-iSPAAR population, and our design may have overlooked strong AA-specific pQTL because we could not identify an appropriate AA replication population. Future work is necessary to attempt to replicate AA-specific plasma sRAGE pQTL. In addition, we acknowledge that plasma sRAGE was tested at only one time-point. Prior studies have indicated that plasma sRAGE levels are dynamic in the setting of ARDS (26). Repeated measures might have captured a larger proportion of plasma sRAGE variance during sepsis. However, this early time-point may be most useful for possible plasma sRAGE-driven approach to ARDS preventive therapeutics. While we have examined one biomarker in this study, we have applied MR to a biomarker with a demonstrated relationship to epithelial barrier function in ARDS (27, 28). As MR gains prominence in critical care research, we anticipate the application of causal inference methodologies to additional candidate biomarkers. Last, while our study represents an early application of MR to biomarkers in ARDS, our group previously described plasma ANG2 as a causal factor in ARDS development (10). This study has several important distinctions from the prior one in that 1) new, robust MR statistical methodologies were used, 2) two independent populations were used to derive the genetic instruments, and 3) a marker of epithelial injury rather than endothelial activation was analyzed. Overall, the identification of plasma sRAGE as a causal intermediate is complementary to our plasma ANG2 knowledge since each biomarker possesses a distinct pathobiology in ARDS.

As an early application of Mendelian randomization for causal inference during critical illness, our study demonstrates that plasma sRAGE may act as a causal, intermediate conferring risk for ARDS in patients with European and African ancestry, addressing a critical need for genetic studies in diverse populations (47, 63). Elucidation of the AGE-RAGE axis has several important areas that require further research to develop a full knowledge of the actions of the pathway including investigation into the RAGE isoforms and

RAGE ligands during stress. Prior to designing a clinical trial to leverage the AGE-RAGE pathway by modifying plasma sRAGE as a novel ARDS therapy, it is imperative to develop a precision approach using plasma sRAGE as a biomarker to

identify the patients with sepsis who would have the highest potential to benefit from such anti-RAGE axis therapies. ■

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

Acknowledgment: The authors acknowledge the patients and families of the MESSI and iSPAAR cohorts who graciously agreed to participate in this research study. They also acknowledge the nurses, physicians, and staff in the medical and surgical ICUs who participated in the clinical care of the enrolled patients.

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