

# A Missense Genetic Variant in *LRRC16A/CARMIL1* Improves Acute Respiratory Distress Syndrome Survival by Attenuating Platelet Count Decline

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## Abstract

**Rationale:** Platelets are believed to contribute to acute respiratory distress syndrome (ARDS) pathogenesis through inflammatory coagulation pathways. We recently reported that leucine-rich repeat-containing 16A (*LRRC16A*) modulates baseline platelet counts to mediate ARDS risk.

**Objectives:** To examine the role of *LRRC16A* in ARDS survival and its mediating effect through platelets.

**Methods:** A total of 414 cases with ARDS from intensive care units (ICUs) were recruited who had exome-wide genotyping data, detailed platelet counts, and follow-up data during ICU hospitalization. Association of *LRRC16A* single-nucleotide polymorphisms (SNPs) and ARDS prognosis, and the mediating effect of SNPs through platelet counts were analyzed. *LRRC16A* mRNA expression levels for 39 cases with ARDS were also evaluated.

**Measurements and Main Results:** Missense SNP rs9358856G>A within *LRRC16A* was associated with favorable survival within 28 days (hazard ratio [HR], 0.57; 95% confidence interval

[CI], 0.38–0.87;  $P = 0.0084$ ) and 60 days ( $P = 0.0021$ ) after ICU admission. Patients with ARDS who carried the variant genotype versus the wild-type genotype showed an attenuated platelet count decline ( $\Delta$ PLT) within 28 days (difference of  $\Delta$ PLT,  $-27.8$ ;  $P = 0.025$ ) after ICU admission. Patients with  $\Delta$ PLT were associated with favorable ARDS outcomes. Mediation analysis indicated that the SNP prognostic effect was mediated through  $\Delta$ PLT within 28 days (28-day survival: HR<sub>Indirect</sub>, 0.937; 95% CI, 0.918–0.957;  $P = 0.0009$ , 11.53% effects mediated; 60-day survival: HR<sub>Indirect</sub>, 0.919; 95% CI, 0.901–0.936;  $P = 0.0001$ , 14.35% effects mediated). Functional exploration suggested that this SNP reduced *LRRC16A* expression at ICU admission, which was associated with a lesser  $\Delta$ PLT during ICU hospitalization.

**Conclusions:** *LRRC16A* appears to mediate  $\Delta$ PLT after ICU admission to affect the prognosis in patients with ARDS.

**Keywords:** SNP; platelets; ARDS outcome; causal mediation analysis

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## At a Glance Commentary

### Scientific Knowledge on the

**Subject:** Acute respiratory distress syndrome (ARDS) is a deadly, complex disease with an in-hospital mortality rate that exceeds 30%. Platelets are believed to contribute to the pathogenesis of ARDS and affect patients' prognosis.

### What This Study Adds to the

**Field:** Leucine-rich repeat-containing 16A appears to mediate platelet count decline after admission to the intensive care unit, and, in turn, affect ARDS patients' prognosis. The findings may aid in identifying novel therapeutic targets for ARDS within platelet cellular processes.

Acute respiratory distress syndrome (ARDS), which is characterized by acute hypoxemic respiratory failure and bilateral pulmonary infiltrates with an amplified inflammatory response, is a deadly complex disease with an in-hospital mortality rate that exceeds 30% (1–3). Several factors affect ARDS outcomes. For example, worsening oxygenation, multiple organ dysfunction, and progressive underlying diseases are common risk factors for ARDS mortality (4). Recently, platelets have been increasingly recognized for their roles in ARDS outcomes, which are likely mediated by their involvement in inflammatory responses and disseminated intravascular coagulation (5–7). Genetic risk factors may also determine ARDS outcomes (8); studies have identified a few potential prognostic biomarkers (9–13). However, the roles of genetic susceptibility in ARDS outcome and its interplay with micro-environmental factors remain incompletely understood (14).

Recently, we reported that a novel genetic variant in leucine-rich repeat-containing 16A (*LRRC16A*) is associated with reduced ARDS risk, and this phenomenon is partially mediated through platelet counts (15). These findings might aid in identifying novel therapeutic targets for ARDS within platelet cellular processes (16). However, no studies have yet examined the interplay of *LRRC16A* and platelets on ARDS outcomes. The Gene Nomenclature Committee of the Human Genome Organization might rename

*LRRC16A* as a capping protein regulator and myosin 1 linker 1 (*CARMIL1*) to reflect its homology and functional similarity to the genes currently approved as *CARMIL2* and *CARMIL3*.

We hypothesized that *LRRC16A* influences ARDS outcomes by regulating platelets after intensive care unit (ICU) admission, and we explored this association using mediation analysis. Mediation analysis is one of several causal inference approaches that can identify the potential mechanism by which an independent variable (e.g., genetic variant) affects the outcome (e.g., ARDS death) via an explanatory factor or mediator (e.g., platelet count decline [ $\Delta$ PLT] during ICU hospitalization). This approach has been widely applied in epidemiological studies and in lung disease research (15, 17–19). Using exome-wide genotyping data, we evaluated the association of genetic variants in *LRRC16A* with baseline platelet count at ICU admission and  $\Delta$ PLT up to 28 days after ICU admission. Significant genetic variants were further evaluated for an association with ARDS survival and whether their effects were mediated through platelet counts using causal mediation analysis. Some of the results have been previously reported in the form of an abstract for the 2015 American Thoracic Society meeting (20).

## Methods

### Clinical and Genotyping Study Population

The original study titled “Molecular Epidemiology of ARDS Study (MEARDS)” was approved by the institutional review boards of Harvard T. H. Chan School of Public Health, Massachusetts General Hospital (MGH), and Beth Israel Deaconess Medical Center (BIDMC) (approval number: 1999P0086071/MGH). All participants or their surrogates gave written informed consent.

Cases with ARDS were selected from the MEARDS study. As described previously (15), we screened each ICU admission at MGH and BIDMC for eligible subjects, who were defined as critically ill without any of the exclusion criteria (age younger than 18 yr, diffuse alveolar hemorrhage, chronic lung diseases other than chronic obstructive pulmonary disease or asthma, directive to withhold intubation, immunosuppression

not secondary to corticosteroids, and treatment with granulocyte colony-stimulating factor) and with at least one predisposing condition for ARDS: sepsis, septic shock, trauma, pneumonia, aspiration, or massive transfusion of packed red blood cells (RBCs) (defined as >8 packed RBC units during the 24 h before admission). After enrollment, subjects were followed daily for the development of ARDS, as defined by the American-European Consensus Committee, which generally corresponds to moderate to severe ARDS by the Berlin definition (21, 22). Patient demographics and baseline clinical characteristics were collected at ICU admission. Patients who died or were discharged within 24 hours after ICU admission were excluded. All enrolled patients were followed until death, hospital discharge, or 28 days after ICU admission. Patients were also followed until 60 days after ICU admission for secondary outcomes. Lowest platelet counts were collected daily during ICU hospitalization for up to 28 days.

As described in the previous report (15), the genotyping was performed using the Infinium HumanExome BeadChip (Illumina, Inc, San Diego, CA) followed by standard quality control processes. The 414 cases with ARDS were selected from the existing exome-wide genotyping data, which had complete survival follow-up data and detailed platelet count data during ICU hospitalization. Five common single-nucleotide polymorphisms (SNPs) within *LRRC16A* of the 414 cases with ARDS were available, and they were included in the analysis.

### Gene Expression Study Population

In addition, *LRRC16A* mRNA expression data of 39 cases with ARDS from the MEARDS cohort were included from an existing gene expression study and were further analyzed. The 39 cases were aged  $64.2 \pm 15.1$  years old, 82% were men, 85% had declined platelet counts, and Acute Physiology and Chronic Health Evaluation (APACHE) III scores were averaged at  $87.9 \pm 22.7$ . Gene expression levels were evaluated by Custom TaqMan Arrays (probe #: Hs01084245\_m1; Thermo Fisher Scientific, Inc, Waltham, MA). Protocols for sample collection, processing, and whole blood total RNA extraction were described previously (23). Each sample was run in triplicate, and cycle threshold (Ct)

**Table 1.** Study Population Demographic and Clinical Characteristics at Intensive Care Unit Admission

Variable	ARDS Cases (n = 414)	
	Survivor	Nonsurvivor*
N	298 (72.0)	116 (28.0)
APACHE III score	72.8 ± 21.3 <sup>†</sup>	91.2 ± 19.9
Age, yr	55.8 ± 17.5	68.0 ± 13.7
Sex		
Male	191 (64.1)	71 (61.2)
Female	107 (35.9)	45 (38.8)
Baseline platelet count <sup>‡</sup> , 10 <sup>3</sup> /μl	216.1 ± 139.3 <sup>§</sup>	189.2 ± 158.6
ΔPLT <sub>28d</sub> <sup>  </sup> , 10 <sup>3</sup> /μl	57.5 ± 107.3 <sup>§</sup>	85.8 ± 119.9
ΔPLT <sub>7d</sub> <sup>¶</sup> , 10 <sup>3</sup> /μl	40.6 ± 101.5 <sup>**</sup>	76.5 ± 113.8
Ethnicity		
Caucasian	290 (97.3)	113 (97.4)
African American	3 (1.0)	0 (0.0)
Hispanic	2 (0.7)	2 (1.7)
Asian/Pacific Islander	2 (0.7)	0 (0.0)
Other	1 (0.3)	1 (0.9)
Physiology		
Heart rate, beats/min		
Lowest <sup>††</sup>	79.6 ± 19.2	79.9 ± 17.9
Highest <sup>††</sup>	120.2 ± 22.9	120.9 ± 22.1
Respiratory rate, breaths/min		
Lowest <sup>††</sup>	15.7 ± 5.2 <sup>§</sup>	16.9 ± 5.6
Highest <sup>††</sup>	30.3 ± 8.9	31.8 ± 8.8
Systolic blood pressure, mmHg		
Lowest <sup>††</sup>	81.8 ± 15.9	77.1 ± 14.6
Highest <sup>††</sup>	146.8 ± 24.4	143.9 ± 21.9
Mean arterial pressure, mmHg		
Lowest <sup>††</sup>	57.9 ± 10.0 <sup>†</sup>	54.1 ± 8.3
Highest <sup>††</sup>	99.8 ± 16.6	96.5 ± 13.9
ABG testing in 24 h after ICU admission		
PaO <sub>2</sub> , mmHg	118.0 ± 73.6	118.1 ± 73.0
FiO <sub>2</sub> , mmHg	0.79 ± 0.23	0.80 ± 0.23
P/F ratio	157.4 ± 87.0	156.9 ± 93.3
ARDS predispositions		
Sepsis	261 (87.6) <sup>†</sup>	113 (97.4)
Pneumonia	207 (69.5)	82 (70.7)
Aspiration	30 (10.1)	8 (6.9)
Multiple transfusion	25 (8.4)	8 (6.9)
Pulmonary contusion	12 (4.0)	1 (0.9)
Multiple fractures	12 (4.0)	1 (0.9)
Complications		
Diabetes mellitus	57 (19.3)	27 (23.3)
Immune suppression <sup>§§</sup>	21 (7.1)	14 (12.1)
Chronic dialysis or peritoneal dialysis	25 (8.5)	12 (10.3)
Cirrhosis	10 (3.4)	16 (13.8)
Hepatic failure with coma or encephalopathy	9 (3.1)	8 (6.9)
Solid tumor with metastasis	4 (1.4)	5 (4.3)
Non-Hodgkin's lymphoma	0 (0.0)	2 (1.7)

*Definition of abbreviations:* ABG = arterial blood gas; APACHE = Acute Physiology and Chronic Health Evaluation; ARDS = acute respiratory distress syndrome; ICU = intensive care unit; P/F = PaO<sub>2</sub>/FiO<sub>2</sub>; ΔPLT = platelet count decline.

Continuous variables are described as mean ± SD and compared by *t* test or Kruskal-Wallis rank sum test (platelet variables); categorical variables are described as number (%) and compared by Fisher's exact test.

\*Patients dead within 28 days after ICU admission.

<sup>†</sup>*P* < 0.001.

<sup>‡</sup>Lowest platelet count at ICU admission.

<sup>§</sup>*P* < 0.05.

<sup>||</sup>Maximal platelet count decline within 28 days (ΔPLT<sub>28d</sub>) after ICU admission compared with baseline platelet count.

<sup>¶</sup>Maximal platelet count decline within 7 days (ΔPLT<sub>7d</sub>) after ICU admission compared with baseline platelet count.

<sup>\*\*</sup>*P* < 0.01.

<sup>††</sup>Minimal value in first 24 hours of ICU admission.

<sup>‡‡</sup>Maximal value in first 24 hours of ICU admission

<sup>§§</sup>Immune suppression within the past 6 months including radiation or chemotherapy with ≥0.3 mg/kg/d prednisone or equivalent.

values were averaged (<sub>a</sub>Ct) to represent Ct values for *LRRC16A* (<sub>a</sub>Ct<sub>LRRC16A</sub>) and reference gene *18S* (<sub>a</sub>Ct<sub>18S</sub>), which was used to normalize *LRRC16A* expression by  $\Delta\text{Ct}_{LRRC16A} = \text{aCt}_{LRRC16A} - \text{aCt}_{18S}$ . The 39 cases had complete platelet count data during ICU hospitalization and survival follow-up data, and were carried forward for further analysis. Of these, a subset of 27 cases had ExomeChip data and were used to explore the expression quantitative trait locus (eQTL) relationship.

### Statistical Analyses

Baseline characteristics, including demographics and baseline measurements, were described as mean ± SD for continuous variables and number (%) for categorical variables. Student's *t*-test or Fisher's exact test was used for comparisons between groups for continuous or categorical variables, respectively. All SNPs were encoded in additive genetic format (0: wild type; 1: heterozygosity; 2: homozygosity; minor allele as effect allele), unless stated otherwise. Baseline platelet count was the lowest platelet count on the day of ICU admission (PLT<sub>baseline</sub>). Maximal ΔPLT was estimated as PLT<sub>baseline</sub> minus the lowest platelet count measured within 28 days (ΔPLT<sub>28d</sub> for primary purpose) or the lowest platelet count within 7 days (ΔPLT<sub>7d</sub> for sensitivity evaluation) after ICU admission. Primary prognostic outcome was ARDS survival (time-to-death) within 28 days (survival<sub>28d</sub>) after ICU admission, whereas the secondary prognostic outcome was survival within 60 days (survival<sub>60d</sub>) after ICU admission.

To test the association between *LRRC16A* variants and ARDS outcomes and the underlying causal mediation pathway through platelets, we split the analyses into four main scenarios in the predefined order of: (1) testing the associations between *LRRC16A* genetic variants and ARDS outcomes; (2) evaluating the associations between the identified genetic variant(s) and platelet count variables (PLT<sub>baseline</sub>, ΔPLT<sub>28d</sub>, and ΔPLT<sub>7d</sub>); (3) analyzing the associations between the selected platelet count variable(s) and ARDS outcomes; and (4) causal inference analysis. A sequential testing strategy was used in which an analysis would move forward only if the previous one reached statistical significance (24).

Associations of *LRRC16A* SNPs on ARDS survival<sub>28d</sub> (primary outcome) and survival<sub>60d</sub> (secondary outcome) were evaluated by Cox regression with adjustment for common covariates, including age, sex, multiple transfusions, and APACHE III score. Results are described as hazard ratio (HR), 95% confidence interval (CI), and *P* value. False discovery rate (FDR) was used for multiple comparison correction (25). Any SNP with an FDR-*q* value  $\leq 0.05$  was carried forward. The identified SNP was further analyzed using Kaplan-Meier survival curves in additive and dominant (0: wild type, 1: heterozygosity or homozygosity) genetic models, respectively.

The identified SNP tested the association with platelet count variables (PLT<sub>baseline</sub>,  $\Delta$ PLT<sub>28d</sub>, and  $\Delta$ PLT<sub>7d</sub>) using linear regression. For correlations between SNP and  $\Delta$ PLT, sensitivity analysis was performed with additional adjustment for PLT<sub>baseline</sub>. The platelet count variables with *P* values  $\leq 0.05$  were carried forward.

Cox regression was used to test the associations between platelet count variables and ARDS outcomes (survival<sub>28d</sub> and survival<sub>60d</sub>), respectively, with adjustment for common covariates. For the associations with  $\Delta$ PLT<sub>28d</sub> and  $\Delta$ PLT<sub>7d</sub>, models were additionally adjusted for PLT<sub>baseline</sub>. Results are described as HR per  $100 \times 10^3/\mu\text{l}$  increment, 95% CI, and *P* value.

Causal inference analysis is detailed in the online supplement. Briefly, the mediation model by Valeri and VanderWeele was applied to evaluate the

indirect effect of a SNP on ARDS survival that was mediated through  $\Delta$ PLT (HR<sub>indirect</sub>) and the proportion of the effect mediated (26). Covariates included age, sex, multiple transfusion, APACHE III score, and baseline platelet count. The statistical *P* value for HR<sub>indirect</sub> was obtained by permutation test with 10,000 permutations.

Associations of the gene expression on  $\Delta$ PLT variables ( $\Delta$ PLT<sub>28d</sub> and  $\Delta$ PLT<sub>7d</sub>) were tested using linear regression, with adjustment for baseline platelet count. The eQTL relationship between SNP and gene expression was also analyzed using linear regression.

All analyses were performed in R Version 3.0.1 (The R Foundation, Vienna, Austria) or Stata Version 14 (StataCorp LP, College Station, TX).

## Results

Distributions of demographic and baseline clinical characteristics among the 414 cases with ARDS are described in Table 1.

Ninety-seven percent of cases were Caucasian. A total of 116 (28%) cases were dead within 28 days after ICU admission. Most of the patients had  $\Delta$ PLT within 28 days (324 of 414; 78%) or the first 7 days (311 of 414; 75%) during ICU hospitalization. Seventeen SNPs in *LRRC16A* were genotyped on the Illumina ExomeChip; however, only five common SNPs (minor allele frequency  $\geq 0.05$ ) were included in the analysis to ensure adequate statistical power (see Figure E1 in the online

supplement). The overall analysis workflow is described in Figure E2.

First, associations between five common SNPs and outcomes of ARDS patients were analyzed (Table 2). Only missense SNP rs9358856 (p.Val77Ile) showed a significant association with both the primary outcome: 28-day survival (HR, 0.57; 95% CI, 0.38–0.87; *P* = 0.0084; FDR-*q* = 0.04) (Figure 1A), and the secondary outcome: 60-day survival (HR, 0.55; 95% CI, 0.38–0.81; *P* = 0.0021; FDR-*q* = 0.01) (Figure 1A). The results retained statistical significance with additional adjustment for sepsis (see Table E1 in the online supplement). Because only a small number of patients were homozygous (AA) for SNP rs9358856, we also tested the association between SNP rs9358856 in a dominant genetic model and ARDS survival, which gave consistent results (Figure 1B). SNP rs7766874, which was previously reported to be associated with ARDS risk (15) was only modestly associated with 28-day survival, with borderline significance (HR, 0.80; *P* = 0.0610) (Table 2). Thus, SNP rs9358856 was analyzed further.

Next, we evaluated the association between SNP rs9358856 and platelet count-related variables, including PLT<sub>baseline</sub>,  $\Delta$ PLT<sub>28d</sub>, and  $\Delta$ PLT<sub>7d</sub> (see Figure E3). There was no significant association between SNP rs9358856 and the baseline platelet count (*P* = 0.335) (see Figure E3A). However, patients carrying the A allele of rs9358856 (GA or AA genotype) showed a significantly less decline of platelet count versus the wild-genotype (GG) group within 28 days (difference of  $\Delta$ PLT<sub>28d</sub> between GA/AA and GG groups,  $\beta_{\text{GA/AA-vs.-GG}} = -27.8$ ; 95% CI  $-52.0$  to  $-3.6$ ; *P* = 0.025), which retained statistical significance with further adjustment for PLT<sub>baseline</sub> (*P* = 0.036) (see Figure E3B). Similar results were observed for  $\Delta$ PLT within 7 days after ICU admission ( $\beta_{\text{GA/AA-vs.-GG}} = -29.9$ ; *P* = 0.018; adjustment for PLT<sub>baseline</sub>, *P* = 0.030) (see Figure E3C).  $\Delta$ PLT variables further tested the associations with ARDS outcomes. Both  $\Delta$ PLT<sub>28d</sub> and  $\Delta$ PLT<sub>7d</sub> were significantly associated with ARDS outcomes (Table 3), which retained statistical significance after additional adjustment for sepsis (see Table E2).

Furthermore, because SNP rs9358856 was a significant QTL marker of  $\Delta$ PLT and was associated with ARDS survival,  $\Delta$ PLT after ICU admission might act as an

**Table 2.** Association between *LRRC16A* SNPs and Survival of Patients with Acute Respiratory Distress Syndrome

SNP <sup>‡</sup>	Survival <sub>28d</sub> <sup>*</sup>		Survival <sub>60d</sub> <sup>†</sup>	
	HR (95% CI)	<i>P</i> Value (FDR- <i>q</i> )	HR (95% CI)	<i>P</i> Value (FDR- <i>q</i> )
rs9358856	0.57 (0.38–0.87)	0.0084 (0.04)	0.55 (0.38–0.81)	0.0020 (0.01)
rs2274089	0.63 (0.38–1.04)	0.0714 (0.12)	0.69 (0.42–1.11)	0.1260 (0.25)
rs7766874	0.80 (0.63–1.01)	0.0610 (0.12)	0.85 (0.68–1.06)	0.1528 (0.25)
rs1012899	1.12 (0.82–1.52)	0.4762 (0.48)	1.10 (0.81–1.50)	0.5344 (0.67)
rs742132	1.12 (0.87–1.45)	0.3684 (0.46)	1.05 (0.82–1.36)	0.6816 (0.68)

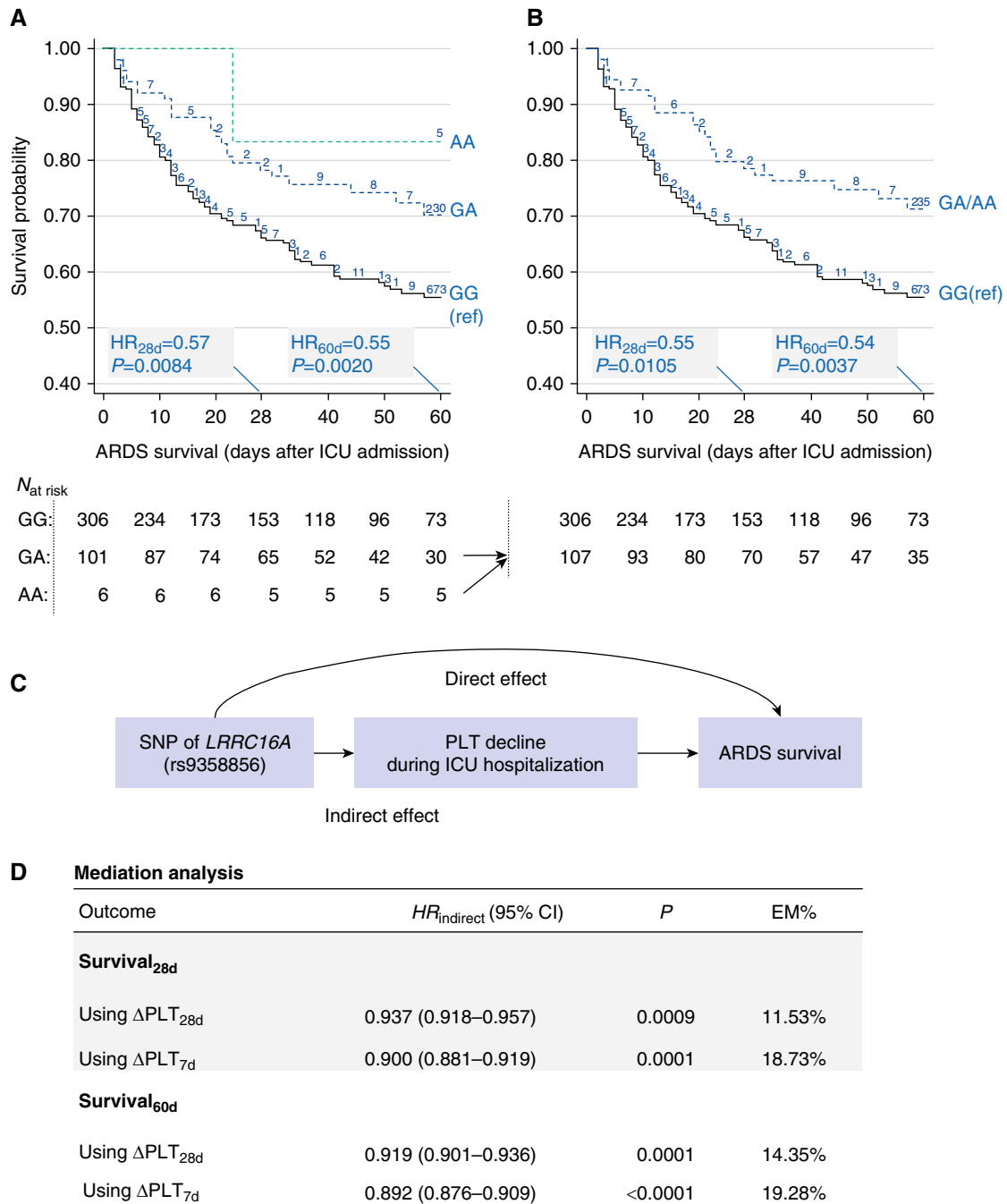
Definition of abbreviations: CI = confidence interval; FDR-*q* = false discovery rate-adjusted *P* value; HR = hazard ratio per minor/effect allele; *LRRC16A* = leucine-rich repeat-containing 16A; SNP = single-nucleotide polymorphism.

The model was adjusted for age, sex, blood transfusion, baseline platelet count, and Acute Physiology and Chronic Health Evaluation III score.

\*Primary outcome.

†Secondary outcome.

‡Analyzed in additive genetic model.



**Figure 1.** Association between single-nucleotide polymorphism (SNP) rs9358856 and acute respiratory distress syndrome (ARDS) survival and the effects mediated through platelet count. (A) Kaplan-Meier survival curves stratified by SNP rs9358856 genotypes in additive genetic model—wild type (GG; black line), heterozygous (GA; blue dashed line), and homozygous (AA; green dashed line). (B) Due to the small sample size of the AA group, AA was merged with GA group. Numbers on the Kaplan-Meier curves represent censored cases. Hazard ratios (HRs) per minor allele, 95% confidence intervals (95% CIs), and statistical *P* values were estimated by Cox regression with adjustment for age, sex, blood transfusion, baseline platelet count, and Acute Physiology and Chronic Health Evaluation III score. (C) The mediation model. (D) Results of mediation analyses for ARDS 28-day survival and 60-day survival. Results are described as observed indirect prognostic effect (indirect hazard ratio [HR<sub>indirect</sub>]) of the SNP that was mediated through platelet count decline, 95% CI, *P* value, and the proportion of effect mediated (EM%). Two mediators were considered: maximal platelet count decline within 28 days (ΔPLT<sub>28d</sub>) and within 7 days (ΔPLT<sub>7d</sub>) after intensive care unit (ICU) admission compared with the baseline platelet count. *LRRC16A* = leucine-rich repeat-containing 16A.

**Table 3.** Platelet Count Decline and Prognosis of Patients with Acute Respiratory Distress Syndrome

Variable	Survival <sub>28d</sub> <sup>*</sup>		Survival <sub>60d</sub> <sup>†</sup>	
	HR (95%CI)	P Value	HR (95%CI)	P Value
$\Delta\text{PLT}_{28d}^{\ddagger}$	1.53 (1.08–2.16)	0.0172	1.73 (1.25–2.39)	$8.4 \times 10^{-4}$
$\Delta\text{PLT}_{7d}^{\S}$	1.73 (1.26–2.39)	$7.3 \times 10^{-4}$	1.82 (1.36–2.44)	$6.8 \times 10^{-5}$

Definition of abbreviations: CI = confidence interval; HR = hazard ratio per  $100 \times 10^3/\mu\text{l}$  increment; PLT = platelet count.

Cox model was used with adjustment for age, sex, blood transfusion, and baseline platelet count and Acute Physiology and Chronic Health Evaluation III score.

<sup>\*</sup>Primary outcome.

<sup>†</sup>Secondary outcome.

<sup>‡</sup>Maximal platelet count decline within 28 days ( $\Delta\text{PLT}_{28d}$ ) after intensive care unit admission compared with baseline platelet count.

<sup>§</sup>Maximal platelet count decline within 7 days ( $\Delta\text{PLT}_{7d}$ ) after intensive care unit admission compared with baseline platelet count.

important mediator of the genetic effect of *LRRC16A* on ARDS outcomes. To test this hypothesis, we performed causal mediation analysis (Figure 1C), which detected a significant indirect effect for SNP rs9358856 on favorable ARDS 28-day survival that was mediated through  $\Delta\text{PLT}_{28d}$  (HR<sub>indirect</sub>, 0.937 per effect allele; 95% CI, 0.918–0.957;  $P = 0.0009$ , 11.53% effects mediated) (Figure 1D). Sensitivity analysis using  $\Delta\text{PLT}_{7d}$  revealed a higher proportion of indirect effects (HR<sub>indirect</sub>, 0.900; 95% CI, 0.881–0.919;  $P < 0.0001$ , 18.73% effects mediated) (Figure 1D). Causal mediation analysis on 60-day

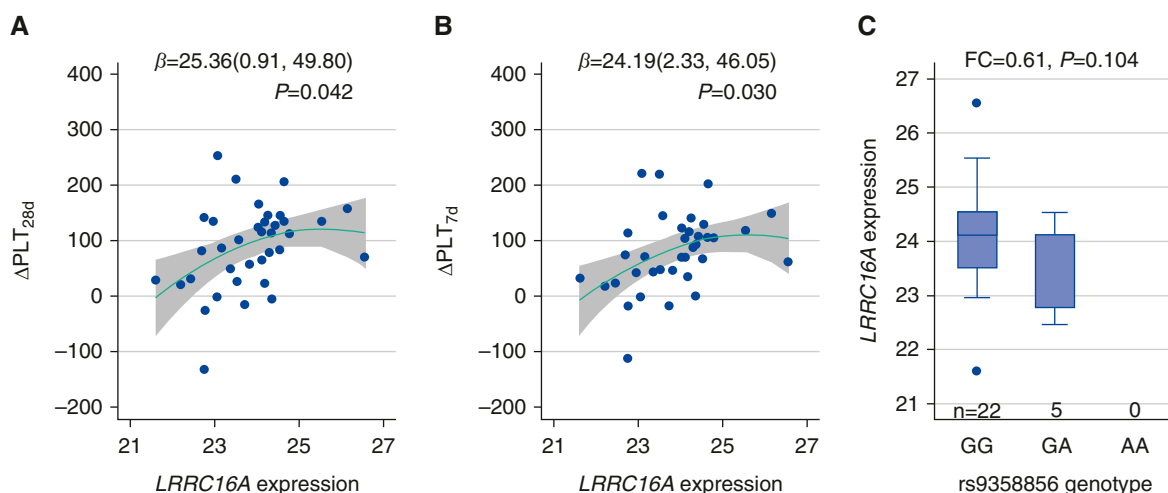
survival showed similar results (Figure 1D). The indirect effects were also tested with additional adjustment for sepsis, which retained statistical significance (see Table E3).

Furthermore, to explore the functionality of SNP rs9358856 and the gene, we evaluated *LRRC16A* mRNA expression levels at ICU admission of 39 cases with ARDS who had complete survival follow-up and platelet count data during ICU hospitalization. A higher *LRRC16A* gene expression at ICU admission was significantly correlated with a higher  $\Delta\text{PLT}$  within both 28 days

( $P = 0.042$ ) (Figure 2A) and 7 days ( $P = 0.030$ ) (Figure 2B) after ICU admission. Interestingly, 27 of the 39 cases with ARDS had both gene expression and genotyping data, which showed a trend between SNP and reduced gene expression ( $P = 0.104$ ) (Figure 2C).

## Discussion

As previously reported, genetic variants in *LRRC16A* are associated with platelet counts among relatively healthy populations and critically ill patients in the ICU (15, 27). Interestingly, in this study, we identified a novel missense SNP in *LRRC16A* that downregulated the gene expression and was associated with attenuated  $\Delta\text{PLT}$  after ICU admission among cases with ARDS. The previously identified SNP rs7766874 is located in the intron of *LRRC16A*, but little is understood about its function. However, the newly identified SNP rs9358856 is a missense SNP located in exon #5 that causes valine-to-isoleucine amino acid change. It is also located at the exonic splicing enhancer, which indicates SNP rs9358856 may affect the process of *LRRC16A* pre-messenger RNA splicing (28). In contrast, although the intron SNP rs7766874 was associated with baseline platelet count at ICU admission as reported in our previous study



**Figure 2.** Relationship among single-nucleotide polymorphism (SNP) rs9358856, leucine-rich repeat-containing 16A (*LRRC16A*) gene expression, and platelet count. *LRRC16A* mRNA expression levels at intensive care unit (ICU) admission of 39 cases with acute respiratory distress syndrome were used to analyze the associations with platelet count decline within (A) 28 days ( $\Delta\text{PLT}_{28d}$ ) and (B) 7 days ( $\Delta\text{PLT}_{7d}$ ) after ICU admission, respectively. Unilinear relationship (solid line and confidence band) was demonstrated. Linear regression was used to estimate  $\beta$ , 95% confidence interval, and  $P$  value with adjustment for the baseline platelet count. (C) A subset of 27 cases had both expression and genotyping data. Sample size (n) of each genotyping group: 22 were wild type (GG), 5 were heterozygous (GA), and none were homozygous (AA). Box plot and expression fold change (FC) were used to explore the relationship between SNP rs9358856 and expression level.

(15), the presently identified missense SNP rs9358856 was associated with  $\Delta$ PLT after ICU admission rather than the platelet count at ICU admission. The two SNPs are not linked among Caucasians ( $r^2 = 0.02$ ;  $D' = 0.31$ ) (see Figure E2), which suggests different underlying mechanisms that affect the gene and warrants further well-designed functional experiments. Taken together, these studies suggest that *LRRC16A* plays a role in the platelets of critically ill patients in the ICU.

Furthermore, we provided evidence that a lower baseline platelet count or larger decline of platelet count after ICU admission was associated with worse outcomes in patients with ARDS in the ICU, which was consistent with a previous report (29). Thrombocytopenia is the most common coagulation problem in the ICU, and is generally caused by the following mechanisms: increased destruction or consumption of platelets, decreased production, dilution, and sequestration of platelets (30, 31). Clinical events related to these mechanisms, such as disseminated intravascular coagulation, trauma, or massive transfusions, are considered risk factors that affect ARDS outcomes (7, 32, 33). Thus, treatments for thrombocytopenia might have benefits for patients with ARDS, whereas the treatments should be given in respect to the etiology (34). For example, systemic activation of blood coagulation results in consumption and subsequent exhaustion of coagulation proteins and platelets that causes thrombocytopenia (30). For this etiology, anticoagulant therapy is an option for thrombocytopenia, which also has a potential benefit for ARDS outcomes (35). However, in this study, we could not distinguish among the potential roles of platelet production, consumption, or functional disorders in ARDS outcomes; therefore, future experimental studies are warranted.

The primary finding of this study suggested that *LRRC16A* is associated with ARDS outcomes, which appear to be partially mediated through a declining platelet count after ICU admission. The protective effect of SNP rs9358856 for ARDS outcomes might be mediated through reduced *LRRC16A* expression, which attenuates declines in platelet count. Capping protein ARP2/3 and myosin-I linker (CARMIL), encoded by *LRRC16A*, binds capping protein Z, which acts as an

inhibitor of F-actin polymerization (36). Animal models demonstrated that the binding of the CARMIL protein to capping protein decreases its affinity for the actin filament barbed end, which leads to F-actin polymerization (37). F-actin polymerization is crucial during platelet activation through involvement in the formation of dynamic structures such as filopodia and lamellopodia (38, 39). The missense SNP that reduces *LRRC16A* expression might result in moderate F-actin polymerization, which is related to decreased platelet consumption after ICU admission, which leads to a favorable prognosis. Although this mediation pathway is statistically significant, only 10 to 20% of the prognostic effect of the SNP rs9358856 is mediated through  $\Delta$ PLT, which implies that *LRRC16A* has an effect beyond  $\Delta$ PLT during ICU stay. *LRRC16A* might therefore affect other pathways that contribute to ARDS survival. However, the mechanisms underlying the effects of the missense SNP on CARMIL protein quantity and/or activity, and, in turn, on dynamic platelet counts (or function) after ICU admission, are not well understood, and therefore, highlight the need for further well-designed functional studies.

Accumulating evidence demonstrates a role of platelets in ARDS morbidity and mortality (5, 40, 41). Platelets promote lung injury via inflammatory signaling and contribute to basal barrier integrity of alveolar capillaries (42, 43). Thus, reduced or inactivated platelets might reduce lung inflammation and permeability. As a result, there is emerging interest in therapies that target platelet function among at-risk patients with ARDS. Aspirin treatment in mouse models promotes increased levels of lipoxin (ATL; 15-epi-lipoxin A4) and blocking of the lipoxin A4 receptor, which prevents neutrophil-platelet aggregation and attenuates acute lung injury (44). Accordingly, prehospital aspirin use in patients at high risk for ARDS is associated with reduced ARDS risk (45–49). Even patients in the ICU with ARDS potentially receive therapeutic benefits from aspirin usage to reduce ICU mortality (50). However, a recent published LIPS-A (Lung Injury Prevention Study with Aspirin) randomized clinical trial did not demonstrate a benefit to administration of aspirin to either reduce the risk of ARDS at 7 days or improved secondary outcomes

(51). Interestingly, as commented by Reilly and Christie, one possible explanation for the inefficacy is timing of delivery (52). Identifying biomarkers for uncovering patient subtypes might predict treatment responsiveness and prognosis, which could be incorporated into the design of clinical trials (53). Enhancing our understanding of the mechanisms of how platelets mechanisms contribute to ARDS outcome, by integrating with genetic information, might aid in identifying novel therapeutic targets or identifying more sensitive subgroups for antiplatelet therapy.

We acknowledge some limitations in our study. First, statistical power was insufficient to study the association of rare functional SNPs, which could be examined using target sequencing in larger cohorts. We also were aware that the mRNA study population (39 cases) did show, to some extent, imbalances in terms of age, sex, severity of disease and platelet decline, and comorbidities compared with the genotyping study population, which might be partially due to sampling and small sample size. For this reason, the results of the present gene expression study should be taken with caution and validated using a more comparable population and larger sample size. Second, functional studies are needed to evaluate the mechanisms that underlie the associations between *LRRC16A* genetic variants and ARDS outcome and the mediating pathway through platelets. Third, we only evaluated platelet count as a potential causal mediator, whereas platelet function likely plays a comparable mediation role in this pathway. Therefore, more detailed platelet information should be measured in future studies, including immature platelet fractions, platelet function, aggregation, and neutrophil adhesion (16). Fourth, thrombocytopenia is a marker for common conditions in the ICU (e.g., disseminated intravascular coagulation, hemodilution, or advanced liver disease) and could result in clinical consequences (e.g., bleeding, immunomodulation) that might be the biologic drivers for ARDS poor outcomes. Thus, related underlying causal pathways should be explored in the future to provide better understanding of ARDS etiology. In addition, our cases with ARDS were recruited from an ICU at-risk population with a high proportion of sepsis, and all cases with ARDS corresponded to moderate

to severe ARDS according to the Berlin definition, which showed a higher mortality than those with mild ARDS (2). Therefore, the findings in the study were mainly pertinent to patients with septicemia in the ICU who developed ARDS, and should be validated before generalization in cohorts

with ARDS with different precipitating conditions.

In summary, our findings suggest a role of *LRRC16A* in ARDS outcomes, and its prognostic effect appears to be partially mediated through a progressive decline of platelet counts after ICU admission. ■

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