Platelet Count Mediates the Contribution of a Genetic Variant in *LRRC16A* to ARDS Risk

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BACKGROUND: Platelets are believed to be critical in pulmonary-origin ARDS as mediators of endothelial damage through their interactions with fibrinogen and multiple signal transduction pathways. A prior meta-analysis identified five loci for platelet count (PLT): *BAD*, *LRRC16A*, *CD36*, *JMJD1C*, and *SLMO2*. This study aims to validate the quantitative trait loci (QTLs) of PLT within *BAD*, *LRRC16A*, *CD36*, *JMJD1C*, and *SLMO2* among critically ill patients and to investigate the associations of these QTLs with ARDS risk that may be mediated through PLT.

METHODS: ARDS cases and at-risk control subjects were recruited from the intensive care unit of the Massachusetts General Hospital. Exome-wide genotyping data of 629 ARDS cases and 1,026 at-risk control subjects and genome-wide gene expression profiles of 18 at-risk control subjects were generated for analysis.

RESULTS: Single-nucleotide polymorphism (SNP) rs7766874 within *LRRC16A* was a significant locus for PLT among at-risk control subjects ($\beta = -13.00$; 95% CI, -23.22 to -2.77 ; $P = .013$). This association was validated using *LRRC16A* gene expression data from at-risk control subjects ($\beta = 77.03$ per 1 SD increase of $\log_2$ -transformed expression; 95% CI, 27.26 - 126.80 ; $P = .005$). Further, rs7766874 was associated with ARDS risk conditioned on PLT (OR = 0.68; 95% CI, 0.51-0.90; $P = .007$), interacting with PLT (OR = 1.15 per effect allele per $100 \times 10^3/\mu\text{L}$ of PLT; 95% CI, 1.03-1.30; $P = .015$), and mediated through PLT (indirect OR = 1.045; 95% CI, 1.007-1.085; $P = .021$).

CONCLUSIONS: Our findings support the role of *LRRC16A* in platelet formation and suggest the importance of *LRRC16A* in ARDS pathophysiology by interacting with, and being mediated through, platelets.

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ABBREVIATIONS: ALI = acute lung injury; DIC = disseminated intravascular coagulation; MAF = minor allele frequency; MGH = Massachusetts General Hospital; PLT = platelet count; QC = quality control; QTL = quantitative trait locus; SNP = single-nucleotide polymorphism

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ARDS is characterized by acute hypoxemic respiratory failure with bilateral pulmonary infiltrates. Its in-hospital mortality rate is 41.1%, placing a large burden on public health.^{1,2} The most common underlying causes of ARDS include pulmonary risk factors, such as pneumonia or pulmonary contusion, and nonpulmonary risk factors, including sepsis, burn, severe trauma, and multiple transfusions.³ Studies have focused on the role of genetics in ARDS pathophysiology and have identified dozens of loci related to ARDS outcome (eg, *IL1RN* [IL1 receptor antagonist], *ANGPT2* [angiopoietin-2], *PI3* [peptidase inhibitor 3], and *PPFIA1* [protein tyrosine phosphatase, receptor type, f polypeptide]).⁴⁻⁸ However, the roles of genetic susceptibility and its interactions with microenvironmental factors remain incomplete.^{9,10}

Platelets are believed to make an important contribution to pulmonary-origin ARDS among critically ill patients acting in conjunction with fibrinogen to mediate endothelial damage through multiple signal transduction pathways.^{11,12} Indeed, if patients with ARDS develop thrombocytopenia or systemic coagulation disorders, such as platelet consumption and/or disseminated intravascular coagulation (DIC), they are at increased risk for a poor prognosis.¹³ Genetic factors contributing to platelet count (PLT) are beginning to be uncovered. In particular, a meta-analysis of seven genome-wide association studies of 16,388 individuals from seven

population-based cohorts identified five quantitative trait loci (QTLs) associated with PLT: *BAD* at 11q13, *LRRC16A* at 6p22, *CD36* at 7q11, *JMJD1C* at 10q21, and *SLMO2* at 20q13.¹⁴ Although the identification of these

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loci provides a foundation for understanding the genetic contributions to PLT, no studies have yet examined the role of PLT-associated loci in ARDS pathophysiology.

Our hypothesis is that PLT may act as an important causal mediator of genetic effect on ARDS development. In causal inference, the mediation modeling is a rigorous approach to identify and explain the mechanism by which an independent variable (eg, genetic variant) affects the outcome (eg, ARDS risk) via a third explanatory variable, known as a mediator variable (eg, PLT) (Fig 1). This approach has been successfully applied in research on several lung diseases.¹⁵⁻¹⁷

Using exome-wide genotyping data (Illumina ExomeChip), in this study we validated QTLs of PLT within five candidate genes—*BAD*, *LRRC16A*, *CD36*, *JMJD1C*, and *SLMO2*—among critically ill patients. We further evaluated the findings by independent gene expression experimental data. Finally, we explored the effect of these genetic variants on ARDS risk and whether their effects are causally mediated through PLT.

Materials and Methods

Study Population

The study was reviewed and approved by institutional review boards of Massachusetts General Hospital (MGH) and Harvard School of Public Health (project approval number: 1999P0086071/MGH). All participants or their surrogate care providers gave written informed consent.

ARDS cases and control subjects were identified from critically ill patients from the ICU at MGH as described previously.⁵ Briefly, we screened each ICU admission for eligible subjects, who were defined as critically ill patients with at least one predisposing condition for ARDS: (1) sepsis, (2) septic shock, (3) trauma, (4) pneumonia, (5) aspiration, or (6) massive transfusion of packed RBCs (defined as > 8 packed RBC units during the 24 h before admission) and without any of the exclusion criteria (age < 18 years, diffuse alveolar hemorrhage, chronic lung diseases other than COPD or asthma, directive to withhold intubation, immunosuppression not secondary to corticosteroid, and treatment with granulocyte colony-stimulating factor). After enrollment, subjects were followed daily for the development of ARDS, as defined by the American-European Consensus Committee.^{18,19} Control subjects were identified as at-risk patients who did not meet criteria for ARDS during the ICU stay and had no prior history of ARDS. Patient demographic and baseline clinical characteristics including sepsis, pneumonia, trauma, transfusion, and PLT were collected at ICU admission.

Genotyping

DNA was extracted from peripheral WBCs using the protocols as described previously.²⁰ Subjects were genotyped using the Infinium HumanExome BeadChip (Illumina, Inc). Before analysis, a systematic

quality evaluation was conducted on the raw genotyping data according to the general quality control (QC) procedure described by Anderson and colleagues.²¹ Briefly, unqualified samples were excluded if they fit the following QC criteria: (1) overall genotype completion rates < 95%, (2) sex discrepancies, (3) unexpected duplicates or probable relatives (based on pairwise identity by state value, PI_HAT in PLINK > 0.185), or (4) heterozygosity rates more than six times the SD from the mean. Unqualified SNPs were excluded when they fit the following QC criteria: (1) SNPs had a call rate < 95% in all samples, (2) the genotype distributions of SNPs deviated from those expected by Hardy-Weinberg equilibrium ($P < .000001$), or (3) SNPs were too rare to detect variants under our sample size. After quality evaluation, 629 ARDS cases and 1,026 at-risk control subjects and 19 SNPs were included in the initial analyses.

Gene Expression Microarray

A validation study of genome-wide gene expression profiles were generated from total RNA samples extracted from peripheral blood collected at ICU admission of 18 at-risk control subjects from the MGH ARDS study cohort. Protocols for sample collection and processing, whole

Figure 1 – A mediation model.

blood total RNA extraction, and quality assessment were described previously.²² RNA samples were hybridized to Affymetrix Hu133A 2.0 GeneChips (Affymetrix, Inc). Expression values for four probe sets within *LRRC16A* were extracted, normalized, and log₂-transformed using dChip software.²³ Three probe sets were excluded from the analysis because they had absent calls in > 50% of samples. Only one probe set of *LRRC16A* (230793_at) was included in the analysis.

Statistical Analyses

Baseline characteristics including demographics and baseline measurements are described as mean $\pm$ SD (minimum, maximum) for continuous variables and number (%) for categorical variables. The Student *t* test or Fisher exact test were used for comparisons between groups for continuous or categorical variables, respectively. All SNPs were encoded at additive genetic format (0: wild type; 1: heterozygosity; 2: homozygosity, minor allele as effect allele). This study included three main scenarios of analyses in the predefined order of (1) QTL analysis of PLT, (2) ARDS risk analysis of SNP variants, and (3) causal inference analysis. The sequential testing strategy—borrowed from serial gate-keeping procedure—was used, in which the analysis would move forward only if the previous one reached a reasonable significance.^{24–26} This strategy is broadly used to control for family-wise type 1 error in clinical trials with multiple comparisons that have a predefined sequence. All models were adjusted for covariates of age, sex, sepsis, and pneumonia at admission, unless stated otherwise.

The Sequence Kernel Association Test was used for gene-based QTL analysis.²⁷ The log₂-transformed gene expression was further standardized as *z* score (*z*) for QTL analysis using linear regression. The results are indicated by the β coefficient (explained by the change of PLT per 1 SD increase of log₂-transformed expression level) and the 95% CI. For SNP-level QTL analysis, linear regression was used for analyzing of five common SNPs, respectively. The results are indicated by β coefficient (the change of PLT per effect allele of SNP) and the 95% CI. The SNP with false discovery rate (described as *q*-value) < .10 was selected for ARDS risk analysis. Only one SNP (rs7766874) reached the criteria. The proportion of PLT variance explained by the SNP rs7766874 was estimated using analysis of variance as the ratio of the sum of squares of SNP (SS_{SNP}) to the sum of squares total (SS_{total}).

Results

The distributions of demographic and clinical characteristics at ICU admission among 629 patients with ARDS and 1,026 at-risk control subjects are described in Table 1. All the control subjects and 97.77% of the ARDS cases were white. No significant differences were observed in age or sex distributions between ARDS and at-risk control groups. PLT and number of subjects with sepsis or pneumonia (as risk factors for ARDS) were significantly different between cases and control subjects.

Since the design of the Illumina ExomeChip focuses on functional exonic SNPs, most of the covered SNPs are either low-frequency or rare alleles (minor allele frequency [MAF] < 0.05).²⁹ No SNPs were mapped within *BAD*, *CD36*, or *SLMO2*. Two rare SNPs were mapped within *JMJD1C*; these were excluded from further analysis to ensure adequate statistical power. The 17 SNPs identified within *LRRC16A* were included in the analysis (Table 2). The analysis workflow is described in Figure 2.

For ARDS risk analysis, the association of SNP, PLT, and the interaction with ARDS risk were evaluated by logistic regression and are described by OR (the exponential function of the coefficient) and the 95% CI:

$$\text{logit}(p) = \theta_0 + \theta_{SNP} \times SNP + \theta_{PLT} \times PLT + \theta_{Interaction} \times SNP \times PLT + B \times Covariates \quad (1)$$

where θ_{SNP} , θ_{PLT} , and $\theta_{Interaction}$ represent the coefficients of SNP, PLT, and SNP-PLT interaction, respectively; *B* is a vector of coefficients of covariates. The analysis would move forward if the statistical *P* value of θ_{SNP} or $\theta_{Interaction}$ test < .05.

For causal inference analysis, the VanderWeele mediation model was applied to evaluate the causal indirect effect of a SNP that was mediated through PLT.¹⁷ The results are described by indirect OR ($OR_{Indirect}$, per effect allele of SNP) and the 95% CI:

$$OR_{Indirect} = e^{(\theta_{PLT} \times \beta_{SNP} + \theta_{Interaction} \times \beta_{SNP})} \quad (2)$$

where β_{SNP} was estimated by the following weighted linear regression model of PLT on SNP:

$$PLT = \beta_0 + \beta_{SNP} \times SNP + B \times Covariates + e, \text{ weights} = w \quad (3)$$

In model (3), the weights (*w*) were calculated for cases or control subjects, respectively:

$$w = \begin{cases} prevalence / r & , \text{ for cases} \\ (1 - prevalence) / (1 - r) & , \text{ for controls} \end{cases} \quad (4)$$

where the *prevalence* represents the prevalence of ARDS among the at-risk critically ill population, and *r* represents the proportion of ARDS cases in the analytical dataset. Bootstrapping with 1,000 replications was used to estimate the 95% CI of $OR_{Indirect}$ and statistical significance. The Imai causal inference method was also used with the same weights for cross-validation with the VanderWeele mediation model.²⁸ The statistical significance level was 0.05. All analyses were performed using R statistical software, v 2.15.1 (The R Foundation).

We initially conducted a gene-based QTL analysis of PLT on *LRRC16A* genetic variants (Table 3). Five common SNPs (MAF $\geq$ 0.05) showed a significant joint association with PLT among at-risk control subjects (*P* = .036). We validated this finding by using independent whole-genome gene expression data from 18 at-risk control subjects. A linear regression model detected a significant association between *LRRC16A* expression level and PLT (β = 77.03 per 1 SD increase of expression level; 95% CI, 27.26–126.80; *P* = .005) (Fig 3). After adjustment for common covariates including age, sex, sepsis, and pneumonia using stepwise regression, the association remained significant (*P* = .011).

We then performed a single SNP QTL analysis for each common SNP within *LRRC16A* (Table 4). Only SNP rs7766874 was significantly associated with PLT among at-risk control subjects (β = −13.00 per effect allele of SNP; 95% CI, −23.22 to −2.77; *P* = .013; false discovery rate adjusted *q*-value = .065), which explained around

TABLE 1] Study Population Demographic and Clinical Characteristics at ICU Admission

Variable	ARDS Case Subjects (n = 629)	At-Risk Control Subjects (n = 1,026)	P Value
Age, y	60.65 ± 17.27 (18, 97)	62.19 ± 16.84 (18, 97)	.074
Sex			.567
Male	394 (62.64)	627 (61.11)	
Female	235 (37.36)	399 (38.89)	
Ethnicity			<.001
White	615 (97.77)	1,026 (100.00)	
Black, not of Hispanic origin	5 (0.79)	0 (0.00)	
Hispanic	5 (0.79)	0 (0.00)	
Asian/Pacific Islander	2 (0.32)	0 (0.00)	
Other	2 (0.32)	0 (0.00)	
PLT (10 ³ /μL)	200.71 ± 126.18 (9, 691)	213.52 ± 124.68 (16, 695)	.043
0: ≥ 500	21 (3.34)	33 (3.22)	.027 ^a
1: 450-499	9 (1.43)	28 (2.73)	
2: 400-449	16 (2.54)	39 (3.80)	
3: 350-399	17 (2.70)	45 (4.39)	
4: 300-349	48 (7.63)	78 (7.60)	
5: 250-299	68 (10.81)	91 (8.87)	
6: 200-249	90 (14.31)	138 (13.45)	
7: 150-199	121 (19.24)	226 (22.03)	
8: 100-149	96 (15.26)	178 (17.35)	
9: 50-99	99 (15.74)	132 (12.87)	
10: <50	44 (7.00)	38 (3.70)	
Sepsis			<.001
0: No	82 (13.04)	220 (21.44)	
1: Yes	547 (86.96)	806 (78.56)	
Pneumonia			<.001
0: No	209 (33.23)	632 (61.60)	
1: Yes	420 (66.77)	394 (38.40)	
Transfusion			.090
0: No	577 (91.73)	914 (89.08)	
1: Yes	52 (8.27)	112 (10.92)	
Chronic dialysis or peritoneal dialysis			.004
0: No	569 (91.04)	969 (94.72)	
1: Yes	56 (8.96)	54 (5.28)	
Leukemia ^b			.304
0: No	625 (100.00)	1,019 (99.61)	
1: Yes	0 (0.00)	4 (0.39)	
Non-Hodgkin's lymphoma			1.000
0: No	621 (99.36)	1,017 (99.41)	
1: Yes	4 (0.64)	6 (0.59)	
Solid tumor with metastasis			1.000
0: No	607 (97.12)	993 (97.07)	
1: Yes	18 (2.88)	30 (2.93)	

(Continued)

TABLE 1] (continued)

Variable	ARDS Case Subjects (n = 629)	At-Risk Control Subjects (n = 1,026)	P Value
Immune suppression ^c			.586
0: No	575 (92.00)	932 (91.10)	
1: Yes	50 (8.00)	91 (8.90)	
Hepatic failure with coma or encephalopathy			.002
0: No	602 (96.32)	1,010 (98.73)	
1: Yes	23 (3.68)	13 (1.27)	
Cirrhosis			.019
0: No	587 (93.92)	987 (96.48)	
1: Yes	38 (6.08)	36 (3.52)	
Diabetes mellitus			.128
0: No	481 (76.96)	752 (73.51)	
1: Yes	144 (23.04)	271 (26.49)	

Continuous variables are described as mean $\pm$ SD (minimum, maximum), and categorical variables are described as No. (%). PLT = platelet count.

^aWilcoxon rank-sum test.

^bIncluding acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, and multiple myeloma.

^cImmune suppression within the past 6 mo including radiation, chemotherapy ≥ 0.3 mg/kg/d prednisone or equivalent.

1% of PLT variance. To evaluate more potential confounders, we did a sensitivity analysis with adjustment for all covariates in Table 1 using stepwise regression. The results consistently showed a significant QTL relationship ($\beta = -12.70$; 95% CI, -22.62 to -2.78 ; $P = .012$). After excluding 14 non-white cases, the results remained significance (results not shown). Among 1,026 at-risk control subjects, there were 93 patients with acute lung injury (ALI; $200 \text{ mm Hg} < \text{PaO}_2/\text{FiO}_2 \leq 300 \text{ mm Hg}$, defined as mild ARDS in new Berlin definition³⁰). After excluding those 93 patients with ALI as highly heterogeneous “control subjects”, the sensitivity analysis showed a consistent association between SNP rs7766874 and PLT ($\beta = -11.08$ per effect allele of SNP; 95% CI, -21.59 to -0.57 ; $P = .039$).

For SNP rs7766874, we conducted an ARDS risk analysis in which we identified significant associations of the rs7766874A > G variant that was conditioned on PLT ($OR_{\text{SNP}} = 0.68$ per effect allele; 95% CI, 0.51-0.90; $P = .007$), of PLT conditioned on rs7766874 ($OR_{\text{PLT}} = 0.72$ per $100 \times 10^3/\mu\text{L}$ increase in PLT; 95% CI, 0.62-0.84; $P = .001$), and SNP-PLT interaction ($OR_{\text{Interaction}} = 1.15$ per effect allele per $100 \times 10^3/\mu\text{L}$ increase of PLT; 95% CI, 1.03-1.30; $P = .015$) (Table 5). To evaluate potential impact from the other clinical confounders, we did a sensitivity analysis with adjustment for all covariates in Table 1 using stepwise regression. The results were consistent ($OR_{\text{SNP}} = 0.68$; 95% CI, 0.51-0.90; $P = .007$; $OR_{\text{PLT}} = 0.75$; 95% CI, 0.65-0.87; $P = .001$; $OR_{\text{Interaction}} = 1.16$;

95% CI, 1.03-1.30; $P = .013$). By excluding 14 non-white cases, the results remained significance (results not shown). An analysis stratified by sepsis or pneumonia status showed consistent results among sepsis-positive or pneumonia-negative samples. Our population included 181 (28.77%) ARDS cases who were diagnosed on the day of ICU admission (day 1); ARDS could have occurred before the platelet test. A sensitivity analysis excluding those 181 ARDS cases diagnosed on the day of ICU admission showed better results ($OR_{\text{SNP}} = 0.62$; 95% CI, 0.46-0.84; $P = .002$; $OR_{\text{PLT}} = 0.69$; 95% CI, 0.59-0.82; $P = .001$; $OR_{\text{Interaction}} = 1.21$; 95% CI, 1.06-1.37; $P = .004$). We performed another sensitivity analysis excluding 93 patients with ALI from the control group; the results of this ARDS risk analysis also remained consistent ($OR_{\text{SNP}} = 0.69$; 95% CI, 0.52-0.92; $P = .012$; $OR_{\text{PLT}} = 0.73$; 95% CI = 0.63-0.85; $P = .001$; $OR_{\text{Interaction}} = 1.15$; 95% CI, 1.02-1.30; $P = .021$).

Because SNP rs7766874 was a significant QTL marker of PLT (Table 4), and was associated with ARDS risk by interacting with PLT (Table 5), PLT may act as an important causal mediator of the genetic effect of *LRRIC16A* on ARDS outcome. To test this hypothesis, we performed a causal inference test using mediation analysis. Based on previously reported studies³¹⁻³³ and considering that our cases were all Berlin moderate or severe ARDS,³⁰ the ARDS prevalence among the at-risk population was set as 5%; this was in turn used to calculate the weights for mediation analysis. Significant causal

TABLE 2] SNPs in the Genes Identified From Meta-analysis¹⁴

SNP	Chr	Position ^a	Minor Allele/Effect Allele	Major Allele	MAF	Gene	Result
rs9358856	6	25426768	A	G	0.1373	<i>LRRC16A</i>	Missense, V[Val]→I[Ile]
rs73734005	6	25435805	C	G	0.0009	<i>LRRC16A</i>	Missense, R[Arg]→T[Thr]
rs189044678	6	25471485	A	G	0.0006	<i>LRRC16A</i>	Missense, T[Thr]→I[Ile]
rs2274089 ^b	6	25488583	A	G	0.0890	<i>LRRC16A</i>	Intron
rs12207840	6	25515904	A	G	0.0003	<i>LRRC16A</i>	Missense, P[Pro]→L[Leu]
rs41271819	6	25538140	C	G	0.0006	<i>LRRC16A</i>	Missense, G[Gly]→R[Arg]
rs12527029	6	25538188	A	C	0.0080	<i>LRRC16A</i>	Missense, R[Arg]→S[Ser]
rs181145814	6	25554271	A	G	0.0009	<i>LRRC16A</i>	Missense, D[Asp]→N[Asn]
rs193225623	6	25556962	A	G	0.0012	<i>LRRC16A</i>	Missense, P[Pro]→S[Ser]
rs200785124	6	25556995	A	G	0.0003	<i>LRRC16A</i>	Missense, E[Glu]→K[Lys]
rs202056331	6	25581495	A	G	0.0006	<i>LRRC16A</i>	Missense, A[Ala]→V[Val]
rs7766874	6	25589165	G	A	0.4759	<i>LRRC16A</i>	Intron
rs200533860	6	25594653	A	G	0.0003	<i>LRRC16A</i>	Missense, A[Ala]→V[Val]
rs9885914	6	25600772	G	A	0.0054	<i>LRRC16A</i>	Missense, N[Asn]→S[Ser]
rs201967397	6	25605058	A	G	0.0006	<i>LRRC16A</i>	Missense, V[Val]→I[Ile]
rs1012899	6	25605091	G	A	0.2369	<i>LRRC16A</i>	Missense, G[Gly]→S[Ser]
rs742132 ^c	6	25607571	G	A	0.2878	<i>LRRC16A</i>	Intron
rs199600117	10	63173236	A	C	0.0003	<i>JMJD1C</i>	Missense, Q[Gln]→H[His]
rs35796382	10	63450379	A	G	0.0027	<i>JMJD1C</i>	Synonymous, E[Glu]→E[Glu]

Chr = chromosome; MAF = minor allele frequency; SNP = single-nucleotide polymorphism.

^aIn GRCh37/hg19 assembly.

^bPrevious study showed association with blood transferrin ($P = .00000032$).

^cPrevious study showed association with uric acid ($P = .00000001$).

indirect effect was detected for SNP rs7766874 on the increased risk of ARDS that was mediated through PLT (VanderWeele method, $OR_{\text{Indirect}} = 1.045$ per effect allele; 95% CI, 1.007-1.085; $P = .021$) (Table 6). Significance was confirmed by the Imai method ($P = .038$) (Table 6). We also performed two sensitivity analyses that excluded the control subjects who could be reclassified as patients with ALI or excluded the ARDS cases who were diagnosed on the day of ICU admission. Consistently significant or borderline significant associations were observed (Table 6). To evaluate the impact of different ARDS prevalence on our results, we set the ARDS prevalence at 10% or 15% for further sensitivity analyses; these results were consistent with that at 5% ARDS prevalence (Table 6).

Discussion

Genetic variants within *LRRC16A* were previously reported to be associated with PLT among relatively healthy African American populations.¹⁴ In this study, we replicated these results by validating the association between a common intronic SNP (rs7766874, G allele frequency = 47.59%) within *LRRC16A* and PLT in a

white population of 1,655 critically ill patients. The SNP rs7766874 showed limited population diversity in the National Center for Biotechnology Information dbSNP database (G allele frequency in African American = 51%). Interestingly, among ARDS cases and at-risk control subjects, the association was inconsistent. The mechanisms for this are not well understood. It is possible that *LRRC16A* plays a different role in at-risk control subjects vs ARDS cases and needs to be further studied. In this study, we demonstrate strong evidence that a low platelet count contributes to a higher risk of ARDS. A low PLT could be caused by thrombocytopenia or DIC; both are common complications among critically ill patients and are independent risk factors affecting prognosis.³⁴⁻³⁷ However, we could not rule out the role of low platelets in ARDS pathophysiology from platelets consumption in DIC.

Notably, our results suggest that *LRRC16A* plays an important role in ARDS risk mediating through PLT. The indirect hazard effect of SNP rs7766874 on ARDS risk may be explained because SNP rs7766874 causes a

Figure 2 – Analysis design and workflow. QTL = quantitative trait locus; SNP = single-nucleotide polymorphism.

decrease in PLT among at-risk control subjects that would, in turn, increase the risk of ARDS. Interestingly, SNP rs7766874 has nonsignificant independent effect

on ARDS risk as illustrated by removing the interaction term from Model 1 ($OR_{SNP} = 0.91$, $P = .211$) or removing both PLT and interaction term ($OR_{SNP} = 0.92$,

TABLE 3] Gene-Based QTL Analysis of *LRRC16A* and PLT

Samples	No. of SNPs ^a			P Value of Joint Test ^b		
	Total	Low Frequent SNPs	Common SNPs	Low Frequent Only	Common Only	All SNPs
All samples	17	12	5	.536	.543	.686
Case only	13	8	5	.202	.162	.347
Control only	14	9	5	.658	.036	.084

QTL = quantitative trait locus. See Table 1 and 2 legends for expansion of other abbreviations.

^aLow frequent SNP was defined by $MAF < 0.05$.

^bP value was estimated using Sequence Kernel Association Test with adjustment for age, sex, sepsis, and pneumonia.

Figure 3 – *LRRC16A* gene expression level and PLT among at-risk control subjects ($n = 18$). In the figure, the $\log_2$ -transformed expression level was dichotomized by median (Low: $<$ median; High: $\geq$ median). The boxplot of PLT was demonstrated stratified by dichotomized expression level. To increase statistical power, the QTL relationship between *LRRC16A* gene expression and PLT was evaluated by linear regression model of PLT on continuous expression level and described as regression coefficient (β) per 1 SD increase of expression level, its 95% CI and P value. See Figure 2 legend for expansion of other abbreviation.

$P = .231$, also known as total effect in mediation analysis). It is possible to have a significant indirect effect in the absence of total effect.^{38,39} This can be explained by the presence of several mediating paths (multiple potential mediators) that conflict with each other and become noticeable when one of the cancelling mediators is controlled for. Thus, the indirect effect identified in our study is a “partial” mediation effect. However, this mechanism needs to be better understood through well-controlled experiments. Also the interaction between *LRRC16A* and PLT implies that *LRRC16A* has effect beyond platelet count but also on function and, in turn, on ARDS risk. Extensive evidence demonstrates a role of platelet function in increased morbidity and mortality among patients in the ICU,^{40–43} whereas the precise mechanism of how the genetic variants impact platelets and in turn affect ARDS risk requires further studies of functional genomics.

LRRC16A encodes capping protein ARP2/3 and myosin-I linker (CARMIL), a cytoskeletal scaffold protein that binds *CAPZA2* (capping protein muscle Z-line α 2), with high affinity and significantly decreases the affinity of *CAPZA2* for actin barbed ends.⁴⁴ *CAPZA2* is the β subunit of the barbed-end actin binding protein, the

TABLE 4 | QTL Analysis of PLT on Common SNPs Within *LRRC16A*

SNP	Case Only		Control Only		Control Only, Patients With ALI Excluded ^a	
	β (95% CI) ^b	P Value (q) ^c	β (95% CI) ^b	P Value (q) ^c	β (95% CI) ^b	P Value (q) ^c
rs7766874	10.09 (–3.21, 23.39)	.138 (.345)	–13.00 (–23.22, –2.77)	.013 (.065)	–11.08 (–21.59, –0.57)	.039 (.195)
rs2274089	5.75 (–18.55, 30.05)	.643 (.804)	–4.78 (–22.88, 13.32)	.605 (.756)	2.15 (–16.62, 20.93)	.822 (.871)
rs742132	–14.26 (–29.32, 0.81)	.064 (.320)	6.65 (–4.67, 17.96)	.250 (.417)	8.18 (–3.52, 19.87)	.171 (.428)
rs9358856	–1.73 (–21.54, 18.07)	.864 (.864)	–0.92 (–16.09, 14.26)	.906 (.906)	–1.32 (–17.30, 14.66)	.871 (.871)
rs1012899	6.32 (–10.15, 22.79)	.452 (.753)	10.97 (–1.34, 23.27)	.081 (.203)	7.06 (–5.70, 19.82)	.279 (.465)

Analysis was performed using linear regression with adjustment for age, sex, sepsis, and pneumonia. ALI = acute lung injury. See Table 1–3 legends for expansion of other abbreviations.

^aThere were 93 control subjects reclassified as ALI (defined as mild ARDS in new Berlin definition), who were excluded for sensitivity analysis.

^bRegression coefficient and its 95% CI, representing the change in PLT per effect allele.

^c q represents adjusted P value by false discovery rate method.

TABLE 5] Association Between SNP rs7766874, PLT, and ARDS Risk

Samples Used	OR_{SNP} (95% CI) ^a	<i>P</i> Value	OR_{PLT} (95% CI) ^b	<i>P</i> Value	$OR_{Interaction}$ (95% CI) ^c	<i>P</i> Value
All samples	0.68 (0.51, 0.90)	.007	0.72 (0.62, 0.84)	<.001	1.15 (1.03, 1.30)	.015
Subgroup or sensitivity analysis						
Sepsis-negative	0.58 (0.29, 1.18)	.134	0.64 (0.36, 1.15)	.137	1.38 (0.91, 2.11)	.132
Sepsis-positive	0.69 (0.50, 0.94)	.019	0.73 (0.63, 0.85)	<.001	1.15 (1.01, 1.30)	.032
Pneumonia-negative	0.58 (0.39, 0.88)	.011	0.68 (0.54, 0.86)	.002	1.24 (1.01, 1.51)	.037
Pneumonia-positive	0.74 (0.50, 1.10)	.138	0.77 (0.63, 0.93)	.007	1.11 (0.96, 1.29)	.162
ALI patients excluded from control group ^d	0.69 (0.52, 0.92)	.012	0.73 (0.63, 0.85)	<.001	1.15 (1.02, 1.30)	.021
ARDS diagnosed after day 1 ^e	0.62 (0.46, 0.84)	.002	0.69 (0.59, 0.82)	<.001	1.21 (1.06, 1.37)	.004

Logistic regression was performed with adjustment for age, sex, sepsis, and pneumonia at ICU admission; the SNP was encoded in an additive genetic model, and the PLT was included in a continuous format. See Table 1, 2, and 4 legends for expansion of abbreviations.

^aMain effect of SNP per effect allele conditioned on PLT.

^bMain effect of PLT (rescaled in per $100 \times 10^3/\mu\text{L}$ of PLT) conditioned on SNP.

^cInteractive effect of SNP and PLT (rescaled in per $100 \times 10^3/\mu\text{L}$ of PLT).

^dThere were 93 control subjects reclassified as ALI (defined as mild ARDS in new Berlin definition) who were excluded for sensitivity analysis.

^eOnly ARDS cases that were diagnosed later than the day of admission to ICU were included in the analysis.

heterodimeric actin-capping protein that blocks actin filament (F-actin) assembly and disassembly at the fast-growing (barbed) filament ends and functions in regulating actin filament dynamics as well as in stabilizing actin filaments. Actin-based cellular processes are essential for end-amplification of proplatelet processes during megakaryocyte maturation.^{45,46} The intronic SNP rs7766874 may be in linkage disequilibrium with latent functional variants that alter the activity of CARMIL and thereby result in abnormal megakaryocyte maturation

and altered platelet formation. Interestingly, platelets contain actin-binding proteins that can bind to and shorten actin filaments.⁴⁷⁻⁵⁰ Moreover, F-actin acts as a key step in ARDS induced by systemic inflammatory response syndrome via increased blood neutrophil adhesion and migration and by the production of inflammatory factors.⁵¹ An alternative postulated mechanism of *LRRC16A* on ARDS is platelet independent that the variant *LRRC16A* prevents actin polymerization, which impacts the alveolar capillary barrier function.⁵²

TABLE 6] Causal Inference Analysis for the Indirect Effect of SNP rs7766874 on ARDS Risk Mediated Through PLT

Prevalence ^a	Samples Used	$OR_{Indirect}$ (95% CI) ^b	<i>P</i> Value ^c	<i>P</i> Value ^d
0.05	All samples	1.045 (1.007, 1.085)	.021	.038
	Patients with ALI excluded from control group ^e	1.048 (1.003, 1.095)	.037	.046
	ARDS diagnosed after day 1 ^f	1.036 (0.999, 1.075)	.055	.068
0.10	All samples	1.039 (1.001, 1.078)	.045	.048
	Patients with ALI excluded from control group ^e	1.040 (0.996, 1.086)	.073	.070
	ARDS diagnosed after day 1 ^f	1.032 (0.996, 1.070)	.083	.078
0.15	All samples	1.033 (0.997, 1.071)	.074	.080
	Patients with ALI excluded from control group ^e	1.033 (0.995, 1.072)	.091	.126
	ARDS diagnosed after day 1 ^f	1.028 (0.995, 1.062)	.099	.128

See Table 1, 2, and 4 legends for expansion of abbreviations.

^aThe prevalence of ARDS among the ICU at-risk population.

^bThe indirect effect of SNP rs7766874 on ARDS risk that was mediated through PLT.

^cStatistical *P* value of VanderWeele mediation analysis by bootstrapping with 1,000 replications.

^dStatistical *P* value of Imai mediation model using the same weights as VanderWeele method.

^eThere were 93 control subjects reclassified as ALI (defined as mild ARDS in new Berlin definition) who were excluded for sensitivity analysis.

^fOnly ARDS cases that were diagnosed later than the day of admission to ICU were included in the analysis.

We acknowledge some limitations in our study. The Illumina ExomeChip includes a high percentage of low-frequency alleles. Our study was limited in power to study the association of these low-frequency functional SNPs with ARDS outcome. Additionally, further investigation is needed to evaluate the mechanisms that underlie the mediation of platelets in the association between *LRRC16A* genetic variants and risk of ARDS. Considering the heterogeneity and various manifestations of ARDS, these study results should be examined

in even larger samples to evaluate our findings among specific subgroups. Another limitation is that the attribution of causality is solely based on the results of mediation analysis, which requires follow-up well-designed functional studies.

In summary, we validated *LRRC16A* as associated with platelet count among critically ill patients. Our findings suggest that *LRRC16A* plays an important role in ARDS pathophysiology by interacting with and being causally mediated through platelets.

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References

1. Rubenfeld GD, Caldwell E, Peabody E, et al. Incidence and outcomes of acute lung injury. *N Engl J Med*. 2005;353(16):1685-1693.
2. Camporota L. The public health burden of acute lung injury. *Thorax*. 2006;61(1):1.
3. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med*. 2000;342(18):1334-1349.
4. Meyer NJ, Feng R, Li M, et al. IL1RN coding variant is associated with lower risk of acute respiratory distress syndrome and increased plasma IL-1 receptor antagonist. *Am J Respir Crit Care Med*. 2013;187(9):950-959.
5. Su L, Zhai R, Sheu CC, et al. Genetic variants in the angiopoietin-2 gene are associated with increased risk of ARDS. *Intensive Care Med*. 2009;35(6):1024-1030.
6. Lin X, Dean DA. Gene therapy for ALI/ARDS. *Crit Care Clin*. 2011;27(3):705-718.
7. Tejera P, O'Mahony DS, Owen CA, et al. Functional characterization of polymorphisms in the PI3 (elafin) gene and validation of their contribution to risk of ARDS. *Am J Respir Cell Mol Biol*. 2014;51(2):262-272.
8. Christie JD, Wurfel MM, Feng R, et al; Trauma ALI SNP Consortium (TASC) investigators. Genome wide association identifies PPFA1 as a candidate gene for acute lung injury risk following major trauma. *PLoS ONE*. 2012;7(1):e28268.
9. Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. *J Clin Invest*. 2012;122(8):2731-2740.
10. Devaney J, Contreras M, Laffey JG. Clinical review: gene-based therapies for ALI/ARDS: where are we now? *Crit Care*. 2011;15(3):224.
11. Dixon JT, Gozal E, Roberts AM. Platelet-mediated vascular dysfunction during acute lung injury. *Arch Physiol Biochem*. 2012;118(2):72-82.
12. Zarbock A, Singbartl K, Ley K. Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. *J Clin Invest*. 2006;116(12):3211-3219.
13. Wu J, Sheng L, Wang S, et al. Analysis of clinical risk factors associated with the prognosis of severe multiple-trauma patients with acute lung injury. *J Emerg Med*. 2012;43(3):407-412.
14. Qayyum R, Snively BM, Ziv E, et al. A meta-analysis and genome-wide association study of platelet count and mean platelet volume in African Americans. *PLoS Genet*. 2012;8(3):e1002491.
15. Yang L, Yang X, Ji W, et al. Effects of a functional variant c.353T>C in *snai1* on risk of two contextual diseases. Chronic obstructive pulmonary disease and lung cancer. *Am J Respir Crit Care Med*. 2014;189(2):139-148.
16. Vanderweele TJ, Vansteelandt S. Odds ratios for mediation analysis for a dichotomous outcome. *Am J Epidemiol*. 2010;172(12):1339-1348.
17. VanderWeele TJ, Asomaning K, Tchetgen Tchetgen EJ, et al. Genetic variants on 15q25.1, smoking, and lung cancer: an assessment of mediation and interaction. *Am J Epidemiol*. 2012;175(10):1013-1020.
18. Gong MN, Thompson BT, Williams P, Pothier L, Boyce PD, Christiani DC. Clinical predictors of and mortality in acute respiratory distress syndrome: potential role of red cell transfusion. *Crit Care Med*. 2005;33(6):1191-1198.
19. Bernard GR, Artigas A, Brigham KL, et al. The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am J Respir Crit Care Med*. 1994;149(3 pt 1):818-824.
20. Sheu CC, Zhai R, Wang Z, et al. Heme oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome. *Intensive Care Med*. 2009;35(8):1343-1351.
21. Anderson CA, Pettersson FH, Clarke GM, Cardon LR, Morris AP, Zondervan KT. Data quality control in genetic case-control association studies. *Nat Protoc*. 2010;5(9):1564-1573.
22. Wang Z, Beach D, Su L, Zhai R, Christiani DC. A genome-wide expression analysis in blood identifies pre-elafin as a biomarker in ARDS. *Am J Respir Cell Mol Biol*. 2008;38(6):724-732.
23. Li C. Automating dChip: toward reproducible sharing of microarray data analysis. *BMC Bioinformatics*. 2008;9:231.
24. Dmitrienko A, Tamhane AC, Wang X, Chen X. Stepwise gatekeeping procedures in clinical trial applications. *Biom J*. 2006;48(6):984-991.
25. Dmitrienko A, Tamhane AC. Gatekeeping procedures with clinical trial applications. *Pharm Stat*. 2007;6(3):171-180.
26. Dmitrienko A, Wiens BL, Tamhane AC, Wang X. Tree-structured gatekeeping tests in clinical trials with hierarchically

- ordered multiple objectives. *Stat Med*. 2007;26(12):2465-2478.
27. Ionita-Laza I, Lee S, Makarov V, Buxbaum JD, Lin X. Sequence kernel association tests for the combined effect of rare and common variants. *Am J Hum Genet*. 2013;92(6):841-853.
28. Imai K, Keele L, Tingley D. A general approach to causal mediation analysis. *Psychol Methods*. 2010;15(4):309-334.
29. Grove ML, Yu B, Cochran BJ, et al. Best practices and joint calling of the HumanExome BeadChip: the CHARGE Consortium. *PLoS ONE*. 2013;8(7):e68095.
30. Ranieri VM, Rubenfeld GD, Thompson BT, et al; ARDS Definition Task Force. Acute respiratory distress syndrome: the Berlin Definition. *JAMA*. 2012;307(23):2526-2533.
31. Goyal M, Houseman D, Johnson NJ, Christie J, Mikkelsen ME, Gaieski DF. Prevalence of acute lung injury among medical patients in the emergency department. *Acad Emerg Med*. 2012;19(9):E1011-E1018.
32. Roupie E, Lepage E, Wysocki M, et al. Prevalence, etiologies and outcome of the acute respiratory distress syndrome among hypoxemic ventilated patients. SRLF Collaborative Group on Mechanical Ventilation. Société de Réanimation de Langue Française. *Intensive Care Med*. 1999;25(9):920-929.
33. Esteban A, Ferguson ND, Meade MO, et al; VENTILA Group. Evolution of mechanical ventilation in response to clinical research. *Am J Respir Crit Care Med*. 2008;177(2):170-177.
34. Chang JC, Aly ES. Acute respiratory distress syndrome as a major clinical manifestation of thrombotic thrombocytopenic purpura. *Am J Med Sci*. 2001;321(2):124-128.
35. Housinger TA, Brinkerhoff C, Warden GD. The relationship between platelet count, sepsis, and survival in pediatric burn patients. *Arch Surg*. 1993;128(1):65-66.
36. Lopez-Delgado JC, Rovira A, Esteve F, et al. Thrombocytopenia as a mortality risk factor in acute respiratory failure in H1N1 influenza. *Swiss Med Wkly*. 2013; 143:w13788.
37. Zou Z, Yang Y, Chen J, et al. Prognostic factors for severe acute respiratory syndrome: a clinical analysis of 165 cases. *Clin Infect Dis*. 2004;38(4):483-489.
38. Rucker DD, Preacher KJ, Tormala ZL, Petty RE. Mediation analysis in social psychology: current practices and new recommendations. *Social & Personality Psychology Compass*. 2011;5(6):359-371.
39. Hayes AF. Beyond Baron and Kenny: statistical mediation analysis in the new millennium. *Communication Monographs*. 2009;76(4):408-420.
40. Vanderschueren S, De Weerd A, Malbrain M, et al. Thrombocytopenia and prognosis in intensive care. *Crit Care Med*. 2000;28(6):1871-1876.
41. Dengler V, Downey GP, Tudor RM, Eltzschig HK, Schmidt EP. Neutrophil intercellular communication in acute lung injury. Emerging roles of microparticles and gap junctions. *Am J Respir Cell Mol Biol*. 2013;49(1):1-5.
42. Kuebler WM. Selectins revisited: the emerging role of platelets in inflammatory lung disease. *J Clin Invest*. 2006;116(12):3106-3108.
43. Pittet JF, Mackerse RC, Martin TR, Matthay MA. Biological markers of acute lung injury: prognostic and pathogenetic significance. *Am J Respir Crit Care Med*. 1997;155(4):1187-1205.
44. Yang C, Pring M, Wear MA, et al. Mammalian CARMIL inhibits actin filament capping by capping protein. *Dev Cell*. 2005;9(2):209-221.
45. Raslova H, Kauffmann A, Sekkaï D, et al. Interrelation between polyploidization and megakaryocyte differentiation: a gene profiling approach. *Blood*. 2007; 109(8):3225-3234.
46. Watkins NA, Gusnanto A, de Bono B, et al; Bloodomics Consortium. A HaemAtlas: characterizing gene expression in differentiated human blood cells. *Blood*. 2009;113(19):e1-e9.
47. Xu W, Xie Z, Chung DW, Davie EW. A novel human actin-binding protein homologue that binds to platelet glycoprotein Ib α . *Blood*. 1998;92(4):1268-1276.
48. Vidal C, Geny B, Melle J, Jandrot-Perrus M, Fontenay-Roupie M. Cdc42/Rac1-dependent activation of the p21-activated kinase (PAK) regulates human platelet lamellipodia spreading: implication of the cortical-actin binding protein cortactin. *Blood*. 2002;100(13):4462-4469.
49. Heise H, Bayerl T, Isenberg G, Sackmann E. Human platelet P-235, a talin-like actin binding protein, binds selectively to mixed lipid bilayers. *Biochim Biophys Acta*. 1991;1061(2):121-131.
50. Lind SE, Yin HL, Stossel TP. Human platelets contain gelsolin. A regulator of actin filament length. *J Clin Invest*. 1982;69(6):1384-1387.
51. Du L, Zhou J, Zhang J, et al. Actin filament reorganization is a key step in lung inflammation induced by systemic inflammatory response syndrome. *Am J Respir Cell Mol Biol*. 2012;47(5):597-603.
52. Safdar Z, Wang P, Ichimura H, Issekutz AC, Quadri S, Bhattacharya J. Hyperosmolarity enhances the lung capillary barrier. *J Clin Invest*. 2003;112(10):1541-1549.