

ORIGINAL ARTICLE

Distinct and replicable genetic risk factors for acute respiratory distress syndrome of pulmonary or extrapulmonary origin

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ABSTRACT

Background The role of genetics in the development of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) from direct or indirect lung injury has not been specifically investigated. The aim of this study was to identify genetic variants contributing to ALI/ARDS from pulmonary or extrapulmonary causes.

Methods We conducted a multistage genetic association study. We first performed a large-scale genotyping (50K ITMAT-Broad_CARE Chip) in 1717 critically ill Caucasian patients with either pulmonary or extrapulmonary injury, to identify single nucleotide polymorphisms (SNPs) associated with the development of ARDS from direct or indirect insults to the lung. Identified SNPs ($p \leq 0.0005$) were validated in two separated populations (Stage II), with trauma (Population I; $n=765$) and pneumonia/pulmonary sepsis (Population II; $n=838$), as causes for ALI/ARDS. Genetic variants replicating their association with trauma related-ALI in Stage II were validated in a second trauma-associated ALI population ($n=224$, Stage III).

Results In Stage I, non-overlapping SNPs were significantly associated with ARDS from direct/indirect lung injury, respectively. The association between rs1190286 (*POPDC3*) and reduced risk of ARDS from pulmonary injury was validated in Stage II ($p < 0.003$). SNP rs324420 (*FAAH*) was consistently associated with increased risk of ARDS from extrapulmonary causes in two independent ALI-trauma populations ($p < 0.006$, Stage II; $p < 0.05$, Stage III). Meta-analysis confirmed these associations.

Conclusions Different genetic variants may influence ARDS susceptibility depending on direct versus indirect insults. Functional SNPs in *POPDC3* and *FAAH* genes may be driving the association with direct and indirect ALI/ARDS, respectively.

INTRODUCTION

Two different pathogenic pathways can lead to the development of acute lung injury (ALI) and its more severe manifestation, acute respiratory distress syndrome (ARDS): a direct or pulmonary insult that directly affects lung parenchyma, and/or an indirect or extrapulmonary injury that results from an acute systemic inflammatory response and yields pulmonary endothelial damage.¹ Since this distinction was

posed by the American European Consensus Conference (AECC) in 1994, the question of whether ARDS of different origins represents two different syndromes, and the possible clinical implications of this differentiation, have been widely debated. Conflicting results have been reported among different clinical studies, largely due to the fact that the classification of the type of injury that leads to ARDS is not always straightforward. Furthermore, it is possible that direct and indirect insults coexist simultaneously in the same patient, and patients in each category can also present different degrees of severity of lung injury.^{2–5} In spite of these contradictory results, there is a growing body of evidence suggesting that pathophysiological characteristics differ between the two types of primary insults. Clinical data and experimental models support differences in pathophysiology, lung morphology, respiratory mechanics and response to different ventilator strategies and pharmacological agents between ARDS from pulmonary and extrapulmonary origin.^{4–19} Genetic factors are known to play an important role in ARDS development.^{20–22} While several studies have indicated an effect modification by the type of injury in the genetic associations with the risk of ARDS,^{23–26} the potential role of genetics underlying the differences between ARDS resulting from pulmonary and extrapulmonary injury has not been investigated in detail. In this study, we explore the hypothesis that different genetic susceptibility profiles could underlie the development of ARDS from different insults. To identify common genetic variants contributing to the development of ARDS from different origins, we conducted a large-scale genomic association study involving ~2100 genes on a critically ill patient population of 1717 subjects with either direct or indirect lung injury as predisposing conditions for ARDS. Three critically ill populations with severe trauma or pneumonia/pulmonary sepsis as the risk factor for ALI/ARDS were used to validate our primary results.

METHODS

Study populations

The initial phase of the study included subjects admitted to an adult intensive care units (ICU) at the Massachusetts General Hospital (MGH) and

the Beth Israel Deaconess Medical Center (Boston) with pulmonary or extrapulmonary injury as predisposing condition for ARDS. Details of the study design have been described previously.^{27–28} Stage II consisted of two independent replication populations. Population I included patients admitted to the Harborview Medical Center (HMC, Seattle, Washington, USA) ICU for 48 h or longer following major trauma.²⁹ Population II consisted of ARDS cases with pneumonia/sepsis from pulmonary sources as a risk factor for ARDS, collected as part of the Fluid and Catheter Treatment Trial (FACTT), Albuterol for the treatment of ALI (ALTA) and EDEN-Omega trials conducted by the NHLBI ARDS Network (<http://www.ardsnet.org/clinicians/studies>). Controls for this population were non-ARDS patients with pulmonary injury from the discovery set (MGH). Stage III consisted of subjects admitted to the surgical ICU of Hospital of the University of Pennsylvania (HUP) after a major trauma and with an injury severity score (ISS) ≥ 16 , corresponding to severe trauma.^{30–32}

At each stage, eligible patients were followed for the development of ARDS as defined by AECC criteria.¹ At each site, the institutional review board and/or human subjects committee reviewed and approved the study. Full description of the cohorts is provided in the online supplementary material (see also online supplementary figure S1).

Genotyping strategy and quality control

Genotyping of the discovery population was carried out using the 50K single nucleotide polymorphism (SNP) ITMAT-Broad_CARE (IBC) array (Illumina, San Diego, California, USA).³³ As a candidate gene chip designed to capture variation in loci important to inflammatory, metabolic and vascular phenotypes, the IBC chip also includes many genes with plausible role in ALI development (<http://bmic.upenn.edu/cvdsnp>) (further justification for the use of this platform is provided in the online supplementary material). Patients in Stage II were genotyped using the Infinium II HumanHap610K-quadrant BeadChip (Illumina).^{34–35} Genotyping data were filtered for only those SNPs passing the threshold for significant association at Stage I ($p \leq 0.0005$).^{36–37} Patients in Stage III were also genotyped using the IBC chip (Illumina),³³ and genotyping data were filtered for SNPs passing Stages I and II (see online supplementary figure S2, study overview). For those IBC SNPs not typed on the genome-wide array, genotype imputation was carried out using MACH V3.0³⁸ and 1000 Genomes European ancestry samples as reference panel. Genotype data were subjected to rigorous quality control measures in order to remove poor quality SNPs as well as individuals of non-European ancestry. Further details about genotyping strategy and quality control are provided in the online supplementary material.

Statistical analysis

We used logistic regression to perform SNP-based association analyses with ALI/ARDS risk as implemented in PLINK.³⁹ The genotype-specific OR for ALI/ARDS susceptibility were estimated using the χ^2 test. An additive model of genetic risk was assumed, adjusting for clinical covariates available at each stage. Analyses were restricted to subjects of European ancestry. The impact of population stratification was evaluated by calculating the genomic control inflation factor⁴⁰ in Stage I, and by using principal components analysis⁴¹ and multidimensional scaling analysis^{41–43} in Stages II and III, respectively. A three-stage association study was performed.^{36–37–43} We used a p value $\leq 5 \times 10^{-4}$ to pass Stage I (instead of 10^{-6} (0.05/50 000 SNPs in the IBC Chip)) in order to reach satisfactory power for our cohort. The significance of the associations observed in Stage I was then

established by independent replication of our findings in Stages II and III of the study. The statistical power at each stage was determined using Quanto software (<http://hydra.usc.edu/gxe/>). Further details of power calculation and selection of significance thresholds at each stage are provided in the online supplementary material. Aggregate effects of common SNPs were assessed by calculating polygenic risk score, using a 'count method' as previously described.⁴⁴ Meta-analysis of the discovery and replication cohorts was performed using an inverse variance-weighted method under fixed and random-effects models as implemented in PLINK³⁹. A p value < 0.0005 in the meta-analysis was considered as suggestive evidence of significance. Heterogeneity among study populations was assessed with the Cochran's Q-statistic.³⁹ Correlation between SNP associated with ARDS and gene expression levels was examined in silico using the Gene Expression Variation (GENEVAR) project database at the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/resources/software/genevar/>) and expression data from three cell types (fibroblast, lymphoblastoid cell line and T-cell) derived from umbilical cords of 75 Geneva GenCord individuals.⁴⁵ The correlation between the number of risk alleles, and normalised mRNA levels was examined by linear regression using the Genevar V3.1.1 Java tool.⁴⁶ Further details of our analyses are presented in the online supplemental material.

RESULTS

Stage I

After quality control, 1717 critically ill Caucasian patients at risk for ARDS, and with only one type of lung injury, were included in the first stage of the study (see online supplementary figure S2). Among them, 417 were ARDS cases and 1300 were non-ARDS. The demographics and baseline clinical characteristics for these subjects are shown in table 1. A total of 29 483 autosomal SNPs passed quality control in Stage I (see online supplementary table S1). SNP-level quality control metrics were: genotyping call rate $> 95\%$, minor allele frequency (MAF) ≥ 0.05 and Hardy-Weinberg equilibrium (HWE) $p \geq 0.001$. The chromosomal distribution of all p values is shown in online supplementary figure S3. The calculated genomic control for the association with pulmonary and extrapulmonary injury-related ARDS ($\lambda = 1.000$ and $\lambda = 1.018$, respectively, see online supplementary figure S4) did not indicate stratification in the discovery population.⁴⁷

Assuming an additive model, and after adjustment by age, gender and Acute Physiology And Chronic Health Evaluation (APACHE) III score, we identified a total of 17 SNPs (annotated to 12 genes) and 8 SNPs (in 7 genes) significantly associated with pulmonary and extrapulmonary injury-related ARDS (p value ≤ 0.0005), respectively (table 2).

Of note, no SNPs associated with pulmonary injury-related ARDS were associated with extrapulmonary injury-related ARDS. The converse was likewise true. No variant exhibited even a marginal association in both types of lung injury (see online supplementary table S2). The same result was observed when the effect of SNPs significantly associated with ARDS ($p \leq 0.0005$) in the pulmonary and extrapulmonary subgroups was evaluated jointly, by the use of a multi-SNP genotypic risk score. Additional details of this analysis are provided in the online supplementary material.

Stages II and III

SNPs demonstrating an association with the development of ARDS in the discovery set (table 2) were tested for validation in Stage II, using two different populations. SNPs associated

Table 1 Baseline characteristics of study populations

Stage I: Boston (MGH) Cohort			
	Patients with pulmonary injury (n = 839)	Patients with extrapulmonary injury (n = 878)	p-value
Age	62.37 ± 18.0	61.40 ± 16.5	0.2276
Male	540 (64.4%)	510 (58.1%)	0.0086
APACHE III score	49.70 ± 18.6	48.55 ± 19.6	0.2199
Predisposing condition			
Trauma	0	63 (7.2%)	<0.001
Multiple transfusion	0	185 (21.1%)	<0.001
Sepsis	710 (84.6%)	665 (75.74%)	<0.001
Bacteremia	101 (12.0%)	202 (23.0%)	<0.001
Pneumonia	775 (92.4%)	0	<0.001
Aspiration	28 (3.3%)	0	<0.001
Pulmonary contusion	47 (5.6%)	0	<0.001
Comorbidities			
Diabetes	196 (23.4%)	230 (26.2%)	0.1796
Liver cirrhosis	41 (4.9%)	42 (4.8%)	1.0000
History of alcohol abuse	114 (13.6%)	85 (9.7%)	0.0127
Developed ARDS	290 (34.6 %)	127 (14.5%)	<0.001
Stage II			
Population I (Harborview Trauma Cohort)			
	ALI (n=597)	No ALI (n=168)	p-value
Age	44.6 ± 20.1	34.1 ± 19.0	< 0.001
Male	439 (73.5%)	130 (77.4%)	0.40
Blunt trauma	538 (90.1%)	145 (86.3%)	0.009
ISS	26.8 ± 10.3	22.5 ± 9.6	< 0.001
APACHE II score	24.8 ± 7.5	16.6 ± 7.7	< 0.001
Population II (MGH/ARDS net, pneumonia/pulmonary sepsis Cohort)			
	ARDS (n = 392)	No ARDS (n = 446)	p-value
Age	52.32 ± 16.14	64.21 ± 16.84	<0.001
Male	199 (50.8%)	161 (36.0%)	<0.001
Predisposing condition			
Sepsis	212 (54.1%)	396 (88.85)	0.068
Pneumonia	392 (100%)	446 (100%)	0.965
Stage III: Penn Trauma Cohort			
	ALI (n=74)	No ALI (n=150)	p-value
Age	41.4 ± 20.5	43.9 ± 20.0	0.27
Male	52 (69.3%)	106 (70.2%)	0.89
Blunt	71 (94.7%)	141 (93.4%)	0.71
ISS	26.4 ± 7.6	25.4 ± 7.3	0.34
Modified APACHE III	/63.9 ± 25.1	59.6 ± 19.8	0.40
Total pRBC 1 st 24 hr	2.59 ± 4.8	1.0 ± 2.3	0.007

with ARDS resulting from extrapulmonary injury were validated in Population I (ill trauma patients) consisting of 597 cases and 168 non-ALI. SNPs associated with ARDS from direct lung injury were validated in Population II consisted of 392 ARDS cases from NHBLI ARDS Network (180 FACTT samples, 84 ALTA samples, 112 Omega samples, and 16 ALTA/Omega coenrolled samples) with pneumonia and pulmonary sepsis as causes of ARDS. Controls for this population were those from discovery population with direct injury (n=446). SNPs replicating the association with the development of extrapulmonary injury-related in Population I were tested in Stage III using the ALI-associated trauma cohort (HUP) (n=224). About 33% of these subjects developed ALI during the first 5 days post-trauma. Characteristics of the replication populations in Stages II and III and available clinical data are shown in table 1.

In Stage II, over 600 000 (Linkage Disequilibrium (LD))-bin-tagging SNPs were assayed using the Human 610-Quad platform, of which 530 459 passed all quality control measures (genotyping call rate $\geq 95\%$; HWE p value $\geq 10^{-4}$; and MAF ≥ 0.01) were included in the analyses (see online supplementary table S1). The genomic inflation factor for this set was 1.027. The results of all genotyped SNPs were filtered for the SNPs significantly associated with ARDS from direct or indirect injury in Stage I ($p \leq 0.0005$). Association results for the SNPs selected for validation in Stage II are summarised in table 3.

Seven of the eight SNPs associated with extrapulmonary injury-related ARDS and tested in Stage II Population I (trauma-related ALI) failed to replicate the association with ALI ($p \geq 0.006$). Only SNP rs324420 in *FAAH* showed significant association with an increased risk of ALI from extrapulmonary sources in Population I with an OR=1.58 (95% CI 1.14 to 2.18), and a $p=0.0007$. The association was robust after adjustment for clinical variables (age, ISS and APACHE II, OR=1.59, $p=0.0131$).

SNP rs324420 has been associated with obesity.^{48–51} Because obesity may influence ALI outcome,^{52–53} we tested whether the rs324420-ARDS association was modified by body mass index (BMI), using logistic regression and BMI data from discovery population. After adjustment, rs324420 remained independently associated with increased risk of ARDS development (OR=1.77; $p=0.0002$).

SNPs associated with extrapulmonary injury-related ARDS in Stage I were replicated using an ALI (as opposed to ARDS) trauma-specific cohort (Population I). ALI and ARDS represent different manifestations of the same syndrome, only the severity of the hypoxaemia differentiates ALI from ARDS.¹ We performed a sensitivity analysis of the results in Stage II to test if differences in the clinical phenotype ALI versus ARDS might influence our findings (see online supplementary material for additional information). Approximately 70% of our ALI cases in the replication population also met the criteria for ARDS. To assess the robustness of the replication results, the association analyses were repeated after recategorising ALI cases (defined as $\text{PaO}_2:\text{FiO}_2 < 300$ mm Hg) in Population I (Stage II) into ARDS cases ($\text{PaO}_2:\text{FiO}_2 < 200$ mm Hg).¹ The association of SNP rs324420 with ARDS in Stage I was replicated in Stage II, without any differences in the magnitude and direction of the association (see online supplementary table S3).

After demonstrating a reproducible association with increased risk of ALI/ARDS from extrapulmonary sources in Stages I and II (Population I) of our study, SNP rs324420 was tested for validation in a third critically ill population (HUP) with severe trauma (ISS > 16) as risk factor for ALI.^{30–32} In Stage III, SNP rs324420 also showed a reproducible association with increased ALI risk: OR=1.85 (95% CI 1.08 to 3.19), $p=0.026$ (adjusted for age, ISS, modified APACHE III score, blunt trauma and total amount of packed red blood cells transfused in the first 24 h post-trauma). Sensitivity analyses looking at ARDS versus ALI as the phenotype were not carried out in Stage III, since approximately 97% of the subjects in this population also met the criteria for ARDS.³²

The association results of the discovery (Stage I) and replication cohorts (Stages I and III) were then combined by meta-analysis. In the combined analysis, rs324420 remained the only significant SNP associated with the development of extrapulmonary injury-related ALI/ARDS, and showed increased statistical significance with a $p=2 \times 10^{-6}$ and

Table 2 Association with extrapulmonary and pulmonary injury-related ARDS in Stage I ($p \leq 0.0005$)

Chr	SNP	Gene	Location	Minor allele	MAF Case/Ctrl.	HWE	OR (95% CI)	p* (additive)
SNPs associated with extrapulmonary injury-related ARDS								
19	rs198977	KLK2	Exon	T	0.34/0.22	0.25	1.74 (1.23 to 2.32)	0.00021
12	rs9645765	VWF	Intron	G	0.14/0.07	1	2.17 (1.43 to 3.28)	0.000276
19	rs2889490	SFRS16	Intron	G	0.58/0.46	0.71	1.67 (1.26 to 2.19)	0.000276
1	rs3128126	ISG15	Intron	G	0.48/0.36	0.032	1.70 (1.28 to 2.26)	0.000278
22	rs16980496	ADRBK2	Intron	A	0.13/0.06	0.75	2.23 (1.44 to 3.45)	0.00034
12	rs2070887	VWF	Intron	G	0.15/0.08	0.44	2.07 (1.38 to 3.10)	0.0004
2	rs10490072	BCL11A	3' near gene	C	0.30/0.21	0.65	1.72 (1.27 to 2.33)	0.000476
1	rs324420	FAAH	Exon	A	0.29/0.19	0.13	1.74 (1.27 to 2.39)	0.000503
SNPs associated with pulmonary injury-related ARDS								
7	rs7807769	PRKAG2	Intron	A	0.48/0.39	0.37	1.58 (1.28 to 1.94)	1.61E-05
7	rs7801616	PRKAG2	Intron	T	0.48/0.39	0.33	1.54 (1.25 to 1.89)	4.37E-05
6	rs1190286	POPDC3	Intron	C	0.13/0.20	0.29	0.53 (0.39 to 0.72)	5.30E-05
18	rs9960450	TNFRSF11A	Intron	C	0.08/0.03	0.38	2.48 (1.56 to 3.93)	0.000114
1	rs2254358	HSPG2	Exon	C	0.25/0.33	0.63	0.63 (0.50 to 0.80)	0.000129
13	rs732821	HTR2A	5' near gene	A	0.54/0.45	1	1.52 (1.22 to 1.88)	0.000136
7	rs6970522	PRKAG2	Intron	G	0.51/0.44	0.26	1.49 (1.21 to 1.82)	0.000167
16	rs3887893	ABCC1	Intron	G	0.45/0.36	0.07	1.48 (1.20 to 1.83)	0.000287
18	rs17069902	TNFRSF11A	Intron	T	0.09/0.04	0.05	2.12 (1.41 to 3.20)	0.000312
2	rs2671222	IL8RA	5' near gene	A	0.02/0.06	1	0.34 (0.19 to 0.61)	0.000325
1	rs12080701	PDE4B	Intron	G	0.14/0.09	0.30	1.85 (1.32 to 2.60)	0.000362
19	rs8112223	HAS1	5' near gene	A	0.45/0.36	0.78	1.48 (1.19 to 1.84)	0.00037
5	rs6451620	GHR	Intron	A	0.09/0.04	0.32	2.15 (1.41 to 3.29)	0.000372
1	rs17419964	PDE4B	Intron	G	0.34/0.26	0.83	1.50 (1.20 to 1.88)	0.000433
7	rs802440	GRM3	Intron	T	0.40/0.30	0.11	1.47 (1.19 to 1.83)	0.000452
1	rs4075731	MAP3K6	Intron	A	0.32/0.40	1	0.67 (0.54 to 0.84)	0.000468
2	rs2854386	IL8RA	3' near gene	C	0.03/0.07	1	0.36 (0.20 to 0.64)	0.000476

*p Values were adjusted for age, gender and APACHE III score in Stage I population.

ARDS, acute respiratory distress syndrome; Chr, chromosome; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism.

OR=1.70 (table 4 and see online supplementary table S4). The regional association plot of *FAAH* (see online supplementary figure S5) revealed rs324420 as the most significant SNP associated with trauma-related ALI in *FAAH* gene (imputed $p=0.00509$). SNP rs324420 is located in exon 3 of *FAAH* gene and leads to a non-synonymous change 385 C/A (P129T).

Among the 17 SNPs associated with pulmonary injury-related ARDS in Stage I, only SNP rs1190286 in *POPDC3* gene validated its association with reduced ARDS risk in Stage II Population II (pneumonia/pulmonary sepsis): OR=0.64 (95% CI 0.49 to 0.83), and a $p=0.0007$. The association was also robust after adjustment for clinical variables (age, gender, top six principal components) (OR=0.65, $p=0.0094$) (table 3).

Five additional SNPs in *PDE4B* (rs12080701, rs17419964) *ABCC1* (rs3887893) and *TNFRSF11A* (rs9960450, rs17069902) were significantly associated with increased risk of pulmonary injury-related ARDS ($p \leq 0.0005$) in the combined analysis (table 4). SNP rs1190286 in *POPDC3* was the most significant association signal from meta-analysis ($p=2.7 \times 10^{-6}$; OR=0.58). In order to refine our association, we imputed genotypes of SNP in *POPDC3* gene. The regional association plot of *POPDC3* showed a block of intronic SNPs (also containing rs1192806) in tight linkage disequilibrium, and significantly associated with a decreased risk of pulmonary injury-related ARDS ($p < 0.003$) (see online supplementary figure S6).

To gain insight into the functional significance of SNPs in *POPDC3* associated with reduced ARDS risk, we investigated their correlation with *POPDC3* expression levels using genotypic and normalised mRNA expression data of three different

cell lines from GENEVAR resource.^{45 46} The probe used for *POPDC3* expression analysis was ILM_1652244 on the Illumina human whole-genome expression array (WG-6 v3). As shown in figure 1, variant allele of rs1190298 and rs9399904 were significantly correlated with a decreased level of *POPDC3* mRNA in fibroblast cell line ($r=0.276$; $p=0.0164$ and $r=0.304$; $p=0.0079$, respectively).

DISCUSSION

Evidence indicates that ALI/ARDS derived from a pulmonary insult has different pathophysiological, biochemical, radiological and mechanical patterns from ALI/ARDS caused by an extrapulmonary injury.⁵⁴ The current study was aimed at gaining understanding of the genetic contribution to the development of ALI/ARDS from extrapulmonary and pulmonary sources. Using a large-scale genotyping approach (50 000 SNPs in ~2000 genes) and a multistage study design, we identified different genetic profiles underlying ALI/ARDS development from different insults to the lung. There was no overlap between SNPs associated with ARDS from direct or indirect insults in our study. No variant exhibited even a marginal association in both types of lung injury either in the individual analysis, or when their effects were combined in a multi-SNP genotypic risk score. Our analyses suggest nonexistence of shared risk factors contributing to the development of ARDS from direct or indirect insults. However, it is possible that variants with smaller effects, not detected in our study, may be contributing to the development of ARDS from both pulmonary and extrapulmonary sources. Therefore, negative findings from Stage I should be interpreted with caution.

Table 3 SNPs associated with ALI/ARDS in Stage II

SNP	Gene	Minor allele	Human 610-quadr*	MAF Case/Ctrl	OR† (95% CI)	p‡
<i>SNPs associated with extrapulmonary injury-related ARDS in Stage I and tested for validation in Stage II using a trauma-related ALI population (Population I, Harborview trauma cohort)</i>						
SNPs replicated in Stage II						
rs324420‡	FAAH	A	Typed	0.23/0.16	1.59 (1.10 to 2.31)	0.0131 (0.0007)‡
SNPs not replicated in Stage II						
rs198977	KLK2	T	Typed	0.38/0.22	1.23 (0.89 to 1.70)	0.2019
rs9645765	VWF	G	Imputed	0.08/0.08	0.98 (0.60 to 1.61)	0.942
rs2889490	SFRS16	G	Imputed	0.48/0.49	1.03 (0.77 to 1.37)	0.8319
rs3128126	ISG15	G	Imputed	0.33/0.36	0.77 (0.52 to 1.16)	0.2112
rs16980496	ADRBK2	A	Imputed	0.07/0.09	1.05 (0.60 to 1.86)	0.8509
rs2070887	VWF	G	Typed	0.08/0.08	1.14 (0.69 to 1.88)	0.5928
rs10490072	BCL11A	C	Imputed	0.24/0.23	1.09 (0.78 to 1.52)	0.6125
<i>SNPs associated with pulmonary injury-related ARDS in Stage I and tested for validation in Stage II using a pneumonia/pulmonary sepsis-related ARDS population (Population II, MGH/ARDS net)</i>						
SNPs replicated in Stage II						
rs1190286‡	POPD3	C	Imputed	0.14/0.20	0.65 (0.46 to 0.90)	0.0094 (0.0007)‡
SNPs not replicated in Stage II						
rs7807769	PRKAG2	A	Imputed	0.42/0.39	1.08 (0.85 to 1.38)	0.5082
rs7801616	PRKAG2	T	Typed	0.42/0.40	1.08 (0.85 to 1.37)	0.5195
rs9960450	TNFRSF11A	C	Typed	0.05/0.03	1.64 (0.91 to 3.00)	0.1009
rs2254358	HSPG2	C	Imputed	0.33/0.31	0.98 (0.75 to 1.28)	0.8977
rs732821	HTR2A	A	Imputed	0.46/0.47	0.99 (0.78 to 1.25)	0.9171
rs6970522	PRKAG2	G	Typed	0.47/0.44	1.07 (0.84 to 1.35)	0.5847
rs3887893	ABCC1	G	Typed	0.39/0.36	1.23 (0.97 to 1.58)	0.0912
rs17069902	TNFRSF11A	T	Typed	0.06/0.04	1.51 (0.91 to 2.50)	0.1098
rs2671222	IL8RA	A	Imputed	0.06/0.07	0.96 (0.59 to 1.58)	0.8890
rs12080701	PDE4B	G	Imputed	0.09/0.09	1.27 (0.81 to 1.98)	0.2204
rs8112223	HAS1	A	Typed	0.40/0.35	1.06 (0.82 to 1.35)	0.6609
rs6451620	GHR	A	Typed	0.05/0.04	0.98 (0.54 to 1.77)	0.9416
rs17419964	PDE4B	G	Imputed	0.25/0.27	1.24 (0.94 to 1.62)	0.12
rs802440	GRM3	T	Imputed	0.32/0.31	0.96 (0.75 to 1.25)	0.7879
rs4075731	MAP3K6	A	Imputed	0.37/0.41	0.93 (0.73 to 1.18)	0.547
rs2854386	IL8RA	C	Imputed	0.06/0.07	0.96 (0.59 to 1.58)	0.8806

SNPs associated with extrapulmonary and pulmonary injury-related ARDS in Stage I ($p \leq 0.0005$) were tested for validation in Stage II using two different populations with indirect (Population I) and direct (Population II) lung injury as risk factor for ALI/ARDS. Both populations were genotyped with the Human 610-quadr platform.

In Stage II, SNPs rs324420 and rs1190286 demonstrated a reproducible association with increased risk of ALI from indirect insult (trauma) and decreased risk of ARDS from pulmonary injury (pneumonia/pulmonary sepsis), respectively, and those associations were robust after adjusting for clinical variables ($p = 0.0131$ and $p = 0.0094$, respectively).

*Indicates whether the SNP was directly genotyped by the Human 610-quadr. For those ITMAT-Broad_CARE, SNPs not directly genotyped on the genome-wide array, imputation was performed.

†OR and p values were adjusted for clinical covariates (age, ISS and APACHE II score in Population I and age, gender and top six principal components in Population II).

‡Only rs324420 in Population I, and rs1190286 in Population II met these thresholds (unadjusted p values displayed in italics).

The threshold of significance in Stage II was established in $p \leq 0.006$ (0.05/8 SNPs) and $p \leq 0.003$ (0.05/17 SNPs) for SNPs previously associated with ARDS from indirect and direct insults, respectively.

ALI, acute lung injury; APACHE, acute physiology and chronic health evaluation; ARDS, acute respiratory distress syndrome; Case/Ctrl, Case/Control; LD, linkage disequilibrium; MAF, minor allele frequency; MGH, Massachusetts General Hospital; SNP, single nucleotide polymorphism.

Among the top SNPs associated with extrapulmonary injury-related ARDS in the discovery phase, SNP rs324420 successfully replicated its association with ALI in the second and third stages of our study (trauma-related ALI), with the same direction and magnitude of association as observed in Stage I (increased risk of ALI). Meta-analysis confirmed this association. SNP rs324420 is located in the exon 3 of the *FAAH* gene that spans 19 582 nucleotides on chromosome 1 and encodes the fatty acid amide hydrolase (FAAH). This enzyme is part of the endocannabinoids (ECs) system⁵⁵ that involves ECs and their receptors, CB1 and CB2, in the nervous system and periphery.⁵⁶ FAAH is a key enzyme in the degradation of ECs and modulates levels of ECs that act at CB1 and CB2 receptors. Overactive signalling at the level of CB1 has been shown to influence body weight and fat metabolisms by modulating energy balance, feeding behaviour and peripheral lipid metabolism.⁵⁷ SNP rs324420 leads to a non-synonymous change 385 C/A (P129T), and produces a mutant enzyme with reduced

expression and activity.⁵⁸ A recent study confirmed direct effect of SNP rs324420 in ECs system activation⁴⁸ suggesting that this SNP may be a risk factor for obesity caused by elevated plasma levels of endocannabinoids. Because obesity may influence ALI outcome,^{52–53} we tested whether BMI represented a confounding bias in the association rs324420-ARDS. After adjustment for BMI, rs324420 remained independently associated with increased risk of ALI from indirect lung injury.

Although previous reports conflict with regard to the effects of genetic variation in *FAAH* and body composition,^{49–51} recent evidence suggests that it has a more direct influence on lipid homeostasis. SNP rs324420 has been recently associated with increased serum triglycerides and reduced high-density lipoprotein cholesterol (HDLc) level among subjects in one of the largest family-based obesity study cohorts.⁵⁹ These results suggest that the defective FAAH protein may affect lipid homeostasis by modifying ECs levels, however, the mechanistic link between genetic variations in *FAAH*, ECs/CB1 signalling

Table 4 Association results for SNPs significantly associated with pulmonary/extrapulmonary injury-related ALI/ARDS in meta-analysis

SNP	Gene	Chr	Minor allele	Discovery phase (Stage I)				Replication phase I (Stage II)				Replication phase II (Stage III)				Meta-analysis		
				MAF Case Ctrl	OR	95% CI	p	MAF Case Ctrl	OR	95% CI	p	MAF Case Ctrl	OR	95% CI	p	OR	P-meta	Q*
SNPs significantly associated with extrapulmonary injury-related ALI/ARDS																		
rs324420	FAAH	1	A	0.29 0.19	1.74 (1.27 to 2.39)	0.000503	0.23 0.16	1.59 (1.10 to 2.31)	0.0131	0.24 0.17	1.85 (1.08 to 3.19)	0.026	1.70	2×10^{-6}	0.89			
SNPs significantly associated with pulmonary injury-related ALI/ARDS																		
rs12080701	PDE4B	1	G	0.14 0.09	1.85 (1.32 to 2.60)	0.000362	0.09 0.09	1.27 (0.81 to 1.98)	0.2997	—	—	—	1.61	0.0005	0.19			
rs17419964	PDE4B	1	G	0.34 0.26	1.50 (1.20 to 1.88)	0.000433	0.25 0.27	1.24 (0.94 to 1.62)	0.12	—	—	—	1.39	0.0002	0.29			
rs1190286	POPDC3	6	C	0.13 0.20	0.53 (0.39 to 0.72)	5.30E-05	0.14 0.20	0.65 (0.46 to 0.90)	0.0094	—	—	—	0.58	2.7×10^{-6}	0.38			
rs3887893	ABCC1	16	G	0.45 0.36	1.48 (1.20 to 1.83)	0.000287	0.39 0.36	1.23 (0.97 to 1.58)	0.0912	—	—	—	1.37	0.0001	0.16			
rs9960450	TNFRSF11A	18	C	0.08 0.03	2.48 (1.56 to 3.93)	0.000114	0.05 0.03	1.64 (0.91 to 3.00)	0.1009	—	—	—	2.12	5.3×10^{-5}	0.28			
rs17069902	TNFRSF11A	18	T	0.09 0.04	2.12 (1.41 to 3.20)	0.000312	0.06 0.04	1.51 (0.91 to 2.50)	0.1098	—	—	—	1.85	0.0001	0.31			

The meta-analysis was performed using a fixed effects-model ($p < 0.1$ for Cochran's Q test); ARDS, acute respiratory distress syndrome; Case/Ctrl, Case/Controls; Chr, chromosome; MAF, minor allele frequency; SNP, single nucleotide polymorphism.

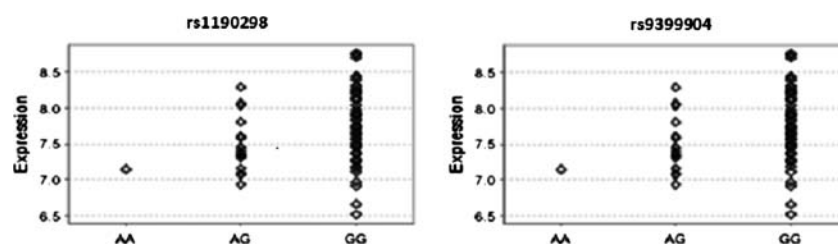
and lipoprotein biology is still poorly understood. Plasma lipoproteins, especially HDL, have been reported to exert immunomodulatory effects in vivo.⁶⁰ HDLs have been suggested to play a crucial role in innate immunity by regulating the inflammatory response as well as reducing the severity of organ injury.⁶¹ HDLc has also been shown to be protective in inflammatory disease models in which local or systemic inflammation are important determinants.⁶² Based on these observations, the association between SNP rs324420 in *FAAH* gene and the development of ALI/ARDS could be explained by the adverse effect of rs324420 on HDLc levels which may lead to a reduction of the protective effect that HDLc exerts against conditions associated with systemic inflammation. Further studies will be needed to investigate the relation between *FAAH* variation, HDLc levels and ALI/ARDS development.

In the discovery and replication cohorts, as well as in the combined meta-analysis, rs324420 showed the strongest association with ALI/ARDS development compared with any other *FAAH* SNP. Based on our association data and the functional nature of the variant rs324420 (C/A, P129T),^{48 58} this polymorphism is a strong candidate to be considered as the causative allele underpinning the association with extrapulmonary injury-related ALI/ARDS. However, it is also possible that this SNP serves only as a marker in linkage disequilibrium with the causal variant. Further functional studies would be necessary to confirm the causality of rs324420 in the development of ALI/ARDS.

Besides rs324420, no other SNP associated with extrapulmonary injury-related ARDS in Stage I replicated its association in the second stage of our study. Since our replication population in Stage II (Population I) is a homogenous trauma population, lack of replication for the remaining SNPs associated with extrapulmonary injury-related ARDS could be indicative of inherent differences between trauma and other causes of extrapulmonary injury. In line with this, several studies have reported improved outcomes for patients with trauma-related ALI than those with non-trauma-related ALI.^{63 64} Less severe lung epithelial and endothelial injury may explain the better outcomes of the trauma population, suggesting a different pathophysiology underlying ALI development in trauma patients than other lung injury patients.⁶⁵

Our study also identified several SNPs associated with pulmonary injury-related ARDS in the discovery set. Among them, rs1190286 in *POPDC3* gene showed a replicated association with decreased risk of ARDS from pulmonary sources in Stage II Population II (pneumonia/pulmonary sepsis). Additional significant associated SNPs were identified in *PDE4B*, *ABCC1* and *TNFRSF11* genes (table 4 and see online supplementary table S5) by meta-analysis. Meta-analysis also confirmed rs1190286 as the most significant SNP associated with decreased risk of ARDS from pulmonary sources. By using imputation, we identified a block of intronic SNPs (containing rs1192806) in tight linkage disequilibrium, and also significantly associated with a decreased risk of pulmonary injury-related ARDS. We found a significant correlation between minor allele of rs1190298 and rs9399904 (in that block) and decreased *POPDC3* mRNA levels. *POPDC3* is one of the three members of the Popeye domain-containing (POPDC) gene family (POPDC1-3). Popdc1-null mice show an impaired ability to regenerate skeletal muscle. Null mutants for Popdc2 and Popdc3 proteins have not been developed yet; however, the fact that Popdc1 phenotype is not lethal suggests a potential redundant role of Popdc2 and Popdc3 in skeletal muscle regeneration.⁶⁶ Our results provide evidence that variants in *POPDC3* gene associated with a decreased *POPDC3* mRNA expression level are protective from

Figure 1 Association of rs1190298 and, rs9399904 with mRNA *POPDC3* levels. Linear regression analyses were performed based on the mRNA expression profiling and genotypic data from fibroblast cell line obtained from the Gene Expression Variation database. The correlation between single nucleotide polymorphisms rs1190298 and rs9399904 and *POPDC3* expression levels was significant ($r=0.276$; $p=0.0164$ and $r=0.304$; $p=0.0079$, respectively).



ARDS development. Due to the LD among SNPs in *POPDC3*, future research will be needed to determine the causal SNPs that is driving the association with ARDS, and to elucidate the role for *Popdc3* in the lung.

Our study includes several strengths. First, we used a large and well-defined discovery ARDS cohort, where patients were carefully assigned into pulmonary and extrapulmonary groups, excluding ambiguous cases, and reducing possible bias from misclassification. Second, we performed a large-scale deep coverage genotyping strategy (IBC Chip) ensuring the coverage of most of the targeted genes with a density greater than the standard genome-wide genotyping platforms.³³ Third, we implemented a multistage study design,^{36–37} and used three separate populations and multiple genotyping platforms to test the validity of our associations. The replication of our findings with the same direction and magnitude as observed in Stage I, and the association with genetic variants affecting protein expression and activity,⁵⁸ reduces the chance of false positive associations and strengthens the chance that the observed genotypes are likely to play a role in development of ALI/ARDS secondary to direct or indirect insults to the lung.

Our study also has several limitations. By contrast to hypothesis-free genome-wide-based platform, the candidate gene approach used in our study limits our findings to those genes in the chip, excluding the discovery of novel loci relevant to ALI/ARDS development.

The statistical threshold to declare significance when using a dense, hypothesis-driven candidate gene SNP array is uncertain.⁶⁷ None of our stage I results would be declared if a conservative Bonferroni method to account for 50 000 SNPs was applied. However, there are limitations to reliance on extreme p values to prioritise candidate gene associations. The Bayesian design of the IBC chip, combined with replication of our association in three different populations, and the functional nature of the ARDS-associated SNP, lend support to *FAAH* and *POPDC3* as novel susceptibility genes for the development of ALI/ARDS from extrapulmonary and pulmonary sources, respectively.

SNPs associated with extrapulmonary injury-related ARDS in Stage I were replicated using trauma-related ALI populations. A total of 90% of subjects in Population I had blunt trauma, and they were classified as having ALI from extrapulmonary origin. However, it is possible that at least some of these patients had a concurrent injury to the thorax. We could not adjust our results for pulmonary contusion because this level of phenotypic data was unavailable. While our a priori hypothesis was that the trauma population would serve as a replication population for indirect-cause ARDS associations, we did test whether any direct-cause ARDS Stage I variants replicated in Population I. No replications were observed lending support for

the classification of blunt trauma as an extrapulmonary insult (see online supplementary table S6). None of the direct-cause ARDS variants were validated in Stage III population either (data not shown).

Population II (pneumonia/pulmonary sepsis) was used in the validation of the SNPs associated with pulmonary injury-related ARDS. None of the indirect-cause ARDS Stage I variants were validated in this population (see online supplementary table S7).

As we mentioned in the Methods section, controls in Population II were non-ARDS patients with pulmonary injury from the discovery set. We selected the same control group as in Stage I since no other population was available at the time of the study. The MAF of rs1190286 in Population II was 0.20/0.14 (controls/cases). The MAF in the control group (0.20) was slightly higher than the MAF reported at HapMap (<http://hapmap.ncbi.nlm.nih.gov/>) and 1000 genomes (<http://www.1000genomes.org/>) datasets (0.14 and 0.15, respectively). These differences may suggest that the observed association between rs1190286 and decreased risk of ARDS from direct lung injury might be spurious, and could be driven by the systematic differences in allele frequencies between our selected control group and cases in Population II. Our analyses did not indicate stratification in the discovery population (either in the pulmonary or extrapulmonary groups: $\lambda=1.000$ and $\lambda=1.018$, respectively). We believe that the differences in MAF of rs1190286 between our control group and HapMap/1000 genomes datasets are due to the very nature of our control population, and might indicate a protective element from the development of ARDS. Unlike the subjects recruited at HapMap/1000 genomes studies, subjects in our control group were not healthy subjects but critically ill patients at risk of ARDS. These subjects were ascertained according to the proposed criteria for the correct design of association studies for complex diseases.^{68–69} Selecting healthy subjects as controls would bias the results by blending the real differences in allelic frequencies between the affected and control populations, reducing the statistical power of our study or yielding false associations.⁷⁰

Finally, our study was also limited to Caucasians. Replication across different populations would be necessary to determine if the observed associations are also present in non-European populations.

To our knowledge, our study represents the first attempt to comprehensively estimate the genetic contribution underlying the differences in the development of ALI/ARDS from pulmonary and extrapulmonary sources. Our data and its replication in three critically ill populations suggest that different injury-related genetic variants may contribute to susceptibility to ALI/ARDS from direct versus indirect insults, lending

support to the concept that ALI/ARDS is not a stereotyped response of the lung to injury. The identification of injury-specific genetic profiles may lead to a better understanding of the range of different pathways that lead to pulmonary dysfunction, and may help to improve the present definitions of the pulmonary and extrapulmonary injury categories. Understanding the pathophysiology of ALI/ARDS caused by different original insults is a necessary first step toward the development of therapeutic interventions that target specific aspects of these disease processes. The inclusion of patients into these two genetically defined injury categories should be considered in the design of future trials in the study of ALI/ARDS.

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Ethics approval At each site, the institutional review board and/or human subjects committee reviewed and approved the study. For the Stages I and II (Population II) of the study, signed informed consent was obtained from all study participants or their appropriate surrogates. Stage II (Population I) and Stage III were granted waiver of informed consent in accordance with institutional and federal regulations.

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