ORIGINAL ARTICLE

Understanding Heterogeneity in Biologic Phenotypes of Acute Respiratory Distress Syndrome by Leukocyte Expression Profiles

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Abstract

Rationale: Two biologic phenotypes of acute respiratory distress syndrome (ARDS) have been identified based on plasma protein markers in four previous studies.

Objectives: To determine if blood leukocyte gene expression is different between the "reactive" and "uninflamed" phenotype.

Methods: This is a new study adding blood leukocyte transcriptomics and bioinformatics analysis to an existing patient cohort of ARDS in patients with sepsis admitted to two ICUs during a 1.5-year period. Canonical pathway analysis was performed.

Measurements and Main Results: A total of 210 patients with sepsis and ARDS were included, of whom 128 had a reactive and 82 an uninflamed phenotype. A total of 3,332/11,443 (29%) transcripts were significantly different between the phenotypes. Canonical pathway analysis showed upregulation of oxidative phosphorylation genes indicative of mitochondrial dysfunction (52% of genes in pathway). The uninflamed phenotype was characterized by upregulation of mitogen-activated protein kinase pathways.

Conclusions: A third of genes are differentially expressed between biologic phenotypes of ARDS supporting the observation that the subgroups of ARDS are incomparable in terms of pathophysiology. These data provide additional support for biologic heterogeneity in patients with ARDS and suggests that a personalized approach to intervention focusing on oxidative phosphorylation is pivotal in this condition.

Keywords: ARDS; phenotypes; personalized medicine

At a Glance Commentary

Scientific Knowledge on the Subject: Two phenotypes of acute respiratory distress syndrome were identified and validated in several randomized trials and observational studies.

What This Study Adds to the Field: This study shows distinct expression profiles between the phenotypes and identifies therapeutic targets for intervention studies in acute respiratory distress syndrome.

The acute respiratory distress syndrome (ARDS) is a biologically heterogeneous syndrome without a singular underlying pathophysiologic mechanism (1–3). Pharmacologic interventions for ARDS by and large did not show any benefit and this may be explained by biologic heterogeneity (4, 5). Two biologic phenotypes (also called subphenotypes) have been identified in four randomized clinical trials (6–8) and one observational study (9). One of the biologic phenotypes, previously referred to as

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"reactive" or "hyperinflammatory," is characterized by high plasma levels of proinflammatory markers, coagulopathy, and endothelial injury. Furthermore, these patients more frequently fulfill criteria for septic shock and have lower plasma levels of bicarbonate. The mortality for patients within this phenotype is astonishingly high compared with patients with the other phenotype (referred to as "uninflamed" or "hypoinflammatory"), namely 40–45% versus 15–20%. Biologic phenotypes are stable over the first 3 days of ICU admission (10).

The biologic phenotype is associated with a differential response to several treatment strategies: positive end-expiratory pressure, fluid management strategy, low-dose macrolide therapy, and simvastatin administration (6, 7, 11, 12). These findings suggest that biologic heterogeneity is not only of academic interest but also a treatable trait important for clinical practice (13). It is pivotal to further understand the pathophysiologic mechanisms involved in generating the biologic phenotypes to choose the right pharmacologic interventions for prospective evaluation (14).

Gene expression signatures are a well-established method to optimize pharmacologic interventions in several fields of medicine and may be used to select molecular specific interventions (15). In line with the observed heterogeneity in plasma biomarker profiles, no consistent diagnostic gene signature was found for ARDS in a metaanalysis of all available whole blood expression data (16). It was suggested that further analysis should focus on a biologic phenotype-based analysis of expression data to uncover pathophysiologic mechanisms within ARDS endotypes (16).

We hypothesized that there are differences in leukocyte gene expression profiles between patients with sepsis with a "reactive" and an "uninflamed" phenotype of ARDS. We postulated that alterations in gene expression correspond to specific pathways that may be indicative of the pathophysiologic changes within that phenotype. To answer these questions, we performed gene expression analysis of blood leukocytes in all consecutive patients with sepsis with ARDS included in the MARS (Molecular Diagnosis and Risk Stratification of Sepsis) study during a 1.5-year period and we externally validated our findings with transcriptomics data stored in an online repository. Some of the results of these studies have been previously reported in the form of an abstract (17).

Methods

Study Design

This was a planned analysis within the wider context of the MARS project (NCT01905033), a prospective observational cohort study in the mixed ICUs of two tertiary teaching hospitals (Amsterdam University Medical Center, the Netherlands; and University Medical Center Utrecht, the Netherlands). The Institutional Review Boards of both hospitals approved the study protocol and opt-out consent method used for this study (IRB: 10-056C); the study was registered at clinicaltrials.gov (registration number NCT01905033). The patients or their legal representatives were presented with a brochure and an opt-out form, to be completed in case of unwillingness to participate. This was preferred over a waiver of consent to allow a patient or representative to remove personal data because of privacy reasons. Parts of the methods were previously described in a report on the discovery and validation of biologic phenotypes and the patients included in this analysis are part of that previously described population (9).

Setting

Both ICUs were closed-format units, in which a team of board-certified critical care physicians, fellows in critical care medicine, and board-certified ICU nurses care for a mixed medical-surgical population of patients. The nurse to patient ratio was 1:1–1:2, depending on disease severity.

Inclusion and Exclusion Criteria

Consecutive adult patients admitted to the ICU with an expected length of stay of more than 24 hours were eligible for participation in the MARS study. Infection likelihood was scored based on adjusted CDC criteria, as described previously (18, 19). For the current analysis only patients with sepsis, defined as probable or definite infection (18) and at least one organ dysfunction (20), were included to minimize the biologic heterogeneity of the population. ARDS was defined according to the Berlin definition (21). Gene expression profiles were available for patients recruited between January 1, 2011, and July 20, 2012. There were no additional exclusion criteria.

Diagnosis of ARDS

A dedicated team of researchers who were regularly trained by studying case vignettes, screened for the presence of ARDS on a daily basis while patients remained in the ICU. Patients were classified as having mild, moderate, or severe ARDS according to the Pa_{O_2}/FI_{O_2} at the moment of diagnosis.

Additional Control Groups

Patients with sepsis without ARDS served as a control group for comparison of gene expression profiles with an ICU population without lung injury in a *post hoc* analysis. A group of healthy, age-matched, elderly control subjects was used to compare expression signatures with the normal situation.

Biologic Phenotype Defined by Plasma Biomarkers

Blood was collected from all patients in a plastic vacuum container filled with ethylenediaminetetraacetic acid. After centrifugation ($1{,}500 \times g$ for 15 min) plasma was frozen at $-80^{\circ}C$ for batch-wise analysis. IL-6 and IFN-γ were measured in all samples with a cytometric bead array Flex Set multiplex assay according to the instructions from the manufacturer (BD Biosciences). Plasminogen activator inhibitor-1 and angiopoetin 1 and angiopoetin 2 were measured in all samples with Luminex according to the manufacturer instructions (BioRad). Plasma concentrations of IL-6, IFN-γ, angiopoetin 1/2, and plasminogen activator inhibitor-1 were used to classify patients within the two phenotypes, as published previously (9).

Microarray Analysis

Blood was collected in PAXgene blood RNA tubes (Becton-Dickinson) within 24 hours of ICU admission, at the same moment as plasma was obtained for biologic phenotype characterization. We also collected blood from the healthy control subjects in PAXgene blood RNA tubes. Gene expression profiles were generated using Human Genome U219 96-array plates and the GeneTitan instrument (Affymetrix) (19, 22). MARS gene expression data are available in the Gene Expression Omnibus, via accession number GSE65682. Data were preprocessed as described previously (19).

Independent Replication Data

The Gene Expression Omnibus database was searched with the following terms: "ARDS" OR "Lung injury" and all human datasets involving patients with ARDS were identified (four hits). Three of the four did not contain data on the patient characteristics (comorbidities/outcomes). The largest (Gene Expression Omnibus; GSE 66890) did contain data on age, sex, Acute Physiology and Chronic Health Evaluation III scores, shock, and 60-day mortality and was downloaded from the online repository as independent replication cohort (23).

Statistical Analysis

The analytical pipeline had five steps. First, biologic phenotypes were identified based on plasma biomarkers, as described previously (9). Second, expression profiles were compared between the two biologic phenotypes using fold-changes and *P* values through the "limma" package for expression data. *P* values were corrected for multiple testing and Q-values were calculated. Benjamini-Hochberg multiple comparison-adjusted probabilities (false-discovery rate <5%) defined significance. These results were visualized in a volcano plot. Third, the top ranking upregulated and downregulated probes were matched to corresponding gene identifiers through the Affymetrix database (absolute fold change >1 and adjusted *P* value <0.05). If multiple probes corresponded to a single gene, the top differentiating probe was selected as top upregulated or downregulated probe. Top differentially expressed markers were listed in a figure with fold change. These genes were matched to the available literature on expression profiles obtained in the first 24 hours of ARDS (23). Fourth, ingenuity pathway analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com) was used to identify enrichment of canonical signaling pathways. Importantly, all probes can be imported into IPA without selection. Top matching pathways were displayed as heatmaps. Upstream analysis was used to predict differences in plasma biomarkers based on expression profiles and was checked with actual biomarker concentrations. Fifth, the four top ranking differentially expressed genes in the MARS cohort were used as predictors of the biologic phenotype in a logistic regression model and this model was applied to the independent replication cohort. The model was internally validated by 1,000 bootstrap iterations and the cross-validated measurements of accuracy were reported.

All previous steps were also taken for the independent replication cohort.

Results

Patient Characteristics in Derivation Cohort

Whole-blood mRNA expression profiles available for 210 patients with sepsis with ARDS. Eighty-two had an "uninflamed" and 128 (62%) had a "reactive" biologic phenotype. Patient characteristics are shown in Table 1 and are comparable with those reported in the whole cohort of patients with ARDS (9). In addition, expression profiles were available for 547 patients with sepsis without ARDS and 42 healthy, age-matched control subjects. Table E1 in the online supplement shows the clinical characteristics of these patients.

Univariate Comparison of Expression Profiles between Biologic Phenotypes

A total of 3,332/11,443 (29%) genes were significantly different between the two phenotypes (Benjamini-Hochberg-adjusted *P* values <0.05). Figure 1 shows the volcano plot with adjusted *P* values and fold changes. Figure 2 shows the top differentially expressed genes in the "reactive" and "uninflamed" phenotype.

Seven of eight genes related to neutrophil activation that were positively associated with ARDS in a previous study (*MMP8*, *OLFM4*, *LCN2*, *HP*, *BPI*, *RETN*, and *TCN1*) (23) were identified as strongly upregulated genes in the "reactive" phenotype of ARDS as compared with the "uninflamed" phenotype in this study. Half of the genes that were previously found to be negatively associated with ARDS were identified to be upregulated in the "uninflamed" phenotype as compared with

Table 1. Patient Characteristics in Derivation Cohort

	Uninflamed (*n* = 82)	Reactive (*n* = 128)	*P* Value
Age, yr	64.5 (58–73)	60.5 (47.8–70)	0.009
Male	48 (58.5)	73 (57)	0.89
Type of admission			0.032
Medical	64 (78)	90 (70.3)	
Elective surgery	10 (12.2)	9 (7)	
Emergency surgery	8 (9.8)	29 (22.7)	
BMI, kg/m^2	25.5 (22.9–27.8)	24.8 (22.6–27.8)	0.619
APACHE IV score	78.5 (63.2–92)	95 (75.8–114)	<0.001
Acute physiology score	64.5 (48.2–79.2)	80.5 (65.8–107)	<0.001
Berlin severity			0.42
Mild	31 (37.8)	37 (28.9)	
Moderate	40 (48.8)	70 (54.7)	
Severe	11 (13.4)	21 (16.4)	
Pulmonary cause for ARDS	63 (76.8)	76 (59.4)	0.013
Maximal airway pressure	26 (19–32.2)	29 (22.8–33)	0.037
Pa_{O_2}/Fi_{O_2}, mm Hg	148 (110–208)	159 (111–198)	0.82
PEEP, cm H_2O	8 (6–12)	10 (8–15)	0.015
APPS	5 (5–6)	5.5 (5–7)	0.39
SOFA: circulation	3 (1–4)	4 (3–4)	<0.001
SOFA: CNS	0 (0–0)	0 (0–1)	0.05
SOFA: coagulation	0 (0–1)	1 (0–2)	<0.001
SOFA: liver	0 (0–0)	0 (0–1)	<0.001
SOFA: renal	0 (0–1)	1 (0–3)	<0.001
SOFA: respiratory	3 (3–4)	3 (3–4)	0.226
SOFA: total	7 (5–9)	10 (8–13)	<0.001
Days of mechanical ventilation	8 (4–12)	9 (4–22)	0.10
ICU length of stay, d	9 (5.2–13.8)	11 (5.8–23.2)	0.073
Ventilator-free days and alive at Day 28	19 (0.8–23)	8.5 (0–21)	0.003
Death in ICU	17 (20.7)	42 (32.8)	0.062
Survival at 30 d	59 (72)	84 (65.6)	0.37

Definition of abbreviations: APACHE = Acute Physiology and Chronic Health Evaluation; APPS = age–Pa_{O_2}/Fi_{O_2}–plateau pressure score; ARDS = acute respiratory distress syndrome; BMI = body mass index; CNS = central nervous system; PEEP = positive end-expiratory pressure; SOFA = Sequential Organ Failure Assessment score.

All continuous variables are shown as median (interquartile range), and all categorical variables are shown as *n* (%).

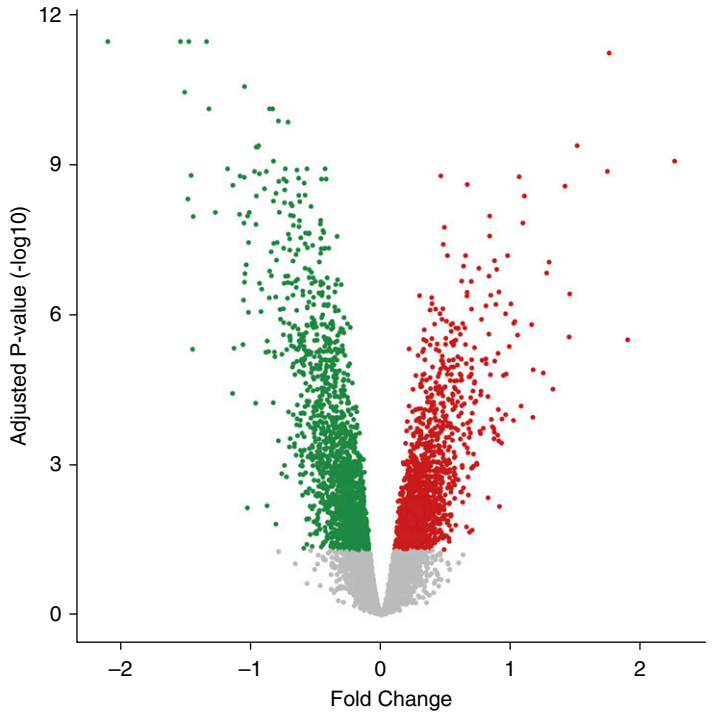

Figure 1. Volcano plot of differential gene expression between the two phenotypes. The *x*-axis shows fold change (log_2 transformed) between the phenotypes. Elevated genes in the "reactive" phenotype are displayed in red; elevated genes in the "uninflamed" phenotype are shown in green. The *y*-axis shows the Benjamini-Hochberg adjusted *P* value ($-log_{10}$).

the "reactive" phenotype (three of six; *HCAR3*, *RBP7*, *MME*) (23). Table E2 provides additional details on the comparison with previous studies.

Pathway Analysis

IPA showed that oxidative phosphorylation was the most enriched pathway in blood leukocytes of patients with the "reactive" phenotype compared with the "uninflamed" phenotype (Figure 3). Furthermore, several pathways involved in cholesterol metabolism were upregulated in the "reactive" phenotype (Figure 3). Compared with the "reactive" phenotype, the uninflamed phenotype was characterized by upregulation of *MAP2K4* (*Mitogen-Activated Protein Kinase 4*) and *RAF1* (*Raf-1 Proto-Oncogene, Serine/Threonine Kinase*)-dependent mitogen-activated protein kinase (MAPK) pathways that coordinately regulate cell proliferation, differentiation, motility, and survival (Figures 3 and E1).

Correlation with Other Biomarkers

Expression levels of *MMP8* were significantly higher in the "reactive" phenotype and strongly correlated with the plasma concentration of MMP8 (Figure E2) ($R^2 = 0.39$; $P < 0.001$). This association was independent of the phenotype of a patient ($R^2 = 0.40$; $P < 0.001$). All genes within the oxidative phosphorylation pathway were positively associated with the plasma concentration of lactate, with *COX17* and *NDUFZ2* showing the highest explained variance (Figure E3) ($R^2 = 0.16/R^2 = 0.17$, both $P < 0.001$), which was independent of the Sequential Organ Failure Assessment (SOFA) circulation score ($P < 0.001$). Gene expression of *LCN2* (also known as NGAL; a biomarker for acute kidney injury) was not associated with higher levels of serum creatinin, when corrected for the biologic phenotype of a patient (β-coefficient = 0.001; $P = 0.15$).

Independent Replication Cohort

The four most differentially expressed genes were used to build a logistic regression model to differentiate between the two phenotypes in the MARS cohort (*MMP8*, *OLFM4*, *MME*, *RBP7*; none involved in the upregulated pathways identified by IPA in either phenotype; receiver operating characteristic curve, 0.82; *see* online supplement for more details). Addition of extra genes did not significantly improve the accuracy of the model significantly. This model was used to predict biologic phenotypes in the independent replication cohort. The publicly available clinical data stratified per biologic phenotype is shown in Table 2. IPA was used to evaluate enrichment of canonical pathways and as shown in Figure 3. Almost all pathways that were enriched in the derivation cohort were also enriched in the independent replication cohort for both phenotypes (Figure 3).

Post Hoc Analysis: Comparison with Patients with Sepsis and Healthy Control Subjects

Figure E4 shows the comparison for the top differentially expressed genes for healthy control subjects, patients with sepsis, and the two biologic phenotypes of ARDS. Principal component (PC) analysis was used to reduce the dimensionality of the most differentially expressed genes (Figure 2). The first two PC explained 56% and 8% of variance, respectively, and were compared between the four groups. The "reactive" phenotype showed significantly different values for PC1 than the other groups (Figure 4) ($P < 0.001$ in all comparisons). PC1 for the "uninflamed" phenotype was not different from that of patients with sepsis without ARDS ($P = 0.31$). All comparisons for PC2 were not statistically significant. These results were confirmed in the independent replication cohort (Figures 4 and E5).

Post Hoc Analysis: Comparison with Other Phenotypes

Table 3 shows that the sepsis endotype MARS3 is more prevalent in the "uninflamed" phenotype, whereas the other sepsis endotypes had a similar prevalence between the ARDS biologic phenotypes. There was no statistically significant difference in Berlin severities of ARDS between the biologic phenotypes of ARDS.

Discussion

The present data further support the postulate that there is considerable biologic heterogeneity among patients with sepsis with ARDS. Almost a third of the measured genes was differentially expressed in blood leukocytes between patients with a "reactive" and "uninflamed" phenotype. The "reactive" phenotype showed

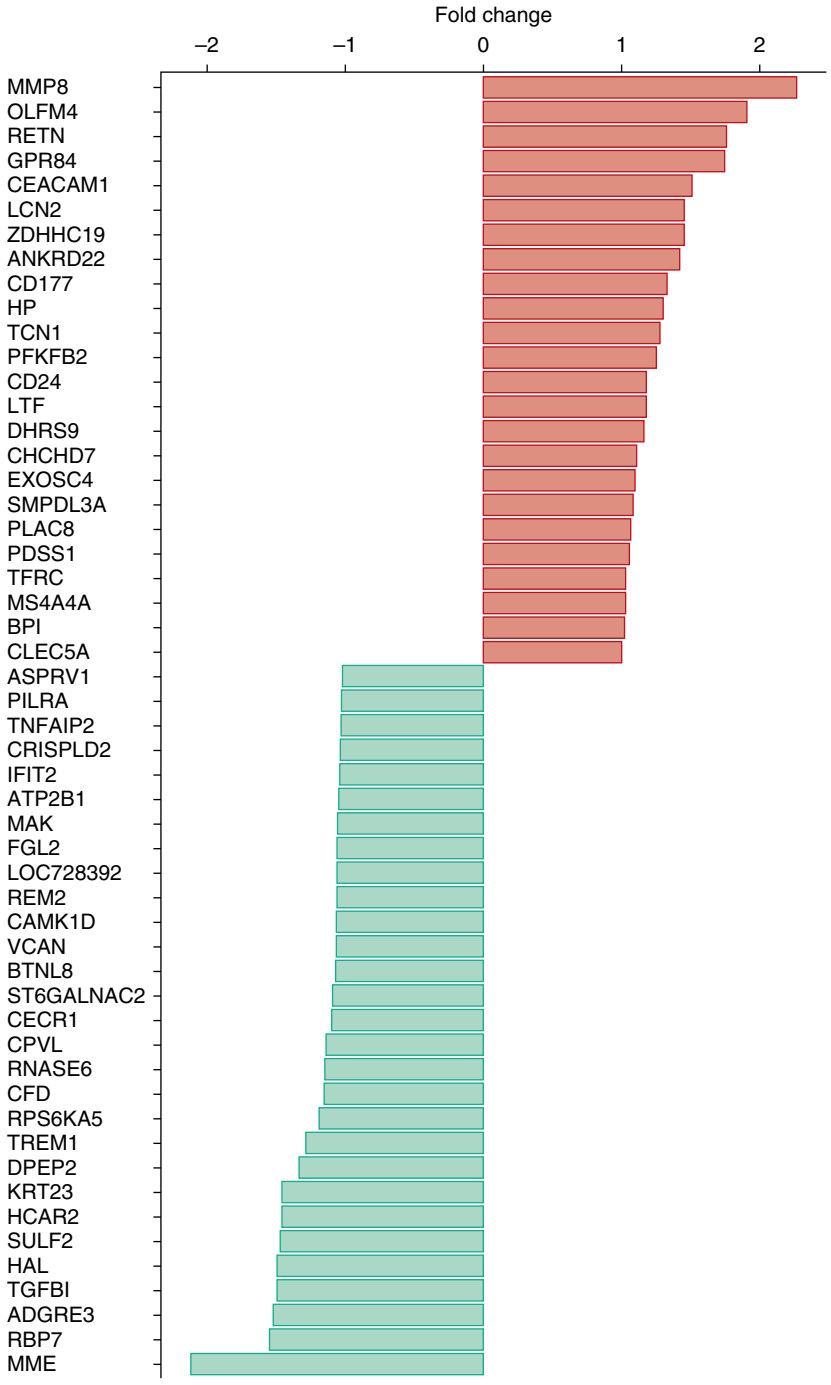

Figure 2. Top differentially expressed genes on the *y*-axis, annotated by standardized gene symbols. The *x*-axis shows the fold change (log_2) between groups. In red are genes that are upregulated in the "reactive" phenotype, and in green are genes that are upregulated in the "uninflamed" phenotype.

upregulation of individual neutrophil-related genes and enrichment of canonical pathways related to oxidative phosphorylation and mitochondrial dysfunction. Compared with the "reactive" phenotype, the "uninflamed" phenotype was characterized by upregulation of MAP2K4- and RAF1-dependent MAPK pathways that coordinately regulate cell proliferation, differentiation, motility, and survival. The same clinical phenotypes could be identified in an independent replication cohort of publicly available expression data based on a subset of four strongly differentially expressed genes suggesting external applicability.

First and foremost, our findings underline the importance of investigating a within phenotype biologic response, rather than investigation of an unselected population. The whole-blood gene-expression studies performed up to now have failed to show a consistent gene signature for an unselected ARDS population (16). These differences can be explained in traditional terms by: variation in *1*) definition of ARDS, *2*) measurement methodology, *3*) timing, *4*) standard ICU care, *5*) false-positive results, and *6*) sample size (24–26). However, a much simpler and more convincing explanation is that cohorts include a different mix of ARDS phenotypes, which inherently results in inconsistent gene expression profiles if, as we have shown, one in three genes is altered by ARDS phenotype.

Top upregulated genes in the "reactive" phenotype were mostly related to neutrophil activation. Several of these genes were previously identified as markers of ARDS in studies using an unselected population. Table E2 clearly shows that genes that are consistently identified show similar trends: if the gene is upregulated in the comparison of unselected patients with ARDS versus patients with sepsis it is also upregulated in the comparison of the "reactive" phenotype versus sepsis or the "uninflamed" phenotype. For the comparison of the "uninflamed" phenotype versus sepsis patients the comparison are shows differential expression in the *other* direction or no statistical significant difference. Taken together, differences in genes related to neutrophil expression can be attributed to the subset of patients with the "reactive" phenotype.

The neutrophil has been established as an important inflammatory cell in the development of acute lung injury in animal models and higher alveolar concentrations of neutrophils and neutrophil activation products were previously observed in bronchoalveolar lavage fluid of patients with ARDS (27). In the current study, no data on the inflammatory response in the lung were available, so we could not validate our suspicions that the upregulation of neutrophil-related genes is associated with a more profound neutrophil response in the lung. In terms of potential therapies, antiinflammatory treatments, such as steroids or Sivelestat (a selective neutrophil

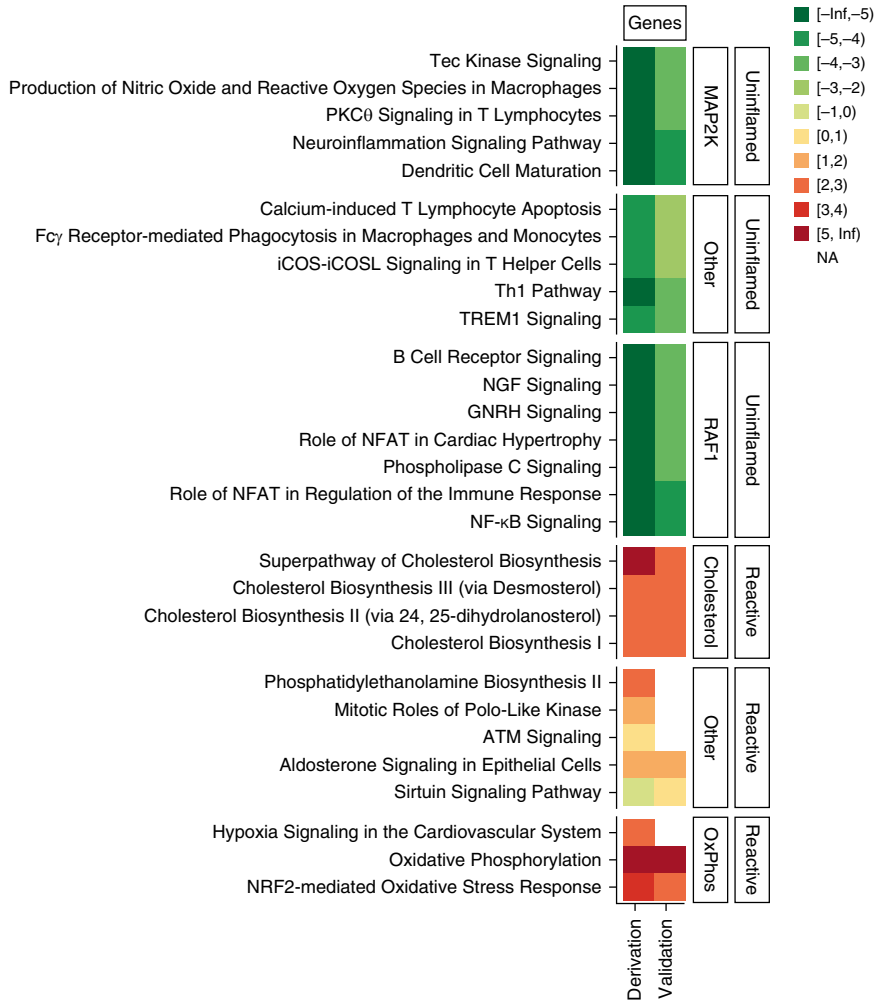

Figure 3. Top significantly different canonical pathways are shown for the derivation cohort, and the same pathways are also shown for the independent replication cohort. Green indicates upregulation of the pathway in the "uninflamed" phenotype; red indicates upregulation in the "reactive" phenotype. ATM = ataxia–telangiectasia mutated; Cholesterol = cholesterol biosynthesis pathways; GNRH = gonadotropin-releasing hormone; ICOS = inducible T-cell costimulator; ICOSL = ICOS ligand; Inf = infinity; MAP2K4 = mitogen-activated protein kinase 4–dependent MAPK pathways; NA = not applicable; NF-κB = nuclear factor κB; NFAT = nuclear factor of activated T cells; NGF = nerve growth factor; NRF2 = nuclear factor erythroid 2–related factor 2; OxPhos = oxidative phosphorylation; PKC = protein kinase C; RAF1 = Raf-1 proto-3 oncogene, serine/threonine kinase–dependent MAPK pathways; Th1 = T-helper cell type 1; TREM = triggering receptor expressed on myeloid cells.

Table 2. Patient Characteristics in Independent Replication Cohort

	Uninflamed (*n* = 19)	Reactive (*n* = 10)	*P* Value
Age, yr	60 (48–70)	64.5 (43.5–75.5)	0.95
Male	9 (47.4)	7 (70)	0.63
BMI, kg/m^2	24.8 (22.6–27.8)	25.5 (22.9–27.8)	0.62
APACHE III score	99 (73.5–138)	135 (131–161)	0.12
Pulmonary cause for ARDS	17 (89.5)	4 (40)	0.01
Shock	11 (57.9)	10 (100)	0.026
Mortality at 60 d	4 (21.1)	5 (50)	0.21

Definition of abbreviations: APACHE = Acute Physiology and Chronic Health Evaluation; ARDS = acute respiratory distress syndrome; BMI = body mass index.
All continuous variables are shown as median (interquartile range), and all categorical variables are shown as *n* (%).

esterase inhibitor), may only be effective in this phenotype, which may explain the inconsistency of results from clinical trials (28, 29). However, our understanding of the pathophysiologic consequences of ARDS phenotypes is still limited and previous studies, such as with rhIL1RA in patients with sepsis, have yielded contraintuitive results (30).

Canonical pathway analysis showed enrichment of oxidative phosphorylation in the "reactive" phenotype compared with the "uninflamed" phenotype and gene expression in this pathway was associated with plasma lactate levels. This relation was independent of SOFA circulation scores and may be indicative of mitochondrial dysfunction. Mitochondrial dysfunction has been hypothesized to be important in the multiorgan failure syndrome (31) and this is in line with the clinical phenotype of these patients. Several potential therapies have been proposed for the treatment of mitochondrial dysfunction in sepsis, some of which have been tested in animal models (32).

Simvastatin treatment was shown to improve outcome in the "hyperinflammatory" phenotype (12) and has a profound antioxidative effect in endothelial cells (33) and blood leukocytes (34). Furthermore, simvastatin decreased H_2O_2-mediated induction of the cellular expression of MMPs and proinflammatory mediators in pulmonary epithelial cells (35). Taken together, the results from this study suggest that the protective effect of simvastatin in the "hyperinflammatory" phenotype may be best explained through mediation of effects on MMPs and oxidative phosphorylation.

The "uninflamed" phenotype showed enrichment of MAP2K4- and RAF1-dependent MAPK pathways compared with the "reactive" phenotype. These pathways are highly conserved and responsible for regulation of cell proliferation, differentiation, motility, and survival. p38 and ERK, two important genes in the involved MAPK pathways, are essential for cytokine release by alveolar macrophages (36). Therefore, it may be surprising to find upregulation of these MAPK pathways in the "uninflamed" phenotype compared with the "reactive" phenotype. Any explanation for this finding is highly speculative and one should keep in mind that this concerns differential expression between two phenotypes within ARDS. An

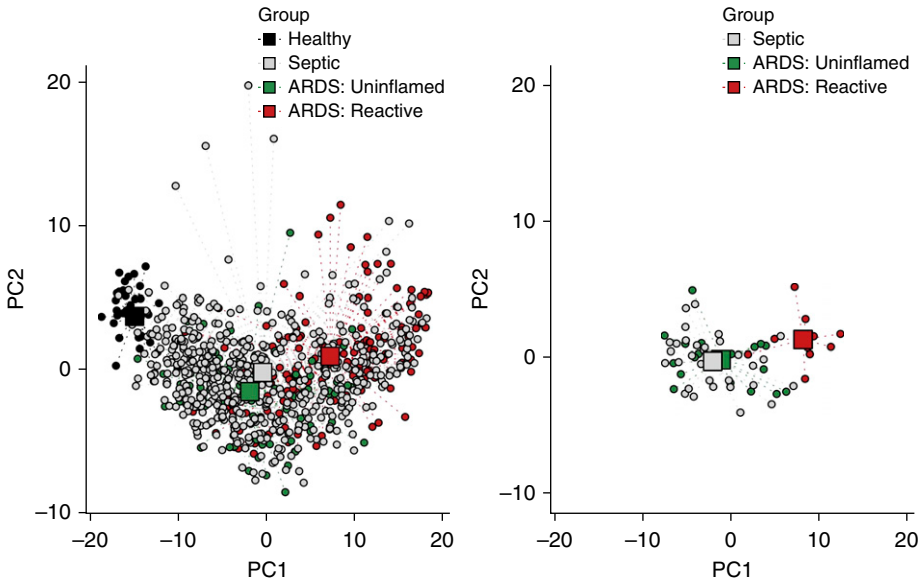

Figure 4. Principal component (PC) analysis. The *x*-axis shows PC1 explaining around 56% of variance of the most differentially expressed genes in the derivation cohort. The *y*-axis shows PC2 explaining around 8% of variance. Dots represent individual patients with colors identifying groups. The larger rectangle shows the centroid per group. The healthy control group was not available for the independent replication cohort. ARDS = acute respiratory distress syndrome.

interesting hypothesis may be that MAPK pathways are essential in neutrophil apoptosis and may work as protective mechanisms against a proinflammatory response in this case (37, 38). This mechanism is actually prevented by oxidative stress, which may explain the differential MAPK pathway expression in the other phenotype (37). Alternatively, MAPK pathway activation is well described in animal models of ventilator-associated lung injury (39–41) and we may speculate that this process is more frequently encountered in the "uninflamed" phenotype, even though there is no circumstantial evidence to support this hypothesis.

In a *post hoc* analysis, we compared ARDS phenotypes with previously identified sepsis endotypes (22) and showed that the sepsis endotype MARS3 was more prevalent in the "uninflamed" ARDS phenotype. The MARS3 endotype is known to be relatively low risk and is associated with increased expression of adaptive immune or T-cell functions. This is in line with the lower risk of mortality seen in the "uninflamed" phenotype. Despite the partial overlap between ARDS phenotypes and sepsis endotypes, they are not fully concordant. Another group of investigators has identified SRS1 and SRS2 phenotypes of sepsis (42) and have shown a differential response to steroid treatment (43). Patients with the immunocompetent SRS2 endotype who were treated with corticosteroids had poorer survival than those who received placebo. Unfortunately, we could not replicate these phenotypes in this study, but because the SRS phenotypes did not show differences in concentrations of proinflammatory cytokines in plasma, which are at the basis for the "uninflamed" and "reactive" phenotype, we do not suspect that there is complete overlap between these phenotypes either. Based on the comparisons of patients with ARDS with patients with sepsis without ARDS, leukocyte expression in patients with "reactive" ARDS should be seen as rather unique within the spectrum of patients with sepsis and this might imply that specific strategies are needed for this highly selected population.

Table 3. Overlap between "Uninflamed" and "Reactive" Phenotype and Other Identified Phenotypes

	Uninflamed (%)	Reactive (%)	*P* Value
Sepsis endotypes (22)			0.009
MARS1	28	33	
MARS2	29	43	
MARS3	33	13	
MARS4	10	11	
Berlin definition (21)			0.072
Mild	39	26	
Moderate	52	55	
Severe	9	19	

Definition of abbreviation: MARS = Molecular Diagnosis and Risk Stratification of Sepsis. "Calfee subphenotypes" could not be identified in this dataset.

Several strengths of the described study should be noted. This is the first study to investigate between-phenotype differences in gene expression. Second, the sample size is larger than any other gene-expression study in patients with ARDS. A background population of patients with sepsis was used to limit bias caused by clinical risk factors for ARDS and allow for better comparison with already published rapports on gene expression. Third, we tested independent replication in a dataset from an online repository, which decreases the chances of false-positive findings because this population differed in location, time of inclusion, sampling protocol, and type of microarray, yet yielded very similar clinical characteristics of the phenotypes. Unfortunately, we did not have access to plasma protein markers in that cohort, thus this cannot be considered definitive external validation. Prospective studies are necessary to validate the proposed predicted of biologic phenotypes with a limited number of genes. Finally, we also included data from patients without ARDS, showing that the "uninflamed" ARDS phenotype is most similar to patients with sepsis without ARDS in terms of gene expression.

This study also has several limitations. Primarily, analysis of leukocyte mRNA expression may provide different results than protein analysis and may therefore not always represent functional changes. However, the most strongly upregulated gene that we identified, *MMP8*, was part of the protein assay that led to the identification of biologic phenotypes and gene expression strongly correlated with protein levels suggesting that this was not a

major concern in our study. Second, although the sample size was sufficient for comparisons between the two biologic phenotypes it was insufficient to use gene expression data to identify novel phenotypes within these subgroups. Third, selection bias could have played a role because gene expression profiles were not measured in all patients with ARDS. Finally, we assume that the "reactive" phenotype and the "hyperinflammatory" phenotype, respectively identified in an observational study and randomized controlled trials, capture the same biologic variation as suggested by similar biologic and clinical features that define the phenotypes. However, this assumption is not yet validated and it should be noted that the prevalence of the "reactive" phenotype was much higher than that of the "hyperinflammatory" phenotype.

To conclude, almost a third of genes are differentially expressed in blood leukocytes between biologic phenotypes of ARDS supporting the observation that the subgroups of ARDS are incomparable in terms of biology. Within ARDS, the "reactive" phenotype is characterized by neutrophil activation and oxidative phosphorylation, whereas the "uninflamed" phenotype is characterized by enrichment of MAP2K4- and RAF1-dependent MAPK pathways. These observations have implications for intervention studies targeting the immune response in patients with ARDS, because it no longer makes biologic sense to target these two populations with the same immune modulatory drugs. Furthermore, our findings potentially explain the positive effects of simvastatin in the "reactive" phenotype. ■

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

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