Whole-Blood Mitochondrial DNA Copies Are Associated With the Prognosis of Acute Respiratory Distress Syndrome After Sepsis

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Acute respiratory distress syndrome (ARDS) is an inflammatory process of the lungs that develops primarily in response to pulmonary or systemic sepsis, resulting in a disproportionate death toll in intensive care units (ICUs). Given its role as a critical activator of the inflammatory and innate immune responses, previous studies have reported that an increase of circulating cell-free mitochondrial DNA (mtDNA) is a biomarker for fatal outcome in the ICU. Here we analyzed the association of whole-blood mtDNA (wb-mtDNA) copies with 28-day survival from sepsis and sepsis-associated ARDS. We analyzed mtDNA data from 687 peripheral whole-blood samples within 24 h of sepsis diagnosis from unrelated Spanish patients with sepsis (264 with ARDS) included in the GEN-SEP study. The wb-mtDNA copies were obtained from the array intensities of selected probes, with 100% identity with mtDNA and with the largest number of mismatches with the nuclear sequences, and normalized across the individual-probe intensities. We used Cox regression models for testing the association with 28-day survival. We observed that wb-mtDNA copies were significantly associated with 28-day survival in ARDS patients (hazard ratio = 3.65, 95% confidence interval = 1.39–9.59, p = 0.009) but not in non-ARDS patients. Our findings support that wb-mtDNA copies at...
sepsis diagnosis could be considered an early prognostic biomarker in sepsis-associated ARDS patients. Future studies will be needed to evaluate the mechanistic links of this observation with the pathogenesis of ARDS.

**Keywords:** ARDS, mitochondria, DAMPs, whole blood, mtDNA, survival

### INTRODUCTION

The acute respiratory distress syndrome (ARDS) is a lung inflammatory process that develops primarily as a response to respiratory or systemic-induced sepsis, which causes a disproportionate mortality burden in the adult intensive care unit (ICU) and has disabling consequences for years in surviving patients (1–3). ARDS occurs in 7 cases per 100,000 people per year, although the estimate varies widely among studies since the clinical diagnostic criteria are nonspecific. Its overall mortality rate remains high in most series, around 30%–40% (2–4). ARDS still has no effective and efficient treatment despite multiple studies that have focused on identifying the pathophysiology and improving the prognosis of these patients since it was first described. To date, lung-protective mechanical ventilation (MV) remains the main standard supportive ARDS treatment (5, 6) and there is no specific pharmacological therapy for it. Thus, identifying specific biomarkers will help to develop early therapeutic and preventive therapies, while assisting in predicting the prognosis of individual ARDS patients (3, 7, 8).

Mitochondria are a bioenergetic and biosynthetic cell organelle and a signaling hub that controls several important cellular functions, including cell survival and differentiation, as well as functioning of inflammatory responses (9, 10). The multiorgan and cellular dysfunction underlying sepsis and leading to ARDS could trigger mitochondrial dysfunction, which is characterized by fragmentation of the mitochondria and loss of integrity of mitochondrial DNA (mtDNA) (11). Previous studies have shown that, while the cellular mtDNA levels decrease, the cell-free mtDNA levels increase in response to a stimulus due to major trauma or a microbial infection (12–16). Based on evidence from animal models (17) and patient studies (18, 19), circulating mtDNA levels have been proposed as a potential biomarker for the systemic inflammatory response and lung injury after major trauma.

Cell-free mtDNA is considered a molecular pattern associated with damage (DAMPs) and could act as a critical activator of the innate immune system and inflammation (7, 9, 20, 21). Circulating cell-free mtDNA, measured by quantitative PCR (qPCR), has been associated with the overall 28-day mortality in ICU patients (22). Among ARDS patients, plasma mtDNA levels measured by qPCR on day 7 after diagnosis were significantly higher among non-surviving patients (23). Similarly, the plasma mtDNA levels among sepsis patients admitted to the emergency room were significantly higher among those who did not survive, and a score combining their levels with plasma lactate concentration considerably improved the 28-day mortality prediction (24). In fact, molecular patterns associated with mtDNA damage in transfusion products significantly contribute to the incidence of ARDS after massive transfusions (25).

Based on this evidence, and following a pragmatic approach, we tested the association of array-based measures of whole-blood mtDNA (wb-mtDNA) copies within 24 h of sepsis diagnosis with 28-day patient survival. We hypothesized that early wb-mtDNA measurements could be associated with mortality in patients with sepsis and ARDS.

### METHODS

#### Study Population

Peripheral blood samples and clinical information from 687 unrelated adult patients of European ancestry aged between 18 and 93 years from the network of Spanish postsurgical units and ICUs (GEN-SEP study) were used for this study (Table 1). The GEN-SEP cohort is a national, multicenter, observational study conducted in Spain between January 2002 and June 2019. For the purpose of this study, sepsis was defined according to the Third International Consensus Definitions for Sepsis (26). ARDS was defined according to the Berlin definition criteria (1). All participants gave written informed consent, and the study was approved by the Research Ethics Committee from all participating centers.

#### Measures of Whole-Blood mtDNA Levels

DNA was purified using a commercial column-based solution (Illustra™ blood genomicPrep Mini Spin Kit) from peripheral blood drawn within 24 h of sepsis diagnosis, and the concentration was measured on the Qubit 3.0 fluorometer with the dsDNA HS Assay kit (Thermo Fisher Scientific). All samples were assessed for single-nucleotide polymorphisms (SNPs) across the genome using the Axiom Genome-Wide Human CEU 1 Array data (Thermo Fisher Scientific) in the National Genotyping Center (CeGen), Universidad de Santiago de Compostela Node, Spain. The intensity data were processed using AffyPipe v2.10.0 (27), following the quality controls recommended by the manufacturer. Further genotyping quality controls were performed with the R environment v3.6.0 and PLINK v1.07 (28). Samples with genotype call rates <95% or with evidence of relatedness (PIHAT > 0.2) were removed from the study. Likewise, we excluded SNPs based on genotyping rate <95%, minor allele frequency (MAF) <0.01, and largely deviating from Hardy–Weinberg expectations (p <1×10⁻⁸). Principal components (PCs) to assess genetic heterogeneity among patients were obtained from a subset of approximately 100,000
Organ Failure Assessment.

TABLE 1 | Demographic and clinical features among sepsis, non-ARDS, and ARDS related to sepsis cases from the GEN-SEP study.

| All sepsis (N = 687) | Non-ARDS (N = 423) | ARDS (N = 264) | p-value* |
|---------------------|--------------------|----------------|----------|
| Gender, % male (N)  | 63 (430)           | 60 (255)       | 66 (175) | 0.133 |
| Age, mean years ± SD| 64 ± 15            | 64 ± 15        | 63 ± 14  | 0.139 |
| BMI, mean ± SD      | 27 ± 6             | 27 ± 5         | 29 ± 7   | 0.029 |
| SAPS, mean ± SD     | 47 ± 15            | 48 ± 15        | 49 ± 14  | 0.071 |
| APACHE II, mean ± SD| 20 ± 7             | 19 ± 7         | 22 ± 7   | <0.001 |
| Comorbidities5, % (N)| 43 (256)           | 45 (174)       | 39 (82)  | 0.237 |
| 28-day mortality, % (N)| 26 (181)         | 20 (85)        | 36 (96)  | <0.001 |
| ICU mortality, % (N) | 28 (194)           | 19 (82)        | 42 (112) | <0.001 |
| Days in hospital, mean ± SD| 35 ± 40          | 33 ± 44        | 38 ± 33  | <0.001 |
| Days in ICU, mean ± SD| 16 ± 23            | 12 ± 22        | 22 ± 23  | <0.001 |
| Organ dysfunction, % (N) | Cardiovacular | 90 (619) | 87 (375) | 92 (244) | 0.137 |
| Neurological systems | 22 (152)           | 20 (85)        | 25 (67)  | 0.128 |
| Coagulation         | 24 (168)           | 23 (98)        | 26 (70)  | 0.088 |
| Hepatic             | 17 (117)           | 18 (75)        | 16 (42)  | 0.579 |
| Renal               | 37 (252)           | 35 (150)       | 39 (102) | 0.476 |
| Respiratory         | 59 (404)           | 37 (157)       | 94 (247) | <0.001 |
| Total SOFA* mean ± SD| 8 ± 4              | 8 ± 4          | 8 ± 4    | 0.257 |
| Partial pressure of oxygen (PaO2), mean ± SD | 109 ± 47 | 116 ± 51 | 96 ± 37 | <0.001 |
| Sepsis of pulmonary origin, % (N) | 34 (229) | 25 (103) | 48 (126) | <0.001 |
| Pathogen, % (N)     | Gram-positive      | 26 (126)       | 24 (74)  | 30 (52)  | 0.151 |
| Gram-negative       | 35 (171)           | 35 (108)       | 36 (63)  | 0.747 |
| Others*             | 29 (139)           | 29 (93)        | 26 (46)  | 0.532 |

*p-value calculated between non-ARDS and ARDS patients. Comparisons for gender, comorbidities, 28-day mortality, ICU mortality, sepsis of pulmonary origin, organ dysfunction, and pathogen were conducted by a chi-square test. The rest of variables were compared using the Mann–Whitney U-test.

5Includes: cancer, age >80 years, hepatopathy, valvular disease, immunodeficiency, severe brain damage, morbid obesity, chronic disease, autoimmune disease, pregnancy, myopathy, pneumonia, and serious recurrent infections.

6Total SOFA: sum of the cardiovascular, neurological systems, coagulation, hepatic, renal, and respiratory SOFA scores.

7Includes: mixed Gram-positive and Gram-negative infection, fungi, virus, and polymicrobial.

APACHE II, Acute Physiology and Chronic Health Evaluation II; BMI, body mass index; ICU, intensive care unit; SAPS, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment.

Statistical Analyses

The statistical power of the study was estimated using the formula $\nu_{\text{event}} = \left(4 \times (Z_{\alpha} + Z_{\beta})^2\right)/\left[\ln(RR)\right]^2$ (30), supporting that as few as 88 events were needed to reach 90% statistical power. The “survival” R package 3.1-12 (31) was used to model the association between wb-mtDNA copies and 28-day survival in all patients with sepsis (N = 687), in those who developed ARDS (N = 264), and in non-ARDS patients (N = 423). Cox regressions and Kaplan–Meier analyses were performed. Sensitivity analyses were used to evaluate the effects of demographic and clinical variables on the Cox regression models. Finally, receiver operating characteristic (ROC) curves and their area under the curve (AUC) estimates were assessed with “pROC” R package 1.17.0.1 (32).

RESULTS

Demographic and clinical features of all sepsis patients and of those with or without ARDS are shown in Table 1. As expected, there were large and significant differences between ARDS and non-ARDS patients for their severity scores, hospital and ICU length of stay, mortality rate, and physiological variables such as the partial pressure of oxygen (PaO2). There were differences in the organ dysfunction among ARDS and non-ARDS patients (ANOVA, p < 0.001). However, as expected, they were due to the lung affection. On the other hand, for the pathogens, we did not observe significant differences between ARDS and non-ARDS patients (ANOVA, p = 0.127).

We first found that the association between wb-mtDNA copies within 24 h of sepsis diagnosis and 28-day survival among all patients with sepsis from GEN-SEP was significant (Table 2). We then tested the same model stratifying the sepsis patients by those who did not develop ARDS and those who developed ARDS. In all models, the proportional risk assumption was held. Although we did not observe any association between wb-mtDNA copies and 28-day survival in septic non-ARDS patients, we found a strong association in septic patients who developed ARDS (hazard ratio [HR] = 3.65, 95% confidence interval [CI] 1.39–9.59, p = 0.009). Taken together, this indicates that the significant association between the wb-mtDNA copies within 24 h of sepsis diagnosis and 28-day survival observed among all GEN-SEP patients could be explained by those developing ARDS. A Kaplan–Meier analysis of the 28-day mortality among the ARDS patients reinforced this observation (log-rank test p = 0.037) (Supplementary Figure S1).

Given that there were demographic and clinical differences between ARDS patients that survived and that did not survive (Table 3), sensitivity analyses were conducted to ensure that

TABLE 2 | Association results of wb-mtDNA copy number with 28-day mortality.

| Cohorts (N/events) | Hazard ratio (95% CI) | p-value |
|--------------------|-----------------------|---------|
| All patients (687/181) | 2.39 (1.18–4.84) | 0.015 |
| Non-ARDS (423/85) | 1.24 (0.44–3.51) | 0.683 |
| ARDS (264/96) | 3.65 (1.39–9.59) | 0.009 |

independent variants using PLINK v1.90 (29), and the first 5 PCs were used for sensitivity analysis.

To obtain a measure of the wb-mtDNA copies, we selected mtDNA probes from the array data and normalized (log R ratio) across the individual-probe intensities of the cohort following the methodology described by Tin and colleagues (11). To ensure specificity of the estimations, we used the average of the GC content-corrected intensities of the array probes targeting the human mtDNA with 100% identity, with the largest number of mismatches against the nuclear sequences based on BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Probes behaving as outliers (beyond 1.5 SD of the mean) for the corrected intensities were then removed from the analysis. These filtering steps left us with a total of 17 probes to obtain the wb-mtDNA copy estimates, which were finally available for a total of 687 sepsis patients, where 181 subjects died in the ICUs within 28 days from sepsis onset. A total of 264 patients developed ARDS, and 96 of them died within 28 days from sepsis onset (Table 1).
TABLE 3 | Demographic and clinical features of the ARDS patients.

| Survivors (N = 168) | Non-survivors (N = 96) | p-value* |
|--------------------|-----------------------|----------|
| Gender, % male (N) | 66 (111) | 67 (64) | 1.000 |
| Age, mean years ± SD | 62 ± 14 | 66 ± 13 | 0.021 |
| BMI, mean ± SD | 29 ± 7 | 27 ± 6 | 0.192 |
| SAPS, mean ± SD | 47 ± 12 | 54 ± 16 | 0.004 |
| APACHE II score, mean ± SD | 21 ± 7 | 24 ± 7 | <0.001 |
| Comorbidities, % (N) | 40 (59) | 37 (23) | 0.707 |
| Days in hospital, mean ± SD | 51 ± 35 | 17 ± 11 | <0.001 |
| Days in ICU, mean ± SD | 29 ± 26 | 11 ± 7 | <0.001 |
| Organ dysfunction, % (N) | | | |
| Cardiovascular | 92 (155) | 93 (89) | 1.000 |
| Neurological systems | 21 (35) | 34 (52) | 0.031 |
| Coagulation | 21 (35) | 36 (55) | 0.009 |
| Hepatic | 12 (20) | 23 (22) | 0.029 |
| Renal | 31 (52) | 52 (60) | 0.001 |
| Respiratory | 94 (158) | 93 (89) | 0.888 |
| Total SOFA†, mean ± SD | 8 ± 4 | 8 ± 5 | 0.834 |
| Partial pressure of oxygen (PaO2), mean ± SD | 95 ± 33 | 100 ± 44 | 0.627 |
| Sepsis of pulmonary origin, % (N) | 51 (84) | 44 (42) | 0.323 |
| Pathogen, % (N) | | | |
| Gram-positive | 29 (32) | 34 (20) | 0.537 |
| Gram-negative | 39 (45) | 31 (18) | 0.320 |
| Others* | 25 (28) | 31 (18) | 0.511 |

*p-value calculated between survivors and non-survivors. Comparisons for gender, comorbidities, sepsis of pulmonary origin, organ dysfunction, and pathogen were conducted by a chi-square test. The rest of variables were compared using the Mann‐Whitney U-test.

†Includes: cancer, age >80 years, hepatopathy, valvular disease, immunodeficiency, severe brain damage, morbid obesity, chronic disease, autoimmune disease, pregnancy, myopathy, pneumonia, and serious recurrent infections.

‡Total SOFA: sum of the cardiovascular, neurological systems, coagulation, hepatic, renal and respiratory SOFA scores.

*Includes: mixed Gram-positive and Gram-negative infection, fungi, virus and polymicrobial.

APACHE II, Acute Physiology and Chronic Health Evaluation II; BMI, body mass index; SAPS, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment.

those differences did not explain the association with wb-mtDNA. Note that there were no overall differences among ARDS patients in the organ dysfunction (ANOVA, p = 0.159) or in the pathogens (ANOVA, p = 0.985), therefore not affecting the sensitivity analyses. We found that the association was robust to model adjustments by variables that were not significantly different between survivor and non-survivor ARDS patients (e.g., gender, comorbidities, and the first five PCs of genetic heterogeneity). Likewise, the results of the univariate model with wb-mtDNA levels were similar to those with independent adjustment by age, SAPS, APACHE II score, and organ dysfunction, where the assumption of proportionality risk was held (Table 4). The only two adjusted models that did not hold for the proportionality assumption were those including as covariates the length of stay in ICU or in the hospital, for which conclusions should be taken with caution. Based on these findings, we tested multivariate models in the sensitivity analyses except those with a high proportion of missing data (i.e., SAPS) or were variable adjustments that turned into violated assumptions of the proportionality of risks in the sensitivity analyses (i.e., length of stay in ICU or in the hospital) (Table 4). We found that when including age and

TABLE 4 | Association results of wb-mtDNA levels with 28-day survival in ARDS patients adjusting the models for the variables that were significantly different by mortality group.

| Hazard ratio (95% CI) | p-value |
|----------------------|---------|
| wb-mtDNA levels | 264 | 3.65 (1.39–9.58) | 0.009 |
| Adjusted by age | 264 | 3.99 (1.48–10.87) | 0.007 |
| Adjusted by SAPS | 131 | 7.93 (1.57–39.93) | 0.012 |
| Adjusted by APACHE II score | 258 | 4.26 (1.56–11.64) | 0.005 |
| Adjusted by organ dysfunction: | | | |
| Neurological systems | 263 | 3.48 (1.30–9.27) | 0.013 |
| Coagulation | 264 | 3.93 (1.47–10.42) | 0.006 |
| Renal | 264 | 3.66 (1.39–9.62) | 0.009 |
| Adjusted by days in hospital* | 228 | 5.77 (1.70–19.53) | 0.005 |
| Adjusted by days in ICU* | 264 | 4.64 (1.67–12.87) | 0.003 |
| Adjusted by age, APACHE II | 258 | 4.51 (1.62–12.57) | 0.004 |
| Adjusted by age, APACHE II, organ dysfunction | 257 | 4.30 (1.55–12.63) | 0.006 |

*Statistically significant for the Schoenfeld test of the proportionality of risks. APACHE II, Acute Physiology and Chronic Health Evaluation II; ICU, intensive care unit; SAPS, Simplified Acute Physiology Score II.

APACHE II score, the association of wb-mtDNA with survival was similar (HR = 4.51, 95% CI = 1.62–12.47, p = 0.004) and the proportionality of risk was held. The results were similar, and the proportionality of risk was also held, when the model adjusting for age and APACHE II also included the organ dysfunction categories that reached nominal significance in the differences between ARDS patients that survived and that did not survive (HR = 4.40, 95% CI = 1.55–12.53, p = 0.006).

Among the ARDS patients, the AUC of the wb-mtDNA copy number for the 28-day survival was 0.612 (95% CI = 0.541–0.683) (Supplementary Figure S2). However, this predictive value was similar to that provided by other clinical scores routinely used in clinical settings. As an example, for the same patients, the prognostic ability of the APACHE II score reached an AUC of 0.634 (95% CI = 0.565–0.704) (Supplementary Figure S2). Combining both the wb-mtDNA copy number and the APACHE II score in the models, the AUC slightly improved to 0.676 (95% CI = 0.609–0.742) although the AUC of the two curves (wb-mtDNA alone and wb-mtDNA plus APACHE II together) were not significantly different (p = 0.062) based on DeLong’s test for two correlated ROC curves.

**DISCUSSION**

Given the multifactorial risks involved in the prognostic trajectories of ICU patients (33), the identification of an ideal biomarker for predicting outcomes is a difficult task. One of the hallmarks of ARDS is the presence of inflammatory, protein-enriched pulmonary edema (1), caused by an increase in the permeability of the lung tissue (34, 35) and elevating the risk of death (2). It has been reported that DAMPs, including mtDNA, increase endothelial permeability through neutrophil-dependent and independent pathways (36). In a sufficiently powered cohort of Spanish patients recruited from a nationwide network of postsurgical ICUs, we describe evidence supporting that...
mtDNA copies measured in peripheral blood within 24 h of sepsis diagnosis were associated with 28-day survival in patients developing ARDS. Given that the association was absent among non-ARDS patients from the same series, our findings might suggest an ARDS-specific effect.

In agreement with our findings, Nakahira and colleagues observed an association between circulating cell-free mtDNA with overall patient 28-day mortality in the ICUs and if they combined mtDNA levels with other clinical parameters, the prediction of the ICU patients improved (22). Other studies have shown that high mtDNA plasma levels could be associated with sepsis and ARDS (19, 23, 24, 37). In one of these studies, high mtDNA plasma levels and a strong association with 28-day survival were observed in patients with sepsis and septic shock (24). Supporting our results, Huang and colleagues also observed a positive association between higher mtDNA plasma levels and 28-day mortality among patients with all-cause ARDS, although their findings revealed an association only with mtDNA measures of day 7 after diagnosis (23). Likewise, mtDNA plasma levels were also analyzed in other critically ill patients, such as the patients affected by the coronavirus disease 2019 (COVID-19) (38) or trauma patients (19), where also high mtDNA levels were associated with poor prognostics or outcomes of these diseases.

Among the strengths of this study, we recognize that it was based on a well-phenotyped and clinically characterized cohort of sepsis and ARDS patients. In addition, the wb-mtDNA estimates were obtained with a method that is not subject to the sample conservation problems linked to the qPCR (39). Related to this, given that we relied on an SNP array platform, we were able to perform model adjustments by the genetic heterogeneity, which is inherent in any heterogeneous patient population. Nevertheless, our study has some major limitations as well. The main weakness is that the models lacked adjustments by platelet count or the different cell populations, precluding determination of the contribution of the different types of white blood cells and platelets to the overall wb-mtDNA copy number estimation (13, 40). Another important limitation is that we were unable to adjust the models for other relevant clinical data that can be prognostic of ARDS such as creatinine, mean arterial pressure, Glasgow coma scale, and urine, among others. The analyses also lacked longitudinal measurements that could have provided dynamical insights into mtDNA levels and outcomes (23). Besides, we have considered all the mtDNA content from the peripheral blood limiting the comparisons with other studies that have focused on the circulating cell-free fraction of mtDNA (22, 41). A final important limitation is that we were not able to infer causality.

CONCLUSIONS

Wb-mtDNA copies measured within 24 h of sepsis diagnosis are significantly associated with 28-day survival in ARDS patients. Further studies should disentangle whether this association is independent of sepsis and whether the causality of wb-mtDNA elevation is involved in the pathogenesis of ARDS.

DATA AVAILABILITY STATEMENT

The raw intensity data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee for Drug Research from the Hospital Universitario de Canarias (Code: CHUNSC_2018-16). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TH-B: data analysis, interpretation, and manuscript drafting. BG-G, HR-P, IM-R, JL-S, AC: performed the experiments, data analysis, and revision of the manuscript. MP-G, AR-P, DC, JB, AA, EG-H, NC, MG-G, EE, AM, DD, AG, JA, MS, JB, JG: performed the experiments, sample and clinical data collection, and data analysis. JV: analysis, interpretation, critical revision of the manuscript. CF: study conception and design, data analysis, interpretation, critical revision of the manuscript and conception of the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.737369/full#supplementary-material
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**Conflict of Interest:** MG-G was employed by SPECTRUM, LLC. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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