CHEST Online Supplement

Plasma Mitochondrial DNA Levels Are Associated With ARDS in Trauma and Sepsis Patients

Hilary E. Faust, MD; John P. Reilly, MD; Brian J. Anderson, MD; Caroline A.G. Ittner, PhD; Caitlyn M. Forker, BA; Peggy Zhang, BS; Benjamin A. Weaver, BS; Daniel N. Holena, MD; Paul N. Lanken, MD; Jason D. Christie, MD; Nuala J. Meyer, MD; Nilam S. Mangalmurti, MD; and Michael G.S. Shashaty, MD

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e-Appendix 1.

Cohort Details

PETROS is an ongoing prospective cohort study of patients admitted to the intensive care unit (ICU) of the University of Pennsylvania Level I trauma center, details of which have been published previously [1,2]. The MESSI cohort is an ongoing prospective cohort study of sepsis patients hospitalized in the Hospital of the University of Pennsylvania medical ICU meeting Sepsis-2 criteria for severe sepsis [3-5]. PETROS cohort inclusion criteria are injury severity score (ISS) > 15, age ≥14, and presentation within 24 hours of trauma. Patients are excluded for death or discharge within 24 hours of ICU admission or isolated severe head injury. In the PETROS cohort, plasma collection began in 2012 and our study included all patients for whom two plasma samples and phenotyping were available. Exclusion due to death prior to 48 hours was rare in PETROS, occurring in <0.3% of patients enrolled in the cohort.

MESSI cohort inclusion criteria included patients hospitalized in the medical ICU of the Hospital of the University of Pennsylvania meeting Sepsis-2 criteria. Review of the subjects after development of Sepsis-3 criteria revealed that over 95% of patients enrolled to date also met criteria for Sepsis-3[3], and those who did not had organ dysfunction that is not captured by the Sequential Organ Failure Score, such as elevated lactate. For the current study, we included only those MESSI patients who presented to the ED with sepsis, given the potentially greater confounding effects of comorbid acute conditions and variable sepsis duration at ICU admission among patients who develop sepsis during hospitalization. We performed power calculations to determine the number of patients needed to detect an association of mtDNA and ARDS based on the difference in plasma mtDNA observed between ARDS and non-ARDS patients in the PETROS cohort and the rate of ARDS in the MESSI cohort, which indicated that 120 patients would be adequate to detect this association. Just under 10% of patients enrolled in MESSI during the study time period died prior to collection of a 48-hour sample and were thus excluded from our analysis. The ARDS rate in those who died was 42.9%, compared with 37.5% for the patients in our analysis. Demographics, medical history, injury mechanism and severity (PETROS) or infectious source (MESSI), transfusion data, and laboratory, physiologic, and treatment data were collected on each patient by review of the medical record.

Mitochondrial DNA Measurement

Sample specimens were sent to the clinical laboratory in citrated vacutainers and processed for plasma within 30 minutes. Residual plasma was stored at 4°C and was transferred to -80°C within 48 hours. Plasma samples were thawed and centrifuged at 700*g* at 4°C for 5 minutes to remove any remaining suspended cells. The supernatant was then centrifuged for 15 minutes at 18,000*g* at 4°C. DNA was extracted using a commercially available kit (DNEasy, Qiagen). Human mitochondrial gene ND1, a

mitochondrial DNA-specific gene not present in nuclear DNA, was measured in triplicate with SYBR green I (Roche, Basel, Switzerland) using quantitative polymerase chain reaction (PCR) assay (Life Technologies, Carlsbad, CA)[6]. Only ND1 was used for mtDNA quantification as correlation between ND1 and other mitochondrial genes used for mtDNA quantification has been excellent in our lab and others[6,7]. Primer sequences for the mtND1 gene were as follows: forward -5'-ATACCCATGGCCAACCTCCT-3' and reverse 5'-GGGCCTTTGCGTAGTTGTAT-3'. PCR standards for NADH were amplified from DNA extracted from endothelial cell lysates and gel purified using QiaEX II Gel Extraction Kit (Qiagen). Copy number per microliter of plasma was calculated using an online copy number calculator http://cels.uri.edu/gsc/cndna.html and log-transformed to ensure normality[6].

Definition of 30-day Mortality in PETROS and MESSI Cohorts

Mortality was defined in both cohorts as death within 30 days. In the PETROS cohort, given the relatively young age of the population and lack of comorbidities, patients discharged to a location other than hospice without subsequent contact with the health system at a later date were assumed to be alive. In the MESSI cohort, mortality for patients discharged prior to 30 days without subsequent contact with the healthcare system is determined using review of records including obituaries and the Social Security Death Index (SSDI).

Definition of blood transfusion in PETROS and MESSI cohorts

In trauma, we defined RBC transfusion as number of RBC units transfused in the first 6 hours as they comprised the large majority of transfusions during resuscitation and because this time point preceded most cases of ARDS. Because transfusions were much less common in sepsis, we used RBC transfusions during the first two days in the ICU in order to capture those given prior to 48-hour mtDNA measurement.

Definition of Acute Kidney Injury

Acute kidney injury was defined in the MESSI cohort using the Kidney Disease Improving Global Outcomes (KDIGO) criteria applied over the first 6 days of admission[8]. Due to the high rate of augmented renal clearance, the Acute Kidney Injury Network (AKIN) criteria were used for the PETROS cohort, again applied over the first 6 days of admission[9].

Statistical Analysis

We limited confounder selection to avoid model overfitting based on the number of ARDS cases in each cohort. Based on our prior findings, we pre-specified mechanism of injury, shock, and injury severity score (ISS) in trauma and lung source of infection, shock, and age in sepsis [1,2,4].

Results

Patient Demographics

For the MESSI cohort, sources of infection are provided in E-Table 3. Sources are representative of critically ill patients, with pulmonary sources as the most common.

Absolute Levels of Plasma mtDNA by ARDS Status in Sepsis and Trauma Cohorts

Log-transformed values of mitochondrial DNA were used in our analyses to satisfy normality assumptions of the statistical models, but reporting these values obscures the more noticeable difference in raw plasma mtDNA levels between ARDS and non-ARDS patients. Median 48h mtDNA levels in ARDS patients approach the 75th percentile of plasma mtDNA concentration in non-ARDS patients (E-Figure 1, E-Table 4). These findings are consistent with the differences reported by Nakahira et al.

Age as a Potential Confounder of mtDNA-ARDS Association

Age has been associated with increases in circulating plasma mtDNA[10]. Age was included as a confounder in the MESSI cohort and did not affect the association of mtDNA and ARDS. In the PETROS cohort, when age was added to the multivariable model for the association of mtDNA and ARDS, age was neither associated with ARDS (OR 1.00 per year of age, 95% CI 0.99-1.02, p=0.68) nor did it change the odds ratio for the association of mtDNA and ARDS (OR for association of 48-hour mtDNA and ARDS 1.59, 95% CI 1.15-2.20, p=0.005).

Infection as a Potential Confounder of mtDNA-ARDS Association in PETROS Patients

Rates of infection in the trauma cohort were low. Forty-five of 224 (20.0%) subjects had strongly suspected infection within the first 48 hours. mtDNA on presentation and at 48-hours did not differ between subjects with infection and those without (at presentation, plasma mtDNA concentration was 12.08 log copies/uL for subjects without infection vs 12.09 log copies/uL for subjects with suspected, p=0.99, and at 48 hours, plasma mtDNA concentration was 11.82 log copies/uL for subjects without infection vs 11.87 log copies/uL for subjects with suspected infection at 48 hours, p=0.76).

Association of Change in Plasma mtDNA and Clinical Outcomes

In the PETROS cohort, change in mitochondrial DNA from presentation to 48 hours was measured in absolute difference from presentation to 48 hours was not associated with (p=0.24) or mortality (p=0.31) in unadjusted analysis. In the MESSI cohort, change in mtDNA was strongly associated with ARDS in unadjusted (p<0.001) and adjusted analyses (OR 0.48/log copy decrease in mtDNA from presentation to 48 hours, p=<0.001). Absolute decrease in mtDNA from presentation to 48 hours was also highly associated with mortality in unadjusted (p=0.007) and adjusted analyses (OR 0.66 per log decrease in

mtDNA, 95% CI 0.48-0.91, p=0.01) in the MESSI cohort. Analysis of percent change from presentation mtDNA yielded similar results for all analyses.

Sensitivity Analysis with Early vs Late ARDS

We performed a sensitivity analysis to determine whether elevated mtDNA at 48 hours preceded development of ARDS in those subjects presenting with ARDS more than 48 hours into their hospitalization. In the PETROS cohort, 23 patients developed ARDS prior to 48 hours versus 19 cases occurring after 48 hours. In unadjusted analysis, 48-hour mtDNA did not differ between patients with late ARDS compared with those without ARDS (11.9 log copies/uL vs 11.7 log copies/uL, p=0.54). There was no significant difference between plasma mtDNA levels at 48 hours in early versus late ARDS patients (p=0.18). In the MESSI cohort, only 5 subjects had ARDS after 48 hours. Plasma mtDNA at 48 hours was not different in patients with ARDS after 48 hours compared with non-ARDS patients in unadjusted analysis (11.8 log copies/uL vs 11.3 log copies/uL, p=0.41).

Association of mtDNA with ARDS Severity

In each cohort, there was not a clear trend on inspection of median mtDNA stratified by ARDS severity (PETROS: no ARDS 11.5 log copies/uL, mild ARDS 13.48 log copies/uL, moderate ARDS 12.02 log copies/uL, severe ARDS 12.09 log copies/ul; MESSI: no ARDS 10.78 log copies/uL, mild ARDS 11.99 log copies/uL, moderate ARDS 11.06 log copies/uL, and severe ARDS 11.51 log copies/uL). This may be related to the fragmentation of our group of ARDS cases when stratifying further by stage; in particular, there were a relative paucity of mild ARDS cases which may be vulnerable to underpowering (n=8 and n=5 cases of mild ARDS in each cohort respectively). Alternatively, if mtDNA is in fact in the causal pathway of ARDS, the relationship may be one of an exposure threshold rather than a dose-response.

Association of mtDNA with Acute Kidney Injury

The association of acute kidney injury (AKI) was assessed in both cohorts after excluding chronic dialysis patients using unadjusted and adjusted analyses. AKI occurred in 66 (29.5%) of PETROS patients and 68 (56.7%) of MESSI patients. Presentation mtDNA levels were not associated with AKI in either cohort. In the PETROS cohort, 48-hour mtDNA was higher in patients with AKI but the difference was not statistically significant (12.01 log copies/uL in patients with AKI vs 11.76 log copies/uL in subjects without AKI, p=0.13). However, in the MESSI cohort, AKI was associated with 48-hour mtDNA levels in both unadjusted (11.75 log copies/uL in patients with AKI vs 10.90 log copies/uL in subjects without AKI, p=0.002) and multivariable logistic regression analysis after adjusting for age, presence of shock, and history of chronic kidney disease (OR 1.57 per log copies/uL, 95% CI 1.15-2.20, p=0.005). Furthermore, 48-hour mtDNA was significantly associated with stage of AKI in ordered logistic regression analysis after adjusting for age, presence of shock and history of CKD (p=0.01).

References

1. Reilly JP, Zhao Z, Shashaty MGS, et al. Low to Moderate Air Pollutant Exposure and Acute Respiratory Distress Syndrome after Severe Trauma. Am J Respir Crit Care Med 2018.
2. Reilly JP, Anderson BJ, Mangalmurti NS, et al. The ABO Histo-Blood Group and AKI in Critically Ill Patients with Trauma or Sepsis. Clin J Am Soc Nephrol 2015;10:1911-20.
3. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 2016;315:801-10.
4. Reilly JP, Anderson BJ, Hudock KM, et al. Neutropenic sepsis is associated with distinct clinical and biological characteristics: a cohort study of severe sepsis. Crit Care 2016;20:222.
5. Bone RC, Sibbald WJ, Sprung CL. The ACCP-SCCM consensus conference on sepsis and organ failure. Chest 1992;101:1481-3.
6. Hotz MJ, Qing D, Shashaty MGS, et al. Red Blood Cells Homeostatically Bind Mitochondrial DNA through TLR9 to Maintain Quiescence and to Prevent Lung Injury. Am J Respir Crit Care Med 2018;197:470-80.
7. Krychtiuk KA, Ruhittel S, Hohensinner PJ, et al. Mitochondrial DNA and Toll-Like Receptor-9 Are Associated With Mortality in Critically Ill Patients. Crit Care Med 2015;43:2633-41.
8. Khwaja A. KDIGO clinical practice guidelines for acute kidney injury. Nephron Clin Pract 2012;120:c179-84.
9. Lopes JA, Jorge S, Silva S, et al. Prognostic utility of the acute kidney injury network (AKIN) criteria for acute kidney injury in myeloablative haematopoietic cell transplantation. Bone Marrow Transplant 2007;40:1005-6.
10. Pinti M, Cevenini E, Nasi M, et al. Circulating mitochondrial DNA increases with age and is a familiar trait: Implications for "inflamm-aging". Eur J Immunol 2014;44:1552-62.

e-Table 1: Infection Source in MESSI Patients

Infection Source	Frequency	Percent
Pulmonary	57	47.5
Abdominal/GI	18	15.0
Genitourinary	15	12.5
Skin, bone or soft tissue	10	8.3
Blood (including catheter related)	8	6.7
Head and neck	1	0.8
Sepsis source unclear	11	9.2
Total	120	100

e-Table 1: Sources of infection by organ system are provided in eTable 3. Pulmonary sepsis was the most common source, while only 9.2% of patients had an unknown source of infection.

e-Table 2: Clinical characteristics of cohorts by ARDS status

Characteristics	Cohort					
Demographics	PETROS			MESSI		
	No ARDS (n=182)	ARDS (n=41)	P value	No ARDS (n=75)	ARDS (n=45)	P value
Race Black White Asian Other	 93 (50.8) 78 (42.6) 8 (4.4) 4 (2.2)	 22 (53.7) 16 (39.0) 2 (4.9) 1 (2.4)	0.9	 35 (47) 36 (48) 0 (0) 4 (5)	 16 (36) 27 (60) 1 (2) 1 (2)	0.4
Age	33 (24-60)	44 (30-60)	0.6	60 (50-68)	62 (52-67)	0.9
Male Sex	142 (77.6)	37 (90.2)	0.07	34 (45)	15 (33)	0.3
Illness and Injury Severity						
APACHE II	16 (11-21)	23.5 (18-30)	<0.0001	33	32	0.6
Pulmonary Source	N/A	N/A		23 (31)	28 (62)	<0.001
ISS	25 (19-29)	29 (22-36)	0.01	N/A	N/A	
Blunt Injury	129 (70.5)	32 (78.0)	0.33	N/A	N/A	
Pre-ICU Shock	80 (43.7)	26 (63.4)	0.02	54 (72)	39 (87)	0.07
30 Day Mortality	5 (2.7)	12 (29.3)	<0.001	25 (33)	25 (56)	<0.001
Medical history						
Diabetes	13 (7.1)	2 (4.9)	0.34	26 (35)	19 (42)	0.4
CHF	6 (3.3)	2 (4.9)	0.21	13 (17)	8 (18)	1.0
CKD	6 (3.3)	0 (0)	0.24	15 (20)	5 (11)	0.3
HTN	44 (24.0)	11 (26.8)	0.43	46 (61)	24 (53)	0.4
Transfusions						
Subjects transfused	79 (43%)	28 (68%)	0.004	24 (32%)	20 (44)	0.2
PRBCs	4 (2-7)	5.5 (3.5-9.5)	0.004	2 (1-4)	2 (2-2)	0.6

e-Table 2. Characteristics of the PETROS and MESSI cohorts by ARDS status. Categorical variables are given with N, (%); continuous variables are reported as median, (interquartile range).

e-Table 3: Raw Plasma mtDNA Concentrations in PETROS and MESSI Patients

Cohort	**Time Point**	**ARDS Patients** (median copies/ul, IQR)	**Non-ARDS Patients** (median copies/ul, IQR)	**Overall Cohort** (median copies/ul, IQR)
PETROS	Presentation	259,735 (103,568-471,503)	152,884 (89,678-309,242)	163,552 (90,378-340,607)
	48 Hours	187,915 (67,662-513,159)	103,976 (66,623-215,115)	113,811 (66,901-244,137)
MESSI	Presentation	68,093 (26,779-140,268)	70,766 (27,950-143,937)	68,486 (27,898-142,102)
	48 Hours	128,239 (47,560-294,483)	48,476 (27,976-126,719)	60,859 (33,957-214,431)

e-Table 3: Raw plasma mtDNA concentrations in PETROS and MESSI patients, stratified by ARDS status.

e-Table 4: Adjusted Association of Plasma Mitochondrial DNA and Mortality by Cohort

Cohort	Sample	Odds Ratio	95% CI	P value
PETROS	Presentation Plasma [mtDNA]	2.06	1.29-3.30	0.003
	48-Hour Plasma [mtDNA]	1.50	0.98-3.20	0.06
MESSI	Presentation Plasma [mtDNA]	0.90	0.66- 1.22	0.494
	48-Hour Plasma [mtDNA]	1.27	0.98-1.66	0.072

e-Table 4: Association of plasma mtDNA and mortality in PETROS and MESSI cohorts, adjusted for clinical covariates. *PETROS cohort association is adjusted for ISS. **MESSI cohort association is adjusted for age, pulmonary source of infection, and pre-ICU shock

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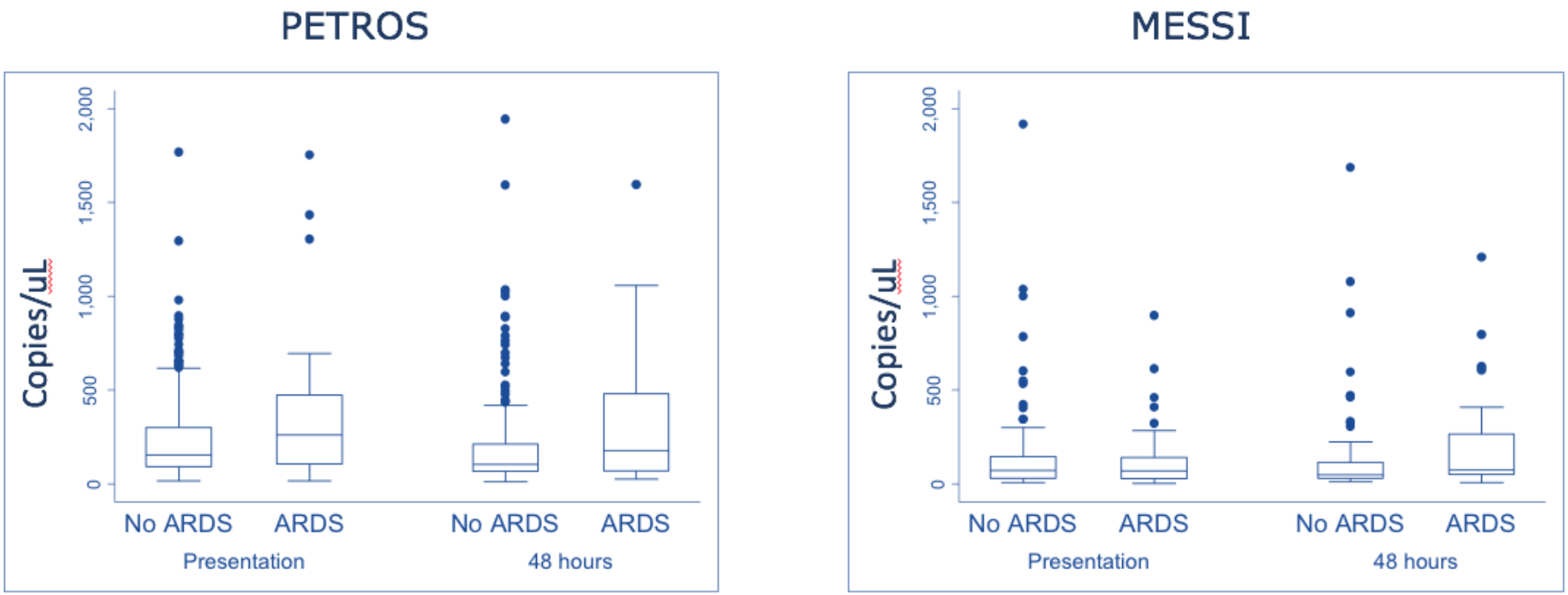
mtDNA Raw Values by ARDS and Time Point



e-Figure 1: Raw values of mtDNA as expressed in copies/uL in PETROS (A) and MESSI (B) cohorts at presentation and 48-h. Median mtDNA in ARDS patients at 48-h approaches or exceeds 75th percentile of mtDNA from non-ARDS patients in both cohorts.