Online Data Supplement

A Novel Assay for Neutrophil Extracellular Traps (NETs) Formation Independently Predicts Disseminated Intravascular Coagulation and Mortality in Critically Ill Patients

Short running head: Monitoring NETosis in critical illness

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eMethods

Patient blood sample collection and measurement

Upon ICU admission, surplus blood samples were collected daily from all patients for the first 96 hours (4 samples per patient: admission, 24 hours, 48 hours and 72 hours post-admission). Plasma was prepared by drawing peripheral blood into citrated vacutainers (4.5 ml 0.109 M + buffered sodium citrate 3.2%, Becton Dickinson, Plymouth, UK) and centrifuged for 20 minutes at 2600xg and 20°C. The resulting plasma supernatant was separated and aliquots stored at -80^{o}C. In some patients, matched sera were also isolated and stored at -80°C.Whole blood platelet, white blood cell and neutrophil counts were measured using a Beckman Coulter DxH800, thrombocytopaenia was microscopically verified. Prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen levels and D-dimers were measured using standard protocols in the coagulation laboratory of the Royal Liverpool University Hospital using an ACL TOP® 700 analyser (Werfen Ltd, UK). A panel of 27 cytokines, chemokines and angiogenic factors (General activation markers: Interleukin (IL)-1β,IL-1ra, IL-2, TNFα, IL-6, IL-15; Chemokines: IL-8, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES; T cell-related: IL-4, IL-5, IL-9, IL-10, IL-12 (p70), IL-13, IL-

17, Eotaxin, INFγ; Bone marrow-derived: IL-7, GM-CSF, G-CSF; Angiogenic factors and endothelial mitogens: bFGF, PDGF-bb, VEGF) were measured by MultiPlex (BioRad) in the plasma of normal donors and critically ill patients upon ICU admission, using a Bio-Plex 100 according to manufacturers' instructions.

Neutrophil isolation

Citrated blood was drawn from healthy donors following written informed consent according to protocol approved by Liverpool University Interventional Ethical Committee (Ref: RETH000685). Neutrophils were purified using two-step gradient centrifugation. Leukocytes were isolated using Histopaque®-1077 (Sigma-Aldrich, UK) and further purified using a Percoll (Sigma-Aldrich**)** gradient to isolate neutrophils (>90% purity).

NETs specific neutralization

To examine the role of interleukin 8 (IL-8) in NETs formation, normal plasma was supplemented with IL-8 (100 pg/ml) and incubated with normal neutrophils for 4 hours prior to fixation and staining. Neutrophils were also pre-incubated for 10 minutes with IL-8 inhibitors: anti-IL-8 mAb (R&D Systems) (1 μg/ml), Cl-amidine (Cambridge biolabs) (10 μM), Reparixin (Dempé) (250 μg/ml) or AZD5069 (AstraZeneca) (10 nM), or a MAPK signalling inhibitor: U0126 (Sigma) (50 μM) prior to adding plasma.

Comparator NETs assays

Circulating histones levels were determined by Western blot, according to our previous publications.(1-3) Cell free DNA (cfDNA) was fluorescently determined using SYTOX green, as previously described.(4) Briefly, 25μl patient plasma was diluted in a final volume of 100 μl and incubated with SYTOX

green (2 μM final concentration). cfDNA was then determined using a fluorescent plater reader (Ex:488nm/Em:523nm) using known concentrations of genomic DNA as standards. Circulating Myeloperoxidase (MPO) (ThermoFisher) were measured by ELISA according to the manufacturer's instructions. Circulating MPO-DNA complex levels were determined using by ELISA using an anti-MPO (SantaCruz Biotech) capture antibody and anti-dsDNA antibody (ROCHE) as a detector. Citrullinated Histone 3 (Cit-H3) was determined in patient plasma by Western blot using a primary antibody against Cit-H3 (Abcam), data were not included due to non-specificity of the antibody.

Western blot analysis of ERK activation

Western blot analysis was performed on normal healthy neutrophils treated with plasma. To investigate the effect of IL-8 treatment on the activation of ERK neutrophils were treated for 0, 15, 30, 45 and 60 minutes with normal plasma supplemented with IL-8 (100 pg/ml). To establish the role of circulating IL-8 patients in activating ERK, normal heatly neutrophils were incubated without or with pre-treatment with anti-IL-8 mAb (1 μg/ml), prior to treatment with septic patient plasm for 15 minutes. Following treatment, samples were lysed and separated by SDS-PAGE followed by transfer onto PVDF membrane. Following blocking, membranes were probed with 1:1000 anti-pERK antibody (Santa Cruz) overnight and 1:10,000 anti-mouse secondary antibody for 45 mins. Bands were visualised using ECL (Enhanced Chemiluminescence). To ensure equal loading, membranes were stripped using stripping buffer for 30 mins at 50°C and blocked. Membranes were probed with 1:1000 anti-ERK antibody (Santa Cruz) overnight and 1:10,000 anti-rabbit secondary antibody for 45 mins. Bands were visualised using ECL and densitometry performed to determine pERK/ERK ratio.

Multivariate logistical regression analysis

Prior to construction of the multivariate model, we selected variables that could plausibly be associated with DIC and mortality. These variables were tested in univariate analysis to determine their association as are displayed in Table E1. For the multivariate, analysis we selected variables independent from one another with a univariate analysis p value of less than 0.1. Following on from this, we constructed the final multivariate model using a standard stepwise approach, sequentially removing variables with a p value of more than 0.1.

eReferences

1. Alhamdi Y, Abrams ST, Cheng Z, Jing S, Su D, Liu Z, Lane S, Welters I, Wang G, Toh CH. Circulating histones are major mediators of cardiac injury in patients with sepsis. *Crit Care Med* 2015;43:2094-2103.

2. Abrams ST, Zhang N, Manson J, Liu T, Dart C, Baluwa F, Wang SS, Brohi K, Kipar A, Yu W, Wang G, Toh CH. Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med* 2013;187:160-169.

3. Alhamdi Y, Abrams ST, Lane S, Wang G, Toh CH. Histone-associated thrombocytopenia in patients who are critically ill. *JAMA* 2016;315:817-819.

4. Fuchs TA, Kremer Hovinga JA, Schatzberg D, Wagner DD, Lämmle B. Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. *Blood* 2012;120:1157-1164.

	Crude Odds ratio	*P* value*
DIC		
Initial diagnosis		
Sepsis	REF	
Cardiovascular	0.000 [0.000-0.000]	.998
CNS	0.000 [0.000-0.000]	.998
Gastro	0.258 [0.058-1.151]	.076
Renal	0.000 [0.000-0.000]	.999
Respiratory	0.087 [0.019-0.661]	**.018**
Trauma	0.222 [0.064-0.770]	**.018**
Hypotension	2.220 [1.001-4.923]	.050
ARDS (P/F)	1.000 [0.996-1.003]	.945
APACHEII	1.144 [1.084-1.208]	**<.0001**
Bacteraemia	7.625 [3.027-19.205]	**<.0001**
Source of infection		
No infection	REF	
Respiratory	7.843 [2.625-23.431]	**<.0001**
Abdomen	5.337 [1.702-16.736]	**.004**
Neuro	11.091 [2.724-45.150]	**.001**
Other	10.893 [2.953-40.183]	**<.0001**
IL-8	1.000 [1.000-1.000]	**.049**
Age	0.988 [0.967-1.009]	.255
Gender	0.538 [0.249-1.165]	.116
Mortality		
Initial diagnosis		
Sepsis	REF	
Cardiovascular	1.036 [0.442-2.427]	.935
CNS	0.184 [0.024-1.442]	.107
Gastro	0.461 [0.167-1.273]	.135
Renal	0.000 [0.000-0.000]	.999
Respiratory	0.395 [0.155-1.003]	.051
Trauma	0.417 [0.182-0.957]	**.039**
Hypotension	1.113 [0.641-1.933]	.704
ARDS (P/F)	0.998 [0.996-1.001]	**.204**
APACHEII	1.087 [1.047-1.128]	**<.0001**
Bacteraemia	2.109 [1.228-3.623]	**.007**

Source of infection		
No infection	REF	
Respiratory	2.914 [1.421-5.973]	**.004**
Abdomen	1.457 [0.651-3.233]	.355
Neuro	1.166 [0.317-4.280]	.817
Other	2.914 [1.194-7.109]	**.019**
IL-8	1.000 [1.000-1.000]	.380
Age	1.014 [0.997-1.031]	.102
Gender	0.850 [0.498-1.451]	.551

Table E1. Univariate analysis for the prediction of DIC and mortality.
To construct the multivariate model an independent variable was included if univariate analysis indicated a $p<0.1$ and gender (convention). NETosis, IL-8, APACHEII, source of infection (categorical) and gender within the initial multivariate analysis and removed non-significant variables ($p>0.1$) in a stepwise method until all remaining variables were significant. We performed multivariate analysis with the dependent variables of DIC and Mortality. Stepwise regression for DIC, IL-8 ($p=0.117$), source of infection ($p = 0.825$, 0.361, 0.679 and 0.936 respectively) and gender ($p=0.175$) were removed. Stepwise regression for mortality, IL-8 ($p=0.984$), source of infection ($p = 0.814$, 0.348, 0.535 and 0.510 respectively) and gender ($p=0.826$) were removed. Our final models for predicting DIC and mortality are adjusted for APACHE II (Table 3).
* P value for crude odds ratio to predict DIC and mortality.

	Normal	Correlation (R value)	Absent NETs	Mild NETs	Moderate NETs	Strong NETs	P value*
Total number (n)			75	170	49	47	
NETs-related markers							
cfDNA (ng/ml), Median [IQR]	245.70 [154.63-443.21]	-.134	617.9 [378.8-971.3] †	521.8 [237.6-1015.8]	530.3 [367.9-990.5]	496.0 [316.8-1237.4]	.864
MPO (ng/ml), Median [IQR]	12.40 [4.55-35.39]	.327	97.4 [36.8-180.8] †	65.5 [39.4-96.1] †	154.4 [51.7-312.2] †, §	101.1 [33.1-192.2] ‡	.204
DNA-MPO (AU), Median [IQR]	0.97 [0.89-1.11]	.158	0.89 [0.83-1.16]	0.96 [0.84-1.19]	0.94 [0.84-1.08]	0.92 [0.82-1.10]	.982

Table E2. Circulating NETs-related markers in absent, mild, moderate and strong NETs formation in ICU patients

* P value for comparisons of absent vs mild vs moderate vs strong NETs patients collectively. Performed using Kruskall-Wallis test for continuous variables and Chi-squared test for categorical variables. † Significant vs normal controls. ‡ Significant vs absent NETs patients. § Significant vs mild NETs patients. R correlation with percentage NETs performed using Spearman's rank.

	Normal	Correlation (R value)	Absent NETs	Mild NETs	Moderate NETs	Strong NETs	P value*
Total number (n)			75	170	49	47	
General activation							
IL-1β (pg/ml), Median [IQR]	5.65 [4.65-6.34]	-.293	5.86 [3.61-8.98]	5.51 [3.33-7.88]	4.95 [3.72-9.37]	4.51 [2.88-8.62]	.773
IL-1ra (pg/ml), Median [IQR]	177.58 [153.24-247.02]	-.286	1098.86 [318.56-6046.29] †	1025.10 [270.46-5705.21] †	443.08 [190.69-2598.12] †	522.78 [170.83-5580.57] †	.485
IL-2 (pg/ml), Median [IQR]	0.00 [0.00-3.38]	-.297	0.00 [0.00-7.78]	0.00 [0.00-3.53]	0.00 [0.00-0.00] §	0.00 [0.00-4.23]	.176
TNFα (pg/ml), Median [IQR]	96.50 [61.56-125.53]	-.265	113.80 [60.41-158.11]	70.51 [51.83-138.10]	94.47 [43.99-150.35]	100.57 [40.56-171.85]	.663
IL-6 (pg/ml), Median [IQR]	15.15 [12.36-19.52]	.265	161.99 [69.65-1123.54]	445.30 [61.09-1216.67] †	146.80 [40.43-608.98] †	343.86 [85.97-2449.15] †, ‖	.177
IL-15 (pg/ml), Median [IQR]	0.00 [0.00-12.59]	-.241	12.53 [0.00-79.01]	20.91 [0.00-45.54] †	0.00 [0.00-32.41]	24.55 [0.00-55.59] †	.279
Chemokines							
IL-8 (pg/ml), Median [IQR]	29.31 [26.74-55.93]	.529	63.27 [39.28-143.31] †	128.89 [48.47-255.64] †	114.36 [52.76-314.29] †	127.53 [73.50-331.65] †, ‡	.069
IP-10 (pg/ml), Median [IQR]	540.68 [379.22-656.62]	.221	1118.65 [668.75-6877.97] †	1037.15 [527.40-1914.35] †	1632.67 [494.98-7669.59] †	1077.73 [683.36-2954.90] †	.529
MCP-1 (pg/ml), Median [IQR]	4.90 [0.00-19.81]	.235	58.00 [22.67-167.01] †	87.17 [27.21-200.48] †	31.54 [2.35-181.35] §	112.28 [33.96-237.29] †	.172
MIP-1a (pg/ml), Median [IQR]	5.93 [4.14-7.62]	-.230	5.14 [2.98-7.81]	4.37 [2.98-6.09]	4.92 [3.33-7.61]	4.45 [2.36-6.47]	.768
MIP-1b (pg/ml), Median [IQR]	23.64 [14.72-34.15]	-.307	92.75 [71.93-240.96] †	100.55 [61.86-172.69] †	96.33 [68.23-164.86] †	91.12 [60.66-139.34] †	.649
RANTES (pg/ml), Median [IQR]	3142.25 [1278.65-3558.20]	-.560	4690.15 [2372.52-6221.61]	5085.97 [3566.83-7098.21] †	3164.76 [1716.22-4301.35] §	2905.09 [14445.22-4311.39] ‡, §	**<.0001**
T cell-related							
IL-4 (pg/ml), Median [IQR]	6.19 [5.74-7.23]	-.463	6.36 [4.65-8.42]	6.90 [5.30-8.08]	5.86 [4.12-7.94]	5.00 [3.19-7.60] §	.126
IL-5 (pg/ml), Median [IQR]	20.18 [8.99-21.52]	-.608	11.36 [4.79-16.28] †	10.55 [4.79-15.18] †	9.08 [1.90-15.29] †	1.63 [0.00-6.40] †, ‡, §, ‖	**.001**
IL-9 (pg/ml), Median [IQR]	17.54 [14.05-44.76]	-.476	56.71 [23.00-94.63] †	47.95 [26.42-84.40] †	30.53 [21.40-49.81] §	28.25 [18.08-59.16] ‡, §	**.043**
IL-10 (pg/ml), Median [IQR]	17.81 [12.79-26.34]	-.266	31.99 [17.63-136.96]	33.80 [20.01-43.01] †	21.20 [13.85-74.45]	37.54 [16.94-79.03]	.432
IL-12 (p70) (pg/ml), Median [IQR]	8.88 [2.73-26.86]	-.576	16.05 [7.46-21.45]	13.95 [9.04-18.96]	11.09 [6.10-19.36]	7.74 [3.10-13.60] ‡, §	**.004**
IL-13 (pg/ml), Median [IQR]	8.62 [3.77-13.82]	-.528	5.31 [2.14-7.81]	5.15 [3.91-9.85]	5.31 [2.19-8.98]	2.71 [0.11-4.39] †, §, ‖	**.009**
IL-17 (pg/ml), Median [IQR]	29.13 [7.53-33.46]	-.512	39.07 [26.70-71.62]	44.29 [21.38-71.00] †	25.41 [15.91-60.50]	21.74 [5.07-39.31] ‡, §	**.007**
Eotaxin (pg/ml), Median [IQR]	87.97 [62.29-113.57]	-.200	95.45 [58.38-127.69]	93.91 [65.52-123.15]	81.49 [58.91-133.11]	91.30 [61.74-119.68]	.960
INFγ (pg/ml), Median [IQR]	162.57 [124.31-201.07]	-.369	162.97 [119.33-269.09]	146.16 [122.53-205.00]	163.95 [119.55-241.74]	134.46 [79.72-223.60]	.415
Bone marrow-derived							

IL-7 (pg/ml), Median [IQR]	22.05 [15.07-29.12]	-.223	17.72 [8.73-22.44]	14.91 [8.57-23.56]	16.36 [7.48-23.51]	14.38 [5.43-30.81]	.973
GM-CSF (pg/ml), Median [IQR]	0.00 [0.00-0.00]	-.365	90.08 [0.00-177.10] †	66.70 [31.51-143.91] †	0.00 [0.00-55.59] ‡, §	51.78 [0.00-143.58] †, ‖	.006
G-CSF (pg/ml), Median [IQR]	112.28 [108.85-139.09]	-.077	152.70 [112.77-318.01]	226.19 [90.74-821.29]	117.67 [79.94-364.42]	178.59 [76.70-738.95]	**.509**
Angiogenic factors and endothelial mitogens							
bFGF (pg/ml), Median [IQR]	32.85 [10.92-153.67]	-.529	64.80 [50.95-92.73]	77.01 [51.64-99.15]	41.55 [18.20-62.48] ‡, §	48.86 [22.89-76.03] ‡, §	<.0001
PDGF-bb (pg/ml), Median [IQR]	636.75 [152.18-863.18]	-.458	528.68 [147.68-1308.47]	662.11 [284.66-888.55]	455.37 [138.82-825.18]	324.95 [122.72-653.54] §	.196
VEGF (pg/ml), Median [IQR]	11.99 [2.21-62.04]	-.464	78.93 [29.29-112.58] †	65.28 [35.64-107.89] †	54.48 [20.10-98.32] †	49.91 [14.05-82.45] §	.105

Table E3. Circulating cytokine levels in absent, mild, moderate and strong NETs formation in patients on ICU admission.

* P value for comparisons of absent vs mild vs moderate vs strong NETs-formation in ICU patients. Performed using Kruskall-Wallis test for continuous variables and Chi-squared test for categorical variables. † Significant vs Normal controls. ‡ Significant vs absent NETs patients. § Significant vs mild NETs patients. ‖ Significant vs moderate NETs patients. R correlation with percentage NETs in patient samples performed using Spearman's rank.

Figure E1. CONSORT diagram illustrating patients' initial recruitment, excluded groups and final study number

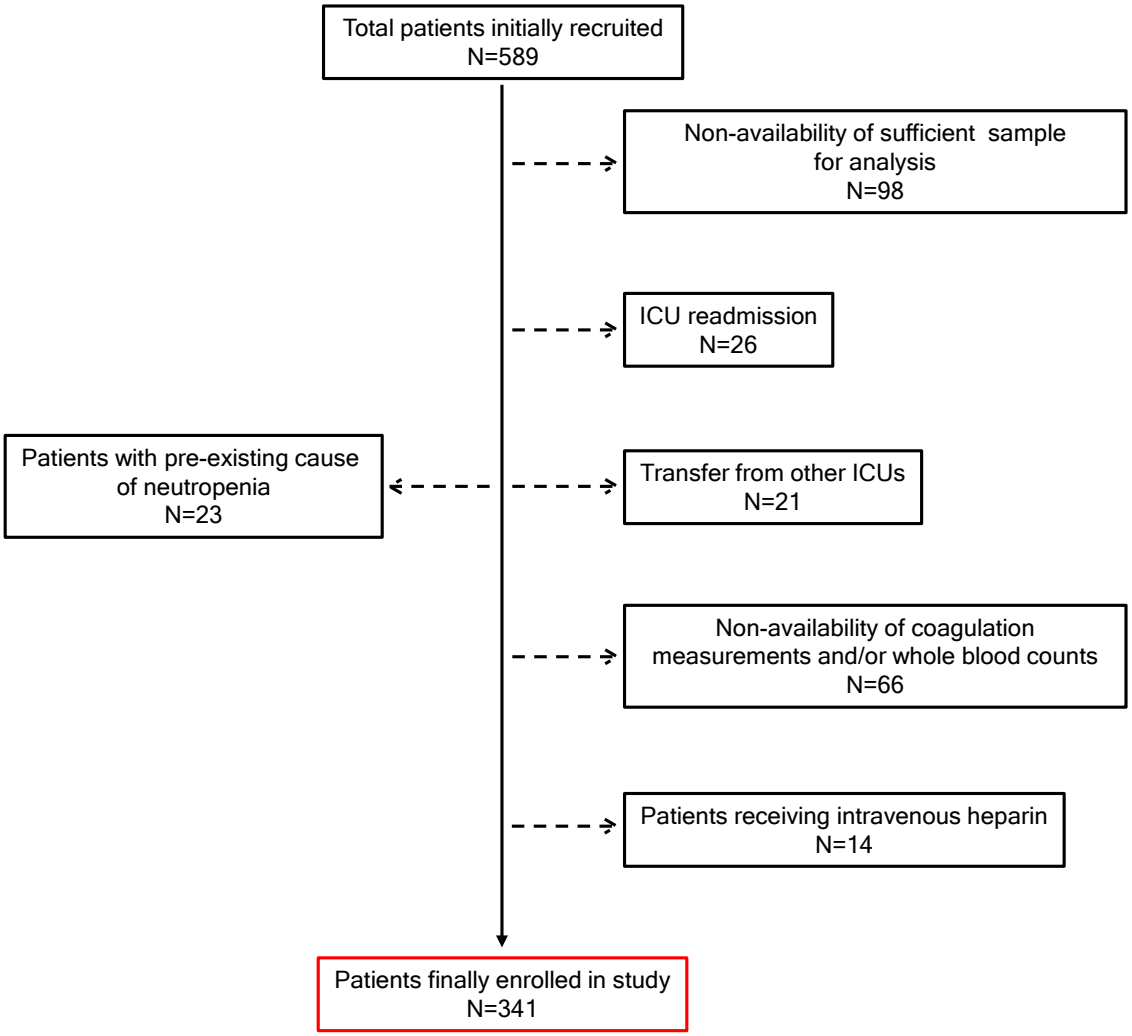

Figure E2. IL-8 induces MAPK activation in neutrophils.
Isolated neutrophils were treated with normal plasma supplemented with IL-8 (100 pg/ml) for indicated time duration. Western blot analysis of ERK activation (pERK/ERK ratio) was then determined relative to T=0.

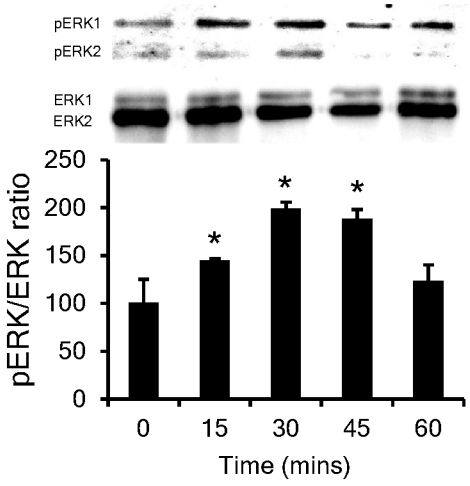

Figure E3. The assay measuring NETs-formation in patient plasma is distinct from NETs-related assays