ERJ Express. Published on March 8, 2018 as doi: 10.1183/13993003.01389-2017

EUROPEAN RESPIRATORY *journal*

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Original article

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Please cite this article as: Ebrahimi F, Giaglis S, Hahn S, *et al*. Markers of Neutrophil Extracellular Traps Predict Adverse Outcome in Community-Acquired Pneumonia. *Eur Respir J* 2018; in press (https://doi.org/10.1183/13993003.01389-2017).

Markers of Neutrophil Extracellular Traps Predict Adverse Outcome in Community-Acquired Pneumonia

Secondary Analysis of a Randomised Controlled Trial

Fahim Ebrahimi MD[1], Stavros Giaglis PhD[2,3], Sinuhe Hahn PhD[3], Claudine A. Blum MD[1,4], Christine Baumgartner MD[5], Alexander Kutz MD[4], Shane Vontelin van Breda[2,3], Beat Mueller MD[4], Philipp Schuetz MD[4], Mirjam Christ-Crain MD[1*] and Paul Hasler MD[2*†]

[1] Department of Internal Medicine, Division of Endocrinology, Diabetes and Clinical Nutrition, University of Basel Hospital, Basel, Switzerland

[2] Department of Rheumatology, Kantonsspital Aarau, Aarau, Switzerland

[3] Department of Biomedicine / University Women's Hospital, University of Basel Hospital, Basel, Switzerland

[4] University Department of Medicine, Kantonsspital Aarau, Aarau, Switzerland

[5] Department of General Internal Medicine, Inselspital, Bern University Hospital, Bern, Switzerland.

*These authors share senior authorship.

† **Corresponding author:**
b) Prof. Paul Hasler
Medical University Department, Division of Rheumatology Kantonsspital Aarau
Tellstrasse, CH-5000 Aarau, Switzerland.
Phone: + 41 62 838 4692
Fax: +41 62 838 4630
E-Mail: paul.hasler@ksa.ch

This article has an online data supplement, which is accessible from this issue's table of content online.

Take Home Message:

Neutrophil extracellular traps predict higher risk for clinical instability and 30-day mortality in pneumonia.

Abstract

Neutrophil extracellular traps (NETs) are a hallmark of the immune response in inflammatory diseases. However, the role of NETs in community-acquired pneumonia is unknown. This study aims to characterize the impact of NETs on clinical outcomes in pneumonia.

This is a secondary analysis of a randomized-controlled, multicentre trial. Patients with community-acquired pneumonia were randomly assigned to either prednisone 50 mg or placebo for 7 days. The primary endpoint was time to clinical stability; main secondary endpoints were length of hospital stay and mortality.

In total 310 patients were included in the analysis. Levels of cell-free nucleosomes as surrogate markers of NETosis were significantly increased at admission and declined over 7 days. NETs were significantly associated with reduced hazards of clinical stability and hospital discharge in multivariable adjusted analyses. Moreover, NETs were associated with a 3.8-fold increased adjusted odds ratio of 30-day mortality. Prednisone treatment modified circulatory NET levels and was associated with beneficial outcome.

Community-acquired pneumonia is accompanied by pronounced NET formation. Patients with elevated serum NET markers were at higher risk for clinical instability, prolonged length of hospital stay and 30-day all-cause mortality. NETs represent a novel marker for outcome and a possible target for adjunct treatments of pneumonia.

Keywords: Innate immunity, Pneumonia, Time to clinical stability, Mortality, Corticosteroids.

Support statement: This study was supported by grants from the Swiss National Science Foundation (SNSF PP0P3_123346) to MCC and by the Nora van Meeuwen Häfliger Stiftung and the Gottfried & Julia Bangerter-Rhyner Stiftung. PH received support from the Research Council of the Kantonsspital Aarau. PS was supported by the SNSF (PP00P3_150531 / 1). FE was supported by Research Funds of the University of Basel.

Conflict of interest: Disclosures can be found alongside this article at erj.ersjournals.com

Introduction

As a severe disease with high frequencies of morbidity and mortality, community-acquired pneumonia (CAP) ranks as one of the leading causes of hospital admission and is responsible for major socioeconomic costs [1]. Despite advances in antibiotic treatment and adjunct measures such as corticosteroids, hospitalized patients have persistently high mortality rates in the range of 5% to 15% [2]. Limited respiratory function and pulmonary, cardiovascular or metabolic comorbidities increase the likelihood of a severe course of CAP and associated complications [3].

The immediate immune response to the inoculation, proliferation and spread of pathogenic microbes in the lower airways is primarily borne by polymorphonuclear neutrophil granulocytes (neutrophils), the most abundant leukocytes in humans. Once primed, they migrate from the blood vessels through the bronchial and alveolar interstitium to engage the offending organisms within the airspaces [4]. They also target microbes invading the pulmonary parenchyma or crossing into the bloodstream. The weapons at their disposal are the generation of reactive oxygen species (ROS), phagocytosis, release of granular enzymes, and, as recently discovered, the extrusion of chromosomal DNA in a reticular array termed neutrophil extracellular traps (NETs) [5]. In addition, they release chemokines and cytokines that recruit further cells of the innate and specific arms of the immune system, firing up inflammation that can be harmful and cause pulmonary dysfunction.

NETs consist of chromatin complexes of cell-free DNA (cfDNA) and histones, with attached neutrophil granular proteins (e.g., neutrophil elastase (NE), myeloperoxidase (MPO), and cathepsin G), which trap and kill infectious microbes

[5]. The process of NET formation entails a novel form of cell death that has been termed NETosis [6].

NETs have been extensively investigated in the laboratory and in inflammatory conditions such as preeclampsia, coagulation disorders, atherosclerosis and autoimmune diseases (e.g. rheumatoid arthritis, lupus erythematodes) [7, 8]. However, clinical data on the role of NETs in infectious diseases are limited to case series in septicemia. The characteristics of neutrophil activation to generate NETs in the initiation and resolution of pulmonary inflammation during the course of lower respiratory tract infections including CAP remain unknown.

Local and systemic effects of inflammation giving rise to measurable changes of mediators in the blood may be clinically useful for the guidance of decisions on therapy and for predicting the outcome of severe lower respiratory tract infections. Therefore, we aimed to explore the pattern and temporal dynamics of markers of NET formation and their associations with disease severity and relevant outcome measures of CAP. To investigate these questions, we chose a well-defined cohort of patients with CAP from a previous multicentre study, in which we recently showed that adjunct corticosteroids meaningfully shorten the time to clinical stability (TTCS) and length of hospital stay (LOS) compared to placebo [9].

Materials and Methods

STUDY SUBJECTS AND DESIGN This is a secondary analysis of an investigator-initiated, double-blind, randomised, placebo-controlled multicentre trial, in which adjunct treatment with prednisone in CAP was investigated. Details of the trial design and results have been published [9].

In brief, consecutive patients (18 years or older) presenting with CAP were enrolled at emergency departments or medical wards of seven tertiary care hospitals in Switzerland within 24 h of presentation.

The conduct of the trial adhered to the declaration of Helsinki and Good Clinical Practice Guidelines, and ethical committees of all participating hospitals approved the study before patient recruitment. The trial was registered on ClinicalTrials.gov (study ID: NCT00973154).

METHODS Eligible patients were randomly assigned (1:1 ratio) to receive either 50 mg of prednisone or placebo daily for 7 days. Patients, treating physicians, investigators, and data assessors were masked to treatment allocation.

Informed consent was obtained within 24 h of admission to hospital. All patients were treated according to international CAP consensus guidelines [10]. Baseline data included medical history, relevant comorbidities, clinical variables relating to pneumonia and all variables required for the calculation of the pneumonia severity index (PSI) [11]. Blood samples obtained over the course of 7 days after admission were used to investigate the temporal dynamics of systemic NET surrogate markers. Clinical study data were gathered up to 30 days after admission. Structured follow-up telephone interviews for secondary outcomes after discharge were performed on day 30.

ANALYSIS

The primary endpoint was TTCS, defined as time to clinical stabilization of vital signs at two consecutive measurements ≥ 12 h apart [9]. Secondary endpoints included time to effective hospital discharge, all-cause mortality, duration of intravenous and overall antibiotic treatment and CAP complications (including recurrence, acute respiratory distress syndrome, empyema, nosocomial infections until day 30, severe adverse events possibly related to CAP, intensive care unit [ICU] admission and re-admission to hospital).

Analysis of surrogates of neutrophil extracellular traps Blood samples (plasma and serum) from each patient were collected upon emergency department admission and on days 3, 5, and 7, respectively, and processed as previously described [9].

Cell-free nucleosomes as surrogate of NETosis were measured in serum samples of the total study population using the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics, Basel, Switzerland). The concentrations of neutrophil elastase (NE) and myeloperoxidase (MPO) were measured in sera and plasma by sandwich ELISA, utilizing the Elastase/a1-PI complex ELISA Kit (Calbiochem m/EMD, Gibbstown, NJ, USA) and the human MPO ELISA kit (Hycult Biotech, Plymouth Meeting, PA, USA), respectively. To specifically characterize the source of cell-free nucleosomes as NET-associated MPO/DNA complexes, MPO-specific capture and subsequent DNA-specific detection antibodies were used as previously described [12]. For detailed methodology see online supplementary material (**Figure S3**).

Statistical analysis Unless stated otherwise, categorical variables are expressed as n (percentage) and continuous variables as medians [interquartile range (IQR)]. In order to assess an integrated value of NETs surrogates over 7 days, area under

the concentration curves (AUC) were calculated. For AUC calculations missing NETs values were imputed using multiple imputation.

For temporal changes in biomarker levels, a multilevel mixed-effects linear regression model was applied. Associations of comorbidities with baseline levels of cell-free nucleosomes were analyzed by univariate and multivariate linear regression models. For the primary endpoint, we used a multivariable Cox proportional hazards model based on a binary outcome of achieving or not achieving clinical stability. For all secondary endpoints, we calculated unadjusted and adjusted estimates of the effect size and corresponding 95% confidence intervals (CIs) using linear, logistic, or Cox proportional hazards regression as appropriate. All multivariate models were adjusted for the same variables: treatment group, patient age, gender, BMI, smoking status, pneumonia severity index (PSI), and comorbidities (congestive heart failure, chronic kidney disease, cerebrovascular disease, peripheral artery occlusive disease, COPD, asthma), as well as clinical variables (days of symptoms before admission, SIRS criteria (systemic inflammatory response syndrome), C-reactive protein and white blood cell count). Kaplan–Meier curves were used to illustrate TTCS based on NET quartiles (highest versus lower three).

All statistical analyses were performed using STATA 14.2 (Stata Corp, College Station, TX, USA) and tests were done at a two-sided 5% significance level with two-sided 95% CIs.

Results

Patient characteristics Overall, 310 patients were randomly selected from the total study population of 785 patients for the current analysis. Baseline characteristics of treatment and placebo subgroups are presented in **Table 1**. The median age was 75 years and 64% were male. The burden of pre-existing pulmonary disease was relatively low, with a positive smoking history in 23.2% and a history of chronic obstructive pulmonary disease (COPD) in 17.4%. At hospital admission, the median duration of symptoms was 4 days, and approximately one-fifth (21.9%) of the patients had been pre-treated with an antibiotic. Overall, 78.5% of the patients had 2 or more SIRS criteria and about half (53.6%) had severe pneumonia classified in the high-risk PSI classes IV and V, respectively.

Temporal dynamics of neutrophil extracellular traps At baseline, serum cell-free nucleosomes as surrogates of NETs formation were highly increased (2.67 ± 1.32 absorbance units [AUs]) compared with values in healthy controls reported as approximately 0.2 AUs [13, 15, 17, 18]. They subsided gradually until day 7 (day 3, 1.48 ± 1.03 AUs, *p<0.0005*; day 5, 1.19 ± 0.87 AUs, *p<0.0005*; day 7, 0.98 ± 0.79 AUs, *p=0.042*; **Figure 1**), when they were still markedly elevated compared to values of randomly selected healthy controls (characteristics of healthy controls in Supplementary **Table S1**). In the plasma, cell-free nucleosomes were much lower and maximum values were reached at day 3 (data not shown). Quantification of MPO-DNA complexes showed a high correlation with values of cell-free nucleosomes, indicating that they represent NETs (r=0.55, R^2=0.3, *p=0.012;* online data supplement **Figure S3**).

Associations of initial NETs surrogates with demographic characteristics, comorbidities and clinical variables Multivariate linear regression models were used to investigate predictors of increased markers of NETosis at baseline. In univariate analysis, advanced age was associated with decreased levels of NETs (data not shown), though after

full adjustment for multiple covariates, this association was no longer significant (**Table 2**). Smokers with a greater past exposure to tobacco, as measured by packyears, had significantly lower NET levels on hospital admission (-0.05 AU per 10 packyears, *p=0.042*). There was likewise an inverse association of NET levels with heart failure. Patients with a history of heart failure had significantly lower NET levels on hospital admission compared to patients without heart failure (2.33 ± 1.45 AUs vs. 2.76 ± 1.28 AUs; *p=0.036*). This association remained robust in a fully adjusted model. Since NETs are generated by neutrophils, NET levels were correspondingly associated with elevations of white blood cell counts as well as neutrophil counts (**Table 2**).

Association between markers of NETs and disease outcome measures Relevant disease outcome measures were analyzed for NETs surrogates at baseline as well as for the AUCs, integrating values of NETs surrogates over 7 days. The primary endpoint, median TTCS was significantly longer in patients from the highest AUC NET quartile (5.0 days; IQR 2.6–9.0) compared to the lower three quartiles (4.0 days; IQR 2.0–7.4) with an adjusted hazard ratio (HR) of 0.97 (95% CI 0.94–0.99, *p=0.041*; **Figure 2** and **Table 3**); a HR < 1.0 corresponding to prolonged TTCS. For high baseline NET levels, a trend towards prolonged TTCS was noted (adjusted HR 0.91, 95% CI 0.82–1.01, *p=0.088*).

Median time to effective discharge from hospital was longer in patients with highest quartile of AUC NET levels (9.0 days; IQR 5.0–14.0) compared to the three lower quartiles (7.0 days; IQR 5.0–11.0) with an adjusted HR of 0.90 (95% CI 0.82–0.99, *p=0.042*). In fully adjusted logistic regression analyses, increased NET levels at admission were associated with a 3.8-fold odds ratio of 30-day mortality. Also, a step-wise increase in mortality was found with mounting quartiles of NETs.

Higher markers of NETs at baseline were associated with a 0.52 day prolongation of intravenous antibiotic treatment (regression coefficient 0.52, 95% CI 0.1–0.94, *p=0.015*).

There was no association of NETs with a composite endpoint of complications connected with community-acquired pneumonia or the probability of ICU admission (**Table 3**).

Effects of corticosteroids on NETs In accordance with the main results from the original cohort, randomised treatment with prednisone over 7 days was associated with a significantly shorter TTCS (3.4 days, IQR 2.0–6.0 vs. 5.4 days, IQR 3.0–9.0) and length of hospital stay (7.0 days, IQR 5.0–11.0 vs. 8.0 days, IQR 5.0–13.0).

Corticosteroid treatment correlated with a slower decrease in levels of NETs surrogates over time (**Figure 3**). While there was no significant difference in mean values of cell-free nucleosomes as NET markers up to 3 days, corticosteroid treatment correlated with significantly higher NET formation on day 5 compared to placebo (1.09 ± 0.88 AUs in placebo group vs. 1.31 ± 0.84 AUs in steroid group, *p<0.023*) and day 7 (0.79 ± 0.74 AUs vs. 1.19 ± 0.80 AUs, *p<0.0005*). However, overall AUC values were not influenced by adjunct corticosteroid treatment compared to placebo (11.27 ± 4.7 AUs vs. 10.74 ± 5.1 AUs, p=0.267). Likewise, patients from the placebo group presenting stable or rising markers of NETs after day 3 had higher hazards of clinical stability (adjusted HR 1.75 [95% CI 1.06–2.89], *p=0.027*).

With regard to the beneficial effects of prednisone on clinical outcomes, we noted a significant effect modification by NET markers on TTCS (*p for interaction 0.008*). Patients with levels of NETs in the highest quartile shared a 1.8-fold higher adjusted HR of clinical stability upon prednisone treatment compared to patients in the three lower quartiles (adjusted HR 1.79, 95% CI 1.03–3.12).

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Discussion

The key findings of this study on NETs in patients with CAP are threefold: First, serum levels of cell-free nucleosomes as a surrogate marker for NETs are vastly increased in the context of CAP and remain at remarkably elevated levels even after 7 days. Since the values for the ELISA of nucleosomes reflect a logarithmic scale, the actual magnitude of NET formation will be inclined to be underestimated, as it is in the range of mean elevations to the power of two to even four, when compared to healthy volunteers [13, 15, 17, 18] and is considerably higher than that observed in autoimmune diseases such as rheumatoid arthritis with well-controlled activity [13]. While NETs are formed to fight infectious agents by entrapping and killing a wide variety of bacteria [21] and other microbes [22, 23], excessive activation of neutrophils and consecutive production of NETs are associated with the pathology of a number of airway disorders [24], such as COPD [25–27], impairment of alveolar gas exchange, lung dysfunction [28–31], and ultimately increased risk of early and late death.

Additionally, as measurements of NET surrogate markers were performed in serum samples, the results not only represent pre-formed systemic NETs, but also allow estimation of the propensity of pre-activated neutrophils to release NETs during coagulation. The magnitude of NETosis measured in the serum at baseline correlates with neutrophil and leukocyte cell count, but was reduced with the presence of heart failure. This observation may be due to pooling of neutrophils in the pulmonary circulation on account of reduced cardiac output [32]. While nicotine has been described to be a potent inducer of NETosis [33–35], in our cohort the number of packyears was negatively associated with baseline levels of NETs, which could possibly be explained by exhaustion due to chronic activation of NETosis-related pathways.

The second important finding is that high levels of NETs at admission as well as a high integral of NETs during the course of CAP over 7 days (AUC) were strong independent risk factors for adverse outcome. Patients in the highest quartile had a median prolongation of TTCS by 1 day and of the length of hospital stay by 2 days, accompanied by increased duration of intravenous antibiotic treatment by 0.5 days. The differences in TTCS seemed to be less prominent in patients with very short (<5 days) or prolonged (>15 days) time to clinical stability, respectively.

Remarkably, the results show that NET values at admission are associated with an almost fourfold odds of all-cause mortality in CAP patients, representing a novel biomarker for outcome prediction, i.e. for identification of patients at risk.

These findings are consistent with previous observational studies that examined the association between cell-free DNA or nucleosomes and the risk of mortality in ICU patients: In a prospective observational cohort study from Finland on 225 patients with severe sepsis, admission concentrations of cfDNA were higher in ICU non-survivors compared to survivors [36]. Likewise, a retrospective observational study on 80 severe sepsis patients revealed high prognostic utility of cfDNA levels to predict ICU and hospital mortality, respectively [37]. Further observational studies support evidence of NETosis as a hallmark in the pathogenesis of systemic inflammation, hypercoagulability and sepsis [38–40]. These observations are in line with experimental data showing that intense pulmonary NET generation determines the disease severity in both viral and bacterial pneumonia [22, 41]. Seeking evidence that measured cell-free nucleosomes effectively mirrored NETs, we quantified complexes of MPO and DNA, measuring the typical components of NETs, DNA in combination with histones and neutrophil granule proteins. High correlations indicated that circulating nucleosomes were, in the least to a large extent, derived from NET-forming neutrophils (online data supplement, **Figure S3**).

Last but not least, our results suggest a bidirectional interaction of corticosteroids and NETs. While a 7-day treatment with prednisone was associated with a beneficial effect to achieve clinical stability, an overall reduction of length of hospital stay and a reduction in duration of intravenous antibiotic treatment [9], corticosteroids modulated NETosis, leading to a slower and more sustained decline of markers of NETs without increasing overall AUC after 3 days. Similarly, the subgroup of patients in the placebo group presenting with more sustained NETs levels from day 3, achieved clinical stability more rapidly than those with a faster decline. Accordingly, the temporal dynamics seem to exert a pivotal influence on clinical outcome: while excessive NETosis at baseline with consecutive exhaustion was associated with adverse outcome, moderate initial NET levels of sustained character were associated with a more beneficial outcome (online data supplement, **Figure S4**). Against this background, beneficial effects of corticosteroids as adjunct in CAP treatment may partly be driven by modulation of NET formation or pre-activation of neutrophils in the course of infection. In fact, significant effect modification of corticosteroid effects on TTCS by NET levels supports this assumption. Yet, known effects of glucocorticoids on neutrophils prolonging the survival, increasing the mobilization from the bone marrow, and reducing marginalisation in the vasculature could in part account for enhanced NETosis [42, 43]. Since there are no data on the effects of corticosteroids on NETosis to date, the underlying pathophysiological mechanisms are yet to be determined.

In conclusion, our findings suggest that cell-free nucleosomes serve as a surrogate marker of NETosis, which appear to be a highly dynamic and prognostic cellular antimicrobial defense mechanism in CAP. The data contribute fundamentally to the understanding of the pathophysiology of lower respiratory tract infections, which are largely driven by neutrophil activation. While NET formation is understandably necessary in microbial defense, modulators of NETosis may have potential therapeutic benefits to limit excessive neutrophil recruitment and activation while preserving host defense.

The main strengths of our study include (i) the well-characterized cohort of patients with CAP of different severities representative for patients usually treated in emergency departments and hospitals, (ii) the large number of patients, (iii) the assessment of clinically relevant endpoints, and (iv) the high accuracy and reproducibility of the measurement methods. However, limitations that need to be considered include: (i) this was a secondary analysis of a randomly selected subgroup, (ii) measured cell-free nucleosomes may not only mirror NET formation, but could partly reflect other mechanisms of cell death such as necrosis or necroptosis, (iii) quantified markers of NETs could be overestimated due to possible fragmentation, and (iv) the results may not be applicable to patients with less severe CAP, who do not require hospitalization.

Further studies are required to characterize NETosis in lower respiratory tract infections and to determine the relative importance to the progression and remission of the condition and whether targeting this pathway would be of therapeutic benefit to patients' outcomes.

Acknowledgments We thank the members of the STEP Study Team (names and affiliations listed in the online data supplement) for the patient recruitment and data organization.

Autor contributions Concept and design of the study, organization and interpretation of data: BM, FE, MCC, PH, PS, SG, and SVB. Experiments: SG, SVB. Drafting the manuscript for important intellectual content: FE, PH, PS, SG, AK, CAB, CB and SH. All authors approved the final version to be published. FE and PS had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

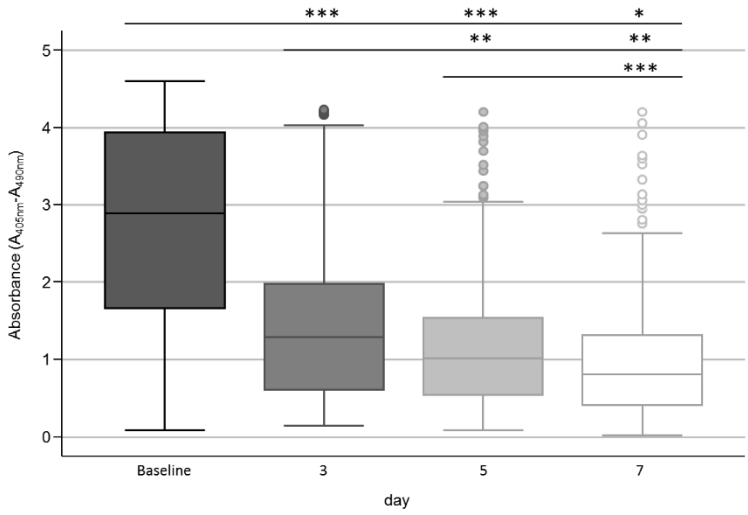

Figure legend 1:

Boxplots for levels of cell-free nucleosomes as a surrogate marker of neutrophil extracellular traps (NETs) in serum samples of the whole cohort (n=310) at four different time points (baseline, day 3, 5, and 7) in the course of community-acquired pneumonia. Each box signifies the upper and lower quartiles, while the median is represented by a line within the box. Whiskers represent the upper and lower adjacent values, outliers are depicted as circles; mixed-effects linear regression model *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

At admission, mean levels of NETs were markedly increased (2.67 ± 1.32 absorbance units [AU]) and decreased gradually over time (day 3, 1.48 ± 1.03, $p<0.0005$; day 5, 1.19 ± 0.87, $p<0.0005$; day 7, 0.98 ± 0.79, $p=0.042$).

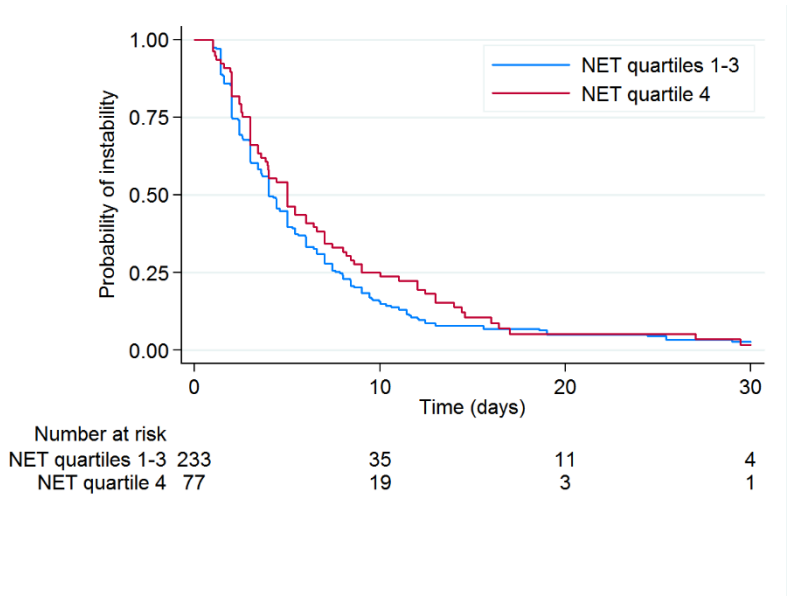

Figure legend 2:

Kaplan-Meier-curve of time to clinical stability for area under the concentration curves of neutrophil extracellular traps (NETs), an integrated value over 7 days: NET quartile 4 (highest quartile) vs. NET quartile 1-3. Higher NET levels are associated with prolonged time to clinical stability, adjusted hazard ratio (HR) of 0.97 (95% CI 0.94–0.99, *p=0.041*).

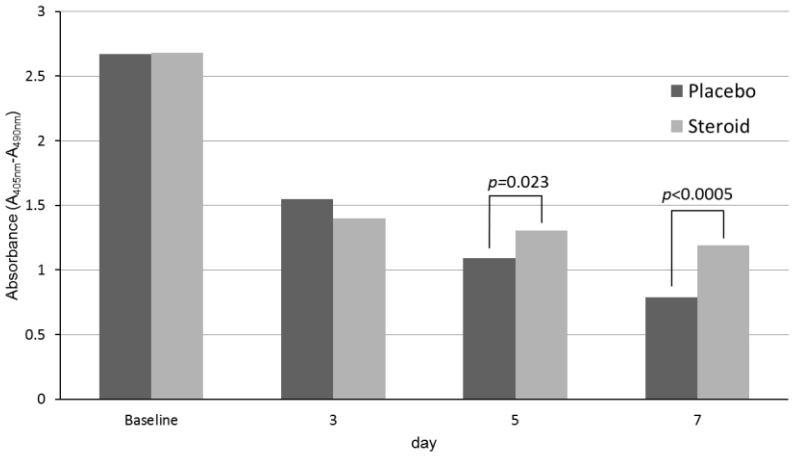

Figure legend 3:

Mean absorbance units of neutrophil extracellular traps (NETs): placebo group (dark gray) vs. prednisone group (light gray). On day 5 (1.09 ± 0.88 absorbance units (AU) in placebo group vs. 1.31 ± 0.84AU in steroid group, *p<0.023*) and day 7 (0.79 ± 0.74AU vs. 1.19 ± 0.80AU, *p<0.0005*) prednisone treatment was associated with significantly higher NET formation compared to placebo.

Tables

Table 1 - Baseline characteristics and clinical variables of enrolled patients

Characteristic/variable	**Total (n=310)**	**Prednisone (n=151)**	**Placebo (n=159)**	***p*-value**
General characteristics				
Age, years	75.0 (63.0, 83.0)	77.0 (63.0, 84.0)	73.0 (62.0, 83.0)	0.19
Male sex	199 (64.2%)	96 (63.6%)	103 (64.8%)	0.83
BMI, kg/m^2	25.9 (23.1, 29.3)	25.1 (22.5, 28.1)	26.4 (23.7, 30.2)	0.009
Smoking status	72 (23.2%)	36 (23.8%)	36 (22.6%)	0.80
Packyears, years	10.0 (0.0, 40.0)	10.0 (0.0, 40.0)	10.0 (0.0, 40.0)	0.80
Comorbidities				
Diabetes mellitus	68 (21.9%)	31 (20.5%)	37 (23.3%)	0.56
Insulin treatment	25 (8.1%)	12 (7.9%)	13 (8.2%)	0.94
COPD	54 (17.4%)	32 (21.2%)	22 (13.8%)	0.088
Asthma	13 (4.2%)	5 (3.3%)	8 (5.0%)	0.45
Heart failure	62 (20.0%)	31 (20.5%)	31 (19.5%)	0.82
Hypertension	176 (56.8%)	88 (58.3%)	88 (55.3%)	0.60

Cerebrovascular disease	28 (9.0%)	13 (8.6%)	15 (9.4%)	0.80
Peripheral artery occlusive disease	20 (6.5%)	8 (5.3%)	12 (7.5%)	0.42
Renal insufficiency	107 (34.5%)	53 (35.1%)	54 (34.0%)	0.83
Neoplastic disease	19 (6.1%)	9 (6.0%)	10 (6.3%)	0.90
Steroid pre-treatment	7 (2.2%)	2 (1.4%)	5 (3.1%)	0.43
Antibiotic pre-treatment	68 (21.9%)	31 (20.5%)	37 (23.3%)	0.56
Clinical variables				
Days with symptoms, days	4.0 (2.0, 7.0)	4.0 (2.0, 7.0)	4.0 (2.0, 7.0)	0.50
Systolic blood pressure, mmHg	121.0 (109.0, 139.0)	122.0 (109.5, 137.0)	120.0 (109.0, 140.0)	0.93
Diastolic blood pressure, mmHg	66.0 (58.0, 74.0)	65.0 (57.5, 74.0)	67.0 (58.0, 73.0)	0.77
Pulse, bpm	85.0 (74.0, 98.0)	85.0 (76.0, 97.0)	85.0 (72.0, 99.0)	0.86
Respiratory frequency, breaths/min	20.0 (18.0, 24.0)	20.0 (18.0, 24.0)	20.0 (16.5, 24.5)	0.56
Temperature, °C [in-ear]	38.0 (37.4, 38.5)	38.0 (37.4, 38.5)	38.0 (37.4, 38.4)	0.52
SIRS, points.				
0	6 (1.9%)	4 (2.6%)	2 (1.3%)	0.86
1	61 (19.7%)	27 (17.9%)	34 (21.4%)	
2	114 (36.8%)	56 (37.1%)	58 (36.5%)	
3	91 (29.4%)	45 (29.8%)	46 (28.9%)	
4	38 (12.3%)	19 (12.6%)	19 (11.9%)	
PSI score[†]				
I	32 (10.3%)	14 (9.3%)	18 (11.3%)	0.55
II	47 (15.2%)	25 (16.6%)	22 (13.8%)	0.50
III	65 (21.0%)	29 (19.2%)	36 (22.6%)	0.46
IV	118 (38.1%)	60 (39.7%)	58 (36.5%)	0.55
V	48 (15.5%)	23 (15.2%)	25 (15.7%)	0.90
PSI, points	94.7 (36.7)	95.6 (37.9)	93.8 (35.6)	0.66
Laboratory values				
Procalcitonin, ng/dL	0.4 (0.2, 2.4)	0.6 (0.2, 2.5)	0.4 (0.2, 2.0)	0.67
C-reactive protein, mg/L	153.6 (80.2, 237.5)	153.0 (80.5, 237.5)	154.0 (73.7, 240.0)	0.98
White blood cell count, G/L	11.9 (8.7, 15.9)	12.3 (8.8, 16.1)	11.6 (8.7, 15.5)	0.27
Neutrophil granulocytes, G/L	9.9 (7.0, 13.2)	10.3 (7.0, 13.4)	9.6 (6.9, 13.1)	0.26
Fasting glucose, mmol/L	6.6 (5.8, 7.5)	6.4 (5.6, 7.5)	6.6 (5.9, 7.6)	0.50

Data are presented as median (IQR) for continuous or n (%) for categorical variables, unless otherwise stated. BMI, body mass index; COPD, chronic obstructive pulmonary disease; SIRS, systemic inflammatory response syndrome; PSI, pneumonia severity index. [†]The PSI is a clinical prediction rule to calculate the probability of morbidity and mortality in patients with community-acquired pneumonia [11]; PSI risk class I corresponds to age ≤50 years, and no risk factors (≤50 points), risk class II to <70 points, risk class III to 71–90 points, risk class IV to 91–130 points, and risk class V to >130 points.

Table 2 - Association of baseline neutrophil extracellular trap (NET) levels with demographic characteristics, comorbidities and clinical variables

	Regression analysis coefficient (95% CI)	***p* value**
General characteristics		
Age at study entry	-0.001 (-0.02, 0.006)	0.292
Male sex	0.118 (-0.22, 0.463)	0.502
BMI	-0.001 (-0.03, 0.026)	0.892
Smoking status	0.147 (-0.23, 0.529)	0.450
Packyears (per 10 years)	**-0.05 (-0.10, -0.00)**	**0.042**
Comorbidities		
Diabetes mellitus	0.160 (-0.23, 0.559)	0.428
Insulin treatment	0.318 (-0.25, 0.895)	0.279
COPD	-0.01 (-0.42, 0.395)	0.943
Asthma	-0.24 (-0.99, 0.504)	0.522
Heart failure	**-0.46 (-0.89, -0.02)**	**0.036**
Hypertension	0.041 (-0.28, 0.370)	0.802
Cerebrovascular disease	-0.23 (-0.79, 0.326)	0.410
Peripheral artery occlusive disease	-0.26 (-0.93, 0.409)	0.442
Renal insufficiency	-0.04 (-0.38, 0.290)	0.775
Neoplastic disease	-0.36 (-1.0, 0.283)	0.270
Clinical variables		
Steroid pre-treatment	0.327 (-0.41, 1.07)	0.385
Antibiotic pre-treatment	-0.02 (-0.40, 0.355)	0.891
Days with symptoms	0.004 (-0.01, 0.021)	0.648
Temperature (°C)	-0.01 (-0.18, 0.164)	0.902
Oxygen saturation (%)	0.006 (-0.04, 0.056)	0.790
PSI score	0.001 (-0.001, 0.007)	0.886
Positive blood culture	0.115 (-0.38, 0.615)	0.649
Pathogen detection	-0.05 (-0.41, 0.312)	0.782
Neutrophil granulocytes	**0.033 (0.006, 0.061)**	**0.016**
Leucocyte count	0.019 (-0.00, 0.043)	0.116

Data for adjusted (multivariate) linear regression analyses are given as regression coefficient (95% CI). Baseline NET levels were negatively associated with the quantity of packyears and a history of heart failure, but positively associated with counts of neutrophils.

Table 3 - Overview of primary and secondary endpoints: Neutrophil extracellular traps

			NETs at baseline		**Area under the concentration curve of NETs**	
	Quartiles 1-3 AUC NETs (n=233)	Quartile 4 AUC NETs (n=77)	Multivariable adjusted regression analysis, OR, HR, coefficient (95% CI)	*p* value	Multivariable adjusted regression analysis, OR, HR, coefficient (95% CI)	*p* value
Primary endpoint						
TTCS, days	4.0 (2.0-7.4)	5.0 (2.6-9.0)	0.91 (0.82, 1.01)[a]	0.088	**0.97 (0.94, 0.99)[a]**	**0.041**
Secondary endpoints						
Time to effective hospital discharge, days	7.0 (5.0-11.0)	9.0 (5.0-14.0)	**0.90 (0.82, 0.99)[a]**	**0.042**	**0.96 (0.94, 0.99)[a]**	**0.012**
Death (30-days)	9 (3.9%)	4 (5.2%)	**3.81 (1.39, 10.4)[b]**	**0.009**	**1.27 (1.05, 1.54)[b]**	**0.012**
Total duration of antibiotic treatment, days	10.0 (7.0-12.0)	11.0 (9.0-14.0)	0.38 (-0.01, 0.84)[c]	0.099	0.07 (-0.01, 0.20)[c]	0.253
Intravenous antibiotic treatment, days	5.0 (4.0-7.0)	6.0 (4.0-10.0)	**0.52 (0.10, 0.94)[c]**	**0.015**	**0.13 (0.01, 0.24)[c]**	**0.021**
CAP complications[d]	77 (33.1%)	17 (22.1%)	0.97 (0.78, 1.22)[b]	0.853	0.99 (0.93, 1.06)[b]	0.92
ICU admission	21 (9.0%)	6 (7.8%)	0.90 (0.58, 1.39)[b]	0.645	0.97 (0.87, 1.09)[b]	0.707

Data are median (IQR) or *n* (%) unless otherwise stated and adjusted for multiple variables (see Materials and Methods).

[a] Hazard ratio, [b] Odds ratio, [c] Regression coefficient. ICU=intensive care unit.

[d] CAP complications: recurrence; acute respiratory distress syndrome; empyema; nosocomial infections until day 30; serious adverse events possibly related to CAP; ICU stay; re-admission to hospital.

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Online Supplemental Material

Markers of Neutrophil Extracellular Traps Predict Adverse Outcome in Community-Acquired Pneumonia

Secondary Analysis of a Randomised Controlled Trial

Contents

1. Detailed Methodology Neutrophil Extracellular Traps

Chromosomal DNA (cfDNA) is a main component of NETs, and cell-free nucleosomes representing chromatin have been reported to serve as a surrogate parameter of NET formation. The provision is that both MPO and NE are detected in quantities proportional to the cell-free nucleosomes. Although they do not detect NET–derived products exclusively, cell-free nucleosomes have been widely used as a surrogate marker for NETosis in rheumatoid arthritis, preeclampsia, coagulation disorders, cancer patients or type 1 and 2 diabetes mellitus [13–19].

While plasma samples contain actual circulating NETs that were formed *in vivo* during the course of infection, serum samples add a functional assessment. Since coagulation represents a potent stimulus of NET induction, pre-activated neutrophils with a predisposition to release NETs give rise to increased NET components in serum measurements, while neutrophils without a previous pre-activation release only low quantities of NET components [13, 17, 20]. Based on these observations, measurements of NETs within the selected cohort were performed in serum samples.

In an independent analysis from a subgroup of 20 randomly selected patients we sought to validate NETs as the primary source of cell-free nucleosomes (**Figures S1 to S3**). MPO, NE and MPO-DNA complexes and nucleosomes were measured in serum samples and values were compared to quantities in plasma. The correlation between MPO-DNA complexes and nucleosomes in the serum is given in **Figure S3**. Cell-free nucleosome levels are given as absorbance units [AUs].

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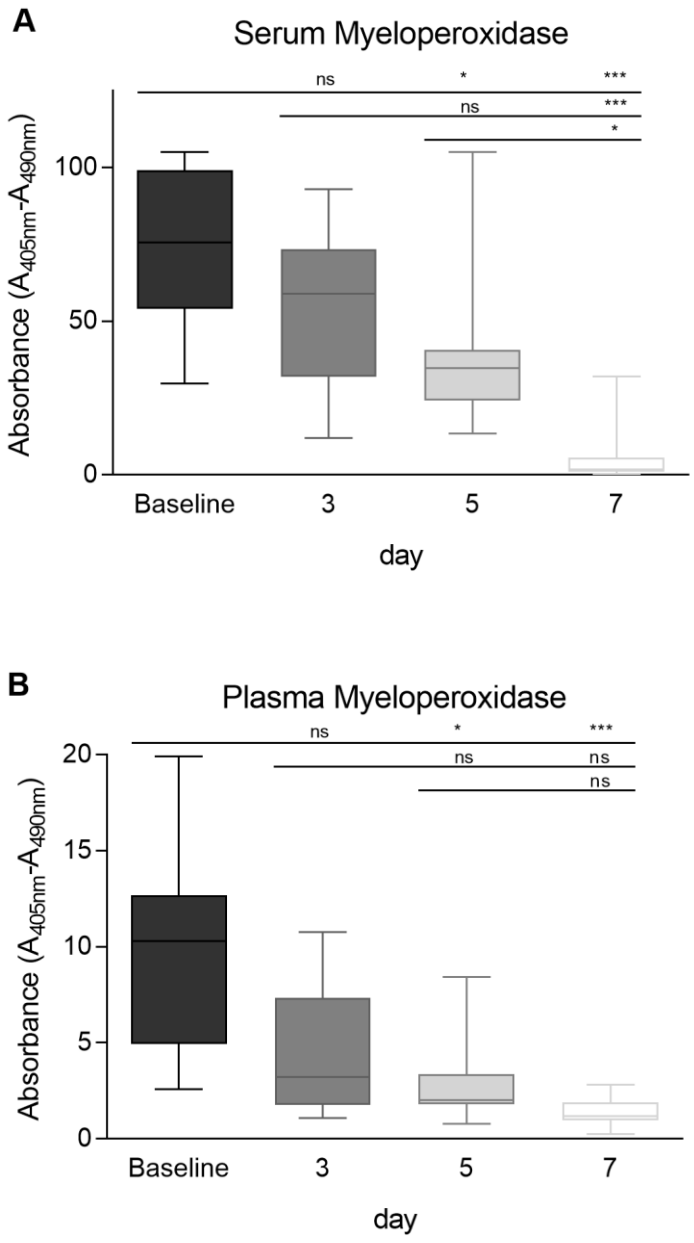
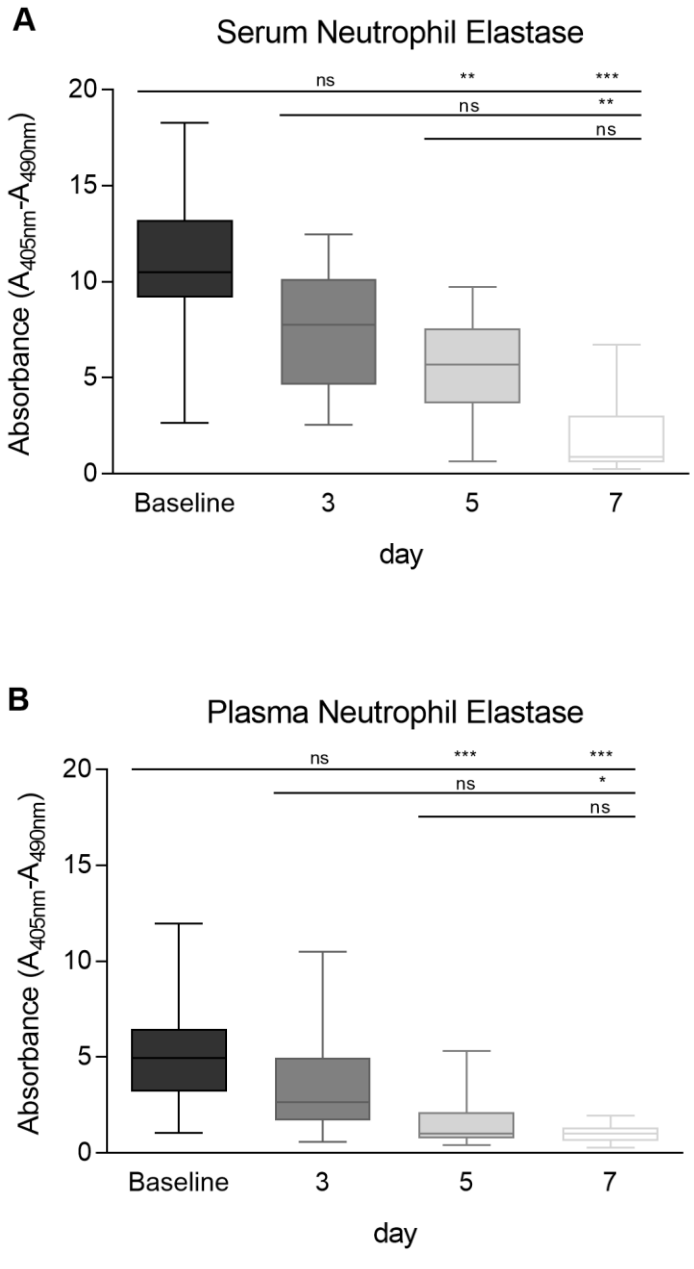
2. Figure S1. Temporal Dynamics of Myeloperoxidase in Serum and Plasma



Boxplots for levels of myeloperoxidase (MPO) as surrogate marker of neutrophil extracellular traps (NETs) (A) in serum and (B) in plasma samples of a subgroup of 20 patients at baseline and days 3, 5, and 7 during the course of community-acquired pneumonia. MPO is significantly increased at hospital admission and steadily declines

over 7 days, almost reaching the level of controls. Serum MPO levels are markedly higher than in plasma, since coagulation leads to NET-formation of pre-activated neutrophils. ns, not significant; * p < 0·05; ** p < 0·01; ***p < 0·001.

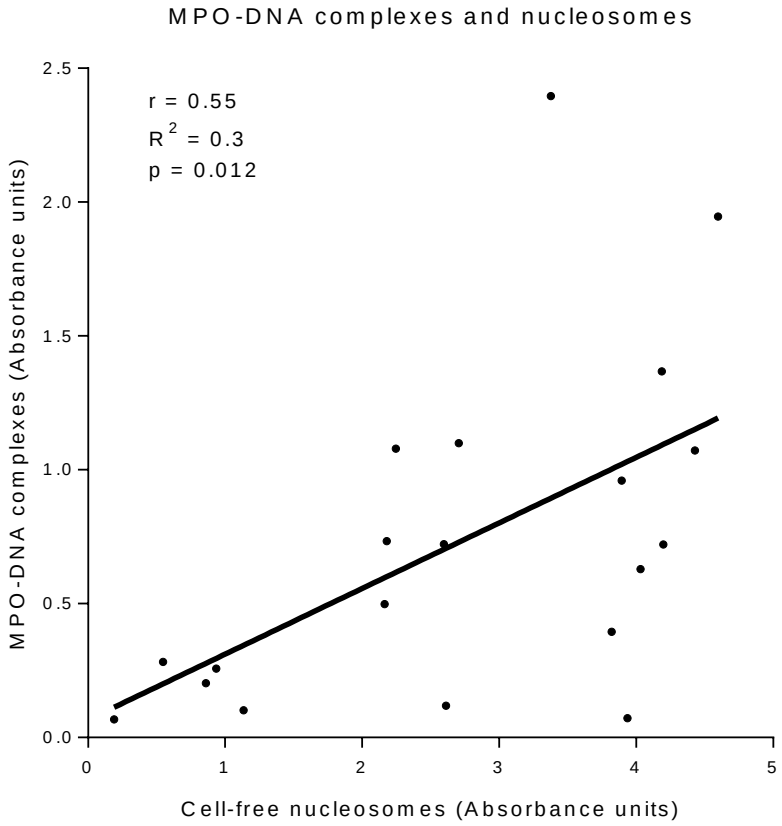
3. Figure S2. Temporal Dynamics of Neutrophil Elastase in Serum and Plasma



Boxplots for levels of neutrophil elastase in (A) serum and (B) plasma of a subgroup of 20 patients at baseline and days 3, 5, and 7 during the course of community-acquired pneumonia.

While plasma samples reveal in vivo circulating NE levels, serum samples offer an added functional testing, since coagulation represents a potent stimulus of formation of NETs, which incorporate MPO and NE during their release. Hence, pre-activated neutrophils with a higher propensity to release NETs are unveiled in serum measurements.

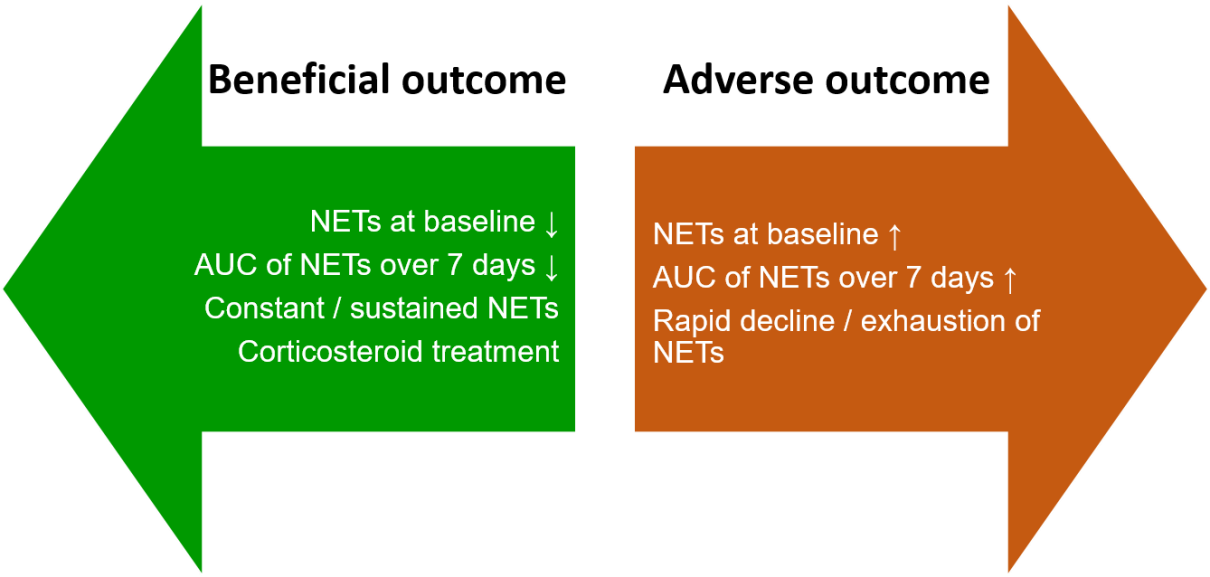
4. Figure S3. Correlation of serum cell-free nucleosomes with MPO-DNA complexes



Scatter plot with fitted linear regression line of quantified serum cell-free nucleosomes and MPO-DNA complexes. Seeking *in vivo* evidence of NET formation, we measured MPO-DNA complexes using a modified capture ELISA which detects the typical components of NETs, DNA in combination with histones and coated with neutrophil MPO.

There is a high correlation between cell-free nucleosomes and MPO-DNA complexes, indicating that circulating nucleosomes are, at least in part, derived from NET-forming neutrophils.

5. Figure S4. NETs and Outcome in Community-Acquired Pneumonia



High baseline levels and AUC of NETs over 7 days were associated with adverse outcome (orange arrow). Moderate NETosis at baseline, but sustained over time, for instance associated with corticosteroid therapy, was correlated with a favorable outcome (green arrow).

6. Table S1. Baseline characteristics of controls

Characteristic/variable	Total (n=20)
General characteristics	
Age, years	59.5 (56, 66.5)
Male sex, %	9 (45%)
BMI, kg/m^2	26.5 (23.5, 31.2)
Body weight, kg	83 (68.5, 96.5)
Systolic blood pressure, mmHg	128.5 (121, 146)
Diastolic blood pressure, mmHg	83.5 (76.5, 89)
Laboratory parameters	
Hemoglobin, g/L	145 (142, 155)
White blood cell count, G/L	5.6 (4.9, 6.25)
Neutrophil count, G/L	3.1 (2.8, 3.8)

Data are presented as median (IQR) for continuous or n (%) for categorical variables, unless otherwise stated. BMI, body mass index.

The controls were 20 randomly selected regular blood donors of the blood donation center Basel without any acute or chronic comorbidities and without regular pharmacological therapies that would influence immune responses.

7. STEP Study Group Members

We thank the members of the STEP Study Team: Nicole Nigro, MD[1] and PD Matthias Briel, MD[2] for study design and protocol writing of the original study. Elke Ullmer, MD[4], Hanno Elsässer, MD[4], Isabelle Suter-Widmer, MD[1], Bettina Winzeler, MD[1], Prof. Roland Bingisser, MD[5], Daniel Drozdov, MD[3], Birsen Arici, MD[2,3], Sandrine Andrea Urwyler, MD[2,3], Julie Refardt, MD[2], PD Philipp E. Tarr, MD[6], Sebastian Wirz, MD[6], Robert Thomann, MD[7], Hervé Duplain, MD[8], Christine Baumgartner, MD[9], and Prof. Nicolas Rodondi, MD,[9] for study coordination and patient recruitment. Furthermore, we thank Prof. Marc Donath, MD[1], as a member of the data safety and monitoring board.

1: Department of Endocrinology, Diabetology, and Metabolism, Department of Internal Medicine and Department of Clinical Research, University Hospital Basel, Switzerland

2: Basel Institute for Clinical Epidemiology and Biostatistics, Department of Clinical Research, and Department of Clinical Epidemiology and Biostatistics, Basel, Switzerland; and McMaster University, Hamilton, ON, Canada

3: Medical University Clinic, Departments of Internal and Emergency Medicine and Department of Endocrinology, Diabetology and Clinical Nutrition, Kantonsspital Aarau, Aarau, Switzerland

4: Medical University Clinic, Kantonsspital Baselland/Liestal, Liestal, Switzerland

5: Emergency Department, University Hospital Basel, Basel, Switzerland

6: Medical University Clinic, Kantonsspital Baselland/Bruderholz, Bruderholz, Switzerland

7: Department of Internal Medicine, Bürgerspital, Solothurn, Switzerland

8: Clinic of Internal Medicine, Hôpital du Jura, Site de Delémont, Delémont, Switzerland

9: Department of General Internal Medicine, Inselspital, Bern University Hospital, Bern, Switzerland