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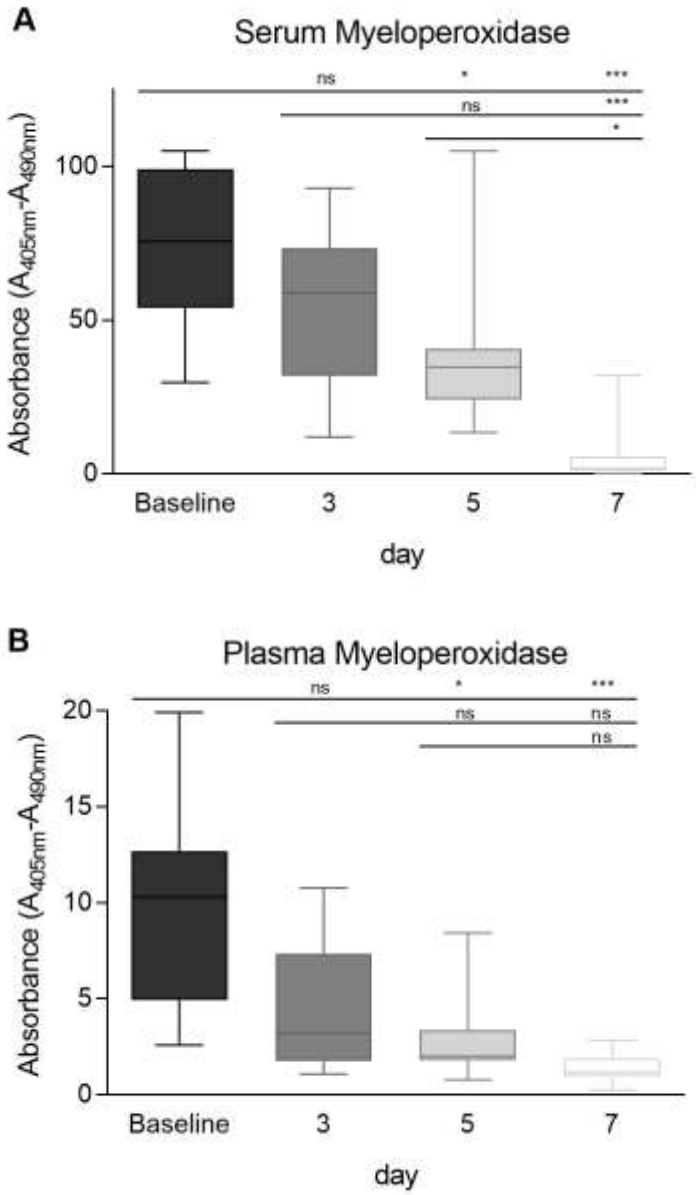
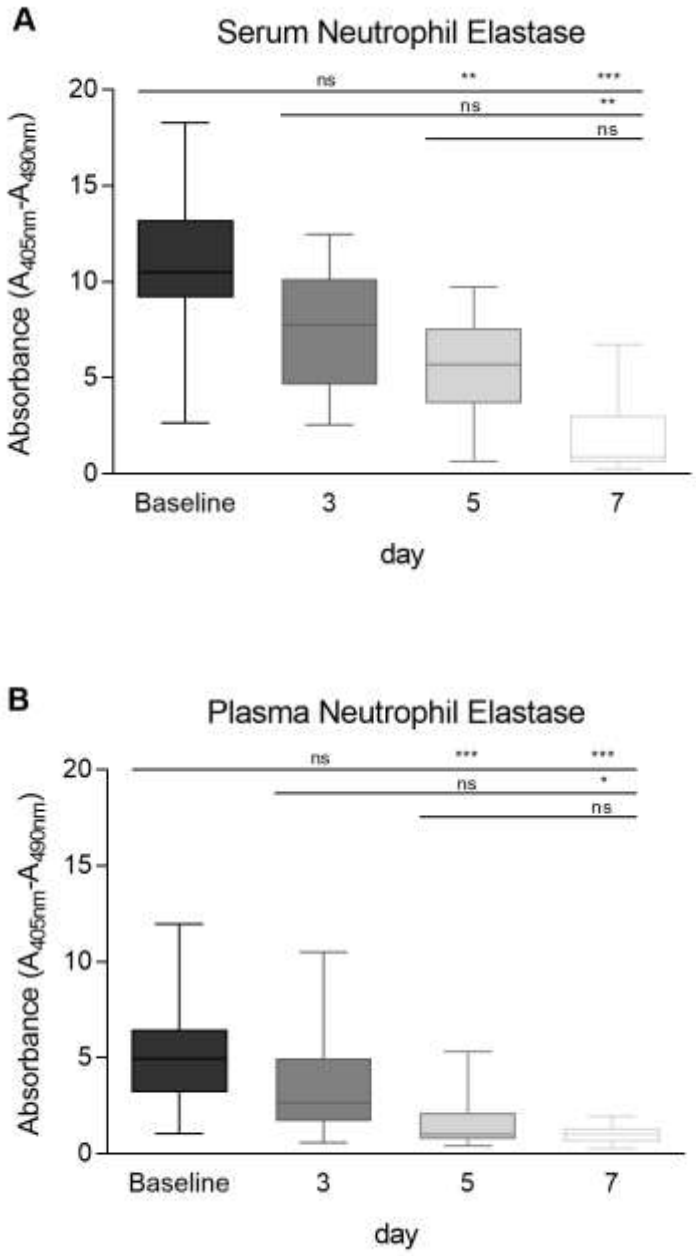
2. Figure S1. Temporal Dynamics of Myeloperoxidase in Serum and Plasma



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; * $p < 0{\cdot}05$; ** $p < 0{\cdot}01$; ***$p < 0{\cdot}001$.

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

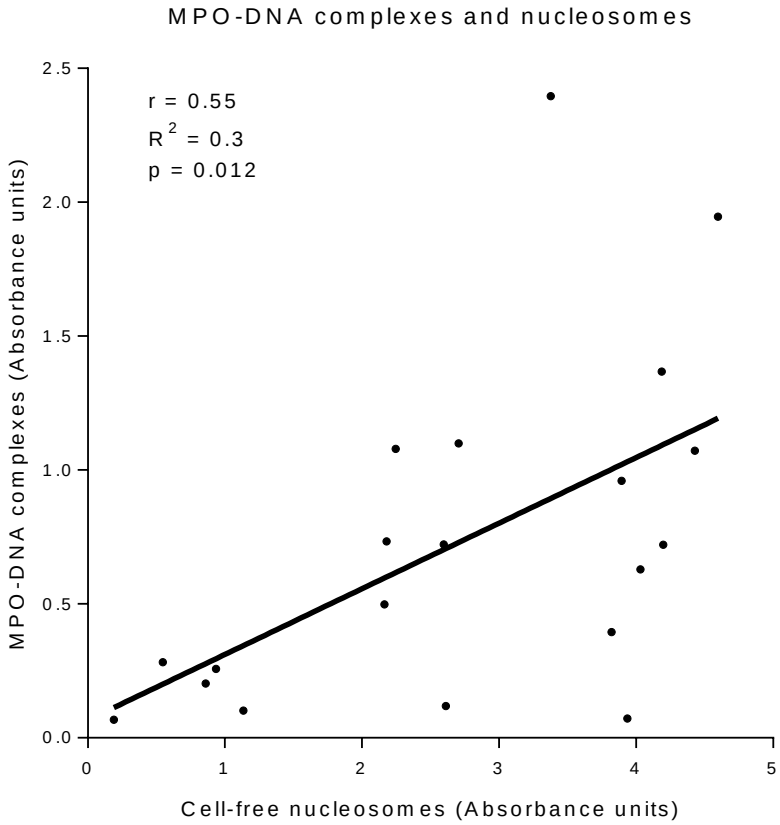


Boxplots for levels of neutrophil elastase in (A) serum and (B) plasma of a subgroup of 20 patients at baseline and days 3, 5, and 7 during the course of community-acquired pneumonia.

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.

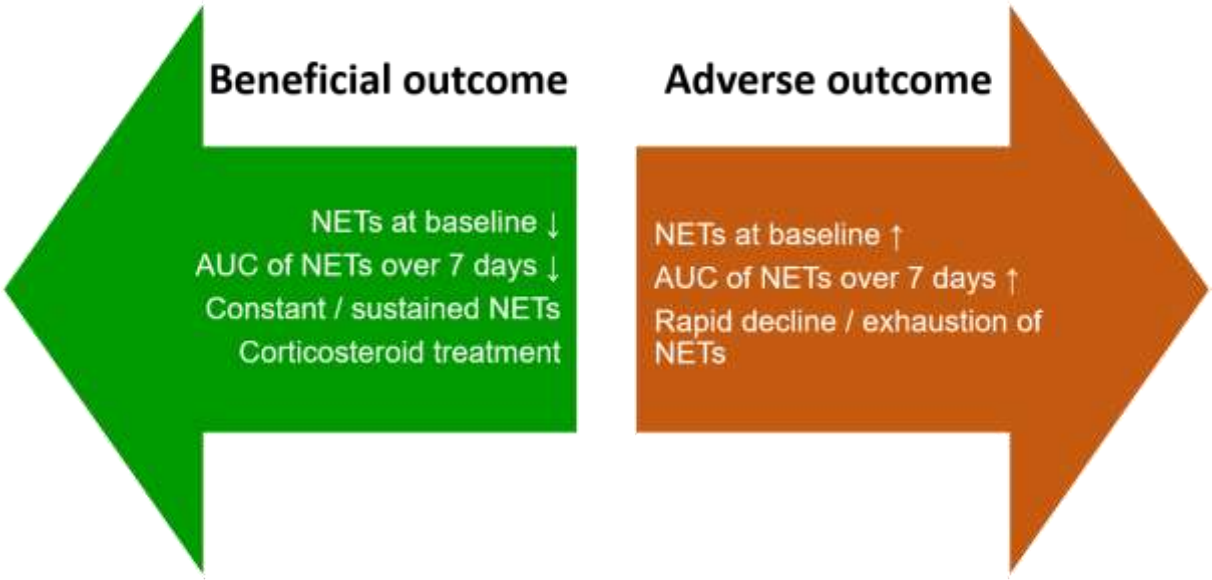
4. **Figure S3. Correlation of serum cell-free nucleosomes with MPO-DNA complexes**



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.

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