Clinical Track

Coronary Neutrophil Extracellular Trap Burden and Deoxyribonuclease Activity in ST-Elevation Acute Coronary Syndrome Are Predictors of ST-Segment Resolution and Infarct Size

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Rationale: Mechanisms of coronary occlusion in ST-elevation acute coronary syndrome are poorly understood. We have previously reported that neutrophil (polymorphonuclear cells [PMNs]) accumulation in culprit lesion site (CLS) thrombus is a predictor of cardiovascular outcomes.

Objective: The goal of this study was to characterize PMN activation at the CLS. We examined the relationships between CLS neutrophil extracellular traps (NETs), bacterial components as triggers of NETosis, activity of endogenous deoxyribonuclease, ST-segment resolution, and infarct size.

Methods and Results: We analyzed coronary thrombectomies from 111 patients with ST-elevation acute coronary syndrome undergoing primary percutaneous coronary intervention. Thrombi were characterized by immunostaining, flow cytometry, bacterial profiling, and immunometric and enzymatic assays. Compared with femoral PMNs, CLS PMNs were highly activated and formed aggregates with platelets. Nucleosomes, double-stranded DNA, neutrophil elastase, myeloperoxidase, and myeloid-related protein 8/14 were increased in CLS plasma, and NETs contributed to the scaffolds of particulate coronary thrombi. Copy numbers of Streptococcus species correlated positively with dsDNA. Thrombus NET burden correlated positively with infarct size and negatively with ST-segment resolution, whereas CLS deoxyribonuclease activity correlated negatively with infarct size and positively with ST-segment resolution. Recombinant deoxyribonuclease accelerated the lysis of coronary thrombi ex vivo.

Conclusions: PMNs are highly activated in ST-elevation acute coronary syndrome and undergo NETosis at the CLS. Coronary NET burden and deoxyribonuclease activity are predictors of ST-segment resolution and myocardial infarct size. (Circ Res. 2015;116:1182-1192. DOI: 10.1161/CIRCRESAHA.116.304944.)

Key Words: atherosclerosis ▪ coronary occlusion ▪ coronary thrombosis ▪ deoxyribonuclease ▪ extracellular traps ▪ myocardial infarction ▪ neutrophils

Acute coronary syndrome (ACS) is among the leading causes of death. However, precise pathomechanisms of acute coronary occlusion and atherothrombosis are still not well understood. Circulating leukocytes, particularly monocytes, play a central role in atherothrombosis. The role of neutrophils (polymorphonuclear cells [PMNs]) in coronary thrombosis is less explored, although systemic PMN counts are among the most robust predictors of acute coronary events and impact outcomes. We have previously shown PMN accumulation in coronary thrombi, and PMNs have been described in thrombi recovered during surgical thrombectomies and open repair of abdominal aortic aneurysms. PMNs hold powerful effector mechanisms, particularly by the formation of neutrophil extracellular traps (NETs). On activation, PMNs release their nuclear content into the extracellular space to engulf and isolate pathogens. NETs are composed of chromatin, which consists of DNA segments wound in sequence around 8 histone protein cores, and other granule proteins, exerting potent proinflammatory, cytotoxic, and prothrombotic effects.

Editorial, see p 1107
In This Issue, see p 1097
NETs, innate immune cells, and platelets are key components of thrombus formation in experimental deep vein thrombosis. We hypothesized that PMNs may play an important role in ST-elevation ACS (STE-ACS). Therefore, we characterized culprit lesion site (CLS) PMNs and quantified NETs in coronary thrombi. We examined the relationship between NETs, deoxyribonuclease (DNase), and ST-segment resolution (STR) and infarct size as outcome measures. Furthermore, we screened bacterial signatures to understand triggers of NETosis and tested whether human coronary thrombi are susceptible to ex vivo lysis with recombinant DNase.

Methods

Patients

Patients with STE-ACS undergoing primary percutaneous coronary intervention (pPCI) with thrombolysis in myocardial infarction 0 to 1 flow were included (n=111). We followed previously reported protocols and inclusion criteria. Patients undergoing pPCI and fulfilling all of the following criteria were included in the study: (1) chest pain at the time of coronary angiography, (2) new ST elevations of ≥2 mm on >1 chest lead or new ST elevations of ≥1 mm on >1 limb lead within 20 minutes of coronary angiography, and (3) coronary anatomy suitable for thrombectomy. Patients under immunosuppression or treatment with glycoprotein IIb/IIIa-blockers were excluded. All patients were heparinized at an activated coagulation time of >300 seconds (4000–10000 IE) and received 250 mg of acetylsalicylic acid.

General criteria for use of a thrombectomy device were a vessel diameter of ≥3 mm, a large intraluminal contrast medium filling defect, suggestive of thrombus within 50 mm of the respective ostium in the absence of severe tortuosity, calcification, or difficult vascular access. For sample collection, a total of 10 to 20 mL CLS blood was aspirated with a commercial thrombectomy catheter (Pronto [Vascular Solutions, MN], Export [Medtronic, MN], Diver [Invatec, Brescia, Italy], and Thrombuster [Atrium Medical Corporation, NH]). Because of flushing of the aspiration catheter with 2 to 4 mL of 0.9% sodium chloride before thrombectomy, all analyses were normalized for femoral whole-blood hematocrit, resulting in equal total protein concentrations. Particulate thrombus material, if present, was separated using a 40-μm cell strainer (BD Falcon; Becton Dickinson, Franklin Lakes, NJ), washed with phosphate-buffered saline (PBS; PAA Laboratories GmbH, Pasching, Austria), and either processed for histology, flow cytometry (FC), polymerase chain reaction (PCR), or thrombolysis assays. In parallel, femoral blood was drawn from the 6 to 8 French femoral sheath. Whole-blood samples were collected and processed for further assays. Respective sample sizes are listed per experiment, and the sampling scheme is shown in Figure 1. Consecutive thrombi were used. In each instance, coronary thrombi were tested in parallel to the femoral arterial sheath sample from the same patient. No thrombus selection criteria were applied for particular experiments. Because of the small size of coronary

Figure 1. Sampling Scheme. A, Representative angiographic example of a STE-ACS case subjected to coronary thrombectomy in the course of primary percutaneous coronary intervention. The left anterior oblique 45° projection of an occluded right coronary artery is shown (A1). The culprit lesion is visible after thrombus aspiration (A2). After stent implantation (A3), coronary flow is restored to thrombolysis in myocardial infarction III (A4). B, Schematic representation of coronary sampling during thrombectomy and subsequent sample processing. Three biological compartments were addressed: particulate thrombus, coronary blood, and femoral blood. Respective numbers of patients are in parentheses. IHC indicates immunohistochemistry; IF, immunofluorescence; PCR, polymerase chain reaction; PPP, platelet poor plasma; and tPA, tissue-type plasminogen activator.
thrombi (illustrated in Figure 7), specimens could not be used in >1 experiment, except in the lysis experiments for which thrombi were halved. A subset of patients who were in the placebo arm of a prospective randomized controlled study underwent a cardiac magnetic resonance (CMR)–based infarct size measurement (n=30). STR was calculated by measuring STE at the J point in respective leads (for anterior infarction: I, aVL, and V1–V6; for inferior infarction: II, III, aVF, V5, and V6) in the index-electrocardiography and 30 minutes after pPCI. The ratio of the STE sum was calculated and expressed in percent.13 Detailed demographics are listed in the Table.

Thrombus specimens from patients who had deep vein thrombosis (n=7; 57.9±11.4 years; 3 women) were studied as comparators. Venous thrombi were obtained fresh during surgical thrombectomies and were immediately embedded in paraffin for immunohistochemistry. All thrombi were obtained fresh during surgical thrombectomies and were immediately embedded in paraffin for immunohistochemistry. All study participants gave written informed consent under an approval of the Ethics Committee of the Medical University of Vienna, Austria (approval reference numbers 114/2011, 303/2005, and 581/2006).

Thrombus Homogenization
To obtain a single cell suspension of coronary thrombi for FC, the experimental setup described by Wyss et al12 was adapted. Thrombi were processed within 1 hour after coronary aspiration. Coronary thrombi were sliced (n=27), washed with PBS, incubated with AccutaseTM (PAA Laboratories) for 5 minutes at room temperature, and resuspended in RPMI medium containing 5% fetal calf serum and 100 ng/mL tissue-type plasminogen activator (tPA; Sigma-Aldrich, St. Louis, MO). Samples were incubated on a tube shaker at 600 rpm for 1 hour at room temperature. The solution was filtered with a 40-μm cell strainer (BD Falcon) and centrifuged at 300g for 5 minutes. The pellet was washed twice with PBS and resuspended in staining buffer (PBS containing 2% fetal calf serum, 0.01% NaN3, and 0.5 mmol/L EDTA) for FC. The same procedure was applied to the femoral blood samples.

Flow Cytometry
Blood samples and cell suspensions (n=27) derived after homogenization were incubated with fluorochrome-labeled antibodies in staining buffer for 30 minutes in the dark. Cells were stained for human CD11a, CD11b, CD42b, CD45, CD62P, CD66b, and toll-like receptor (TLR)2 and TLR4 (BD Biosciences Pharmingen, San Jose, CA; Biologend, San Diego, CA). Erythrocytes were lysed by addition of BD FACS lysis solution (BD Biosciences); cells were washed 3x with PBS and resuspended for the measurements. Per panel, 50000 events were counted and the percentage of positive cells or mean fluorescence intensity was measured.

PMN gating is illustrated in Online Figure 1. Viability of PMNs was assessed in a subgroup of patients (n=12) using an annexin V/propidium iodide FC staining technique (apoptosis detection kit; BD Biosciences). Viability of gated PMNs was >95% (data not shown). Viable cells were separated from cell debris in forward and sideward scatter. CD45 positive cells were gated and divided into crude subgroups (PMNs, monocytes, and lymphocytes), and PMNs were gated for CD66b positivity.

Neutrophil platelet aggregates were identified by CD45+CD66b+CD42b+CD62P+ using a published protocol.14 For negative controls, samples were stained with isotype-matched antibodies (mouse IgG1, IgG2a, and IgM; Biolegend). Cells were analyzed using a BD FACS Canto II and FACS Diva Software (BD Biosciences).

Plasma Measurements
Neutrophil elastase concentration was determined using a Human PMN Elastase Platinum ELISA (eBioscience, San Diego, CA) with a sensitivity of 1.98 pg/mL (n=35). Myeloperoxidase concentration was determined using a Human MPO Instant ELISA (eBioscience) with a sensitivity of 0.03 ng/mL (n=35). Myeloid-related protein (MRP) 8/14 concentration was determined using a MRP 8/14 ELISA Kit (Buehlmann Laboratories, Schönenbuch, Switzerland) with a sensitivity of 0.4 μg/mL (n=35). For the quantification of double-stranded DNA (dsDNA), a Quant-IT PicoGreen dsDNA Assay (Invitrogen, Carlsbad, CA) with a sensitivity of 0.25 pg/mL was used (n=40). For the detection of DNA–histone complexes (nucleosomes), an ELISA—cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) was used (n=49). Optical density values were normalized to the internal positive control and expressed as arbitrary nucleosome units per milliliter (NU/mL). The intra-assay positive control equals 1000 NU/mL.

Deoxyribonuclease Activity Assay
Endogenous DNase activity was measured using a DNase activity assay (Oncogene Diagnostika, Mainz, Germany). A DNA-coated microplate was incubated with plasma samples for 60 minutes. Coated DNA was degraded in proportion to the DNase activity of the respective sample. The optical density of residual DNA was inversely proportional to DNase activity (n=66).

All assays were performed following the manufacturer’s instructions. All measurements were performed in duplicate. All plates

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<th>Table. Patient Characteristics (n=111)</th>
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Data are presented as mean±SD, median [IQR], or number (percent) of patients. Normal ranges are given in parenthesis. Time delay denotes the minutes between first ischemic symptoms and thrombectomy. BMI indicates body mass index; CAD, coronary artery disease; CK-MB max, maximal creatine phosphokinase isoform MB; CMR, cardiac magnetic resonance; CRP, C-reactive protein; CX, circumflex artery; HDL, high density lipoprotein cholesterol; IQR, interquartile range; LAD, left anterior descending artery; LDL, low density lipoprotein cholesterol; pPCI, primary percutaneous coronary intervention; RCA, right coronary artery; TIMI, Thrombolysis in myocardial infarction; TnT, troponin T; and VD, vessel disease.
were read on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Fluorescence assays were read on a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA).

**Immunohistochemistry**

Coronary thrombi (n=30) were stained for leukocyte subsets, markers of thrombosis, and NETs. For in vitro clots, whole blood from healthy donors (volunteering staff personnel, n=4; 2 women; 32±5.9 years) was used. All thrombus material was stained using immunohistochemistry as described. For NET quantification, cell nuclei were stained by hematoxylin (blue), whereas DNA–histone positive staining (red) was used to stain NETs. Tissue sections were automatically analyzed using TissueFAXS (TissueGnostics, Vienna, Austria), which is a validated, largely observer-independent technology for microscopy-based tissue cytometry. Percentages of DNA–histone positive areas were expressed per square millimeter thrombus and designated as NET burden. A detailed description of the immunohistochemistry methodology can be found in the Online Data Supplement.

**Immunofluorescence**

For NET assessment, paraffin sections of the thrombus material (n=19) were stained for DNA-histone structures (red), myeloperoxidase (green), and cell nuclei (blue). For M1 and M2 macrophage quantification, paraffin sections of thrombus material (n=18) were stained. M1 macrophages were defined as DAPI+CD68+CD163low and M2 macrophages as DAPI+CD68+CD163high. Single-color channel staining is shown in Online Figure III. A detailed description of the immunofluorescence methodology can be found in the Online Data Supplement.

**Microbial Community Profiling**

DNA from 18 CLS aspirates and 5 particulate coronary thrombi was isolated using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MO-BIO, Carlsbad, CA). A 2-step PCR (Illumina, San Diego, CA) was performed to amplify total microbial DNA. We chose 8 target species (Streptococcus species, mainly Streptococcus mitis group, Staphylococcus aureus, Staphylococcus epidermidis, Prevotella intermedia, Fusobacterium nucleatum, Aggregatibacter [née Actinobacillus] actinomycetemcomitans, Dialister pneumosintes, Porphyromonas gingivalis, and Parvimonas micra), yielding the highest copy number levels in CLS aspirates of a most recent study, including their oligonucleotide primers and probes. The detection limit was 40 cycles. All procedures were performed by

**Figure 2. Immunohistological analysis of leukocyte subsets in coronary thrombus.** Representative parallel sections from a single coronary thrombus are shown. Thrombi were stained for polymorphonuclear cells (anti-CD66b [A]; boxed area is magnified in B). C, A merge of immunofluorescence (IF) staining of intracellular DNA in blue (DAPI), extracellular chromatin in red (anti-DNA–histone, corresponding to NETs), and myeloperoxidase (MPO) in green (anti-MPO, highlighted by small white arrows). Furthermore, thrombocytes (anti-CD41 [D]), fibrinogen (anti-fibrinogen [E]), monocytes (anti-CD14 [F]), T lymphocytes (anti-CD3 [G]), B lymphocytes (anti-CD20 [H]), and a negative control (I) are shown. Macrophages were stained using IF (DAPI in blue; anti-CD68 in green [J]). Magnifications of M1 macrophages (CD163low in red [K]) and M2 macrophages (CD163high in red [L]) are displayed. Stars mark auto-fluorescent erythrocytes (K).
Microsynth (http://www.microsynth.ch, Balgach, Switzerland). A detailed description of the microbial community profiling methodology can be found in the Online Data Supplement.

Thrombus Lysis Assay

Human coronary thrombi (n=11) were split into 2 equal parts and incubated for 10, 30, and 60 minutes in PBS in the presence of recombinant tPA (1 \( \mu \)g/mL, Sigma-Aldrich)±DNase-1 (Dornase alfa, 100 IE/mL; Pulmozyme®, F.Hoffmann-La Roche Ltd) on a thermomixer (Thermomixer comfort, Eppendorf; Hamburg, Germany) at 600 rpm and 37°C, leading to thrombolysis. Thrombi were weighed before and after 10, 30, and 60 minutes on an ultraprecision balance (Explorer®, Ohaus Europe GmbH, Nänikon, Switzerland). Lysis was measured as percent decrease of baseline thrombus weight.

Cardiac MRI

All cardiac CMR studies were performed by board-certified physicians at the Medical University of Vienna on a 1.5-T cardiac-dedicated clinical magnetic resonance system (Sonata/Avanto; Siemens Medical Solutions, Erlangen, Germany) 4±2 days after STE-ACS, as part of a randomized clinical trial that used MRI-measured infarct size as a primary study end point.12 A detailed description of the CMR methodology can be found in the Online Data Supplement.

Statistics

Normally distributed data are expressed as mean±SD; otherwise, median and interquartile range (IQR) are presented. Paired Student \( t \) test was applied to compare normally distributed variables; otherwise, Wilcoxon signed-rank test was used. Distribution of data was tested using the Kolmogorov–Smirnov test, the Shapiro–Wilk test, and histograms (data not shown). For comparison of multiple groups, 1-way ANOVA with post hoc Scheffé procedure was performed. Pearson correlation (\( r \)) was applied for normally distributed variables; otherwise, Spearman rank correlation (\( r_s \)) was applied to calculate correlation data. The area under the curve of creatine phosphokinase isoform MB values was expressed in arbitrary units and was calculated using the trapezoidal formula,17 if \( \geq 5 \) consecutive values for a period of at least 3 days post admission were available. Bonferroni–Holm correction was used for multiple testing. A \( P \) value of <0.05 was considered significant. Statistical analyses were performed using IBM SPSS Statistics 20.0 for Windows (New York, NY).

Results

Patient Characteristics

We consented 111 patients who had STE-ACS with angiographic thrombolysis in myocardial infarction flow 0 to 1.
Important patient characteristics are listed in the Table. Eighty-one percent of patients were men, and classical risk factors were similar to those of recently published STE-ACS trials. No study participant died in hospital.

Coronary Thrombus PMNs Are Highly Activated and Form Aggregates With Platelets

We found PMNs to be the predominant cell type (median [IQR], 353.6 [237.2–689.5] cells/mm²; Figure 2A and 2B), and numerous NETs in 17 of 19 immunofluorescence-stained thrombi (Figure 2C), forming the clot matrix together with platelets and fibrin (Figure 2D and 2E). Other leukocyte subpopulations were present in lower numbers in the following order of magnitude: monocytes (median [IQR], 81.2 [58.3–132.6] cells/mm²; Figure 2F), T-cells (median [IQR], 15.2 [5.6–45.8] cells/mm²; Figure 2G), and B cells (median [IQR], 8.3 [3.5–50.3] cells/mm²; Figure 2H).

Furthermore, we determined the distribution of macrophage subtypes (n=17). Macrophages (median [IQR], 55.1 [11.45–92.6] cells/mm² of all cells; Figure 2J) were divided into M1 (median [IQR], 63.2 [30.1–76.6]% of macrophages; Figure 2K) and M2 (median [IQR], 36.8 [18.4–69.8]% of macrophages; Figure 2L) subtypes.

CD66b, CD11a, and CD11b expressions were markedly increased on thrombus PMNs compared with femoral PMNs, indicating increased adhesiveness and migratory potential of CLS cells (Figure 3A, 3C, and 3D; Online Table I). Neutrophil platelet aggregates (CD45+CD66b+CD42b+CD62P+) were accumulated in coronary thrombi (Figure 3B; Online Table I). PMN activation at the CLS site was illustrated by increased TLR2 and TLR4 expressions (Figure 3E and 3F; Online Table I).

Neutrophil Elastase, Myeloperoxidase, and MRP 8/14 Are Increased at the CLS

To determine degranulation of CLS PMNs, we measured the concentrations of major granule proteins. Neutrophil elastase concentration was increased at the CLS (n=35; median [IQR] femoral 75.4 [60.0–104.2] versus CLS 138.2 [92.8–213.0] ng/mL; P<0.0001; Figure 3G). Myeloperoxidase concentration was significantly increased at the CLS (n=35; median [IQR] femoral 80.0 [40.2–144.7] versus CLS 102.3 [71.3–189.0] ng/mL; P=0.0009; Figure 3H). Furthermore, we measured MRP 8/14 concentrations (n=35; median [IQR] femoral 3.6 [1.8–5.2] versus CLS 4.3 [3.0–5.7] μg/mL; P=0.002; Figure 3I), confirming an increase at the CLS.

Coronary Thrombi Contain High Levels of NETs

NETs were quantified in coronary thrombi. Deep venous thrombi and in vitro clots produced with healthy donor blood served as a reference thrombus material. Coronary thrombi (median [IQR], 6.7 [5.8–8.7]%; Figure 4A) contained significantly more NETs than venous thrombi (median [IQR], 3.8 [2.8–4.3]%; P=0.012; Figure 4B) or in vitro clots (median [IQR], 2.8 [1.6–3.4]%; P=0.01; Figure 4C). Data are summarized in Figure 4D.

Surrogate Markers of NETs Are Increased at the CLS and Correlate With Coronary Thrombus NET Burden

Nucleosomes and dsDNA are key components of NETs and have been proposed as sensitive markers for cardiovascular events. Nucleosomes were significantly increased at the CLS (n=48; median [IQR] femoral 5.0 [2.0–15.2] versus CLS 17.0 [5.8–66.8] NU/mL; P=0.0002), as well as dsDNA (n=66; femoral 232.8±72.0 versus CLS 357.3±177.4 ng/mL; P<0.0001).
Both CLS nucleosome \((n=19; r_s=0.573; P=0.013)\) and dsDNA concentrations \((n=30; r_s=0.569; P=0.001; \text{Figure 5A})\) correlated positively with coronary thrombus NET burden.

**Streptococcus Signatures Correlate With Surrogate Markers of NETs**

DNA was isolated from 18 CLS aspirates and 5 particulate coronary thrombi. No bacterial DNA was detected by broadband 2-step PCR (Online Figure II). However, we specifically screened for 8 candidate species\(^{16}\) by quantitative real-time PCR and detected traces of bacterial DNA from *Streptococcus* species \((10/23\) positive samples), *Prevotella intermedia* \((9/23\) positive samples), and *Fusobacterium nucleatum* \((2/23\) positive samples). No further positive signals were detected. dsDNA, a major surrogate marker for NETs \((n=30; r_s=0.569; P=0.001; \text{Figure 5A})\), correlated positively with coronary thrombus NET burden. Streptococcus copy numbers \((n=23; r_s=0.452; P=0.03; \text{Figure 5B})\) correlated positively with NET burden \((n=18; r_s=0.391; P=0.109; \text{Figure 5C})\). No correlations were found between *Prevotella intermedia*, *Fusobacterium nucleatum*, and NET burden or dsDNA.

**Coronary NET Burden and Culprit Site**

Table 2. See Figure 6A for the correlation of coronary NET burden, thrombus, and myocardial area at risk.

Deoxyribonuclease Activity Correlates With STR, Creatine Phosphokinase Isoform MB Area Under the Curve, and CMR-Measured Infarct Size

Coronary NET burden correlated negatively with STR \((n=22; r_s=-0.608; P=0.003; \text{Figure 6A})\) and positively with enzymatic infarct size \((n=30; r_s=0.566; P=0.001; \text{Figure 6B})\). Coronary NET burden correlated positively with CMR-measured infarct size \((n=16; r_s=0.689; P=0.003; \text{Figure 6C})\). Time delay did not correlate with NET burden \((n=30; r_s=-0.198; P=0.29)\). CLS DNase activity correlated negatively with coronary thrombus NET burden \((n=30; r_s=-0.623; P=0.001; \text{Figure 6D})\). Moreover, CLS DNase activity correlated positively with STR \((n=29; r_s=0.579; P=0.001; \text{Figure 6E})\) and correlated negatively with CMR-measured infarct size \((n=30; r_s=-0.475; P=0.008; \text{Figure 6F})\) and myocardial area at risk \((n=26; r_s=-0.488; P=0.011)\). Final thrombolysis in myocardial infarction flow did not correlate with NET burden or DNase activity.

**Deoxyribonuclease Accelerates Lysis of Coronary Thrombi**

Mean baseline weight of coronary thrombi was 10.6±8.8 mg (representative example is shown in Figure 7A). Addition of DNase significantly accelerated thrombus lysis measured as percent of baseline thrombus weight per time point (median [IQR]; \(n=11\)): 10 min: tPA 96.3 [84.5–97.8]% versus tPA+DNase 75.6 [69.0–90.5]%; 30 min: tPA 84.0 [64.9–90.5]% versus tPA+DNase 55.8 [44.4–70.6]%; 60 min: tPA 72.7 [51.4–82.6]% versus tPA+DNase 27.2 [24.4–40.0]%; each \(P=0.003\; \text{Figure 7B}\).

**Discussion**

In this work, we demonstrate that coronary NET burden in STE-ACS is correlated negatively with STR and positively with infarct size. These data suggest that CLS NETs may be propagating thrombosis and inflammation distally into the infarcted myocardium and contribute to myocyte death during atheroembolism.\(^{21}\)

Activated neutrophils shed NETs and microparticles\(^{22}\) that have significant effect on inflammatory cell adhesion, cytokine production, degradation of endothelial cell glycocalyx,\(^{23}\) and chemotaxis.\(^{24}\) For example, neutrophilic MRP 8/14 activates coronary monocytes via TLR4.\(^{25}\) CLS PMNs expressed adhesion markers, such as CD11a, CD11b, and CD66b, indicating a proinflammatory state, increased adhesiveness and cytotoxicity, and the formation of neutrophil platelet aggregates.\(^{26}\) Heparin may influence leukocyte-platelet aggregate

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**Figure 5.** Surrogate markers of neutrophil extracellular trap (NET) burden and associations with culprit lesion site (CLS) Streptococcus copy number. A, Thrombus NET burden (NET-positive area) correlated positively with CLS double-stranded DNA (dsDNA; \(n=30; P=0.004\)). B, Streptococcus copy numbers correlated positively with dsDNA \((n=23; P=0.03)\). C, The correlation of Streptococcus copy numbers and thrombus NET burden showed a trend, but it was not significant \((n=18; P=0.109)\).
formation; however, because all samples were treated in the same manner, differences between femoral and CLS samples are valid. Augmented neutrophil TLR expression at the CLS implicates danger-associated molecular pattern–mediated activation. In accord with these data, products of activated neutrophils, for example, the granule proteins myeloperoxidase and neutrophil elastase, as well as MRP 8/14, all being integral components of NETs, were increased at the CLS.

Activated neutrophils release NETs via NETosis, a cell-death program that is different from apoptosis or necrosis. Neutrophil platelet aggregates and myeloperoxidase are important inducers of NETosis. We detected NETs abundantly throughout coronary thrombi, serving as a primary scaffold for platelets and erythrocytes, as well as for fibrin. NETs are commonly analyzed in tissue by optical techniques. Extracellular nucleosomes and dsDNA have been proposed as surrogate markers for NET burden. We found both nucleosomes and dsDNA to be highly increased in CLS plasma compared with femoral plasma. Moreover, CLS nucleosomes and dsDNA levels correlated positively with thrombus NET burden, which suggests that in STE-ACS, circulating nucleosomes and dsDNA primarily originate from NETs.

The presence of NETs is well described in venous thrombosis literature. Coronary NET burden exceeds that in venous thrombi significantly. However, we cannot exclude that the nature of venous thrombosis differs from that of arterial thrombosis in that generation of venous thrombosis is more delayed, and NETS may have disappeared by the time of harvest. Coronary NET burden correlated negatively with STR. Worst STR corresponds to the no-reflow phenomenon, which is highly associated with intracoronary thrombus, and data consistently demonstrate no-reflow as a strong independent predictor of adverse clinical outcomes. Recent data suggest that STR predicts freedom from repeated revascularization in
a contemporary large patient cohort with STE-ACS undergoing pPCI. Furthermore, coronary NET burden correlated positively both with enzymatic and CMR-measured infarct size. One may argue that a prolonged symptom-to-thrombectomy time may lead to increased thrombus-associated neutrophil numbers, thus increasing NET burden and infarct size. However, neither time delay nor final thrombolysis in myocardial infarction flow grade in this study was correlated with infarct size, and time delay was not correlated with NET burden. Furthermore, older thrombi display less NETs. Surrogate markers for NET burden predict cardiac events, implying the presence of NETs early in coronary atherosclerosis.

Because NETs are chromatin structures usually loaded with antimicrobial molecules, which trap and kill bacteria, it was intriguing to speculate that bacterial components trigger NETosis at the CLS. Furthermore, dental infection and oral bacteria, especially Streptococcus species, have been associated with acute coronary thrombosis. We detected positive bacterial DNA signals of 3 out of 8 candidate species related to dental infection. Streptococcus copy numbers at the CLS correlated positively with dsDNA, a surrogate marker for NETs. Our data suggest a link between bacteria and NETosis at the CLS, providing support to the concept of periodontitis as a risk factor for ACS. However, neutrophil-derived serine proteases or danger-associated molecular patterns are also plausible triggers of NETosis in the setting of STE-ACS.

We were also looking for mechanisms antagonizing NETs in coronary thrombus. Previously, we have shown that classical complement activation is ongoing in acute coronary thrombus, including C1q, which is able to opsonize NETs, facilitating NET clearance. Because macrophages are also able to degrade NETs, we searched for these cells both by immunofluorescence and FC in fresh coronary thrombus. Macrophages accounted for ~5% of all cells in coronary thrombi, with relative abundance of the M1 phenotype, which is in accordance with the acute inflammatory milieu at the CLS.

CLS DNase activity correlated negatively with coronary NET burden and with CMR-measured infarct size and area at risk. Extracellular DNase could serve as an endogenous regulator of NETs. Coronary DNase activity could improve flow by DNase-dependent thrombolysis and prevent downstream injury by NETs, as observed in a mouse model. DNase activity is upregulated within the first 3 hours of ACS. A single-nucleotide polymorphism in the DNase-1 gene, which leads to impaired DNase activity, was independently associated with a higher incidence of myocardial infarction. In animal models, medicinal DNase was successfully used to prevent venous thrombogenesis. Inhibiting peptidylarginine deiminase 4, a key factor in NETosis, prolonged time to carotid artery thrombosis in a photochemical injury model. We tested DNase in addition to tPA to dissolve human coronary thrombi and found substantially accelerated lysis ex vivo.

Activated neutrophils presumably adhere to atherosclerotic plaque with vulnerable endothelial coverage, for example, to plaque erosions where high levels of myeloperoxidase have been described. They undergo NETosis, producing endoluminal NETs, and further amplify leukocyte activation. Endogenous DNase is continuously degrading NETs, thus preventing the formation of a scaffold for platelets and erythrocytes. The balance between NET burden and endogenous DNase activity is one factor determining outcomes. Therapeutic use of DNase to accelerate the lysis of coronary thrombus and facilitate pPCI needs to be explored.

Limitations
An atherosclerotic plaque rupture model replicating human ACS does not exist. Therefore, in a patient setting, findings remain observational, with no proof of causality. We could not test DNase-only treatment in the thrombus lysis assay because of the small size of coronary thrombi. Despite a large number of patients, the effective study size was small because of competing experiments (Figure 1).

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Disclosures
None.

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Coronary Neutrophil Extracellular Trap Burden and Deoxyribonuclease Activity in ST-Elevation Acute Coronary Syndrome Are Predictors of ST-Segment Resolution and Infarct Size

Andreas Mangold, Sherin Alias, Thomas Scherz, Thomas Hofbauer, Johannes Jakowitsch, Adelheid Panzenböck, Daniel Simon, Daniela Laimer, Christine Bangert, Andreas Kammerlander, Julia Mascherbauer, Max-Paul Winter, Klaus Distelmaier, Christopher Adlbrecht, Klaus T. Preissner and Irene M. Lang

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Supplemental material

Supplemental Methods and Results

Immunohistochemistry
Coronary thrombi (n=30) were stained for leukocyte subsets, markers of thrombosis and NETs. For *in vitro* clot production, anticoagulated whole blood from healthy donors (volunteering staff personnel, n=4, 2 female, 32±5.9 years) was incubated with CaCl₂ for 5 minutes at 37°C. All thrombus material was fixed in 7.5% buffered formaldehyde, dehydrated, embedded in paraffin and stained employing IHC as described. Murine antibodies were employed to target CD3, CD14 (Abcam) CD20, CD41, CD66b, fibrinogen, (LifeSpan Biosciences, Seattle, WA, USA) and DNA Histone H1 (EMD Millipore Corporation, Billerica, MA, USA). Histochemical staining was performed utilizing the indirect avidin-biotin horseradish-peroxidase method (Histostain SP Kit, Zymed, Invitrogen) and photographed with an Olympus BX 50 microscope equipped with an AxioCam color digital camera and software AxioVision 3.0.6.1 (Carl Zeiss, München-Hallbergmoos, Germany). Stained cells were counted per mm² thrombus area. For NET quantification, tissue sections were analyzed utilizing HistoQuest software (Version 4.04.0151). Hematoxylin (blue) positive staining reflected cell nuclei, whereas DNA-histone positive staining (red) represented NETs. After manually defining reference shades for blue (Hematoxylin) and red (anti-DNA Histone H1, Millipore), tissue sections were automatically analyzed utilizing TissueFAXS (TissueGnostics) which is a validated, largely observer-independent technology for microscopy-based tissue cytometry. Percentages of DNA-histone positive areas were expressed per mm² thrombus, and designated as ‘NET burden’. Images were processed using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Immunofluorescence
For NET assessment, paraffin sections of thrombus material (n=19) were stained using mouse anti-DNA Histone H1 (Millipore) followed by detection with a donkey anti-mouse secondary antibody (Alexa Fluor 555, Invitrogen). MPO was stained using a rabbit anti-human MPO antibody (Abcam) with a donkey anti-rabbit secondary antibody (Alexa Fluor 488, Invitrogen). DAPI (Sigma-Aldrich) was used for nuclear DNA detection. Images were taken with an Axio Imager 2 fluorescence microscope using AxioVision 3.0.6.1 imaging software (Carl Zeiss).

For M1 and M2 macrophage quantification, paraffin sections of thrombus material (n=18) were stained using mouse anti human CD68 (Dako, Agilent Technologies, Glostrup, Denmark) and a donkey anti-mouse secondary antibody (Alexa Fluor 647, Abcam) and goat anti-human CD163 and a donkey anti-goat secondary antibody coupled to FITC (Santa Cruz Biotechnology, TX, USA). DAPI (Vector Laboratories, Peterborough, UK) was used for nuclear DNA detection. M1 macrophages were defined as DAPI+CD68+CD163low and M2 macrophages as DAPI+CD68+CD163high. The sections were scanned at 20x magnification utilizing TissueFAXS (TissueGnostics). Single color channel staining and analysis are shown in supplemental Figure III. For the analysis, TissueQuest software (Version 4.01.0128) was used.

Microbial community profiling
DNA from 18 CLS aspirates and 5 particulate coronary thrombi was isolated utilizing the PowerLyzer™ PowerSoil® DNA Isolation Kit (MO-BIO, CA, USA). 750µl of bead solution was mixed with 100µl of sample, transferred to the bead beating tubes that were supplied with the kit and followed the recommendations of the protocol. Beating conditions were two times 10 minutes at 30Hz on the TissueLyzer (Qiagen, CA, USA).
Polymerase chain reaction
A 2-step PCR (Illumina, CA, USA) was performed to amplify total microbial DNA. The first PCR step consisted of 20 cycles utilizing the following primers: 5’GTGCCAGCMGCGCGGTAA and 5’GGACTACHVGGGTWTCTAAT. The second PCR step consisted of 12 cycles using index adapter oligos (Illumina). The PCR products were purified utilizing the AMPureXP Kit (Agencourt Bioscience, MA, USA). As a microbial positive control 100mg of compost was used. All procedures were performed by Microsynth (www.microsynth.ch, Balgach, Switzerland).

Quantitative real-time polymerase chain reaction
We performed quantitative qRT-PCR to detect bacterial species with high sensitivity and specificity. Eight target species, which were detected in CLS aspirates ², were chosen and published oligonucleotide primers and probes were used. The presence of specific bacterial DNA (Streptococcus species mainly Streptococcus mitis group, Staphylococcus aureus and epidermidis, Prevotella intermedia, Fusobacterium nucleatum, Aggregatibacter [née Actinobacillus] actinomycetemcomitans, Dialister pneumosintes, Porphyromonas gingivalis and Parvimonas micra) were determined with qRT-PCR on a RotorGene 6000 (Qiagen) using the TaqMan Universal Mastermix (Life technology, NY, USA). The following cycle profile was applied: 50°C for 2 minutes, 95°C for 10 minutes, and 50 cycles of 95°C for 15 seconds and 58°C for 1 minute. The detection limit was 40 cycles. qRT-PCRs were performed in triplicates. qRT-PCR efficiency was determined on a dilution series of a specific artificial synthesized positive control with known copy numbers, and copy numbers of the samples were quantified based on these dilution series. Potential unspecific amplification was ruled out using a no template control.

Cardiac magnetic resonance imaging
The CMR protocol included a functional study and late gadolinium enhancement (LGE) imaging according to standard protocols ³. Left ventricular mass was calculated from the total myocardial volume multiplied by the specific gravity of the myocardium (1.05 g/ml). Late gadolinium enhancement was defined as myocardial areas with signal intensity above the average of apparently normal myocardium plus two standard deviations (SD). Areas of LGE were traced automatically, and total mass of LGE was calculated and expressed as percentage LGE. Myocardial area at risk was determined by T2 weighted imaging, infarct size was determined by T1 weighted imaging. For offline analyses, CMR42 version 3.2 (Circle Cardiovascular Imaging, Calgary, Canada) was used.

Distribution of macrophages in acute coronary thrombi
To complement TissueFAXS results describing M1/M2 macrophage distribution in coronary thrombi, we performed FC with CLS thrombus suspensions as described in the methods section. We stained single cell suspensions (n=9) for human CD14, CD68, CD163, CD206 and IL-12, (Biolegend). M1 macrophages were characterized as CD14+CD68+CD163-IL-12+ cells, and M2 macrophages as CD14+CD68+CD163+CD206+ cells. Macrophages (median [IQR] 4.8 [4.3-7.6] % of all cells) comprised the M1 subtype (median [IQR] 51.4 [48.9-79.5] % of total macrophages, IL-12 MFI median [IQR] 534 [403-713] MFI), and the M2 subtype (median [IQR] 48.6 [20.5-51.1] % of total macrophages, CD206 MFI median [IQR] 3371 [3000-5089]). In femoral blood, very few macrophages were found (median [IQR] 0.2 [0.1-0.5] % of all cells), M1 subtype (median [IQR] 16.9 [8.4-34.8] % of total macrophages, IL-12 MFI median [IQR] 539 [451-631]), M2 (median [IQR] 83.1 [65.2-91.6] % of total macrophages, CD206 MFI median [IQR] 1491 [521-4746]).

In vitro whole blood aspiration
To control for artificial activation/destruction of leukocytes, we tested the influence of aspiration by thrombectomy catheters in vitro. Patient whole blood was stained with FC markers (CD66b, CD11a, CD11b, TLR2, TLR4, CD42b, CD62P, CD14, CD68, CD163, CD206, CD86, IL-12, all Biolegend) and for cell viability (annexin V, propidium iodide, Apoptosis Detection Kit II, BD Bioscience). Aspiration catheters were flushed with PBS, and
then blood samples were aspirated through thrombectomy catheters (Export, Medtronic). The first portion was discarded; 1ml per sample was incubated for 15 minutes at room temperature and stained for the same markers. Ratios were calculated between pre and post aspiration signals. No significant changes in any characterization or activation markers were detected, no apoptotic cells were found after aspiration (data not shown).
Supplemental Table

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<th></th>
<th>n</th>
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<th>femoral blood</th>
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<td>CD66b</td>
<td>27</td>
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<td>CD11a</td>
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<td>mean MFI±SD</td>
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<td>2613.8±1201.0</td>
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<tr>
<td>CD11b</td>
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<td>4384 (3018-6346)</td>
<td>p=0.0006</td>
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<td>TLR2</td>
<td>23</td>
<td>median MFI (IQR)</td>
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<td>1541 (1132-2581)</td>
<td>p=0.024</td>
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<tr>
<td>TLR4</td>
<td>23</td>
<td>median MFI (IQR)</td>
<td>270 (11-934)</td>
<td>749 (306-1436)</td>
<td>p=0.014</td>
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**Supplemental Table I: PMN activation in femoral blood and in corresponding coronary thrombus.** Flow cytometry results refer to the data illustrated in Figure 3. Data are expressed as mean fluorescence intensity (MFI) ± standard deviation (SD) or percent±SD. PMNs were identified by forward and sideward scatter and gated for CD45 and CD66b positivity. The following surface markers on PMNs were quantified: Carcino- embryonic antigen-related cell adhesion molecule 8 (CEACAM-8, CD66b); Neutrophil-platelet-aggregates (NPA) were identified by CD66b, CD42b and CD62P positivity; Lymphocyte function-associated antigen 1 (LFA-1, CD11a); Macrophage-1 antigen (Mac-1, CD11b); Toll like receptor 2 (TLR2); Toll like receptor 4 (TLR4).
Supplemental Figures

**Supplemental Figure I: PMN gating by flow cytometry.** Cell debris was excluded by forward and sideward scatter gating (Panel A). CD45 positive leukocytes were gated and divided in crude subgroups (PMNs, monocytes, lymphocytes, Panel B). PMNs were gated for CD66b positivity (Panel C).

**Supplemental Figure II: Microbial Community Profiling of culprit lesion site aspirates.** The second step broad band PCR electrophoresis gel is shown. No positive signals for bacterial DNA could be detected in CLS whole blood aspirates (lane 1-18) or particulate coronary thrombi (lane 19-23). Positive (lane +) and negative controls (lane −) are displayed.
Supplemental Figure III: Immunofluorescence staining of M1/M2 macrophages. An example of a coronary thrombus staining is shown (panel A). Stainings were automatically analyzed (macrophages are encircled in red; other cells are encircled in green, panel F). Cells were stained for nuclei (DAPI in blue, panels B and G), CD68 (anti-CD68 in green, panel C and H) and CD163 (anti-CD163 in red, panel D and I). Magnifications of DAPI+CD68+CD163low M1 macrophages (panel E) and DAPI+CD68+CD163high M2 macrophages are shown (panel J).
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