Elevated Levels of Circulating DNA and Chromatin Are Independently Associated With Severe Coronary Atherosclerosis and a Prothrombotic State

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Objective—Aberrant neutrophil activation occurs during the advanced stages of atherosclerosis. Once primed, neutrophils can undergo apoptosis or release neutrophil extracellular traps. This extracellular DNA exerts potent proinflammatory, prothrombotic, and cytotoxic properties. The goal of this study was to examine the relationships among extracellular DNA formation, coronary atherosclerosis, and the presence of a prothrombotic state.

Approach and Results—In a prospective, observational, cross-sectional cohort of 282 individuals with suspected coronary artery disease, we examined the severity, extent, and phenotype of coronary atherosclerosis using coronary computed tomographic angiography. Double-stranded DNA, nucleosomes, citrullinated histone H4, and myeloperoxidase–DNA complexes, considered in vivo markers of cell death and NETosis, respectively, were established. We further measured various plasma markers of coagulation activation and inflammation. Plasma double-stranded DNA, nucleosomes, and myeloperoxidase–DNA complexes were positively associated with thrombin generation and significantly elevated in patients with severe coronary atherosclerosis or extremely calcified coronary arteries. Multinomial regression analysis, adjusted for confounding factors, identified high plasma nucleosome levels as an independent risk factor of severe coronary stenosis (odds ratio, 2.14; 95% confidence interval, 1.26–3.63; P=0.005). Markers of neutrophil extracellular traps, such as myeloperoxidase–DNA complexes, predicted the number of atherosclerotic coronary vessels and the occurrence of major adverse cardiac events.

Conclusions—Our report provides evidence demonstrating that markers of cell death and neutrophil extracellular trap formation are independently associated with coronary artery disease, prothrombotic state, and occurrence of adverse cardiac events. These biomarkers could potentially aid in the prediction of cardiovascular risk in patients with chest discomfort. **(*Arterioscler Thromb Vasc Biol*. 2013;33:2032-2040.)**

Key Words: atherosclerosis ■ coagulation, blood ■ chromatin ■ DNA ■ neutrophils ■ nucleosomes ■ thrombin ■ thrombophilia

Atherosclerotic plaque disruption and subsequent intraluminal thrombus formation are the pathological hallmark of both acute coronary syndrome, including myocardial infarction, and ischemic stroke. Discharge of plaque or thrombotic debris from unstable or ulcerated lesions into circulation is considered the inciting cause of arterial thromboembolic complications. Despite all modern advances in pharmacological and interventional therapy, atherothrombosis remains one of the most significant clinical burdens worldwide.[1]

See accompanying article on page 1735

During the progression of atherosclerosis, circulating cells and cellular constituents of the vessel wall become more prone to DNA damage. As a result of inadequate DNA repair capacity, processes such as cellular senescence, necrosis, and apoptosis could prevail, thus inducing extracellular DNA and nucleosome (chromatin fragments/histone–DNA complexes) release.[2] There is also a distinct cell death pathway, named extracellular trap formation (ETosis), via which neutrophils

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and other cell types, such as monocytes, mast cells, and eosinophils, can dismantle and expel nuclear or mitochondrial DNA.[3] This process serves as host defense against infection, wherein highly decondensed chromatin threads, carrying both histones and granule proteins, are cast out, providing an extracellular scaffold to trap and kill microbial pathogens.[4] There is growing evidence showing the relationship among different infective pathogens, atherosclerosis progression, and atherothrombosis.[5] In addition to the well-established antibacterial properties, pioneer experimental studies have documented that excessive generation of circulating DNA, nucleosomes, and histones can be deleterious in several disease settings (eg, sepsis, pulmonary inflammation, thrombocytopenia, venous thrombosis, cancer).[6–11] Given their potent cytotoxic and prothrombotic effects, extracellular DNA may establish a new interface between inflammation and coagulation.[12]

There are different imaging techniques to assess the presence and severity of coronary atherosclerosis. Coronary computed tomographic angiography (CCTA) has evolved as a widely available, highly accurate noninvasive diagnostic imaging tool for the assessment of coronary artery disease (CAD).[13,14] The primary aim of this study was to determine the associations between circulating levels of markers of cell death and NETosis and the severity, extent, and phenotype of CCTA-assessed coronary atherosclerosis in individuals with suspected CAD. We further sought to examine the relationship between extracellular DNA and nucleosomes released in plasma, the presence of a prothrombotic state, and the occurrence of major adverse cardiac events (MACEs) during follow-up.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Clinical Characteristics

We studied 282 patients (183 men; 64.9%) with chest discomfort symptoms, suspected for CAD. The median age of the study population was 60 years (min–max, 34–83 years). A total of 245 patients underwent both coronary calcium score scan and CCTA. In the remaining 37 patients, CCTA was waived because of an extremely high coronary calcium score. Baseline characteristics are presented in Table 1. The prevalence of absent, mild, moderate, and severe CAD was 18.1%, 26.6%, 26.2%, and 16.0%, respectively. The 37 patients (13.1%) who did not undergo CCTA are considered as a separate category and are labeled as extremely calcified in all Tables and Figures.

Increased Levels of Circulating dsDNA, Nucleosomes, and MPO–DNA Complexes in Patients With Severe Coronary Atherosclerosis

Figure 1A shows individual double-stranded DNA (dsDNA) levels in patients according to the presence and severity of CAD. Extreme coronary artery calcification is shown as a separate group of patients. The release of extracellular dsDNA into circulation was significantly greater in patients with severe CAD (69.59 ng/mL [41.25–87.75]; P=0.003) or abundant coronary artery calcification (79.37 ng/mL [57.97–92.60]; P<0.001) compared with individuals with no angiographically detected CAD (50.09 ng/mL [13.91–63.92]). Whereas the median fold change in free

Table 1. Baseline Characteristics of the Study Population Stratified by Severity of CAD

	All Participants (n=282)	No CAD (n=51)	Mild CAD (n=75)	Moderate CAD (n=74)	Severe CAD (n=45)	Extremely Calcified (n=37)*	*P* Value
Age in years, median (min–max)	60 (34–83)	55 (36–76)	60 (34–78)	61 (43–83)	60 (37–79)	64 (42–78)	0.004
Male sex, n (%)	183 (64.9)	31 (60.8)	40 (53.3)	46 (62.2)	37 (82.2)	29 (78.4)	0.017
BMI, kg/m^2	26.7 (24.5–29.0)	26.9 (24.7–28.7)	26.3 (24.4–28.7)	26.4 (24.0–29.3)	27.1 (25.6–28.8)	26.8 (24.0–29.9)	0.595
Creatinine, μmol/L	79 (70–88)	80 (70–88)	78 (70–88)	80 (72–90)	82 (70–91)	76 (65–88)	0.558
Smoking, n (%)	70 (24.8)	9 (17.6)	17 (22.7)	18 (24.3)	14 (31.1)	12 (32.4)	0.482
Diabetes mellitus, n (%)	29 (10.3)	5 (9.8)	9 (12.0)	4 (5.4)	4 (8.9)	7 (18.9)	0.565
Family history of CAD, n (%)	105 (37.2)	16 (31.4)	35 (46.7)	27 (36.5)	15 (33.3)	12 (32.4)	0.280
VKA therapy, n (%)	40 (14.2)	3 (5.9)	14 (18.7)	7 (9.5)	10 (22.2)	6 (16.2)	0.046
Aspirin therapy, n (%)	93 (33.0)	15 (29.4)	24 (32.0)	21 (28.4)	19 (42.2)	14 (37.8)	0.432
Lipid-lowering therapy, n (%)	101 (35.8)	11 (21.6)	30 (40.0)	26 (35.1)	20 (44.4)	14 (37.8)	0.087
Antihypertensive therapy, n (%)	66 (23.4)	5 (9.8)	19 (25.3)	19 (25.7)	12 (26.7)	11 (29.7)	0.114
Involvement score, n of segments	2 (0–5)	0 (0–0)	2 (1–4)	5 (2–6)	5 (3–7)	…	<0.001
Calcified lesions, n	1 (0–3)	0 (0–0)	1 (0–2)	2 (1–4)	2 (1–4)	…	<0.001
Mixed lesions, n	1 (0–2)	0 (0–0)	0 (0–1)	1 (1–2)	2 (0–2)	…	<0.001
Noncalcified lesions, n	0 (0–1)	0 (0–0)	0 (0–0)	0 (0–1)	0 (0–1)	…	<0.001
Coronary calcium score	88 (1–356)	0 (0–0)	44 (5–102)	168 (63–355)	197 (46–501)	1211 (948–2295)	<0.001

Categorical variables are presented as numbers (percentages). Continuous data are expressed as median (interquartile range), unless otherwise indicated. Statistical significance at the P<0.05 level (inclusive of all groups of patients with performed CCTA). BMI indicates body mass index; CAD, coronary artery disease; CCTA, computed tomographic angiography; and VKA, vitamin K antagonists.

*Patients with no CT-angiographically confirmed coronary atherosclerotic plaques as a result of severe coronary calcification.

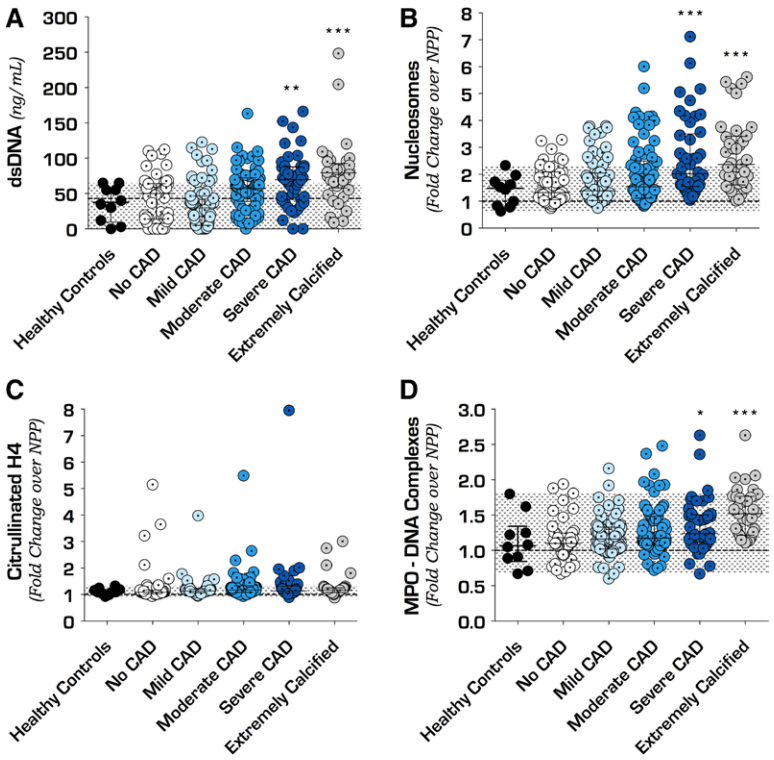

Figure 1. Circulating DNA, nucleosome fragments, and markers of neutrophil extracellular traps according to the presence and severity of coronary artery disease. Patients were divided into categories, based on the severity of coronary artery disease (CAD), as assessed with coronary computed tomographic angiography (CCTA). Patients who did not undergo CCTA because of a high calcium score were considered as a separate category (extremely calcified). A total of 4 markers were measured in all patients (**A**–**D**). Shaded area demonstrates the range of the measured markers in plasma from healthy controls (n=10). Normal pooled plasma (NPP; from n=85 healthy volunteers) is indicated by a horizontal dotted line. **P*<0.05; ***P*<0.01; ****P*<0.001. dsDNA indicates double-stranded DNA.

plasma nucleosomes over normal pooled plasma was 1.32 (1.05–2.09) in the no CAD group, there was a significant fold increase in nucleosome release into circulation in both the severe CAD (2.02 [1.55–3.51]; *P*<0.001) and extremely calcified groups (2.36 [1.60–3.41]; *P*<0.001; Figure 1B). A similar significant increase was observed with respect to plasma levels of myeloperoxidase (MPO)-DNA, Von Willebrand factor (VWF), and thrombin-antithrombin (TAT) complexes (Figures 1D and 2D and 2E). Circulating levels of polymorphonuclear (PMN) elastase–α1-proteinase inhibitor (PI) complexes were significantly higher in the extremely calcified patient group (64.21 ng/mL [20.79–91.04]) compared with the other groups (*P*<0.05 for all comparisons; Figure 2A). Citrullinated histone H4, CXCL4/platelet factor 4, and sCD163 levels in plasma did not significantly differ among the groups (Figures 1C and 2B and 2C). There was a positive association between the severity of luminal stenosis, assessed by CCTA, and several plasma parameters, including dsDNA (Spearman's ρ=0.316; *P*<0.001), nucleosome levels (Spearman's ρ=0.271; *P*<0.001), MPO–DNA (Spearman's ρ=0.215; *P*=0.001), TAT complexes (Spearman's ρ=0.216; *P*=0.001), and VWF (Spearman's ρ=0.186; *P*=0.003; data not shown). To evaluate the strength of associations between all tested variables and CAD severity, we performed multinomial logistic regression, in which a main effects model was implemented (Table 2). Increasing TAT formation robustly predicted all levels of severity of coronary artery stenosis: mild CAD (odds ratio [OR], 1.36; 95% confidence interval [CI], 1.09–1.68; *P*=0.005), moderate CAD (OR, 1.31; 95% CI, 1.06–1.62; *P*=0.013), and severe CAD (OR, 1.33; 95% CI, 1.06–1.68; *P*=0.015). In addition to established risk factors, such as age and male sex, nucleosome (OR, 2.14; 95% CI, 1.26–3.63; *P*=0.005) and VWF (OR, 7.31; 95% CI, 1.33–40.07; *P*=0.022) levels independently predicted the presence of severe coronary stenosis (Table 2).

Associations Between Markers of Cell Death and NETosis and the Extent and Phenotype of Coronary Atherosclerosis

In all patients who underwent CCTA, we were able to assess the number of coronary artery segments affected by atherosclerosis, the degree of luminal stenosis, and characteristics with respect to plaque morphology. In this population, we found a significant positive association between the number of diseased coronary artery segments and plasma dsDNA (Spearman's ρ=0.242; *P*<0.001), nucleosomes (Spearman's ρ=0.219; *P*=0.001), MPO–DNA (Spearman's ρ=0.337; *P*<0.001), TAT complexes (Spearman's ρ=0.330; *P*<0.001), and VWF levels (Spearman's ρ=0.155; *P*=0.015; data not shown). Using a multiple linear regression model (Table I in the online-only Data Supplement) and adjusting for various confounding factors, we examined the independent relationships between candidate determinants and the extent of coronary atherosclerosis in all patients who underwent CCTA. Standardized regression coefficients β are reported in Table I in the online-only Data Supplement. The involvement score (number of affected coronary segments) was mainly determined by male sex, statin use, elevated plasma nucleosomes (β=0.140; *P*=0.026), MPO–DNA (β=0.134; *P*=0.041), and TAT levels (β=0.164; *P*=0.016). Age, family history of premature CAD, and dsDNA remained of borderline statistical significance. With respect to phenotype of coronary plaques, nucleosomes (β=0.152; *P*=0.022) and TAT complexes (β=0.151; *P*=0.036), but also age, male sex, and family history of premature CAD, were found to be independently associated with the number of calcified plaques, quantified by CCTA (Table I in the online-only Data Supplement). We also

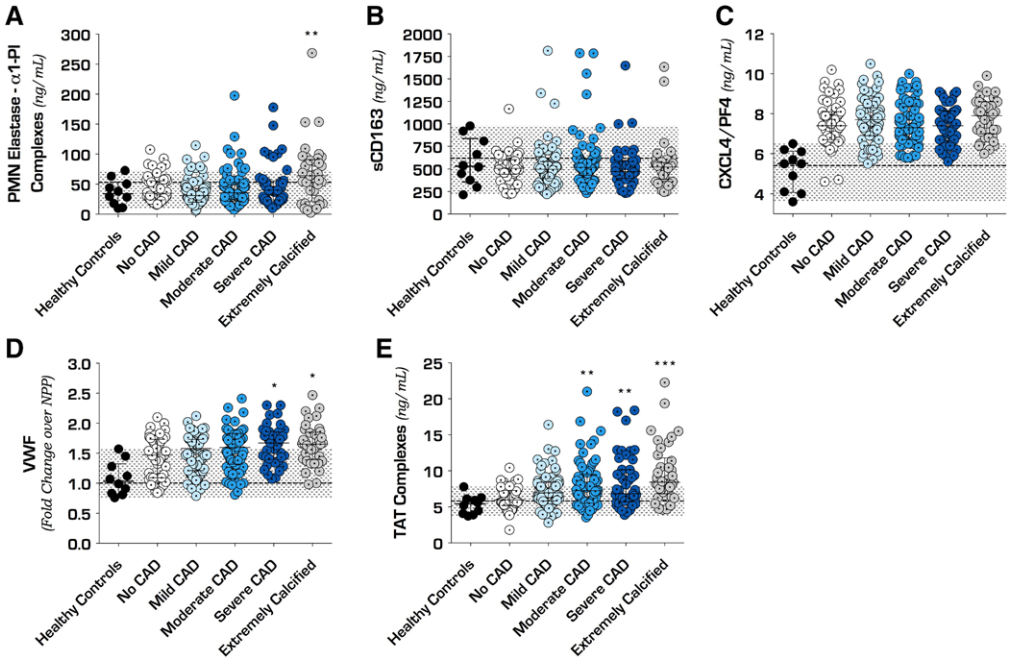

Figure 2. Plasma levels of markers of leukocyte, platelet, endothelial, and coagulation activation according to the presence and severity of coronary artery disease. Patients were divided into categories based on the severity of coronary artery disease (CAD) as assessed with coronary computed tomographic angiography (CCTA). Patients who did not undergo CCTA because of a high calcium score were considered as a separate category (extremely calcified). A total of 5 markers were measured in all patients (**A**–**E**). Shaded area demonstrates the range of the measured markers in plasma from healthy controls (n=10). Normal pooled plasma (NPP; from n=85 healthy volunteers) is indicated by a horizontal dotted line. **P*<0.05; ***P*<0.01; ****P*<0.001. dsDNA indicates double-stranded DNA; MPO, myeloperoxidase; PMN, polymorphonuclear; α1-PI, α1-proteinase inhibitor; PF4, platelet factor 4; TAT, thrombin-antithrombin; and VWF, Von Willebrand factor.

observed an independent relationship among circulating dsDNA (β=0.201; *P*=0.005), TAT complexes (β=0.176; *P*=0.015), CXCL4/platelet factor 4 (β=0.128; *P*=0.042), male sex, and the number of mixed plaques. Free plasma dsDNA (β=0.224; *P*=0.003), MPO–DNA complexes (β=0.150; *P*=0.040), and VWF (β=0.152; *P*=0.022) were further associated with a noncalcified coronary atherosclerotic plaque phenotype.

Extracellular DNA Is an Independent Determinant of a Pronounced Prothrombotic State

Because extracellular DNA, nucleosomes, and neutrophil extracellular traps (NETs) have been functionally implicated in several prothrombotic mechanisms both in vitro and in vivo,[3,12,15] we investigated the associations between extracellular dsDNA and TAT and VWF levels, which are considered well-established prothrombotic markers in various clinical conditions, including atherosclerosis/atherothrombosis.[16] In the entire study population, significant positive correlations were observed between TAT formation and dsDNA (Spearman's ρ=0.367; *P*<0.001), nucleosomes (Spearman's ρ=0.195; *P*=0.001), citrullinated histone H4 (Spearman's ρ=0.231; *P*<0.001), MPO–DNA complexes (Spearman's ρ=0.322; *P*<0.001), PMN elastase–α1-PI complexes (Spearman's ρ=0.274; *P*<0.001), and VWF (Spearman's ρ=0.127; *P*=0.033; data not shown). In a multiple linear regression model, after accounting for various confounding factors (Table II in the online-only Data Supplement), dsDNA (β=0.306; *P*<0.001), MPO–DNA (β=0.230; *P*<0.001), and PMN elastase–α1-PI complexes (β=0.177; *P*=0.003) independently predicted thrombin generation.

Plasma levels of VWF showed significant positive associations with dsDNA (Spearman's ρ=0.171; *P*=0.004), nucleosomes (Spearman's ρ=0.132; *P*=0.026), and citrullinated histone H4 levels (Spearman's ρ=0.322; *P*<0.001). As shown in Table II in the online-only Data Supplement, VWF levels were independently determined by both dsDNA (β=0.146; *P*=0.037) and CXCL4/platelet factor 4 (β=0.200; *P*=0.001) levels in plasma. Overall, there seems to be a strong relationship among extracellular DNA generation (dsDNA), markers of NETosis (MPO–DNA complexes; citrullinated histone H4), and the presence of a prothrombotic state, as determined by the increase in TAT and VWF levels (Figure 3).

High Baseline Levels of Circulating DNA, Nucleosomes, and Markers of NETs Are Significantly Associated With the Occurrence of MACEs

During a median total follow-up of 545 days (interquartile range, 446–666), 27 (9.7%) patients suffered from MACEs (11 percutaneous coronary interventions, 10 coronary artery bypass grafts, 4 acute coronary syndrome, 2 cardiac deaths). MACEs occurred more frequently in men (92.6%), with a median age of 59 years (min–max, 42–76 years), who were overweight (66.7%) and who were less often diabetic (14.8%). Two patients died because of noncardiac causes (Table III in the online-only Data Supplement) and were excluded from the outcome analyses. Significantly elevated baseline levels of circulating dsDNA (*P*=0.0016), nucleosomes (*P*=0.0013), MPO–DNA (*P*=0.0169), TAT (*P*=0.0042), and PMN elastase–α1-PI complexes (*P*=0.0011) were observed in the group

Table 2. Multinomial Logistic Regression Models for CAD Severity as Dependent Variable

	Multinomial Logistic Regression: Main Effects Model					
	Mild CAD Stenosis: 1%–50%		Moderate CAD Stenosis: 50%–70%		Severe CAD Stenosis: >70%	
Variable	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Reference, no CAD (0%)	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Age	1.04 (0.99–1.10)	0.097	1.09 (1.03–1.14)	0.002	1.07 (1.01–1.14)	0.030
Sex (male=1)	1.07 (0.37–3.05)	0.904	1.49 (0.51–4.38)	0.464	5.85 (1.47–23.34)	0.012
BMI	0.94 (0.84–1.05)	0.250	0.99 (0.89–1.11)	0.885	1.06 (0.93–1.20)	0.393
Creatinine	1.00 (0.97–1.04)	0.826	1.00 (0.97–1.04)	0.817	0.99 (0.95–1.03)	0.614
Smoking (yes=1)	1.72 (0.61–4.89)	0.306	1.82 (0.61–5.38)	0.280	2.69 (0.78–9.24)	0.116
Family history of CAD (yes=1)	2.44 (1.02–5.82)	0.045	1.89 (0.76–4.65)	0.168	1.58 (0.52–4.84)	0.420
Diabetes mellitus (yes = 1)	1.13 (0.28–4.65)	0.862	0.44 (0.09–2.11)	0.306	0.82 (0.15–4.60)	0.823
dsDNA	0.99 (0.97–1.00)	0.058	1.01 (0.99–1.02)	0.269	1.01 (1.00–1.03)	0.094
Nucleosomes	1.02 (0.62–1.68)	0.933	1.41 (0.88–2.27)	0.153	2.14 (1.26–3.63)	0.005
Citrullinated histone H4	0.74 (0.33–1.66)	0.469	0.85 (0.44–1.66)	0.638	1.04 (0.50–2.14)	0.925
MPO–DNA complexes	1.04 (0.20–5.37)	0.962	1.40 (0.28–7.00)	0.682	1.74 (0.28–10.78)	0.554
TAT complexes	1.36 (1.09–1.68)	0.005	1.31 (1.06–1.62)	0.013	1.33 (1.06–1.68)	0.015
VWF	1.44 (0.39–5.32)	0.582	1.78 (0.48–6.67)	0.390	7.31 (1.33–40.07)	0.022
PMN elastase–α1-PI complexes	0.99 (0.97–1.00)	0.146	0.99 (0.97–1.01)	0.207	0.99 (0.97–1.01)	0.229
sCD163	1.00 (1.00–1.00)	0.581	1.00 (1.00–1.00)	0.695	1.00 (1.00–1.00)	0.399
CXCL4/PF4	1.11 (0.76–1.62)	0.577	0.95 (0.64–1.42)	0.808	0.77 (0.47–1.27)	0.312
VKA therapy (yes=1)	2.59 (0.59–11.31)	0.205	0.93 (0.19–4.54)	0.933	3.62 (0.67–19.56)	0.135
Lipid-lowering therapy (yes = 1)	2.34 (0.87–6.30)	0.092	1.43 (0.52–3.92)	0.485	1.54 (0.48–4.92)	0.470
Antihypertensive therapy (yes=1)	1.90 (0.59–6.15)	0.282	2.24 (0.68–7.33)	0.184	2.84 (0.75–10.80)	0.126
Aspirin therapy (yes=1)	1.02 (0.41–2.51)	0.973	0.72 (0.28–1.85)	0.494	1.93 (0.60–6.18)	0.266

Statistical significance at the $P<0.05$ level. BMI indicates body mass index; CAD, coronary artery disease; CI, confidence interval; dsDNA, double stranded DNA; MPO, myeloperoxidase; OR, odds ratio; PF4, platelet factor 4; α1-PI, α1-proteinase inhibitor; PMN, polymorphonuclear; TAT, thrombin-antithrombin; VWF, Von Willebrand factor; and VKA, vitamin K antagonists.

who suffered a MACE compared with the event-free group (Figure I in the online-only Data Supplement). To gain further insight into the predictive value of low versus high levels of the tested markers, we dichotomized all continuous predictor variables into 2 groups by using a median split. High baseline values (≥total group median) of dsDNA (OR, 3.12; 95% CI, 1.27–7.63; $P=0.013$), nucleosomes (OR, 2.59; 95% CI, 1.09–6.14; $P=0.030$), MPO–DNA (OR, 3.53; 95% CI, 1.38–9.03; $P=0.009$), TAT (OR, 2.59; 95% CI, 1.09–6.14; $P=0.030$), and PMN elastase–α1-PI complexes (OR, 3.22; 95% CI, 1.31–7.88; $P=0.011$) were significantly associated with the occurrence of MACEs (Table 3).

In addition, we investigated CAD characteristics in all 245 patients who underwent CCTA. Patients developing MACE had a significantly higher involvement score: 5.7 versus 2.8, $P<0.001$. These patients showed significantly more mixed plaques (1.9±1.4 versus 0.9±1.2; $P=0.002$), as well as more calcified plaques (3.2±2.5 versus 1.6±2.0; $P=0.003$). As expected, receiver operating characteristic analysis indicated that computed tomography (CT) score (stenosis >70%) is very useful to discriminate between patients with or without MACEs (area under ROC [AUROC], 0.83; 95% CI, 0.71–0.95; $P<0.001$). In comparison, measurement of dsDNA, nucleosomes, MPO–DNA, TAT, and PMN elastase–α1-PI complexes also showed the potential to significantly predict MACE during follow-up (AUROC, 0.76; 95% CI, 0.68–0.89; $P<0.001$). There was no significant difference between the predictive capacity of CCTA and the aforementioned plasma biomarkers (difference between AUROCs, 0.04; $P=0.571$). However, the addition of these biomarkers to CT score (stenosis >70%) improved its predictive value, whereas the difference between the AUROCs compared with CT score alone indicated a trend toward statistical significance (AUROC, 0.92; 95% CI, 0.87–0.96; $P<0.001$; difference between AUROCs, 0.09; $P=0.086$).

Both neutrophil and monocyte hyperactivation have been demonstrated to play a key role in the initiation of prothrombotic responses.[17] To provide a better understanding of the potential origin of extracellular DNA traps, we performed additional analyses by stratifying all cell death and NET markers by tertiles of either plasma PMN elastase–α1-PI complexes or sCD163, considered sensitive markers of neutrophil and monocyte activation, respectively. Although extracellular DNA might originate from different cell types, here we focused on neutrophils and monocytes because of their significant role in the pathogenesis of atherosclerosis, their involvement in ETosis, but also because of the fact that neutrophils are the most predominant white blood cell type in circulation. Our data suggest that the increase in plasma nucleosomes and MPO–DNA complexes is strictly specific to

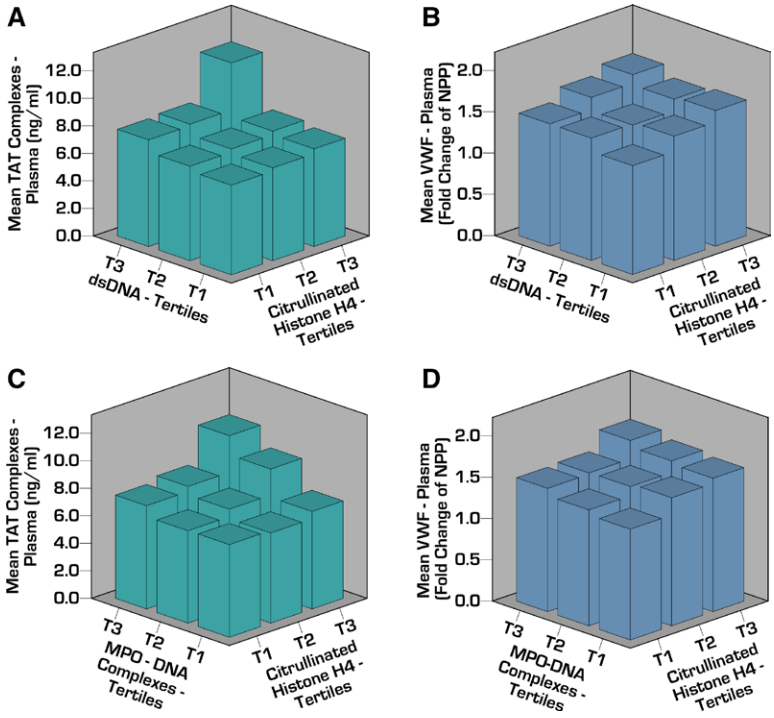

Figure 3. Relationship among extracellular DNA generation, NETosis markers, and a prothrombotic state. **A** and **B**, Relationship among levels of double-stranded DNA (dsDNA), citrullinated histone H4, and mean thrombin-antithrombin (TAT) levels (**A**) or mean Von Willebrand factor (VWF) levels (**B**), respectively. **C** and **D**, Relationship among levels of myeloperoxidase (MPO)–DNA, citrullinated histone H4, and mean TAT levels (**C**) or mean VWF levels (**D**). Elevated levels of both extracellular DNA generation (dsDNA) and NETosis markers (MPO–DNA complexes; citrullinated histone H4) are associated with the presence of a prothrombotic state, defined by increased TAT and VWF levels. NPP indicates normal pooled plasma; and T, tertile.

neutrophil activation and thus might be considered potential markers of NET formation (Table IV in the online-only Data Supplement). Platelet activation, as reflected by CXCL4/platelet factor 4 levels, showed a weak positive correlation with citrullinated histone H4 only (Spearman's ρ=0.138; *P*=0.020). Significant positive associations were observed between neutrophil activation marker PMN elastase–α1-PI complexes and dsDNA (Spearman's ρ=0.257; *P*<0.001), nucleosomes (Spearman's ρ=0.209; *P*<0.001), and MPO–DNA complexes (Spearman's ρ=0.240; *P*<0.001).

Discussion

The current cross-sectional observational prospective clinical study is the first to examine the relationship among plasma markers of extracellular DNA, circulating nucleosomal fragments, NET formation, and the severity, extent, and phenotype of CCTA-defined coronary atherosclerosis in individuals with suspected CAD. The principal finding of this study is the independent association between increased levels of circulating markers of cell death and NETosis and the severity and extent of CAD in patients with chest discomfort symptoms. Furthermore, we provide new data of potential clinical relevance, which demonstrate an independent relationship between extracellular DNA generation and the presence of a prothrombotic state in patients with CAD. Increased baseline levels of circulating dsDNA, nucleosomes, and markers of NETosis were also significantly associated with the occurrence of MACEs during follow-up. Importantly, these biomarkers could potentially aid in the prediction of MACE in patients with chest discomfort.

Table 3. Dichotomized Plasma Markers (High vs Low Values)

Predictors	Relative Risk OR (95% CI)	*P* Values
dsDNA	3.12 (1.27–7.63)	0.013
Nucleosomes	2.59 (1.09–6.14)	0.030
Citrullinated H4	1.51 (0.67–3.43)	0.324
MPO–DNA complexes	3.53 (1.38–9.03)	0.009
TAT complexes	2.59 (1.09–6.14)	0.030
VWF	1.66 (0.73–3.77)	0.225
PMN elastase–α1-PI Complexes	3.22 (1.31–7.88)	0.011
sCD163	1.49 (0.67–3.34)	0.333
CXCL4/PF4	0.51 (0.23–1.17)	0.111

Statistical significance at the *P*<0.05 level. CI indicates confidence interval; dsDNA, double stranded DNA; high values, ≥total group median; low values, <total group median; MPO, myeloperoxidase; OR, odds ratio; PF4, platelet factor 4; α1-PI, α1-proteinase inhibitor; PMN, polymorphonuclear; TAT, thrombin-antithrombin; and VWF, Von Willebrand factor.

Endothelial injury or dysfunction, driven by distinct hemodynamic, oxidative, biochemical, and proinflammatory insults (eg, smoking, perturbed lipid metabolism, or hypertension), precedes the onset of atherosclerosis.[18] In response to tissue injury, the host defense system promotes wound healing by triggering a variety of inflammatory and hemostatic reactions, designed to restore the homeostatic equilibrium.[19] A state of persistent activation and cross talk between inflammation and coagulation can result either in exacerbation of tissue injury (eg, atherosclerotic plaque progression) or thrombosis.[20,21] In fact, chronic inflammation and hypercoagulability are considered integral mechanisms in the pathogenesis of both atherosclerosis and thrombosis.[20,22] A complex network of cellular and molecular interactions, bridging innate and adaptive immunity in atherogenesis, orchestrate all proinflammatory, fibroproliferative, and prothrombotic changes in the arterial vessel wall.[23,24] Neutrophils are the most abundant white blood cell type responsible for the early response to tissue injury. Neutrophils migrate to the site of tissue damage and extrude decondensed chromatin threads (NETs), consisting of nuclear

histones and azurophilic granule proteins, such as MPO and PMN elastase.[4] Histone degradation and citrullination, driven by PMN elastase and peptidylarginine deiminase 4, respectively, are key processes, which comprise the cornerstone of chromatin decondensation and subsequent NET formation.[3,25]

Histological studies have reported the presence of NETs within the luminal portion of human atherosclerotic vessels, but also in coronary thrombosuction specimens obtained from patients after acute myocardial infarction.[26,27] There are various potential pathways via which extracellular DNA traps might induce either initiation or exacerbation of atherosclerosis. Extracellular DNA represents a link between the innate and adaptive immune systems and may aggravate atherosclerosis through activation of T lymphocytes and antigen-presenting cells.[28,29] The chronic inflammatory atherosclerotic environment can induce neutrophil priming, resulting in enhanced neutrophil activation and MPO-dependent respiratory burst.[30] Elevated MPO levels independently predict endothelial dysfunction and the risk of CAD and acute coronary syndrome in patients.[31] Here, we demonstrate an independent association among increased MPO–DNA levels, a marker of NETosis, and the extent of CAD and the presence of a hypercoagulable state.

Neutrophils can undergo apoptosis during inflammation. Macrophages play a crucial role in the clearance of apoptotic neutrophils, thus resulting in resolution of inflammation. This process is also known as efferocytosis. In patients with acute coronary syndrome, delayed neutrophil apoptosis is a commonly observed phenomenon.[36,37] Inflammation can be exacerbated as a result of overloaded efferocytosis.[38] Interestingly, extracellular histones (H3 and H4) significantly impair the clearance of apoptotic neutrophils by macrophages, and activated protein C, known to cleave histones, restores the efferocytotic capacity of macrophages.[6,39] Hence, extracellular DNA trap formation may have deleterious effects by aggravating chronic inflammation during atherosclerosis progression. There are several clinical studies demonstrating a significant increase in circulating deoxyribonuclease I (DNase I) levels during acute coronary events.[32,33] Since DNase I is an endonuclease, which selectively cleaves DNA and contributes to extracellular chromatin degradation, this may be considered additional indirect evidence, suggesting a role for extracellular DNA in the pathogenesis of myocardial infarction.[34] Recent experimental studies show that administration of DNase I prevents thrombus formation in mouse models of deep vein thrombosis.[8,35]

Extracellular DNA and histones exert powerful prothrombotic effects in vitro and in vivo.[3] Nucleosomes and histones can promote thrombin formation through the activation of either extrinsic or intrinsic coagulation pathways and through platelet activation.[7,12,35,40,41] In addition, excess of extracellular histones can affect the function of the anticoagulant protein C pathway by inhibiting protein C activation, thus resulting in enhanced thrombin formation.[42] PMN elastase, which is an integral component of NETs, cleaves tissue factor pathway inhibitor and promotes thrombin generation in a factor Xa–dependent manner.[43] To our knowledge, this is the first clinical study to demonstrate an independent association among increased extracellular DNA generation, TAT formation, and VWF release in cardiac patients. We show that steadily increasing TAT levels are also independently linked to an increased degree of coronary artery stenosis and plaque extent, also comparable with our previous findings.[44] Thrombin is a key molecule that is important not only to hemostasis but also to atherogenesis.[20,45,46] Hence, one can postulate that circulating DNA, chromatin, and possibly NETs might exacerbate atherosclerosis via coagulation activation.

Our data demonstrated that increased levels of plasma nucleosomes, which indicate ongoing chromatin decondensation, predicted the number of calcified plaques and were not associated with any other plaque phenotype. However, it seems that other cell death markers, such as dsDNA, were not phenotype specific. None of the markers more specific to NET formation (citrullinated histone H4 and MPO–DNA complexes) were independently associated with an increased risk of any type of CAD. MPO–DNA complexes seemed a useful tool only in patients with confirmed CAD, as they predicted both the extent and number of noncalcified lesions.[47,48]

Not all studied patients underwent CCTA. In 37 patients, the coronary calcium score scan already revealed an extremely high calcium score. In those patients, CCTA was waived because of the very high a priori chance for a nondiagnostic test result. Predominantly as a result of blooming artifacts, reliable estimation of the severity of the coronary plaque becomes greatly impaired. Furthermore, it is known that blooming can lead to overestimation of the severity of CAD.[49] We at least know that these patients have calcified plaques, but it is conceivable and even plausible that they also have mixed and noncalcified plaques. However, we were not able to prove this because CCTA was waived. Despite the fact that we cannot exactly say to what extent these patients have obstructive CAD, it is known that a high calcium score is associated with an increased risk for severe stenosis and cardiovascular events. In fact, it is even considered to be a better predictor than the Framingham risk score.[50] Instead of excluding these patients, we, therefore, considered them as a separate high-risk group.

Our data indicate that measurement of biomarkers, such as dsDNA, nucleosomes, MPO–DNA, TAT, and PMN elastase–α1-PI complexes, may be useful for the evaluation of patients with chest discomfort. Although the addition of these 5 biomarkers to CT score did not significantly improve risk stratification, we observed a trend in increasing the prediction capacity of traditional CCTA. However, one should consider that CT score provides significant prognostic information, thus it might be difficult to establish an incremental value for any plasma biomarker measured. Larger studies with a longer follow-up are needed to better assess the sensitivity and specificity of all tested biomarkers to identify vulnerable plaques in patients with coronary atherosclerosis, as well as to study their potential to predict the occurrence of MACEs. It also remains of interest to further test whether levels of the different markers predict adverse outcomes within a single CAD phenotype as determined by CCTA. Because some of these tests (eg, dsDNA) are inexpensive and technically simple to determine, a broader use might be considered even before CCTA, if they prove useful as diagnostic and prognostic tools in patients suspected of having CAD.

Study Limitations

The relatively short-term follow-up may be considered a limitation, which resulted in smaller numbers of recorded composite end points. Although not established in this cohort of

patients, it remains of interest to also study the relationship among extracellular DNA and chromatin release, NETosis, and neutrophil counts. High neutrophil counts are considered a potent inflammatory marker for risk stratification in patients with coronary atherosclerosis. Although we found independent associations between elevated markers of cell death and NETosis and the occurrence of MACEs, it is too early yet to use these markers in daily clinical practice. Longitudinal prospective studies will unravel their prognostic power, whereas mechanistic studies are needed to establish whether there is a causal relationship between NET formation and atherosclerotic plaque progression.

Conclusions

Our report provides evidence demonstrating that elevated levels of markers of extracellular DNA, chromatin, and NETosis are independently associated with the severity, extent, and phenotype of coronary atherosclerosis and with the occurrence of MACEs. Our data reveal potential clinical application of these biomarkers to predict cardiovascular risk in patients. Further experimental and clinical studies are necessary to explore the involvement of NETs in the pathogenesis of atherosclerosis and atherothrombosis.

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Disclosures

None.

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Significance

During the progression of atherosclerosis, circulating cells and cellular constituents of the vessel wall become prone to DNA damage and undergo different forms of cell death, thus resulting in enhanced release of chromatin fragments/nucleosomes into circulation. The principal finding of this cross-sectional observational prospective clinical study is the independent association between elevated levels of markers of cell death and neutrophil extracellular trap formation and the severity, extent, and phenotype of coronary atherosclerosis. Furthermore, this study provides evidence indicating an independent relationship between extracellular DNA generation and the presence of a prothrombotic state in patients with coronary artery disease. Increased baseline levels of circulating double-stranded DNA, nucleosomes, and markers of NETosis were also significantly associated with the occurrence of major adverse cardiac events during follow-up. Overall, our data reveal potential clinical application of these biomarkers to predict cardiovascular risk in patients.