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Neutrophil extracellular traps in thrombi retrieved during interventional treatment of ischemic arterial diseases

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ABSTRACT

Introduction: The ultrastructure and cellular composition of thrombi has a profound effect on the outcome of acute ischemic stroke (AIS), coronary (CAD) and peripheral artery disease (PAD). Activated neutrophils release a web-like structure composed mainly of DNA and citrullinated histones, called neutrophil extracellular traps (NET) that modify the stability and lysability of fibrin. Here, we investigated the NET-related structural features of thrombi retrieved from different arterial localizations and their interrelations with routinely available clinical data.

Patients and methods: Thrombi extracted from AIS (n = 78), CAD (n = 66) or PAD (n = 64) patients were processed for scanning electron microscopy, (immune)stained for fibrin, citrullinated histone H3 (cH3) and extracellular DNA. Fibrin fiber diameter, cellular components, DNA and cH3 were measured and analyzed in relation to clinical parameters.

Results: DNA was least present in AIS thrombi showing a 2.5-fold lower DNA/fibrin ratio than PAD, whereas cH3 antigen was unvaryingly present at all locations. The NET content of thrombi correlated parabolically with systemic inflammatory markers and positively with patients' age. The median platelet content was lower in PAD (2.2%) than in either AIS (3.9%) or CAD (3.1%) and thrombi from smokers contained less platelets than non-smokers. Fibrin fibers were significantly thicker in male patients with CAD (median fiber diameter 76.3 nm) compared to AIS (64.1 nm) or PAD (62.1 nm) and their diameter correlated parabolically with systemic inflammatory markers.

Conclusions: The observed NET-related variations in thrombus structure shed light on novel determinants of thrombus stability that eventually affect both the spontaneous progress and therapeutic outcome of ischemic arterial diseases.

1. Introduction

Cardiovascular disorders remain the leading cause of death worldwide, coronary artery disease (CAD) and acute ischemic stroke (AIS) representing the two medical conditions with the strongest impact on mortality and disability [1]. The prevalence of peripheral artery

Abbreviations: AIS, acute ischemic stroke; ASA, acetylsalicylic acid; CAD, coronary artery disease; cH3, citrullinated histone H3; CRP, C-reactive protein; FD50, median of fluorescent signal ratio of fibrin/extracellular DNA; FH50, median of fluorescent signal ratio of fibrin/citrullinated histone H3; NET, neutrophil extracellular trap; PAD, peripheral artery disease; SEM, scanning electron microscopy; sF, surface fibrin fiber occupancy; sPlt, surface platelet occupancy; WBC, white blood cell

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disease (PAD) has also increased in the past years [2], thus contributing to the growing disease burden of arterial thrombotic disorders.

Intravascular clot formation has long been known to be linked to inflammation, but in the past decade a series of findings related to neutrophil extracellular traps (NETs) revealed new aspects of the role of inflammatory cells in hemostasis [3]. The scaffold of these web-like structures released by neutrophil granulocytes during a specific program of cell-death called NETosis [4] is composed mainly of DNA [5]. During the early phase of NETosis the arginine residues of histone proteins are being deiminated (citrullinated) by the enzyme peptidyl-arginine-deiminase 4, thus cH3 is considered to be a specific marker of NETs [6]. Strong evidence supports the role of NETs in both deep venous [7–11] and arterial thrombi [12–14], and they are now regarded as an additional scaffold of thrombus formation tightly intertwined with the fibrin matrix. In a previous study, we demonstrated massive presence of extracellular DNA and histones in thrombi removed with surgery from patients with PAD [15] and recent studies showed the presence of NETs in ischemic stroke thrombi [16,17].

Tissue damage in ischemic cardiovascular disease is often a consequence of embolization from unstable thrombi [18], whereas the clinical outcome of the interventional or lytic treatment in AIS, CAD and PAD is also affected by the structural phenotype of fibrin, the primary matrix of thrombi [19]. The structural features of fibrin are greatly modified in the presence of NET or extracellular matrix components and this modification determines the mechanical and lytic stability of thrombi [15,20,21]. These facts justify the growing interest in investigating thrombus composition [22,23], but very few data are available concerning the fibrin structure in relation to the cellular and NET content of thrombi in the clinical setting of AIS. In view of the expanding application of mechanical thrombectomy to treat large vessel occlusions causing AIS, elaborate characterization of NET components in the culprit clot composition could refine the need for new therapeutics targeting the NETs [17], whereas associations of clot composition and routinely available clinical data, as well as comparisons with thrombi from other therapeutic interventions (CAD, PAD) could help the identification of etiology- and localization-specific predictors for optimal post-interventional treatment to prevent re-occlusion.

2. Patients and methods

The detailed methods section is available as an Online Resource.

Between 2014 and 2016, 208 consecutive patients (66 CAD, 64 PAD and 78 AIS) were prospectively enrolled (for patients' characteristics see Online Resource Table I). Thrombi were collected during acute therapeutic catheter interventions in CAD, open surgery in PAD or via stent-retriever thrombectomy in AIS [24]. Informed written consent was obtained from all individual participants or their legal guardians. Our study was approved by the institutional and regional ethical board (Ref. #8/2014/18.09.2014). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and regional research committee and with the 1964 Helsinki declaration and its later amendments.

Thrombus occupancy by cells and fibrin was determined from morphological examination of scanning electron microscopy (SEM) images. Thrombus composition was then expressed as percentage of regions occupied by each component out of the total area of the image. The morphometric analysis also included the manual measurement of 300 fibrin fiber diameters followed by evaluation of their distribution using scripts running under Image Processing Toolbox of MATLAB R2015a (MathWorks, Natick, MA) as previously described [25–27].

Multiple (6 to 15, depending on the size of the thrombus sample) sections of each thrombus were fluorescently stained with the TOTO-3 nucleic acid dye for extracellular DNA and with indirect immunofluorescence staining for cH3 and fibrin. Confocal laser scanning microscopic images were taken and quantitatively analyzed based on the fibrin/DNA and fibrin/cH3 ratios determined from the image area covered by the respective fluorescent signal. For statistical evaluation each thrombus was characterized by a single parameter, the median of these area ratios in the separate sections (FD50 and FH50 for fibrin/DNA and fibrin/cH3, respectively).

Regression analysis and statistical hypothesis testing were performed on an array of thrombus composition and routine clinical data of each patient. Previously described algorithms were used (Kuiper statistical test for equality of distribution [28], one-tail statistical test for median equality [25], linear regression models with response variable described as quadratic function of one or two explanatory variables [25]) with modifications detailed in the Online Resource.

3. Results and discussion

3.1. Extracellular DNA and cH3

Our previous in vitro [15] and a recent ex vivo [16] study provided evidence that NET components (DNA, histones) modify the structure of fibrin, increase its mechanical stability and render clots less sensitive to lysis with tissue-type plasminogen activator/plasminogen. In line with the recently reported findings in ischemic stroke thrombi [16,17], our present work confirms the presence of NET components in AIS thrombi, but we also demonstrate that extracellular DNA content – the major meshwork-forming constituent of NETs – of AIS (median FD50 of 0.208) thrombi is similar to CAD and 2.5-fold lower than in PAD thrombi (median FD50 of 0.082, $p = 0.0013$, Fig. 1). Thus, compared to other artery locations, in AIS the NET meshwork impedes the least the fibrinolytic therapeutic approach to restore the patency of occluded vessels, a fact that deserves attention when making therapeutic decisions in stroke. Diabetes also had a significant effect on the relative DNA content in PAD, the FD50 ratio was 2-fold higher in diabetic than in non-diabetic patients (0.0654 vs. 0.1383, $p = 0.0131$).

During the early phase of NETosis the arginine residues of histone proteins are being deiminated (citrullinated) by the enzyme peptidyl-arginine-deiminase 4, thus cH3 is considered to be a specific marker of NETs [6]. This antigen was present in all clots (FH50, Fig. I in the Online Resource) – in line with the recently reported findings in ischemic stroke thrombi [16]. No significant quantitative differences were observed between cH3 content at different vascular locations, which could be attributed to the role of citrullination limited to triggering NET formation and thus differences in the tiny quantities of citrullinated histones sufficient to initiate NETosis are not necessarily detectable after the completion of the process when we performed the observations. Because thrombin is able to degrade citrullinated histones [29], extracellular DNA appears to be a more stable marker of NETosis in thrombi. Such an interpretation of the FD50 and FH50 findings is in line with our results regarding the effects of oral anticoagulants, as the treatment with such drugs leads to a two-fold higher relative cH3 content in all thrombi (FH50 decreased from 1.431 to 0.665, $p = 0.0174$). Because of the common use of statins in hypercholesterolemia, we evaluated the effects of these drugs on the NET markers in dyslipidemic patients: neither FH50, nor FD50 was changed by this cholesterol-lowering medication.

In view of the stabilizing impact of NETs on thrombi, an important observation from our work was that both the DNA and the cH3 content, as hallmarks of NETs, correlated positively with the patients' age (except for cH3 in AIS). The regression models used to evaluate the associations are described in the Online Resource (Tables II–III). FD50 showed an inverse correlation in all main groups up to the age of 57 years ($R^2_{adj} = 0.30$ and 0.33 in AIS and PAD and a stronger association in CAD, $R^2_{adj} = 0.99$) (Table 1, Fig. III in the Online Resource). In AIS no further associations with FD50 were revealed, but in CAD an inverse correlation was found between FD50 and fibrinogen level, and a parabolic association between FD50 and WBC count with a minimum at $11 \times 10^3/\mu l$ (Figure IVB in the Online Resource). A parabolic regression

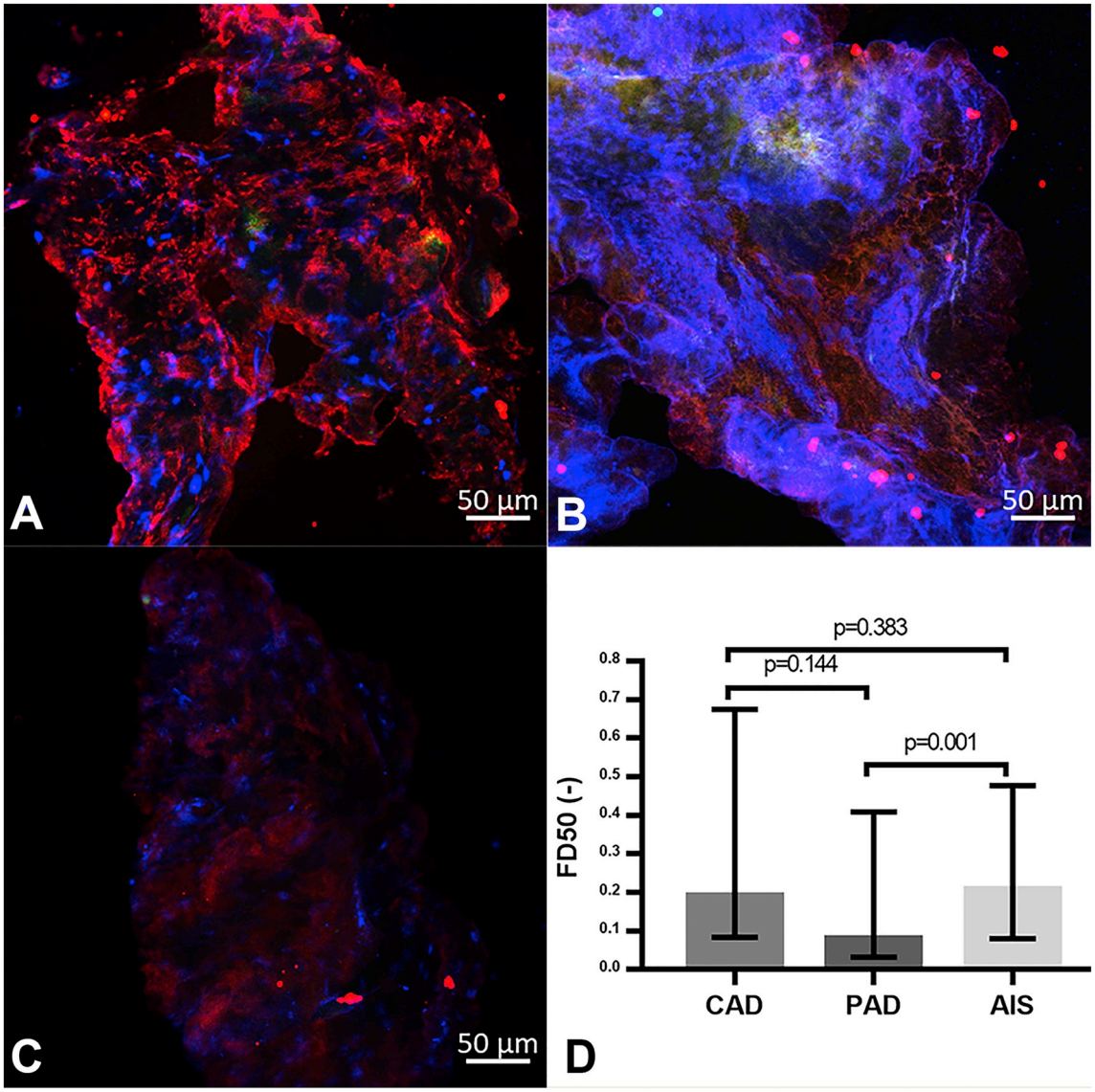

Fig. 1. Indirect immunofluorescent imaging and fibrin/DNA area ratio in arterial thrombi from coronary (CAD), peripheral artery disease (PAD), and acute ischemic stroke (AIS). Following interventional extraction of thrombi from coronary artery disease (A), peripheral artery disease (B) and acute ischemic stroke (C) patients, cryosections were prepared and treated with the fluorescent DNA dye TOTO-3, mouse anti-human fibrin and rabbit anti-human cH3 antibodies followed by the respective species-specific fluorescent anti-IgG antibodies as described in Patients and methods. Images were taken with a confocal laser microscope (red, fibrin; green, cH3; blue, extracellular DNA). Based on the fluorescent signal the ratio of cross-section area occupied by fibrin and DNA was determined in 6–15 regions of each thrombus and the median values of these ratios for each thrombus (FD50) were evaluated (D). A lower value FD50 indicates a higher relative DNA content in clots. The columns and bars represent median and IQR values. The patient number in each group was $n_{CAD} = 49$, $n_{PAD} = 57$ and $n_{AIS} = 64$. *p*-Values result from one-tailed hypothesis testing for medians (significant if p-value < 0.05) using Bootstrap resampling of $n' = 10{,}000$ for each statistical test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

model described the dependence of FD50 on C-reactive protein (CRP) in the range of 0–7.2 mg/l in PAD thrombi (Figure IVC in the Online Resource). Absolute neutrophil count showed similar, parabolic interrelation with FH50, with a minimum at about 5 to 8×10^3/µl (Fig. VI and Table II in the Online Resource). These data suggest the local inflammatory process accompanied by NET release in thrombi is associated with mild to moderate increase in the systemic signs of inflammation (WBC, CRP), whereas their extremely high systemic levels are probably related to additional loci, distinct from the thrombi.

In AIS no significant association was found between FH50 and age at intervention, whereas in the CAD group FH50 showed an inverse correlation with age ($R^2_{adj} = 0.71$). The same association pattern could be observed in PAD between ages 50 and 80, and a positive correlation above 80 years ($n = 11$, $R^2_{adj} = 0.52$, $p_{ANOVA} = 1.7 \times 10^{-2}$). Because histones are known to confer mechanical and lytic resistance to fibrin [15], this data can be interpreted as an age-dependent clot-stabilizing effect, which suggests an improved response to lytic treatment in younger patients, as well as that stabilization of the structure in older patients could favor the success of mechanical intervention. In addition, in PAD the strength of association between the NET content of thrombi and patients' age was increased by atherosclerotic etiology or systemic indicators of inflammation (CRP, leukocytosis, plasma fibrinogen) (Table II).

The age trend in FH50 of AIS patients was reinforced by malignant co-morbidities, known to exert an independent NET-promoting effect [30], to an R^2_{adj} value of 0.90 which points to an increased lytic resistance in this subgroup of patients (Table II). Generally (evaluating all main groups together) the interrelation of the local NET-marker FH50 and the systemic inflammatory indicators followed a rather complex trend in thrombi removed from patients with malignancy (Fig. 2).

Table 1
Strength of association between median fibrin/DNA ratio (FD50), median fibrin/cH3 ratio (FH50) and patient age or inflammatory laboratory markers. The regression models and their coefficients for each dependence are presented as an Online Supplement (Tables II & III). WBC = white blood cell count, CRP = C-reactive protein, n = sample size, R^2_{adj} = adjusted coefficient of determination, p_{ANOVA} = *p*-value of the analysis of variance (significant if p-value < 0.05), (–), no considerable dependence (R^2_{adj} < 0.3), n = sample size constrained to variable range for which the dependence is valid (defined in Table II).

	FD50			FH50		
	R^2_{adj}	n	p_{ANOVA}	R^2_{adj}	n	p_{ANOVA}
All patients						
Neutrophil count	–	–	–	0.38	28	1.22×10^{-3}
Coronary artery disease						
Age at intervention	0.99	21	2.98×10^{-11}	0.71	21	6.09×10^{-5}
WBC	0.48	35	1.50×10^{-5}	–	–	–
Neutrophil count	0.89	13	1.81×10^{-4}	–	–	–
Fibrinogen level	0.58	22	6.19×10^{-4}	–	–	–
Peripheral artery disease						
Age at operation	0.33	18	3.68×10^{-2}	0.39	41	4.33×10^{-5}
CRP	0.75	12	6.31×10^{-3}	–	–	–
Acute ischemic stroke						
Age at intervention	0.3	18	5.71×10^{-2}	–	–	–
Neutrophil count	–	–	–	0.63	23	3.36×10^{-5}

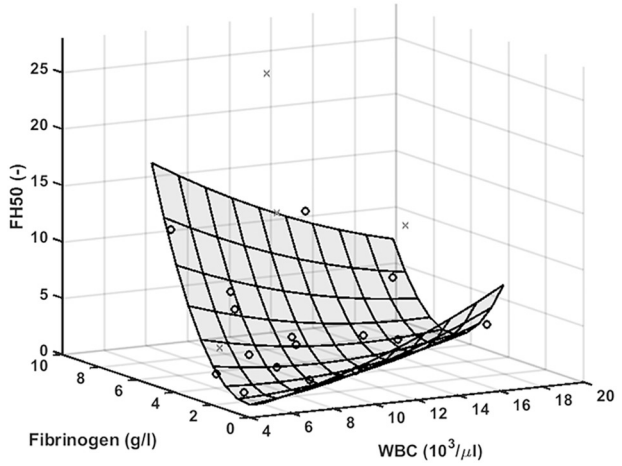

Fig. 2. The joint impact of fibrinogen and white blood cell count on the ratio of fibrin/cH3 in thrombi extracted from patients with malignancy. The regression model and its coefficients are presented as an Online Supplement (Table III). Regression surface is shown for data points (o) after outlier (x) rejection. FH50 = The median of fluorescent signal ratio of fibrin/cH3, WBC = White blood cell count.

Although FH50 correlated positively with WBC count at fibrinogen levels < 4 g/l, the correlation was inverted at higher fibrinogen concentrations ($n = 20$, $R^2_{adj} = 0.65$, $p_{ANOVA} = 1.9 \times 10^{-4}$).

3.2. Platelets in thrombi

Because of the known direct [31–35] and indirect (through von Willebrand factor [36]) NET interactions with platelets and the role of platelets in fibrin formation [37], it was of interest to look at platelet content of the clots. Although in this study we did not observe any associations between measured platelet occupancy and NET components, the SEM analysis of cellular components of thrombi retrieved from AIS, CAD and PAD patients revealed a marked difference in their platelet content (sPlt, Fig. 3). AIS thrombi showed 1.8-fold higher median platelet occupancy than PAD thrombi (sPlt 3.9 vs. 2.2%, $p < 0.001$), whereas sPlt difference from CAD thrombi was observed only in male patients (4.9% in AIS vs. 2.8% in CAD, $p = 0.0032$). These several-fold differences in sPlt can lead to major downstream alterations through the signal amplification of the coagulation cascade as platelets usually initiate clotting in arteries. Our sPlt results in CAD were similar to those reported before by others [38], however concerning AIS other studies found higher platelet content in thrombi [39,40]. The inconsistency of these data could be a result of many factors: group heterogeneity, the smaller sample size in the earlier AIS studies, variations in the rheological conditions of the culprit sites of emboli causing stroke.

Several factors could be identified as modulators of platelet content (Table 2). In AIS with atherosclerotic etiology the median of the sPlt values (2.7%) was 2-fold lower than in the rest of the AIS cases (5.4%), but no such etiology-related difference was seen in PAD thrombi. The presence of a malignant neoplasm was associated with a decreased median sPlt (1.9%) compared to patients with no malignant comorbidity (4.4%) only in AIS thrombi, but not in CAD and PAD. In thrombi removed from diabetic patients we measured lower sPlt values (2.1% compared to 3.2% in non-diabetic patients, $p < 0.001$). Renal insufficiency had no significant effects on the investigated structural parameters. Smoking is a recognized risk factor in ischemic cardiovascular diseases, but a recent study showed that in ST-elevation myocardial infarction the platelet content of thrombi was not altered by smoking [41]. In the current study we observed that in all main groups, the size of which allowed for statistical evaluation, the thrombus occupancy by platelets was more than two-fold lower in active smokers compared to non-smokers, median sPlt 3.6% vs. 1.6% in CAD and 3.2% vs. 1.6% in PAD (there were only 2 documented non-smokers in the AIS group, thus no statistical evaluation could be done). This surprising finding could be attributed to a phenomenon observed in studies designed to evaluate platelet responsiveness to clopidogrel. Whole blood aggregation-based assays indicated improved drug-response in smokers compared to non-smokers ("smokers' paradox"). However, a careful analysis of the laboratory data revealed that this apparent improvement in the inhibition of platelet reactivity in whole blood aggregation is related to an off-drug effect of hematocrit [42,43]. Smokers are known to have higher hemoglobin levels [44] and in our study a similar difference was observed with blood hemoglobin of 8.6 mmol/l in smokers ($n = 74$) versus 7.9 mmol/l in non-smokers ($n = 61$, $p = 0.0124$), which could contribute to the observed difference in the platelet content of thrombi. Interestingly, blood platelet content was not associated with sPlt.

3.3. Fibrin content and fibrin fiber thickness

Because of the known effects of NET components on purified fibrin structure [15], in the present study we investigated the alterations in fibrin content and fiber thickness in thrombi. Although the fibrin fiber occupancy (sF) was statistically higher in PAD thrombi (98.1 ± 5.6%) compared to AIS (97.1 ± 7.93%, $p = 0.043$) or CAD (96.8 ± 8.1%, $p = 0.0081$) groups, these differences were not considerable in magnitude and thus fibrin content could be used as a reference value for the content of NET constituents (justifying the interpretation of the FD50 and FH50 ratio values as measures of DNA and cH3 content of thrombi).

Fibrin fiber thickness has a direct effect on the viscoelastic properties of the clots and their mechanical stability [45]. The median of fibrin fiber diameter measured in this study was significantly higher in CAD thrombi compared to AIS or PAD samples (Fig. 4). These tendencies in the fiber size of the three main groups were due to the structural pattern of fibrin in male patients (median fiber diameter in CAD: 76.3 nm [67.2–90.8], $n = 41$; AIS: 64.1 nm [58.6–85.3] $n = 46$, $p = 0.0093$ compared to CAD; PAD: 62.1 nm [57.4–75.2], $n = 34$, $p = 0.0002$ compared to CAD), whereas fiber diameter did not differ in females from these groups. Because the mechanical stability of thicker fibrin fibers is higher [45], this data indicates that thrombi of male CAD

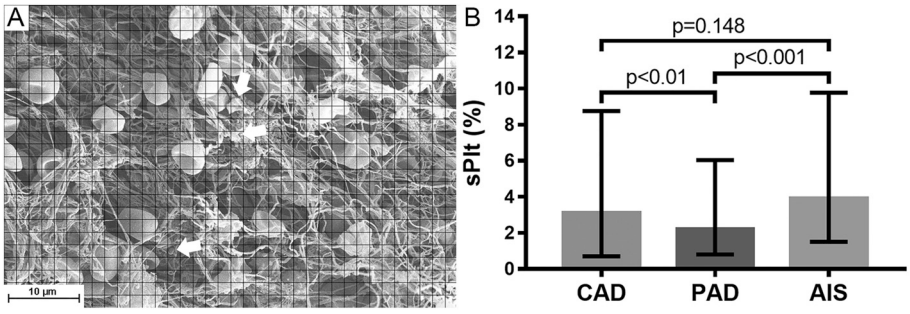

Fig. 3. Platelet content of arterial thrombi from coronary (CAD), peripheral artery disease (PAD), and acute ischemic stroke (AIS). The area of SEM images occupied by platelets or platelet conglomerates (indicated by arrows) was determined as percentage of the total thrombus area (sPlt) as described in Patients and methods (A). The number of observations (evaluated thrombus images, #O) were $\#O_{CAD} = 301$ (from $n = 62$ patients), $\#O_{PAD} = 298$ ($n = 61$) and $\#O_{AIS} = 376$ ($n = 77$). B: The columns and bars represent median and IQR values. *p*-Values result from one-tailed hypothesis testing for medians (significant if p-value < 0.05) using Bootstrap resampling of n′ = 10,000 for each statistical test.

Table 2
Platelet content (sPlt) of thrombi from coronary (CAD), cerebral (AIS) and peripheral (PAD) arteries in the presence and absence of comorbidities and risk factors. The area occupied by platelets on SEM images of thrombi was determined as percentage of the total thrombus area, as described for Fig. 3.

Main group Comorbidity/risk factor	sPlt % med_{no}	sPlt % med_{yes}	p_{med}	p_{distr}	$\#O_{no}$	$\#O_{yes}$
All patients						
Diabetes	3.2	2.1	< 0.001	1.000	724 (148)	248 (50)
CAD						
Smoking	3.6	1.6	0.011	0.669	166 (33)	99 (21)
Malignancy	2.5	3.9	0.139	0.047	244 (50)	57 (12)
PAD						
Atherosclerosis	3.0	2.0	0.097	0.728	60 (13)	233 (47)
Smoking	3.2	1.6	0.004	0.935	115 (24)	178 (36)
Malignancy	2.0	3.4	0.059	0.207	258 (53)	35 (7)
AIS						
Atherosclerosis	5.4	2.7	< 0.001	0.072	175 (37)	196 (39)
Smoking	8.0	2.4	0.164	0.771	11 (2)	67 (13)
Malignancy	4.4	1.9	< 0.001	1.000	308 (64)	63 (12)
Diabetes	4.2	2.5	0.001	1.000	293 (61)	78 (15)

#O = number of observations (evaluated thrombus images); the numbers in parentheses after the #O indicate the numbers of patients included in the respective groups; med = median; no/yes in subscript refers to the presence of the respective comorbidity/risk factor; p_{med} = *p*-value resulting from one-tailed hypothesis testing for medians (significant if p-value < 0.05); p_{distr} = p-value from Kuiper-test for distributions performed with Bootstrap resampling of $n = 10{,}000$ for each statistical test.

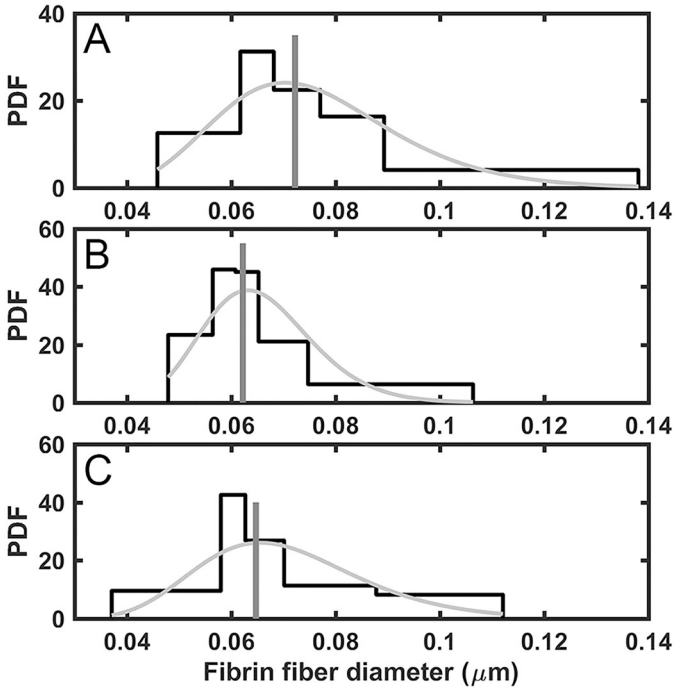

Fig. 4. Fibrin fiber diameter in thrombi removed from different localizations. Manual measurement of 300 fibrin fiber diameters was performed on 5 SEM images of each thrombus followed by evaluation of their distribution. A: Coronary artery disease (CAD), B: Peripheral artery disease (PAD), C: Acute ischemic stroke (AIS). The graphs show the probability density function (PDF) of the empiric distribution (black histogram) and the fitted theoretical log-normal distribution (gray curve). Median values are indicated by vertical lines. The number of thrombi in each group was $n_{CAD} = 62$, $n_{PAD} = 61$, $n_{AIS} = 77$. Both the PAD ($p = 0.0026$) and the AIS group ($p = 0.0132$) had a significantly lower median fibrin fiber diameter, as compared to CAD samples according to one-tailed hypothesis test (significant if *p*-value < 0.05) using Bootstrap resampling of n′ = 10,000.

patients are the most stable. Furthermore, chronic acetylsalicylic acid (ASA) treatment prior to the acute ischemic event was associated with an increased fiber diameter only in males (No ASA: 64.4 nm [58.6–78.4] $n = 45$; ASA: 70.9 nm [61.9–88.8] $n = 68$, $p = 0.022$). ASA had no other significant effects on the measured structural parameters. Regarding comorbidities, accompanying malignant neoplasms reduced fibrin fiber thickness only in PAD (No malignant comorbidity: 64.3 nm [59.2–73.8] $n = 53$ vs. malignant comorbidity: 57.9 nm [54.1–58.7], $n = 7$, $p = 0.018$), but not in AIS and CAD.

The alterations in fibrin structure cannot be attributed to the effect of NETs, because PAD thrombi showed the highest relative DNA and cH3 content and the thinnest fibers, whereas in purified fibrin clots DNA and histones increase the fiber diameter [15]. This discrepancy could be explained at least in part by differences in pre-procedural medication. While all CAD patients received chronic ASA prophylaxis, only a quarter of the AIS and half of the PAD patients were treated with ASA prior the acute intervention. ASA is known to have platelet-independent effects, such as fibrin acetylation, which leads to thicker fibers [46,47]. Clinical trials demonstrate sex differences in response to ASA treatment [48], which is in agreement with our data showing that ASA treatment results in thicker fibrin fibers only in male patients.

Out of the three main groups only in AIS thrombi a parabolic dependence of fibrin fiber median on CRP was found with a minimum at about 5 mg/l CRP ($n = 16$, $R^2_{adj} = 0.48$, $p_{ANOVA} = 1.1 \times 10^{-6}$). CAD thrombi showed a similar correlation between the median diameter of fibrin fibers in thrombi and another acute phase protein, the plasma fibrinogen with a minimum at 4.0 g/l ($n = 35$, $R^2_{adj} = 0.35$, $p_{ANOVA} = 1.9 \times 10^{-4}$, Fig. II and Table II in the Online Resource). The previously reported data on the association of fibrin structure and CRP are inconsistent and controversial with studies variably showing that: 1) CRP-treated fibrin clots had thicker fibrin fibers [49]; 2) a lower CRP (< 2 mg/l) was associated with greater fibrin fiber diameters compared to CRP > 5 mg/L in thrombi removed from CAD [50]. In our study fibrin fiber diameters did not differ significantly in CAD patients with low CRP (< 5 mg/l), compared to those with higher values (71.88 nm [63.34–86.24] $n = 43$ vs. 72.26 nm [61.12–98.48], $n = 18$,

$p = 0.4825$) probably due to the dominant ASA effect discussed above. Plasma fibrinogen level also showed a correlation with symptom-to-intervention time (Fig. VII and Table II in the Online Resource). A strong parabolic regression ($n = 35$, $R^2_{adj} = 0.34$, $p_{ANOVA} = 8.3 \times 10^{-8}$) was observed in thrombi retrieved in < 24 h after the onset of symptoms, with the minimum value at about 6 h. CRP showed no significant association with time. Similarly, we did not identify any significant associations between symptom-to-intervention time and platelet occupancy, fiber diameter, FD50 or FH50.

3.4. Study limitations

Our study has several limitations arising both from the extraction and the processing of thrombi. We were able to study only thrombi that had been successfully retrieved. Moreover, even removed thrombi could have been partially fragmented, without the embolic debris, which might not be completely representative of the whole in situ clot structure. The extraction of clots was performed using different techniques at the different locations (heart, brain, peripheral artery), which in the case of thrombus aspiration may lead to the distortion of structure. Another source of error is the heterogeneity of thrombi, especially if we consider the old age of some peripheral clots. It is highly probable that these samples contain older, more organized parts as well as fresh sections only recently formed on the surface with distinct structural differences. Finally, there are numerous factors that can affect thrombus structure, and thus it is hard to evaluate the effects of only one isolated determinant. These limitations were at least partially overcome by the number of processed samples, by imaging multiple sections from different parts of the thrombi, and using a robust statistical evaluation, including the homogeneity verification of the compared groups.

4. Conclusion

This study provides comparative data on the NET content and related platelet occupancy and fibrin structure of thrombi extracted from patients with acute ischemic stroke, myocardial infarction and peripheral artery disease. These results further our understanding of the structural determinants of the mechanical and lytic stability of thrombi and thus they may contribute to refine the strategies for interventional or enzymatic treatment of acute thrombotic episodes, as well as to provide clues for targeted secondary prevention.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.01.006.

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