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which in turn may be able to reduce the invasiveness of a conventional autopsy.

OFP-03-006

Netosis in coronary plaque ruptures, plaque erosions and intraplaque hemorrhages of myocardial infarction patients at autopsy

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Objective: Netosis is a form of cell death characterized by formation of neutrophil extracellular traps (NETs). To evaluate its participation in coronary atherothrombosis, we investigated the presence and distribution of NETs in coronary plaque ruptures, plaque erosions and intraplaque hemorrhages (IPHs).
Method: Forty-four coronary plaques were retrieved at autopsies from 36 myocardial infarction patients, of which, in HE-stains, were classified as 9 erosions, 18 ruptures and 17 IPHs. 20 intact plaques were selected as controls. Thrombus material in plaques was graded as either fresh, lytic or organized. Immunohistochemistry was performed to visualize neutrophils (MPO) and NETs (citrullinated histone-3/CitH3 and PAD4). Results of immunostaining were scored semi-quantitatively.
Results: Neutrophils (MPO+) and NETs (CitH3+ and PAD4+) were abundantly present in all types of complicated plaques, with no significant differences in extent between ruptures, erosions and IPHs. NETs were found in the thrombus, the underlying plaque tissue and adventitia, the latter with the highest amount in eroded plaques. Fresh and lytic thrombi contained significantly higher numbers of neutrophils and NETs than organized thrombi. In contrast, intact plaques contained no neutrophils and NETs.
Conclusion: Netosis takes part in all distinct types of atherothrombosis, with presumed role in thrombus progression towards vessel occlusion.

OFP-03-007

Balances of different types of cell death in coronary thrombus in relation to thrombus age and instability, after myocardial infarction

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Objective: Fragile thrombus (instability) is associated with a higher mortality rate after acute myocardial infarction (AMI). We investigated which types of cell death are present and involved in thrombus stabilization, over time.
Method: Coronary thrombosuction aspirates of AMI patients were histologically classified in HE-stains as fresh (15), lytic (13) or early organizing (8). An immunohistochemical sequential triple staining was performed using anti-C-reactive protein (necrosis), anti-caspase-3 (apoptosis) and anti-citrullinated histone H3 (ETosis) as primary antibodies. For each specimen, the presence and most prominent type of cell death were semi-quantatively recorded and presented as a percentage of total observations.
Results: All 3 types of cell death were found to be present in all 3 age categories. The most prominent types of cell death observed in fresh and lytic thrombi were ETosis (44.9 and 40 % of specimens, respectively) and apoptosis (43.6 and 35.7 %, respectively), followed by necrosis (11.5 and 24.3 %, respectively). ETosis appeared the most prominent type of cell death found in organizing thrombi (40 % of specimens), but in these thrombi necrosis (37.5 %) was more dominant than apoptosis (22.2 %).
Conclusion: Cell death, along several pathways, is a prominent mechanism in thrombus tissue of AMI patients, and can lead to thrombus instability / fragility.

OFP-03-008

New formula for cardiothoracic ratio for the diagnostic of cardiomegaly on post-mortem CT

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Objective: The cardiothoracic ratio (CTR) is considered to be a reliable detector of cardiomegaly on the CT for livings. Our study aimed to establish an adjusted CTR based score to predict cardiomegaly at post-mortem computed tomography (PMCT).
Method: We selected adult's autopsy cases between 2009 and 2016. Two groups (normal heart weight and overweighed heart) were considered. The CTR was measured on axial images. Logistic regression analysis was performed to investigate the discriminating power of the CTR between groups when adjusting to the confounding factors.
Results: 120 cases with normal heart weight and 100 cases with overweighed heart were analyzed. The factors associated to the cardiomegaly are CTR (p-value = 0.003, OR = 3.57), BMI (p-value = 0.055, OR = 1.09), age (p-value <0.001, OR = 1.67) and gender (p-value 0.002, OR = 4.85). An integer-based point-scoring system was derived based on their β-Coefficients. The score ranged from 21 to 45 with highest values indicating a more likely cardiomegaly. For a threshold of 33, the sensitivity, specificity and the correctly classified were 0.84, 0.78 and 0.81 respectively.
Conclusion: CTR alone cannot be used to discriminate between normal heart weight and overweighed heart at PMCT. A new formula has been developed, including age, gender and BMI to diagnose the cardiomegaly at PMCT.

OFP-03-009

Extra-pulmonary tuberculosis in Nepal: A tip of an iceberg

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Objective: Tuberculosis is a common condition in the underdeveloped countries like Nepal annual case notification rate (CNR) is 136 per 100,000 populations. Tuberculosis is the 6th leading cause of death in Nepal. Tuberculosis is a preventable disease if diagnosed in time. Extra Pulmonary Tuberculosis is 23.1 % out of total registered Tuberculosis cases in the country. Therefore, objective of this study is to determine the Extra Pulmonary Tuberculosis (EPTB) pattern in the specimen received in pathology lab that may help to understand the prevalence and disease identification.
Method: Pathology Lab Database analysis of Histo-cytology specimens for Extra Pulmonary Tuberculosis during 5 years period from 2011 to 2016 at PAHS, Kathmandu, Nepal.
Results: Out of approximately 20,000 specimens received in the Pathology Department in the 5 year period 1 % was of Extra Pulmonary Tuberculosis (EPTB). Lymph nodes comprised of 58 %, followed by Gastrointestinal and Skin in 10 each and 8 % cases were seen in the urogenital tract.
Conclusion: This study represents facility based data only; so it may reflect the tip of an iceberg of at risk population who dwell in the rural mountainous area where the diagnostic facility are not available. Merely the clinical judgement should not overlook the probability of Extra Pulmonary Tuberculosis.

OFP-03-010

Introducing MiniTEM for ultrastructural pathology

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Objective: MiniTEM is a desk-top transmission electron microscopy and analysis platform with a high degree of automation in the microscope

Netosis in coronary plaque ruptures, plaque erosions and intraplaque hemorrhages of myocardial infarction patients at autopsy

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Objective
Netosis is a form of cell death characterized by formation of neutrophil extracellular traps (NETs). To evaluate its participation in coronary atherothrombosis, we investigated the presence and distribution of NETs in coronary plaque ruptures, plaque erosions and intraplaque hemorrhages (IPHs).
Methods
Forty-four coronary plaques were retrieved at autopsies from 36 myocardial infarction patients, of which, in HE-stains, were classified as 9 erosions, 18 ruptures and 17 IPHs. 20 intact plaques were selected as controls. Thrombus material in plaques was graded as either fresh, lytic or organized. Immunohistochemistry was performed to visualize neutrophils (MPO) and NETs (citrullinated histone-3/CitH3 and PAD4). Results of immunostaining were scored semi-quantitatively.
Results
Neutrophils (MPO^+) and NETs ($CitH3^+$ and $PAD4^+$) were abundantly present in all types of complicated plaques, with no significant differences in extent between ruptures, erosions and IPHs. NETs were found in the thrombus, the underlying plaque tissue and adventitia, the latter with the highest amount in eroded plaques. Fresh and lytic thrombi contained significantly higher numbers of neutrophils and NETs than organized thrombi. In contrast, intact plaques contained no neutrophils and NETs.
Conclusions
Netosis takes part in all distinct types of atherothrombosis, with presumed role in thrombus progression towards vessel occlusion.

Keywords: neutrophils, NETs, atherothrombosis, thrombus, hemorrhage

INTRODUCTION

Plaque disruption elicits subsequent thrombosis and hemorrhage, reserving a life-threatening impact. Coronary atherothrombosis resulted from plaque disruption reveals much more dynamic processes. Previously thought that it directly occludes coronary artery and causes an acute adverse event, the thrombus superimposed on coronary atherosclerosis may also remain clinically silent within days to weeks before the fatal event occur (1, 2). Moreover, it could also undergo healing process which may successfully stabilize the thrombus or may fail and trigger the corresponding clinical manifestation

(3). Thus, it is still not fully understood what factors involve in plaque vulnerability prior to plaque disruption as well as in thrombus stability after plaque disruption.

Nowadays, there is a mounting interest in the roles of neutrophils in atherosclerotic disease. While epidemiological study reported that neutrophil counts associated with the risks of acute myocardial infarction (AMI) (4), research on circulating biology activity markers obtained from blood serum of patients with unstable angina pectoris (UAP) and AMI suggested that activated neutrophils and their inflammatory mediators associate with Acute Coronary Syndrome (ACS) (5, 6). Long time before, the presence of neutrophils had ever been confirmed in plaque rupture (7) and later on was observed in culprit lesion of unstable coronary plaque specimens (8).

Recently, a phenomenon of neutrophil generating Neutrophil Extracellular Traps (NETs) arises in atherothrombosis research. Neutrophil releasing NETs, chromatin fibers decorated with granule-derived antimicrobial peptides and enzymes, causes NETosis, which is an active form of cell death, distinct from neutrophil apoptosis and necrosis (9, 10). In forming NETs, neutrophils required an important enzyme namely peptidyl arginine deiminase (PAD4)(11, 12). NETs were reported in luminal atherosclerotic environment of both mice and human, suggesting their contribution to atherogenesis (13, 14). While netosis has been reported to stimulate thrombosis and influence thrombolysis in deep vein thrombosis (15), study in thrombi specimens of infarct-related coronary artery revealed that neutrophils contribute to thrombosis by tissue factor (TF)-bearing NETs release (16). Previous study of our lab has also suggested that NETs modulate thrombus growth and stabilization, indicated by the presence of colocalisations between neutrophils and NETs in fresh and lytic coronary thrombosuction specimens of post-AMI patients(17).

The above studies, mainly carried out in thrombus specimens, have described the presence and roles of NETs during atherogenesis, thrombus formation and thrombus organization after plaque disruption. We investigated the presence and detailed immunolocalisation of neutrophils, PAD4 and

NETs in the specific location of coronary atherosclerotic plaque vasculature on formalin-embedded autopsied coronary specimens of patients with AMI and further assessed whether neutrophil releasing NETs may contribute to the progression of coronary atherosclerotic plaques that endure hemorrhage and thrombotic complication after disruption.

MATERIALS AND METHODS

In the present study, coronary arteries with- and without thrombotic complications were used. After a database search, H&E sections were retrieved from the archives of the Department of Pathology of the Academic Medical Center, and evaluated for the presence of plaque ruptures, plaque erosions, and intraplaque haemorrhages (IPH). Finally, 18 plaque ruptures, 9 erosions, 17 atherosclerotic plaques with IPH and 20 intact atherosclerotic plaques were selected. All plaques were obtained at autopsy, formalin fixed and paraffin embedded. After being selected, tissue blocks were retrieved from the archive and sections (5µm) were cut. The age of thrombus mass was classified as fresh, lytic and organised, as previously described (17, 21). Organised thrombus was characterized by the ingrowth of smooth muscle cells (SMCs), whereas non-organised thrombus mainly composed of platelets, fibrin and red blood cells (RBCs) without SMCs.

Immunohistochemical single stains were perfomed with the following antibodies: polyclonal rabbit anti myeloperoxidase (MPO, DAKO), polyclonal rabbit anti Citrullinated Histone-3 (CitrH3, Abcam) and monoclonal rabbit anti peptidyl arginine deiminase (PAD4, Abcam). Sections were dewaxed in xylene, rehydrated in graded alcohols, endogenous peroxidase activity blocked with 0.3% H_20_2 in methanol for 20 min, followed by and heat-induced epitope retrieval for 20 min at 98°C with Tris-EDTA pH 9.0 or citrate 6.0. After serum-free protein-block (Dako) for 10 mins, sections were incubated with appropriate dilutions of the different primary antibodies for 60 min at room temperature (MPO, PAD4) or overnight at 4°C (CitrH3) and in corresponding anti-mouse or anti-rabbit (Horse Radish Peroxidase/HRP) secondary antibodies. Single immunostainings were visualized by chromogen

diaminobenzidine (DAB) tetrachloride. All sections were mounted with Vecta Mount (Vector Labs). Negative controls whereby the sections incubated in diluent only without primary antibodies were always included in each of staining protocols.

Immunopositivity staining of MPO, H3 and PAD4 were assessed in different locations of the coronary plaque, namely the plaque and adventitia (all types of plaques), thrombus and interface plaque-thrombus (erodedand ruptured plaque), and hemorrhage (IPH plaque). Numbers of positive-cell in each location was scored as 0: absent; 1: focal or few-scattered positive cells (0-25%), 2: multifocal positive cells (25-50%), and 3: diffuse positive-cells (>50%). The assessment was conducted by two observers (KP, XL), and disagreements were resolved by consensus.

Statistical analysis was performed with SPSS 22.0 (IBM Corporation, Armonk, NY, USA). The results of immunostaining MPO, H3 and PAD4 were expressed as mean ± standard deviation. Comparison of immunopositivity between all plaque types in each location were evaluated with either analysis of variance (ANOVA) or Kruskal-Wallis test. A p-values of ≤ 0.05 was considered statistically significant.

RESULTS

Presence and localisation of MPO$^+$neutrophils and CitrH3$^+$NETs in coronary plaque vasculature

The highlight findings of this study were abundant MPO$^+$neutrophils and CitrH3$^+$NETs observed in all locations of coronary plaque with complications (IPH, erosion and rupture) while they were only found scarcely and even absent in the plaque and adventitia of intact plaques, respectively ($p<0.05$, Anova, n=64). Representative images of MPO$^+$neutrophils and CitrH3$^+$NETs are presented in Figure 1 panel A (MPO, plaque), B (MPO, adventitia), E (CitrH3, plaque) and F (CitrH3, adventitia). Regardless the plaque type, MPO$^+$neutrophils were found more profuse than CitrH3$^+$NETs in all observed locations as

seen in the plaque (Figure 1A vs 1E) and in the adventitia (Figure 1B vs 1F). Plaque rupture and IPH contained more MPO^+neutrophils and $CitrH3^+$NETs in the plaque but less MPO^+neutrophils and $CitrH3^+$NETs in the adventitia ($p>0.05$, Anova, n=44, Figure 1G and 1I). On the other hand, the most frequent MPO^+neutrophils and $CitrH3^+$NETs in the adventitia were observed in eroded plaques.

Expression of PAD4 in coronary plaque vasculature

Peptidylarginine deiminase 4 (PAD4) is a nuclear enzyme secreted by neutrophils to mediate histone hypercitrullination inducing chromatin condensation, an essential process in NETs formation (11, 22). Thus, we determined whether PAD4 also presents in coronary plaque vasculature and involves in neutrophils releasing NETs. The results were in line with our findings of MPO^+neutrophils and $CitrH3^+$NETs. In all locations, the presence of PAD4 were more pronounced in disrupted than in intact plaque ($p<0.05$, Anova, n=64, Figure 1C-D). Among different types of disrupted plaques, the most frequent $PAD4^+$cells were found in the plaques of IPHs and in the adventitial layers of plaque erosions (Figure1H).

Neutrophils and NETs are more abundant in non organized thrombi

We further studied the presence of neutrophils and NETS in relation to different ages of thrombus and hemorrhage. MPO^+ neutrophils and $CitrH3^+$ NETs were more frequently found in non-organised thrombus and only occasionally seen in organised ones ($p>0.05$, Kruskal Wallis test, n=27, Figure 2G-H). Representative images of MPO^+neutrophils and $CitrH3^+$ NETs in each of thrombus age are presented in Figure 2 (A-D: non-organised, B-E: ongoing and C-F: organised, respectively).

DISCUSSION

Neutrophils are widely known to infiltrate and contribute in acute and chronic inflammation of atherosclerosclerosis (23). Our main findings were the abundant neutrophils in plaques with

complications (IPH, erosion and rupture) but not in intact plaques without complications. All autopsied coronary artery specimens from AMI patients with plaque rupture and erosion has been previously studied and showed distinct neutrophil infiltration, whereas intact plaques from AMI patients and coronary plaques from non-cardiovascular patients contained only lack of neutrophils (8). Together, these findings indicate that coronary injury provokes rapid activation of neutrophils.

In addition, neutrophils were infiltrated in all observed locations of coronary vessel architecture, especially in adventitial and perivascular layer, whereby they were scarcely found in intact plaques without complications. This finding suggests that inflammation provoked by plaque disruption spreads out eccentrically up to the adventitial and perivascular area. Adventitial inflammation was previously reported more pronounced in ruptured plaques than it was in non-ruptured plaque (24). It also has been known that the rupture-prone plaques have greater neovascularisation in the adventitia compared to the stable plaques (25, 26). Hence, one may speculate that upon disruption event, the inflammatory cells such as neutrophils extravagate from the fragile and leaky micro-vessels at the adventitial site due to "outburst" chemokine stimuli.

In response to injury, neutrophils generate condensed chromatin fibres decorated with granule-derived antimicrobial peptides and enzymes such as neutrophil elastase, cathepsin G and MPO, namely neutrophil extracellular traps (NETs) (27, 28). NETs were previously observed in luminal atherosclerotic environment of human endarterectomy specimens (13). However, our study revealed that NETs were more frequent in disrupted plaques than they were in intact plaque. Our finding is in line with a recent study that observed isolated leukocytes of infarct-related coronary arterial blood release higher number of NETs compared to they were of non-infarct related coronary arteries from the same patients or of control individuals (16). Moreover, similar with previous findings on neutrophils, NETs were also observed in all locations of coronary plaque vasculature of disrupted plaques including the adventitial

and perivascular layer whereby they were completely absent in intact plaques. Thus, while NETs were previously thought to contribute during atherogenesis (13), our study underlined that neutrophils releasing NETs associate with plaque instability and its progression towards atherothrombosis.

Furthermore, regardless the plaque type, we identified that not all neutrophils generate NETs. The findings emphasised previous findings from our lab study that only a subpopulation of neutrophils release NETs (17). Based on these observations, we may speculate that NETs generation could be a time-dependent matter or a process that needs various biological activation modulators. These speculations need further investigation.

Upon inflammatory reaction, neutrophils may express peptidyl arginine deiminase (PAD4) which catalyse histone deamination, a pronounced post-translational modification in NETs formation (11, 22, 29). To our best knowledge, this is the first study that observed the presence of $PAD4^{+}$cells in coronary plaque vasculature. Other cardiovascular studies on the presence and roles of PAD4 in NETs formation had been conducted in murine models of atherosclerosis and deep vein thrombosis (30, 31). In this study, we observed that plaques with complications contained higher numbers of PAD4 than intact plaques without complications in all observed locations. We also noted similar immunostaining patterns between PAD4 and CitrH3 of all plaque types in different locations (Figure 1H-I), suggesting that PAD4 involvement is still considered to be crucial in NETs formation.

Plaque disruption may undergo plaque healing process involving thrombus evolution. The healing process, similar with other remodelling mechanism, encompasses several pathways including inflammation, angiogenesis and ingrowth of fibroblasts (33). Non-organised thrombus has the most inflammatory activity as shown by more frequent neutrophils and NETs found, followed by ongoing thrombus, while organised thrombus displays lack of inflammatory activity (Figure 2G-H). Our findings enhanced the previous findings from our lab that fresh and lytic thrombi contained high numbers of

neutrophils and corresponding NETs, but they were rarely and not found in organised thrombi, respectively (17). Our findings were also similar with previous study that reported the absence of NETs and very low numbers of intact neutrophils in thrombi of STEMI patients which undergo resolution than the stable ones (16). Thus, these findings may suggest that neutrophil generating NETs contribute in healing process of atherothrombosis after plaque disruption.

Intaplaque hemorrhage (IPH) is a major contributor to the progression of coronary atherosclerosis (34). Hemorrhage is known to serve as the stimulus for inflammatory reactions (35, 36) and to involve in atherothrombosis evolution (37). Thus, our other interesting findings were the presence of neutrophils and NETs in the plaque, hemorrhage and adventitial sites of IPH. Our findings emphasised earlier study on involvement of IPH in atherothrombosis evolution (37). The study observed colocalisation of hemorrhage-derived constituents with neutrophils in human carotid endarterectomy plaques with hemorrhages and confirmed with ELISA that conditioned media and tissue extracted from carotid culprit lesions with IPH contained greater amounts of neutrophil markers such as MPO and alpha-defensins than lesions without IPH. Thus, those observations including ours, highlight that hemorrhages induce inflammation, elicit rapid movement of neutrophils and subsequently provoke NETs generation.

CONCLUSION

In conclusion, upon plaque disruption, neutrophils infiltrate eccentrically until the adventitial and perivascular tissue of culprit coronary lesions whereby they are also able to generate NETs. The immunocolocalisation of MPO, PAD4 and CitrH3 suggest that PAD4 is needed for neutrophils producing NETS although there may be other activation mechanism involved. Moreover, NETs generation was also likely to participate during the healing process after coronary atherosclerotic plaque endures hemorrhage and thrombotic complications.

Conflicts of interest: None declared.

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LEGENDS TO FIGURES AND TABLES

Figure.1. Representative images of immunohistochemistry staining of MPO, PAD4 and Citr H3 taken from one disrupted plaque. The staining of MPO are shown in panel A (plaque) and B (adventitia), PAD4 are in panel C (plaque) and D (adventitia), while CitrH3 are in panel E(plaque) and F(adventitia). Note the abundant presence of neutrophils compared to PAD4 and NETs, bar= 50µm. On the right side, the bar graphs show the mean score of immunopositivity of MPO (G), PAD4 (H) and CitrH3 (I) in the plaque and adventitia between different plaque types. Note: not all neutrophils express PAD4 and release NETs. Only occasional neutrophils, PAD4 and NETs are found in the plaque of and none were present in the adventitia of intact plaque (panel G, H and I). Similar immunopositivity pattern between MPO, PAD4 and CitrH3 across different plaque types could be seen especially in the adventitia (panel G, H and I).

Figure.2. Representative immunostaining images and bar graphs showing MPO and CitrH3 immunopositivity between various stages of thrombus organization. Fresh (non-organised) are seen in panel A (MPO) and D (CitrH3), ongoing thrombus in panel B (MPO) and E (CitrH3) while fully organised thrombus in panel C(MPO) and F(CitrH3); bar=50µm in all images. The bar graphs represent comparison of immunostaining pattern of MPO and CitrH3 among different stages of thombus organization. Note MPO and CitrH3 showed similar immunopositivity pattern that organised thrombi contain less neutrophils and NETs compared to ongoing and non-organised ones (G and H). Diffuse infiltration of neutrophils and NETs are clearly found in non-organised thrombi, while thrombus with no NETs are more frequently found in non-organised ones although the differences were not significant.

FIGURES

Figure 1.

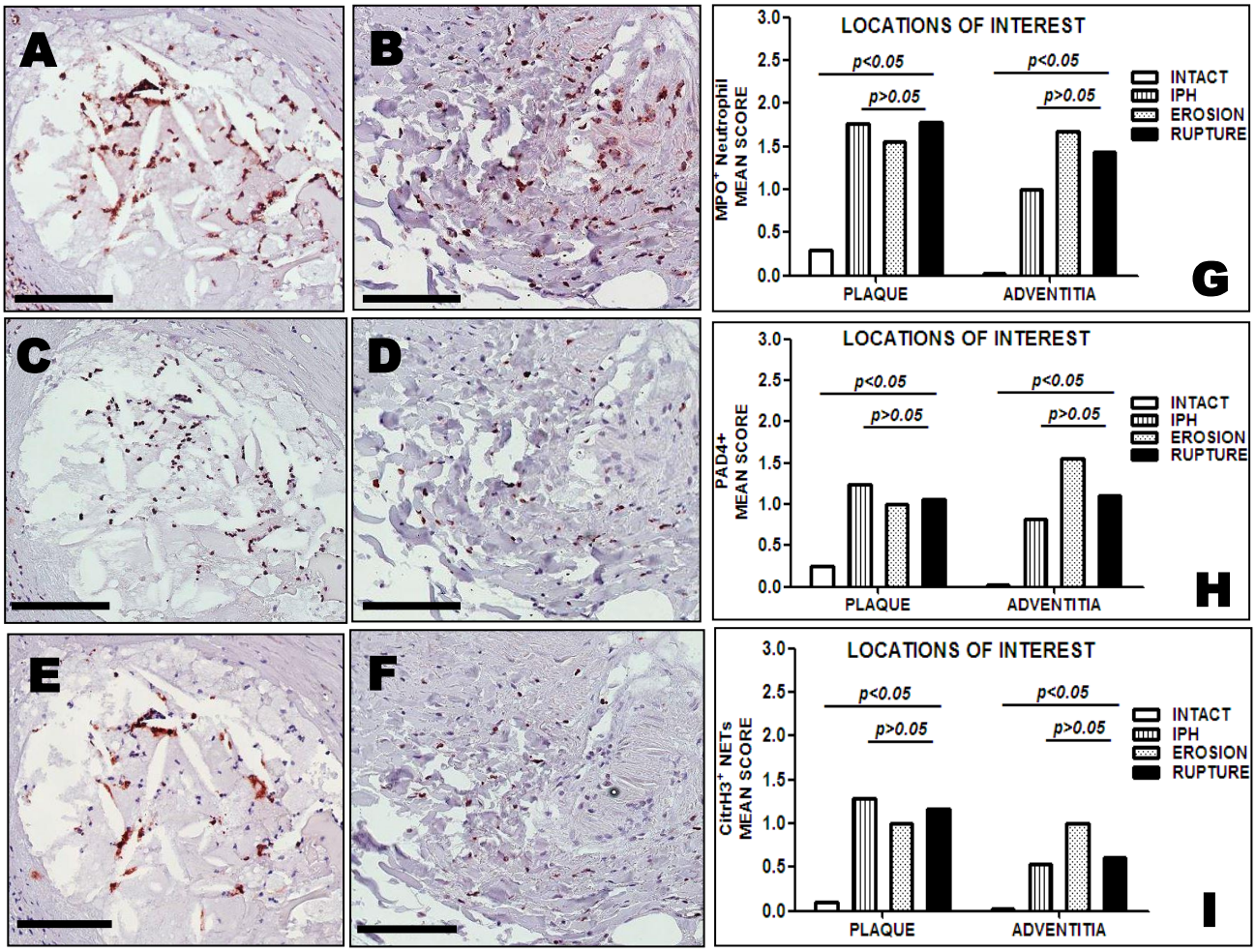
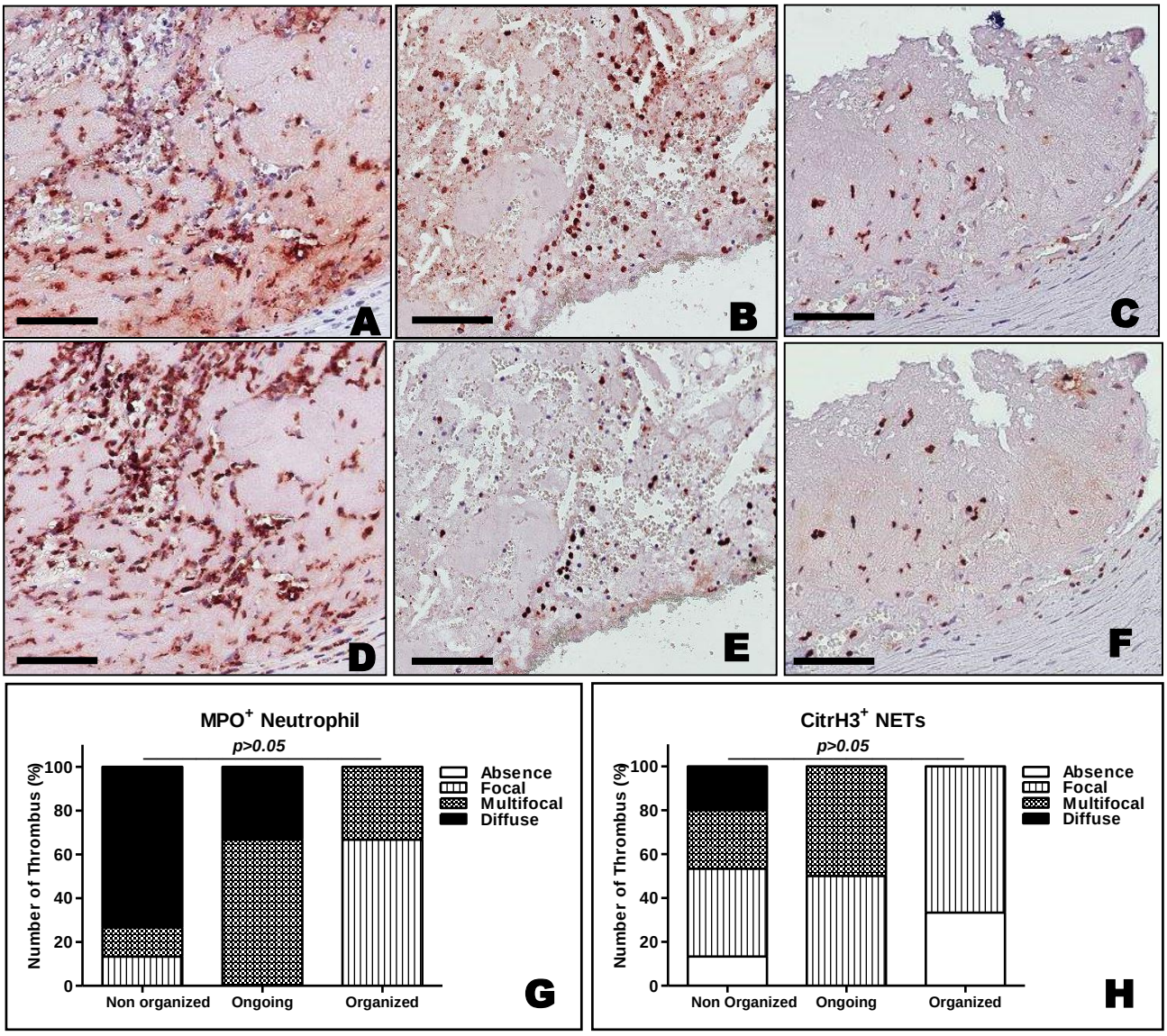


Figure 2.


A
B
C
D
E
F
MPO+ Neutrophil
p>0.05
Number of Thrombus (%)
0
20
40
60
80
100
Non organized
Ongoing
Organized
Absence
Focal
Multifocal
Diffuse
G
CitrH3+ NETs
p>0.05
Number of Thrombus (%)
0
20
40
60
80
100
Non Organized
Ongoing
Organized
Absence
Focal
Multifocal
Diffuse
H