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THROMBOCYTOPENIA AND HYPERINFLAMMATION ARE INDUCED BY EXTRACELLULAR HISTONES CIRCULATING IN BLOOD

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To the Editor,

Circulating histones have been suggested as critical mediators and potential diagnostic/prognostic markers in several hyperinflammatory diseases, including Sars-CoV-2 infection and classical bacterial sepsis, because their levels increase with the worsening of classical and viral sepsis [1-3]. Coagulopathy, thrombocytopenia, platelet aggregation, morphofunctional alterations, immunothrombosis, and thromboinflammation are also critical hallmarks of COVID-19 (Coronavirus Disease-19) and classical sepsis [4].

Several literature data highlighted that histone-platelet interactions could potentially promote thrombocytopenia [5] and platelet activation [6], suggesting that platelets could represent an important target of circulating histones.

Immune cells and platelets are activated to counteract infective stimuli or cell damage, but when they overreact, as during critical sepsis and COVID-19, they could promote a hyperinflammatory response, characterized by an excessive amount of proinflammatory mediators that recruit further immune cells, thus amplifying a dangerous vicious cycle associated with multiorgan failure and death [7].

We recently demonstrated that histones are able to promote morphofunctional alterations in monocytes, thus emerging as critical mediators in monocyte activation during these conditions [8, 9]. Few data are available on the ability of histones to alter platelet indices and associated hyperinflammatory responses. The purpose of this study was to investigate the ability of histones to alter platelet indices (platelet count; Mean Platelet Volume, MPV; Platelet Distribution Width, PDW) and activate hyperinflammatory responses in peripheral blood cells.

Healthy subjects (n=20, age range 24-65) were recruited as volunteers among staff at the Universities of Palermo and Urbino; five samples failed to complete the analytic processes due to

insufficient sample volume. Peripheral venous blood, collected in EDTA-K3 tubes, was treated with a mixture of commercially available histones, including H1, H2A, H2B, H3, and H4 (Histone from calf thymus, Sigma, cod. 10223565001) to evaluate the impact of histones at different times (0, 30, 60, 180 min) and concentrations (0, 50, 100, and 200 μg/mL) on platelet indices and on the release of cytokines from blood cells, as previously described [8, 9].

Routine complete blood cell count, including platelet count (PLT), mean platelet volume (MPV) and platelet distribution width (PDW), were analysed on a UniCell DxH900 Hematology Analyzer (Beckman Coulter), according to the manufacturer's instructions.

Automated slide preparation (unit SP-100, DI-60 system workflow, Sysmex) was used to obtain May-Grunwald-Giemsa-stained blood smears, according to the manufacturer's instructions.

After 3 hours all blood samples were centrifuged (2,000 x g, 15 min), and plasma samples were used to quantify 6 inflammatory biomarkers through the Pro™ Human Cytokine 27-plex assay (including: MIP-1α/CCL3, PDGFbb, RANTES) and the Pro™ Human TGF-β 3-plex assay (including: TGF-β1; TGF-β2, and TGF-β3) through a multiplex suspension immunomagnetic assays, based on the use of fluorescently dyed magnetic beads covalently conjugated with monoclonal antibodies specific for the target proteins, according to the manufacturer's instructions (Bio-Plex, Bio-Rad Labs, Hercules, CA, USA), as previously described [8, 9]. According to the manufacturer's data, the lowest detection limit was 0.5 pg/mL, while the mean inter-assay and intra-variability was 7.4% and 7.1%, respectively.

This observational in vitro study was approved by the local Ethical Committee and all investigations have been conducted according to the Declaration of Helsinki principles.

The *in vitro* treatment of whole blood samples with increasing concentrations of histones (50, 100, and 200 μg\mL) was associated with a very significant reduction in platelet counts, already starting after 30 min from histone treatment, and remaining stably low at 60 and 180 min (Figure 1A), revealing a dose-dependent mechanism confirmed by significant regression lines (Supplemental Table 1).

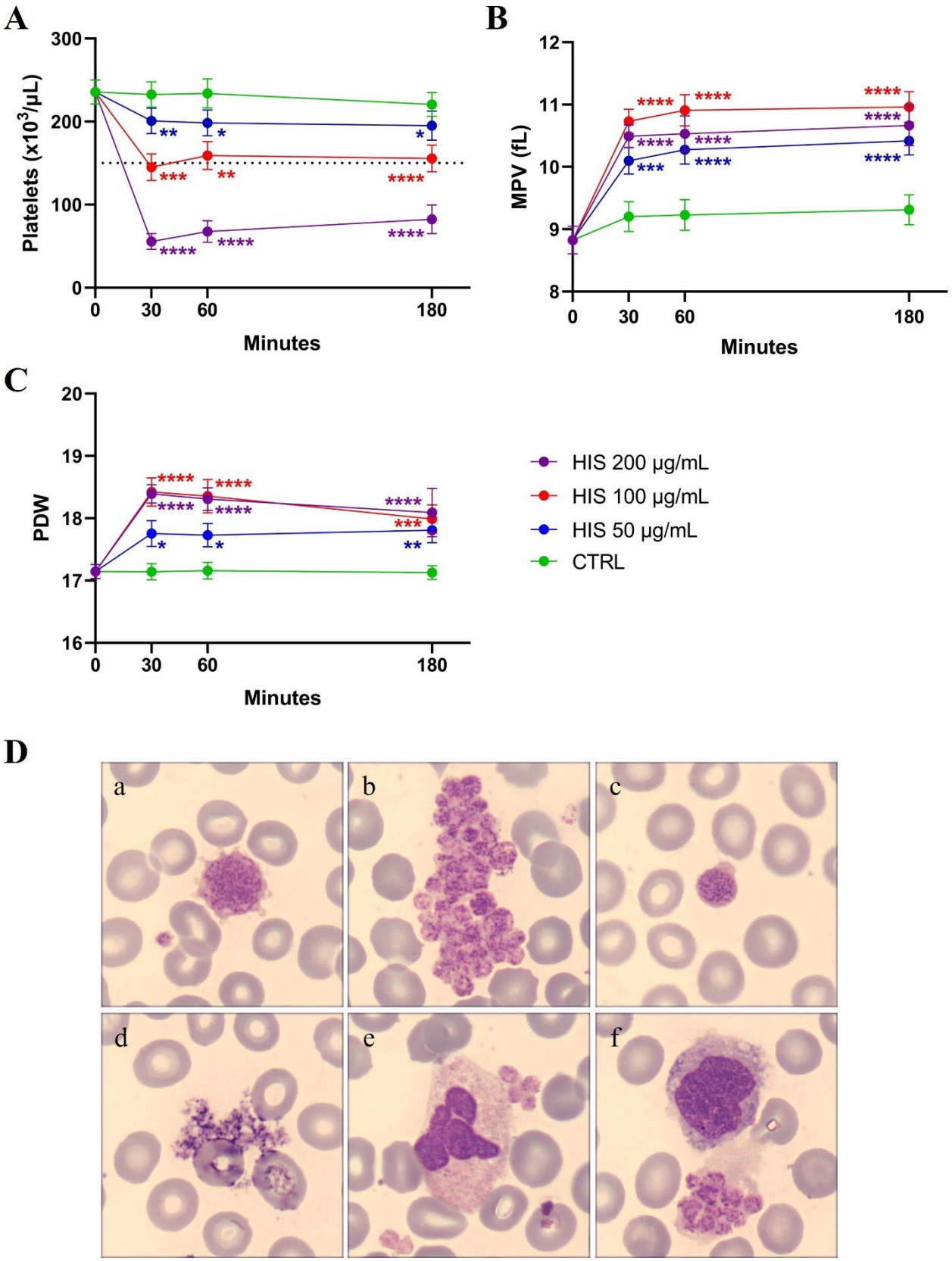

Figure 1. *Platelet index and morphology variations after histone treatments.* ***A)*** *Time- and dose-dependent platelet count variations in histone-treated whole blood samples.* ***B)*** *Time- and dose-*

dependent MPV variations in histone-treated whole blood samples. ***C)*** *Time- and dose-dependent PDW variations in histone-treated whole blood samples. The graphs show the platelet count, MPV and PDW modification in whole blood samples collected from healthy subjects and treated in vitro with 0, 50, 100, and 200 µg/mL of a histone mixture. Values are expressed as mean ± SEM. (Two-way ANOVA: * = p: 0.01-0.05; ** = p: 0.01-0.001; *** = p: 0.001-0.0001; **** = p < 0.0001; the comparisons were calculated among treatments and respective controls at each time). The dotted line in Fig. 1A indicates the lower cut-off level, as reported in the literature.* ***D)*** *Automated light microscopy images of peripheral blood platelets representative of (a) giant thrombocyte; (b) PLT aggregates; (c) Ballooning PLT; (d) PLT-RBC aggregation; (e) PLT-neutrophil interaction; (f) PLT-lymphocyte interaction observed after histone treatments (May-Grunwald-Giemsa, x 100). All statistical tests were performed using GraphPad Prism 9.0. Values are expressed as mean ± standard error mean (SEM) and p values <0.05 were considered significant. Differences among groups were determined using two-way ANOVA followed by Tukey's multiple comparison test.*

MPV was also affected by histone treatments. In fact, besides minimal MPV variations in healthy controls (remaining within the reference range of 7.5-11.7 fL), histone treatments promoted a significant increase in MPV during time-course and at all doses compared to respective controls (Figure 1B).

Moreover, we observed that PDW levels were not significantly changed during time in untreated controls but were significantly increased by treating with all three histone doses, without variations among three time-points (Figure 1C).

The automated digital microscopy of blood smears confirmed morphological changes measured by platelet indices, highlighting platelet alterations related to platelet homotypic and heterotypic aggregations, platelet ballooning, and giant thrombocytes (Figure 1D).

After 3 hours of treatment with 50, 100, and 200 µg\mL of histones we observed a significant and dose-dependent increase of MIP-1α, RANTES, PDGFbb, TGF-β1, TGF-β2, and TGF-β3 as shown in Figure 2.

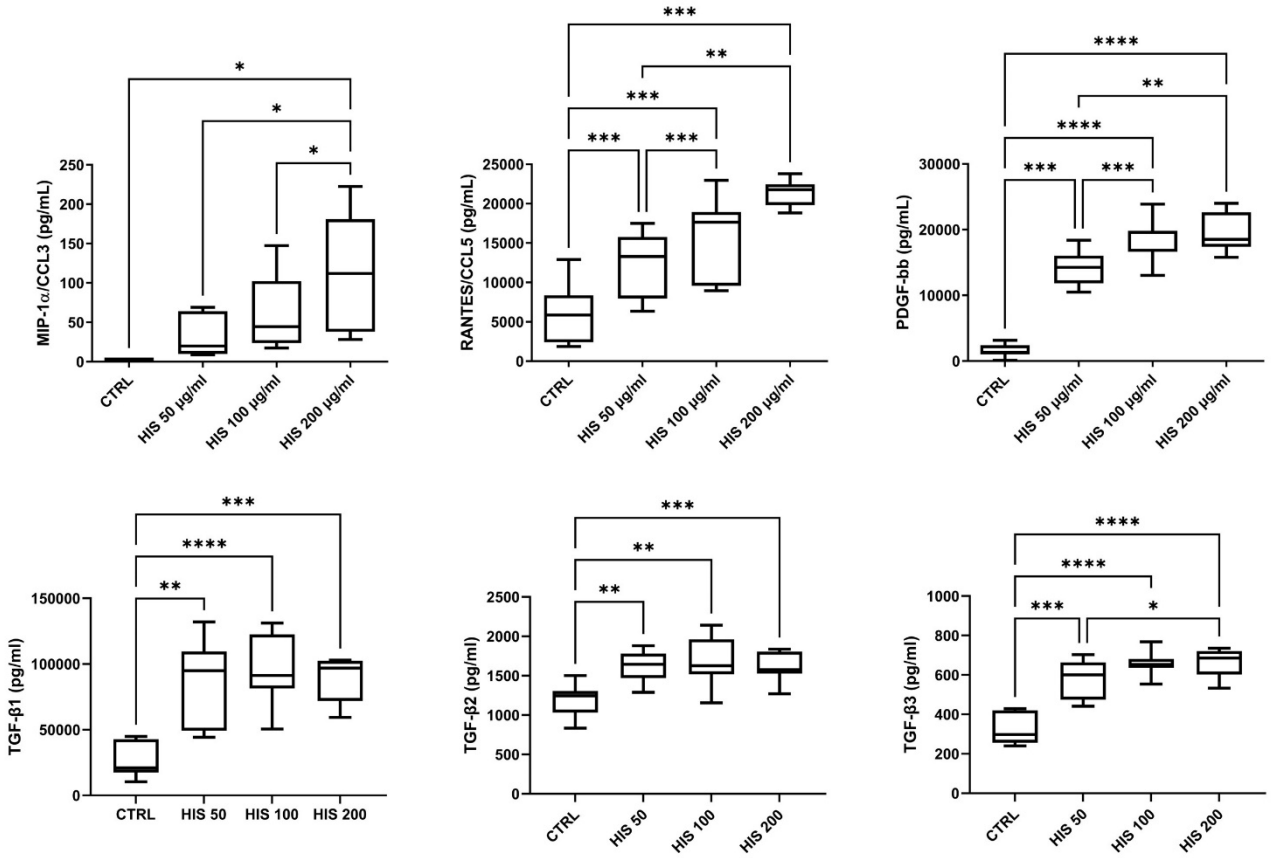

Figure 2. *Cytokine values (mean±SEM) after 3 hours of in vitro treatment with 0, 50, 100, and 200 µg/mL of a histone mixture (One-way ANOVA: *= p: <0.05; **= p: 0.001-0.01; *** =p: 0.001-0.0001; ****= p: < 0.0001; comparisons were calculated among n=8 treatments and respective controls and among each treatment). Values are expressed as pg/mL.*

Studies on the interaction between histones and platelets have become more relevant than ever, as both have been found to be critical cellular and molecular mediators contributing to the immunothrombotic manifestations observed in SARS-CoV-2 infection and sepsis.

We highlighted that the *in vitro* stimulation of whole blood from healthy subjects was associated with a rapid dose-dependent decreased platelet count, and an increase of platelet volumetric indices.

In agreement with our results, several studies reported that high levels of circulating histones are associated with a thrombocytopenic condition in classical sepsis and in COVID-19 infection [2, 10], and that histones induce a rapid thrombocytopenia in mice [5], and activate platelets, promoting their pro-coagulant functions [5, 6].

Histones are also able to induce platelet ballooning, generate platelet-derived microvescicles, and promote platelet aggregation by inducing P-selectin expression on platelet membranes, which act as a binding site for leukocytes and endothelial cells, accordingly to our findings on morphological alterations and homo- and heterotypic aggregates induced by histones.

In our *in vitro* conditions we demonstrated that MPV and PDW variations are the result of the variation of dimension as observed for the platelet ballooning and giant thrombocytes. We can assume that PDW and MPV variations, as markers of platelet heterogeneity and increased volume associated to platelet activation, occur mainly due to circulating platelet activation mediated by histones, which alter platelet morphology, activation, and aggregative status, without involving other platelet maturation mechanisms or direct recruitment of precursors.

Furthermore, we observed that MIP-1α, PDGF-bb, RANTES, TGF-β1, TGF-β2 and TGF-β3 were significantly increased by histone treatments in a dose-dependent manner. These findings are in agreement with our previous results [8], and overall reinforce the hypothesis on the critical contribution of histones in the onset of the hyperinflammatory status found in sepsis conditions.

Although the reduced sample size of our study, it is noteworthy that:

1. the *ex-vivo* whole blood model represents a reliable preclinical model to assess the early modifications of blood cell parameters associated with triggers of bacterial and viral sepsis.

2. this is the first time that histones have been described as inducers of platelet index alterations in whole blood, highlighting that extracellular histone circulating in blood (*i.e.,* cell-free histones) are critical players in both classical (bacterial) and viral sepsis.

3. these findings revealed that histones represent a crucial link between inflammation and thrombosis, suggesting their major role for platelet involvement in infections and non-infectious diseases.

4. the increased concentration of circulating histones in blood affects platelet morphology and activation, inducing thrombocytopenia and thromboinflammation as reported in critical sepsis conditions.

Further studies are ongoing to extend the landscape of blood cell alterations induced by crucial bacterial and viral sepsis triggers.

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Competing interests: Authors state no conflict of interest.

Informed consent: Not applicable.

Ethical approval: This study was approved by the Ethical Committee of University of Palermo (protocol: 07/2019). All investigations have been conducted according to the Declaration of Helsinki principles. In line with non-interventional retrospective design of this in vitro study, investigations were performed on volunteers without clinical indications and no clinical decisions were made based on measured values.

Availability of data and materials: The dataset used in this paper are not publicly available since they are still under elaboration for publication by the Authors but are available from the corresponding author upon reasonable request.

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