The relation between fibrinogen level, neutrophil activity and nucleosomes in the onset of disseminated intravascular coagulation in the critically ill

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Abstract

Background: Nucleosomes and neutrophil extracellular traps (NETs) are important in the pathophysiology of disseminated intravascular coagulation (DIC). Fibrinogen, as an acute phase reactant, may be protective by engaging neutrophils. We hypothesize that DIC can occur when NET formation becomes uncontrolled in relation to low fibrinogen levels.

Patients/method: The ratio of both circulating nucleosomes and human neutrophil elastase alpha-1-antitrypsine complexes (HNE-a1ATc) to fibrinogen was correlated to thrombocytopenia, DIC and organ failure in 64 critically ill coagulopathic patients.

Results: A high nucleosome to fibrinogen ratio correlated with thrombocytopenia and organ failure (ρ - 0.391, p 0.01 and ρ 0.556, p 0.01, respectively). A high HNE-a1ATc to fibrinogen ratio correlated with thrombocytopenia, DIC and organ failure (ρ -0.418, p 0.01, ρ 0.391, p 0.01 and ρ 0.477, p 0.01 respectively).

Conclusion: These findings support the hypothesis that fibrinogen is protective against DIC by counterbalancing excessive neutrophil activation.

Introduction

Disseminated intravascular coagulation (DIC) is a severe complication of critical illness, characterized by consumption coagulopathy with microthrombi formation, contributing to organ failure and mortality[1].

Damage associated molecular patterns (DAMPs) such as extracellular DNA play a role in the pathogenesis of DIC. Nucleosomes, composed of histone-DNA complexes, are released by injured cells or secreted by activated leukocytes as part of neutrophil extracellular traps (NETs). Nucleosomes promote thrombosis by platelet activation and thrombin generation. Accordingly, elevated levels of nucleosomes are associated with thrombosis, DIC and mortality[2-6]. NETs also abundantly contain neutrophil elastase, an inhibitor of physiologic anticoagulants, and are highly procoagulant by providing a scaffold for platelet binding and activating coagulation factors[7-10]**.** In line with this, levels of circulating NETs have been demonstrated to reflect coagulation activation, organ dysfunction and adverse outcome in patients with DIC[11]. Although NET formation is an important contributor to innate immunity, it can become dysregulated during severe inflammation. As a result, high levels of circulating nucleosomes and neutrophil activation could potentially promote consumption coagulopathy and DIC in the critically ill.

Fibrinogen is a key molecule in the coagulation cascade as well as an acute phase reactant since its levels increase manifold in inflammatory states. Why fibrinogen increases is unknown, but it is suggested that fibrinogen is an important regulator of the inflammatory response by engaging leukocytes, independent of clotting function[12, 13]. Consistently, fibrinogen has been shown to delay NET formation in vitro. In addition, fibrinogen has the ability to form complexes with certain DAMPs, thereby neutralizing their damaging effects[14, 15]. We hypothesize that the acute phase reactant

fibrinogen is protective against consumption coagulopathy and DIC in two ways. Firstly, fibrinogen binds circulating nucleosomes, diminishing their thrombotic potential. Secondly, fibrinogen can counterbalance excessive neutrophil activation and subsequent NET induced thrombosis. However, when nucleosome and NET formation becomes dysregulated, levels may increase disproportionately to the increase of fibrinogen, resulting in more DAMPs than fibrinogen can bind. As a result, in the face of overwhelmingly high levels of DAMPs, fibrinogen levels become critically low and DIC can occur.

This exploratory study aims to investigate the relationship between fibrinogen levels and circulating nucleosomes as well as the relationship between fibrinogen and neutrophil activity, as evidenced by HNE-alpha 1 anti trypsine complexes (HNE-a1ATc). To do this, the ratio of nucleosomes and HNE-a1ATc to fibrinogen levels in coagulopathic critically ill patients was correlated to platelet count, DIC score and outcome.

Methods

This study was performed as a post-hoc sub-study of a clinical trial on the efficacy of plasma to reduce the risk of bleeding following an invasive procedure. Patients with a coagulopathy (defined as an INR between 1.5 – 3.0) admitted to the ICU of the Amsterdam UMC, the Netherlands, were included (IRB approval number 10/035 # 10.17.0686) [16]. Written informed consent was obtained from patient or legal representative. For this sub-study, only blood samples that were taken prior to plasma transfusion were used. Patients receiving platelet aggregation inhibitors or anticoagulants as well as those with overt bleeding were excluded.

Coagulation tests included international normalized ratio (INR), activated partial thromboplastin time (aPTT), fibrinogen, platelet count, D-dimer levels and antithrombin by chromogenic substrate method (Sysmex CA 7000 and all reagents, Siemens Healthcare Diagnostics, Germany) using protocols of the manufacturer. Thrombin-antithrombin complexes (TATc) and prothrombin fragment 1 + 2 (F1+2) levels were measured using specific commercially available enzyme linked immunosorbent assay (ELISAs) according to the instruction of the manufacturer (Siemens Healthcare Diagnostics, Germany). Disseminated intravascular coagulation was defined as an ISTH DIC score of ≥ 5 points[17]. Organ failure was assessed with the sequential organ failure assessment (SOFA) score on day of study inclusion.

Nucleosome levels

Nucleosome levels were assessed with an ELISA as described previously [8, 18]. Briefly, monoclonal antibody CLB-ANA/60 that recognizes histone 3 was used as a catching antibody. Biotinylated F(ab)2 fragments of monoclonal antibody CLB-ANA/58, which recognizes an epitope exposed on complexes of histone 2A, histone 2B, and dsDNA, in combination with poly-horseradish peroxidase–labeled streptavidin were used. All reagents are from Sanquin, Amsterdam, The Netherlands. As a standard,

we used culture supernatant of Jurkat cells (1×106 cells/mL), cultured for an additional week, to obtain 100% apoptotic cells. One unit is the amount of nucleosomes released by approximately 100 dead Jurkat cells. The lower detection limit of the assay was 2.5 U/mL. The reference range for circulating nucleosomes in our laboratory is <10.3 U/mL. The inter- and intra-assay coefficient of variation are 8.5% and 4.3%, respectively.

Neutrophil Activation

Human neutrophil elastase - α_1 – antitrypsin (EA) complexes were measured with ELISA[8]. In short, plates were coated with a polyclonal rabbit anti-human neutrophil elastase antibody (1.5 µg/mL; Sanquin, Amsterdam, The Netherlands). Standard and samples were diluted in high-performance ELISA buffer (HPE; Sanquin, Amsterdam, The Netherlands) + 40 µg/mL bovine IgG. Bound complexes were detected by incubation with biotinylated monoclonal anti–α_1-antitrypsin antibody (1 µg/mL) in combination with poly-horseradish peroxidase–labeled streptavidin. Results are expressed in ng/mL by reference to a standard curve of normal human citrated plasma in which EA complexes were generated by incubating with porcine elastase (final concentration 2 µg/mL; Sigma Zwijndrecht, The Netherlands) for 15 minutes at room temperature. The lower detection limit of the assay was 2 ng/mL. The reference range for EA complexes in our laboratory is 8.5 to 55.7 ng/mL. The inter- and intra-assay coefficient of variation are 9.5% and 5.7%, respectively.

Statistical analysis

All variables are expressed as median with interquartile ranges. To compare groups, Mann Whitney U test was used for independent variables. Correlations were assessed using Spearman's rho. Receiver operating characteristics (ROC) curves and corresponding area under the curve (AUC) were computed for HNE-a1ATc to fibrinogen ratio and nucleosome to fibrinogen ratio with DIC development as outcome. $p < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS 26.0 (IBM©, New York, New York, the United States), graphs were made with Prism 8.3.1 (Graphpad Software, San Diego, USA).

Results

In total, 64 patients were included. Coagulopathy in included patients was most frequently caused by sepsis (48%) or liver disease (28%). Thrombocytopenia, defined as a platelet count < 150 x 10^9/L, occurred in 42 patients and 23 patients fulfilled criteria for overt DIC per the ISTH DIC criteria. Patients with DIC were severely ill as demonstrated by a high SOFA score. Moreover, patients classified as DIC had more deranged conventional coagulation test results as well as higher levels of TATc and F1+2 compared to patients without DIC, whereas AT levels were lower (table 1). Collectively, these results underline that patients with DIC in this study have a consumption coagulopathy.

HNE-a1ATc to fibrinogen and platelet ratio and nucleosome to fibrinogen and platelet ratio in DIC

HNE-a1ATc and nucleosome levels did not differ between patients with and without DIC. However, when corrected for fibrinogen levels by using a ratio, the HNE-a1ATc to fibrinogen ratio was significantly higher in patients with DIC than in patients without DIC (152.7 µg/g vs. 39.88 µg/g, p <0.01). Nucleosome levels did not differ between groups, whereas the nucleosome to fibrinogen ratio became more divergent and tended to be higher in DIC than in non-DIC patients. Furthermore, the HNE-a1ATc to platelet ratio (6.4 ug/cellsx10^9 vs 1.1 ug/cellsx10^9, p <0.01) and nucleosome to platelet ratio (11516 u/cellsx10^9 vs 2480 u/cellsx10^9, p <0.01) was significantly higher in patients with DIC compared to non-DIC patients (table 1).

HNE-a1ATc to fibrinogen ratio and nucleosome to fibrinogen ratio in relation to thrombocytopenia and DIC

The HNE-a1ATc to fibrinogen ratio significantly correlated with platelet count and DIC score (ρ -0.418, p 0.01 and ρ 0.391, p 0.01, respectively, figure 1). In addition, the ROC curve of HNE-a1ATc to fibrinogen ratio in relation to DIC had an AUC of 0.75 (95% CI: 0.061 – 0.89, p<0.01, figure 2).

The nucleosome to fibrinogen ratio also correlated with low platelet count (ρ 0.391 p 0.01, figure 1). However, we did not find a correlation between nucleosome to fibrinogen ratio and DIC score (ρ 0.195, p 0.19, figure 1) and ROC analysis showed an AUC of 0.63 (95% CI 0.48 – 0.79, p 0.095, figure 2).

HNE-a1ATc to fibrinogen ratio and nucleosome to fibrinogen ratio in relation to outcome

Both the HNE-a1ATc to fibrinogen and the nucleosome to fibrinogen ratio correlated significantly with organ failure scores (ρ 0.477, p 0.01 and ρ 0.556, p 0.01, respectively, figure 1). In addition, ICU survivors had lower nucleosome to fibrinogen ratios compared to non-survivors (70952 U/g in survivors vs. 186020 U/g in non-survivors, p<0.01). The HNE-a1ATc to fibrinogen ratio was similar in survivors and non-survivors (38 µg/g vs 76 µg/g, p 0.30).

Discussion

DIC development is a multifactorial process, caused by activation of tissue factor dependent coagulation, insufficient control of anticoagulant pathways and suppression of fibrinolysis. Although the pathophysiology of DIC has widely been studied, knowledge of the role of the acute phase reactant fibrinogen in the onset of DIC remains limited. We hypothesize that fibrinogen is protective by neutralizing excessive neutrophil activation. This study demonstrates that the HNE-a1ATc to fibrinogen ratio is significantly increased in patients with overt DIC and that a high HNE-a1ATc to fibrinogen ratio correlates with low platelet count and DIC score. Moreover, a high nucleosome to fibrinogen ratio correlates with low platelet count. In addition, both HNE-a1ATc to fibrinogen ratio and nucleosome to fibrinogen ratio correlate with outcome.

Recent observations have identified an important role for extracellular DNA and NETs in the development of consumption coagulopathy and DIC. Neutrophil elastase, measured as HNE-a1ATc, constitutes one-third of the proteins found on NETs and interferes with both clotting and fibrinolysis, thereby contributing to consumption of coagulation factors[19]. Interestingly, we found that absolute levels of HNE-a1ATc did not differ between patients with and without overt DIC. However, the HNE-a1ATc to fibrinogen ratio was significantly higher in patients with overt DIC, and correlated to platelet count, DIC- and SOFA score. This effect on NET formation is in accordance with a recent study that demonstrated the ability of fibrinogen to delay NET formation in vitro and suggests that fibrinogen may also delay neutrophil activation in vivo[14].

We did not find a similar relation in the nucleosome to fibrinogen ratio, which could be explained by a loss of power due to a high variation in nucleosome levels. Alternatively, the development of DIC may be more driven by neutrophil activation. In line with this, NETs both release nucleosomes to the circulation and provide a platform for nucleosomes to execute their thrombotic potential[10]. Of note, the nucleosome to fibrinogen ratio correlated significantly with adverse outcome, but the mechanism may not be via fuelling a consumption coagulopathy.

The correlations described are modest, which may be due to the multifactorial nature of DIC, in which no single parameter is fully accountable for DIC development. Our results provide further insight into the multifactorial nature of DIC as well as propose a possible intervention for future research. Collectively, these findings support the hypothesis that when an increase in fibrinogen level does not equal NET formation, the amount of circulating fibrinogen can become relatively low in proportion to the amount of circulating cellular damage molecules, thereby possibly contributing to DIC development.

This exploratory study has limitations. Firstly, this study was conducted on a small number of patients. Thereby, significance was not met for all outcomes. Results require further validation in other cohorts. There may also be selection bias, since no patients without coagulopathy were included in this study. In addition, coagulopathy in patients was due to various causes. However, coagulopathy in the included patients was due to consumption rather than decreased production as evidenced by increased TATc levels and decreased levels of AT. Lastly, we did not correct for confounders such as disease severity.

Although causality cannot be inferred from this observational study, these results suggest that low levels of fibrinogen in the presence of excessive neutrophil activation may be a risk factor for consumption coagulopathy and DIC. Whether fibrinogen levels should be kept above a critical level to protect against DIC warrants further research.

Authorship contributions

M.C.A. Müller collected the blood samples, performed the analyses and wrote the manuscript, R.W.G. Dujardin analyzed data and wrote the manuscript, J. Thachil formulated the hypothesis under study, G. van Mierlo assisted with laboratory analysis, S. Zeerleder developed the methodological analyses methods, N. P. Juffermans designed the study and wrote the manuscript. All authors provided feedback on the writing of the manuscript.

Conflicts of Interest

None.

Writing assistance

None.

References

1. Levi M. Pathogenesis and diagnosis of disseminated intravascular coagulation. Int J Lab Hematol. 2018;40 Suppl 1:15-20. doi:10.1111/ijlh.12830
2. Zeerleder S, Zwart B, Wuillemin WA, Aarden LA, Groeneveld ABJ, Caliezi C, van Nieuwenhuijze AEM, van Mierlo GJ, Eerenberg AJM, Lämmle B, Hack CE. Elevated nucleosome levels in systemic inflammation and sepsis*. Critical Care Medicine. 2003;31(7):1947-51. doi:10.1097/01.Ccm.0000074719.40109.95
3. Walborn AT, Patel P, Hoppensteadt D, Mosier M, Statz S, Rondina M, Fareed J. Plasma Nucleosomes Correlate with Markers of Inflammation and Thrombosis in Patients with Sepsis-Associated Coagulopathy. The FASEB Journal. 2017;31(S1):674.15-.15. doi:10.1096/fasebj.31.1_supplement.674.15
4. Duplessis C, Gregory M, Frey K, Bell M, Truong L, Schully K, Lawler J, Langley RJ, Kingsmore SF, Woods CW, Rivers EP, Jaehne AK, Quackenbush EB, Fowler VG, Tsalik EL, Clark D. Evaluating the discriminating capacity of cell death (apoptotic) biomarkers in sepsis. J Intensive Care. 2018;6:72. doi:10.1186/s40560-018-0341-5
5. Chen Q, Ye L, Jin Y, Zhang N, Lou T, Qiu Z, Jin Y, Cheng B, Fang X. Circulating nucleosomes as a predictor of sepsis and organ dysfunction in critically ill patients. Int J Infect Dis. 2012;16(7):e558-64. doi:10.1016/j.ijid.2012.03.007
6. Patel P, Walborn A, Rondina M, Fareed J, Hoppensteadt D. Markers of Inflammation and Infection in Sepsis and Disseminated Intravascular Coagulation. Clin Appl Thromb Hemost. 2019;25:1076029619843338. doi:10.1177/1076029619843338
7. Kimball AS, Obi AT, Diaz JA, Henke PK. The Emerging Role of NETs in Venous Thrombosis and Immunothrombosis. Front Immunol. 2016;7:236. doi:10.3389/fimmu.2016.00236
8. van Montfoort ML, Stephan F, Lauw MN, Hutten BA, Van Mierlo GJ, Solati S, Middeldorp S, Meijers JC, Zeerleder S. Circulating nucleosomes and neutrophil activation as risk factors for deep vein thrombosis. Arterioscler Thromb Vasc Biol. 2013;33(1):147-51. doi:10.1161/ATVBAHA.112.300498
9. Iba T, Miki T, Hashiguchi N, Tabe Y, Nagaoka I. Is the neutrophil a 'prima donna' in the procoagulant process during sepsis? Critical Care. 2014;18(4):230. doi:10.1186/cc13983
10. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Jr., Wrobleski SK, Wakefield TW, Hartwig JH, Wagner DD. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A. 2010;107(36):15880-5. doi:10.1073/pnas.1005743107
11. Kim JE, Lee N, Gu JY, Yoo HJ, Kim HK. Circulating levels of DNA-histone complex and dsDNA are independent prognostic factors of disseminated intravascular coagulation. Thromb Res. 2015;135(6):1064-9. doi:10.1016/j.thromres.2015.03.014
12. Luyendyk JP, Schoenecker JG, Flick MJ. The multifaceted role of fibrinogen in tissue injury and inflammation. Blood. 2019;133(6):511-20. doi:10.1182/blood-2018-07-818211

13. Thachil J. The protective rather than prothrombotic fibrinogen in COVID-19 and other inflammatory states. J Thromb Haemost. 2020;18(8):1849-52. doi:10.1111/jth.14942
14. Locke M, Francis RJ, Tsaousi E, Longstaff C. Fibrinogen protects neutrophils from the cytotoxic effects of histones and delays neutrophil extracellular trap formation induced by ionomycin. Sci Rep. 2020;10(1):11694. doi:10.1038/s41598-020-68584-0
15. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol. 2012;34(1):43-62. doi:10.1007/s00281-011-0290-8
16. Müller MCA, de Jonge E, Arbous MS, Spoelstra -de Man AME, Karakus A, Vroom MB, Juffermans NP. Transfusion of fresh frozen plasma in non-bleeding ICU patients -TOPIC TRIAL: study protocol for a randomized controlled trial. Trials. 2011;12(1):266. doi:10.1186/1745-6215-12-266
17. Taylor FB, Jr., Toh CH, Hoots WK, Wada H, Levi M. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. Thromb Haemost. 2001;86(5):1327-30.
18. Zeerleder S, Zwart B, te Velthuis H, Manoe R, Bulder I, Rensink I, Aarden LA. A plasma nucleosome releasing factor (NRF) with serine protease activity is instrumental in removal of nucleosomes from secondary necrotic cells. FEBS letters. 2007;581(28):5382-8. doi:10.1016/j.febslet.2007.10.037
19. Varjú I, Kolev K. Networks that stop the flow: A fresh look at fibrin and neutrophil extracellular traps. Thromb Res. 2019;182:1-11. doi:10.1016/j.thromres.2019.08.003

Table 1 – Characteristics of patients with DIC and without DIC

	DIC **N=23**	**no DIC** **N=41**	**p**
Sepsis, %	61	43	0.16
Liver disease, %	35	25	0.41
DIC score	6 (5-7)	3 (3-4)	0.000
SOFA score	14 (10 - 16)	11 (8 - 12)	0.014
APACHE II score	27 (21 - 35)	25 (20 - 31)	0.292
Platelet count ($x10^9$/L)	44 (34 - 60)	161 (105 – 258)	0.000
INR [<1.2]	1.96 (1.71 – 2.77)	1.67 (1.52 – 2.08)	0.011
APTT [22 – 32] (sec)	42 (38 - 52)	41 (35 - 49)	0.338
Fibrinogen [1.5 – 3.6] (g/L)	2.30 (1.80 – 3.45)	4.85 (2.93 – 6.33)	0.001
D-dimer [<0.50] (mg/L)	5.6 (4.0 – 19.7)	4.1 (1.8 – 6.3)	0.011
TATc [1.0 – 4.1] (ug/L)	16.8 (10.4 – 28.8)	6.6 (5.1 – 13.0)	0.006
F1+2 [87 – 325] (pmol/L)	441 (211 - 811)	164 (93 - 429)	0.003
Antithrombin [80 – 120] (%)	44 (29 - 54)	69 (38 - 84)	0.007

HNE-a1ATc [8.5 – 55.7] (ng/ml)	376 (90 – 1180)	164 (95 - 322)	0.061
Nucleosomes [<10.3] (u/ml)	375 (198 - 314)	488 (228 - 1120)	0.867
HNE-a1ATc/fibrinogen (µg/g)	152.7 (41.3 – 490.4)	39.88 (17.75 – 82.02)	0.002
Nucleosomes/fibrinogen (u/g)	180000 (75694 – 1095497)	119725 (46868 – 223783)	0.095
HNE-a1ATc/platelets (ug/cellsx10^9)	6.4 (2.1 – 23.1)	1.1 (0.5 – 1.8)	0.000
Nucleosomes/platelets (u/cellx10^9)	11516 (4479 – 92353)	2480 (1150 – 7262)	0.002
ICU LOS, days,	5 (2 - 14)	9 (2 - 20)	0.240
ICU mortality, %	65	65	0.99

Values represented as median (IQR). p value represents difference between patients with and without DIC, calculated with Mann-Whitney U test. Reference values for laboratory tests are reported between square brackets. Abbreviations: DIC, disseminated intravascular coagulation; SOFA, sequential organ failure assessment score; APACHE, acute physiology and chronic health evaluation; INR, international normalized ratio; aPTT, activated partial thromboplastin time; TATc, thrombin-antithrombin complex; F1+2, prothrombin fragment 1+2, HNE-a1ATc, human neutrophil elastase alpha 1 antitrypsin complex; ICU, intensive care unit.

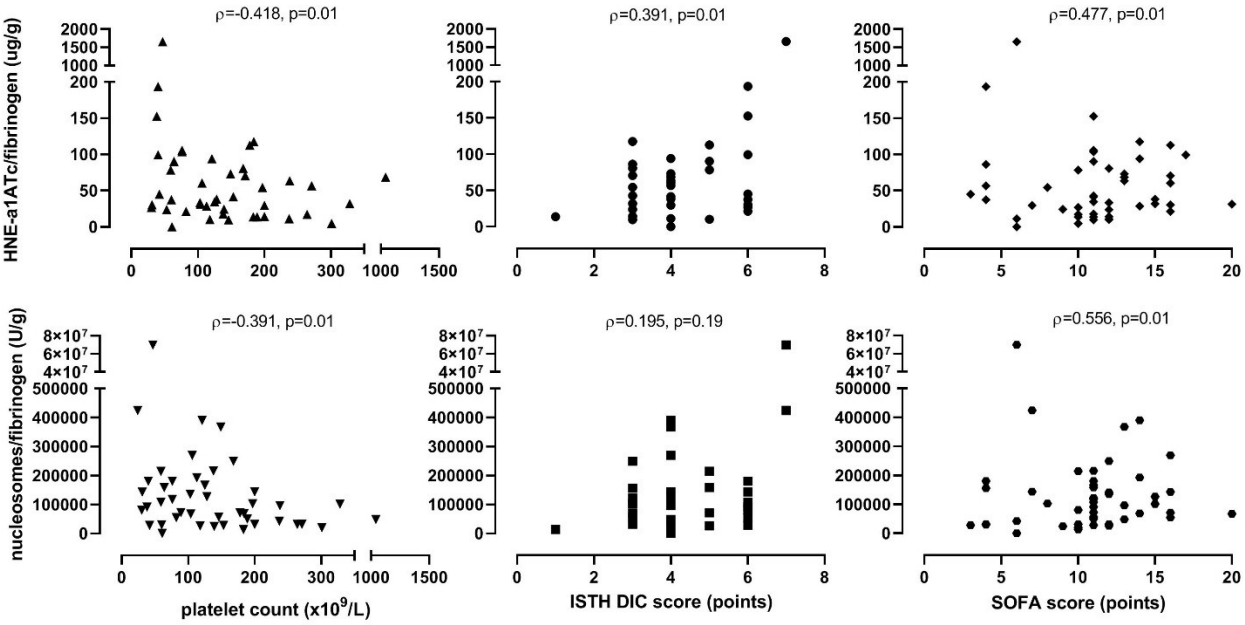

Figure 1. Ratio of HNE-a1ATc and nucleosomes to fibrinogen plotted against platelet count (x10^9/L), ISTH DIC- and SOFA score.

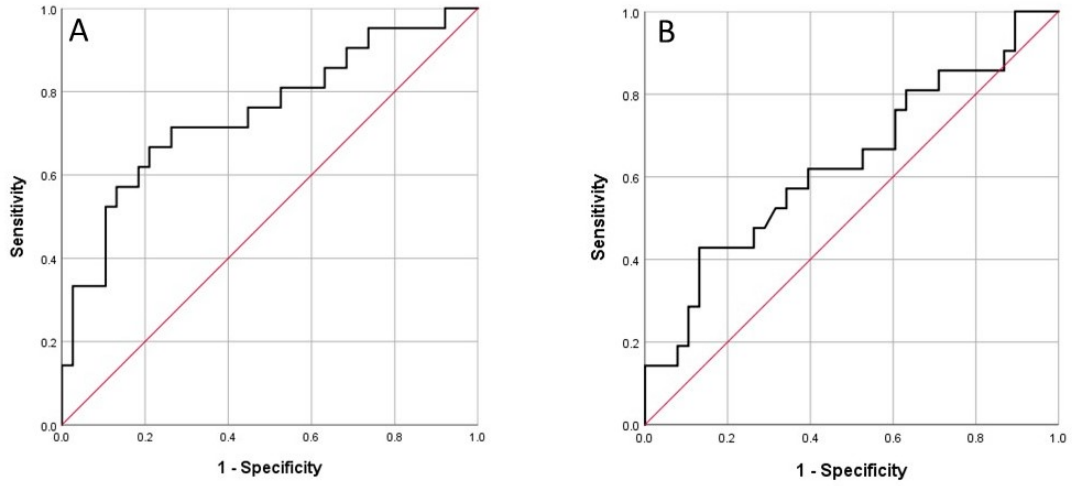

Figure 2. Receiver operating characteristics curves with DIC as outcome for (A) HNE-a1ATc to fibrinogen ratio, AUC 0.75 (95% CI: 0.061 – 0.89) and (B) nucleosomes to fibrinogen ratio, AUC 0.63 (95% CI 0.48 – 0.79).