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Neutrophil extracellular traps: a role in inflammation and dysregulated hemostasis as well as in patients with COVID-19 and severe obstetric pathology

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Abstract

Numerous studies have proven a close relationship between inflammatory diseases and the state of hypercoagulability. In fact, thromboembolic complications represent one of the main causes of disability and mortality in acute and chronic inflammatory diseases, cancer and obstetric complications. Despite this, the processes of hemostasis and immune responses have long been considered separately; currently, work is underway to identify the molecular basis for a relationship between such systems. It has been identified that various pro-inflammatory stimuli are capable of triggering a coagulation cascade, which in turn modulates inflammatory responses. Neutrophil extracellular traps (NETs) are the networks of histones of extracellular DNA generated by neutrophils in response to inflammatory stimuli. The hemostasis is activated against infection in order to minimize the spread of infection and, if possible, inactivate the infectious agent. Another molecular network is based on fibrin. Over the last 10 years, there has been accumulated a whole body of evidence that NETs and fibrin are able to form a united network within a thrombus, stabilizing each other. Similarities and molecular cross-reactions are also present in the processes of fibrinolysis and lysis of NETs. Both NETs and von Willebrand factor (vWF) are involved in thrombosis as well as inflammation. During the development of these conditions, a series of events occurs in the microvascular network, including endothelial activation, NETs formation, vWF secretion, adhesion, aggregation, and activation of blood cells. The activity of vWF multimers is regulated by the specific metalloproteinase ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Studies have shown that interactions between NETs and vWF can lead to arterial and venous thrombosis and inflammation. In addition, the contents released from activated

neutrophils or NETs result in decreased ADAMTS-13 activity, which can occur in both thrombotic microangiopathies and acute ischemic stroke. Recently, NETs have been envisioned as a cause of endothelial damage and immunothrombosis in COVID-19. In addition, vWF and ADAMTS-13 levels predict COVID-19 mortality. In this review, we summarize the biological characteristics and interactions of NETs, vWF, and ADAMTS-13, the effect of NETs on hemostasis regulation and discuss their role in thrombotic conditions, sepsis, COVID-19, and obstetric complications.

Keywords: neutrophils, neutrophil extracellular traps, NETs, thrombosis, fibrin, fibrinolysis, von Willebrand factor, vWF, ADAMTS-13, COVID-19

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Внеклеточные ловушки нейтрофилов: участие в процессах воспаления и дизрегуляции гемостаза, в том числе у пациентов с COVID-19 и тяжелой акушерской патологией

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Резюме

Многочисленными исследованиями доказана тесная связь воспалительных заболеваний с состоянием гиперкоагуляции. Фактически, тромбоэмболические осложнения являются одной из основных причин инвалидности и смертности при острых и хронических воспалительных заболеваниях, онкологических заболеваниях и при акушерских осложнениях. Несмотря на это, процессы гемостаза и иммунные реакции долгое время рассматривались по отдельности; в настоящее время идет работа по выявлению молекулярных основ взаимосвязи между этими системами. Уже известно, что различные провоспалительные стимулы способны запускать коагуляционный каскад который в свою очередь модулирует воспалительные реакции. Внеклеточные ловушки нейтрофилов (англ. neutrophil extracellular traps, NETs) представляют собой сети из гисто-

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нов внеклеточной ДНК, генерируемые нейтрофилами в ответ на воспалительные стимулы. Система гемостаза активируется в ответ на инфицирование с целью минимизировать распространение инфекции и по возможности инактивировать инфекционный агент. Еще одну молекулярную сеть представляет собой фибрин. За последние 10 лет появилось много данных о том, что NETs и фибрин способны формировать единую сеть внутри тромба, стабилизируя друг друга. Сходства и перекрестные молекулярные реакции присутствуют также и в процессах фибринолиза и лизиса NETs. Как NETs, так и фактор фон Виллебранда (vWF) являются участниками и тромбоза и воспаления. В процессе развития этих состояний в микрососудистой сети происходит серия событий, включающая активацию эндотелия, образование NETs, секрецию vWF, адгезию, агрегацию и активацию клеток крови. Активность мультимеров vWF регулируется специфической металлопротеиназой ADAMTS-13 (англ. a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Исследования показали, что взаимодействия между NETs и vWF могут приводить к артериальному и венозному тромбозу, а также воспалению. Кроме того, содержимое, высвобождаемое из активированных нейтрофилов или NETs, вызывает снижение активности ADAMTS-13, что может происходить как при тромботических микроангиопатиях, так и при остром ишемическом инсульте. В последнее время NETs рассматривают как причину повреждения эндотелия и иммунотромбоза при COVID-19. Кроме того, уровни vWF и ADAMTS-13 позволяют прогнозировать смертность от COVID-19. В данном обзоре мы суммируем биологические характеристики и взаимодействия NETs, vWF и ADAMTS-13, влияние NETs на регуляцию системы гемостаза, а также обсуждаем их роль в тромботических состояниях, при сепсисе, COVID-19 и акушерских осложнениях.

Ключевые слова: нейтрофилы, внеклеточные ловушки нейтрофилов, NETs, тромбоз, фибрин, фибринолиз, фактор фон Виллебранда, vWF, ADAMTS-13, COVID-19

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Highlights

What is already known about this subject?

- Various pro-inflammatory agents are known to trigger a coagulation cascade, which in turn modulates inflammatory responses.
- Hemostasis is activated in response to infection for restricting spread of infection and in activating infectious agent.

What are the new findings?

- The article provides an overview of all potential mechanisms regarding an impact of neutrophil extracellular traps (NETs) on hemostasis, especially in case of severe COVID-19, as well as in obstetric complications.

How might it impact on clinical practice in the foreseeable future?

- Evaluation of neutrophil activation markers may be a promising strategy for assessing severity of COVID-19, obstetric complications, diagnosing neonatal sepsis and predicting their course.

Основные моменты

Что уже известно об этой теме?

- Известно, что различные провоспалительные агенты запускают коагуляционный каскад, который в свою очередь модулирует воспалительные реакции.
- Гемостаз активируется в ответ на инфицирование с целью ограничить распространение инфекции и инактивировать инфекционный агент.

Что нового дает статья?

- В статье проведен обзор всех возможных механизмов влияния внеклеточных ловушек нейтрофилов (англ. neutrophil extracellular traps, NETs) на систему гемостаза, особенно в ситуации тяжелых форм COVID-19, а также при акушерских осложнениях.

Как это может повлиять на клиническую практику в обозримом будущем?

- Оценка маркеров активации нейтрофилов может быть многообещающей стратегией для оценки тяжести течения COVID-19, акушерских осложнений, диагностики неонатального сепсиса и прогнозирования их течения.

Introduction / Введение

While the immune system and the hemostasis system fight against pathogen, thrombi are formed and neutrophil extracellular traps (NETs) are released – processes combined into a single concept of immunothrombosis [1, 2].

In lower invertebrates, such as crabs, nuclear immunohemostatic cells – hemocytes are responsible for combating infections, and they also prevent the loss of blood and lymph [3]. In more highly organized organisms, these two systems (the hemostatic and immune systems) are evolutionarily separated. Platelets lose their nuclei and, accompanied by coagulation factors and fibrinogen, form the hemostasis system, where it finally results in formation of fibrin clot [4]. Neutrophils, having preserved their nuclei, participate in immune reactions. They also acquire the ability to synthesize extracellular networks, neutralizing pathogens. Neutrophil nuclei resemble packed NETs, decondensation of which and release into the extracellular space occurs under the influence of microbial and inflammatory stimuli [5]. Thus, both platelets and neutrophils die to the formation of various networks for protecting host from infectious threats.

Recent studies have shown that immune cells such as neutrophils and monocytes are actively involved in the processes of immunothrombosis [2]. Mobilization of pathogens and pathogen-related molecular triggers on the surface of the endothelium induces leukocyte adhesion to such areas. Activated monocytes release tissue factor (TF) that activates the coagulation cascade. The fibrin network formed further contributes to additionally attracted leukocytes and their activation through αMβ2 (Mac-1) integrin [6].

In the 19th century, E. Mechnikov [7] and P. Ehrlich et al. [8] described the microscopic structure of neutrophils. However, their ability to eject the nuclear contents into the extracellular space was discovered only 15 years ago [6]. Since then, NETs have been remained in the spotlight of specialists from various fields of medical science. At present, it is clear that the formation of NETs – NETosis – is not a single event, but consists of multiple processes resulting in the expulsion of the neutrophilic nuclear contents [9]. Suicidal, vital, and mitochondrial types of NETosis have already been described in the literature [5].

Immunothrombosis can be pathological. Studies have shown that both arterial and venous thrombi contain neutrophils and NETs [10]. NETs increase the overall size of the thrombus, retaining platelets and microvesicles [11]. In animal models of thrombosis, with submaximal compression of the inferior vena cava, the thrombus become enriched in NETs [12]. Neutrophils arrive first to the site of injury after laser damage to the vascular wall in mice [13]. NETs are always present in blood clots, especially at the initial organization stage [14]. The same scenario is applied to arterial thrombi in patients with heart attack [15], stroke [16], and peripheral arterial disease [17].

Usually, histones are not detected in the circulating blood. Their concentration increases in pathological conditions such as trauma (230 μg/mL) [18]. In sepsis, the concentration of histone H3 rises up to 60 μg/mL [19], and total histone concentration of more than 75 μg/mL suggest a poor prognosis [20]. Increased concentrations of circulating histones are also coupled to a poor prognosis in stroke, heart attack, venous thrombosis, being detected within blood clots [21, 22].

NETs overproduction or impaired utilization leads to pathological microthrombosis in sepsis [23]. Endogenous and exogenous DNases lead to the degradation of NETs, with a massive release of histones bond to DNA, which is realized in thrombosis [24]. NETs can contribute to thrombogenesis in the large vessels [25], and their circulation is associated with a poor prognosis of cardiovascular and cerebrovascular diseases [21].

Neutrophils are recruited to site of inflammation in several stages, such as activation, adhesion, and extravasation. Attraction to the activated endothelium and activation of neutrophils occur by involving selectins, e. g., P-selectin, and P-selectin glycoprotein ligand 1 (PSGL-1). P-selectin is expressed on the surface of activated endothelial cells and platelets. Integrin αLβ2 and intercellular adhesion molecule 1 (ICAM-1) are involved in neutrophil adhesion. Chemokines are also necessary for neutrophil attraction to the inflamed site, which underlie the final neutrophil extravasation [26] (**Fig. 1**).

T. Yago et al. showed that chemokines and integrins are involved in the recruitment of neutrophils to the site of activated endothelium and thrombosis [27]. One of the strategies being developed for antithrombotic therapy is aimed at inhibiting P-selectin. As early as in 1992, studies demonstrated that the P-selectin suppression leads to lowered leukocyte accumulation and the fibrin deposition in arteriovenous shunts in monkeys [28]. T.W. Wakefield et al. observed other risk factors that reduce the risk of venous thrombosis and effects of inflammatory factors in animal models without increasing the risk of bleeding [29]. Human monoclonal anti-selectin antibody crizanlizumab, which blocks interaction between PSGL-1 and P-selectin, has been proposed as an agent to inhibit P-selectin [30] (**Fig. 1**).

For the complete synthesis of NETs, NADP-oxidize activity is required. Studies have shown that patients with NADP-oxidize deficiency had no formation of NETs [31], while gene correction of the enzyme deficiency can restore the NETosis [32].

NETs affect the hemostasis system in various ways by promoting development of procoagulant state, fibrinolysis disruption, and anticoagulant activity [23].

NETs and coagulation disturbances / NETs и нарушения коагуляции

DNA contribution / Вклад ДНК

DNA triggers a coagulation cascade along the internal pathway because negatively charged surfaces increase activation of factor XII (FXII), the initiating this pathway [33]. Even though in the physiological state, the internal pathway is not the solely involved in hemostasis activation, whereas in pathological conditions coupled to massive DNA release resulting from cell damage and death (for example, as a result of inflammation), it comes to the frontline as a cause of massive fibrin formation [34]. Interestingly, polyphosphates secreted by histone-activated platelets coincide to serve as a negatively charged trigger for the NETs synthesis [35].

In addition to activating the internal coagulation pathway, DNA acts as a cofactor for thrombin-dependent activation of factor XI [36] and contributes to the successful course of reactions of the tissue factor-associated external pathway [37] (**Fig. 2**).

Histones contribution / Вклад гистонов

A great body of publications is devoted to assess an effect of DNA-bound positively charged histones inside

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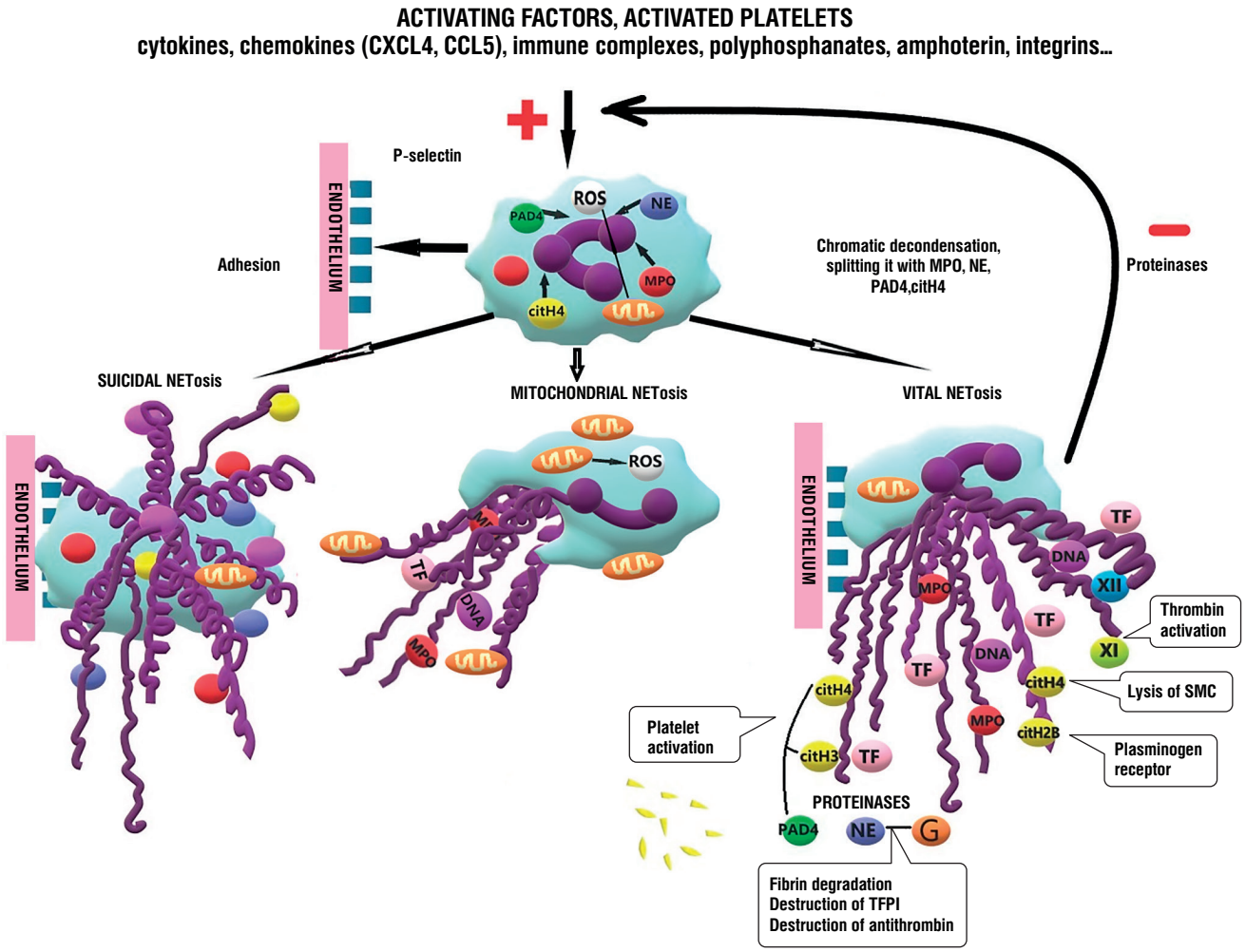

Figure 1. Types and mechanisms of NETosis.

Note: *SMC – smooth muscle cells; ROS – reactive oxygen species; TF – tissue factor; G – cathepsin G; citH – citrulized histone; MPO – myeloperoxidase; NE – neutrophil elastase; PAD4 – peptidyl arginine deiminase 4; P-selectin – P-selectin glycoprotein ligand-1; TFPI – tissue factor pathway inhibitor.*

Рисунок 1. Виды и механизмы нетоза.

Примечание: *SMC – гладкомышечные клетки; ROS – реактивные формы кислорода; TF – тканевой фактор; G – катепсин G; citH – цитрулированный гистон; MPO – миелопероксидаза; NE – эластаза нейтрофилов; PAD4 – пептидил аргинин деиминаза 4; P-селектин – P-селектин гликопротеин лиганд-1; TFPI – ингибитор пути тканевого фактора.*

NETs on hemostasis [38]. Histones secreted within NETs have been shown to be the links in the pathogenesis of arterial, venous thrombosis, as well as thrombosis in the microvasculature.

Histones are substances released by damaged, dying, or activated cells during infectious process, inflammation or injury [39]. The main source of extracellular histones is neutrophils, wherein they are a part of the neutrophil extracellular traps being found along with strands of extracellular decondensed chromatin [6].

Histones destroy the anticoagulant endothelial barrier by forming holes in phospholipid membranes with impaired ion exchange [40, 41]. In the process of endothelial activation and even its death [42] caused by histones, H_2O_2 is released, which further stimulates NETosis [9]. The Weibel-Palade bodies located in the endothelium undergo exocytosis together with the von Willebrand factor (vWF), which binds to platelets and maintains thrombosis.

The interaction of histones with platelet membranes leads to the influx of calcium ions either through the pore formation [43] or by opening pre-existing channels [44], triggering the activation of $\alpha 2b\beta 3$ integrin [45], which promotes fibrin binding. Histones also activate platelets through Toll-like receptors TLR2 and TLR4 [35] and enhance thrombin-dependent platelet activation [46].

Erythrocytes are traditionally considered to be cells that mechanistically [47] and chemically [48] strengthen the thrombus structure and also contribute to increased potential of thrombin generation in whole blood due to exposure to phosphatidylserine [49]. Binding to histones enhances the thrombogenicity of the erythrocyte membrane during NETosis [50].

In addition to interacting with blood cells, histones affect the proteins of the coagulation cascade. Histone H4 while binding to prothrombin, promotes its autoactivation [51]. Histones disrupt antithrombin-dependent thrombin inactivation [52]. Histones interfere with the thrombin-

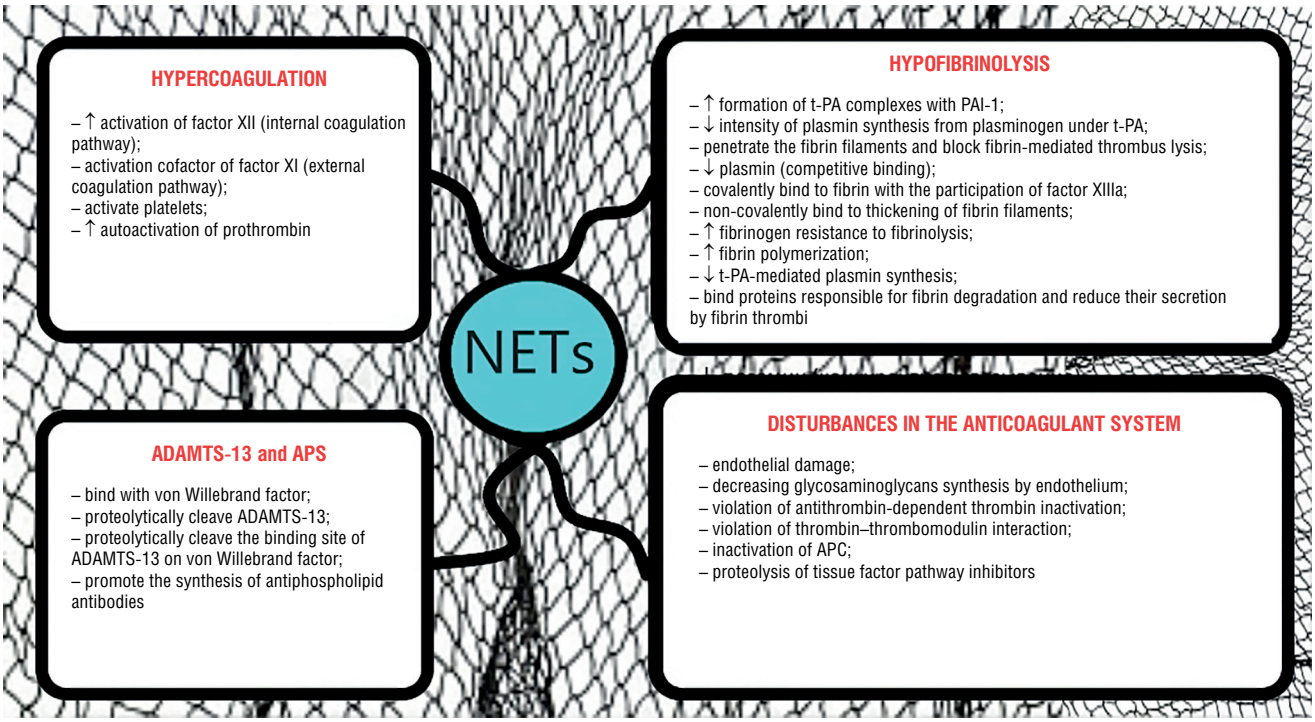

Figure 2. NETs and fully dysregulated hemostasis.

Note: *APS – antiphospholipid syndrome; t-PA – tissue plasminogen activator; PAI-1 – plasminogen activator inhibitor-1; APC – activated protein C.*

Рисунок 2. NETs и полная дизрегуляция гемостаза.

Примечание: *APS – антифосфолипидный синдром; t-PA – тканевой активатор плазминогена; PAI-1 – ингибитор активатора плазминогена-1; APC – активированный протеин С.*

thrombomodulin interaction [53]. Activated protein C (APC) is an anticoagulant capable of inhibiting NETosis via PAR receptors on neutrophils [54]. NET histones trigger the pathways of its inactivation particularly neutrophil oxidase and elastase can inactivate APC. However, speaking about the interaction of thrombin and thrombomodulin, it should not be forgotten that they activate APC and thrombin-activatable fibrinolysis inhibitor (TAFI) [55]. TAFI removes C-terminal lysine moieties in fibrin that serve as plasminogen binding sites [56]. Thus, histones influence to activate interaction between plaminogen and fibrin. The TAFI exerts very short half-life, whereas numerous other procoagulant histone-related effects make such fibrinolysis-stimulating effect negligible (**Fig. 2**).

NETs and fibrinolysis disturbances / NETs и нарушения фибринолиза

Numerous studies are aimed at examining a role for histones and NETs in the thrombosis pathophysiology as well as their impact on coagulation and fibrinolysis, many of which have been focused on the histone procoagulant activity, but only a few of them describe their antifibrinolytic activity.

Histones contribution / Вклад гистонов

Both histones and DNA within NETs display an antifibrinolytic activity [56]. Histones activate not only blood clotting but also enhance thrombus stability via structural changes in fibrin to strengthen it and confer more resistance to fibrinolysis processes. In the 1950s, an interest to polycationic polypeptides and their influence on the formation and degradation of fibrin was ignited. It was shown that polylysine (exerting effects similar to lysine-rich histones such as H1 [57]) inhibits streptokinase-induced fibrinolysis [58] and enhances fibrin formation upon addition of staphylocoagulases and prothrombin [59]. Studies demonstrated the antifibrinolytic effects for plasma and whole blood histones. By activating plasminogen in solution, histones suppress plasmin, acting as competitive substrates. The protection of fibrin from plasminogen action is also enhanced by the covalent binding of histones to fibrin, catalyzed by activated transglutaminase, a clotting factor XIIIa. All histone subtypes (H1, H2A, H2B, H3, and H4) can bind to fibrin. Through non-covalent interactions, histone-associated lateral aggregation of fibrin protofibrils occurs, leading to thickening of fibrin filaments and relevant increased mass-length ratio that results in hindered fibrinolysis processes.

Histones per se would not be so dangerous in suppressing fibrinolysis unless the activated factor XIII was involved. Therapeutic dose of low molecular weight heparins (LMWH) prevent the covalent and non-covalent interaction between fibrin and histones, thereby neutralizing the effect of histones on fibrinolysis.

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Thus, low molecular weight heparins display another antithrombotic mechanism of action unrelated anticoagulant activity.

Fibrinolysis consists of two fundamental processes: the plasmin synthesis from inactive plasminogen, catalyzed by a tissue type-plasminogen activator (t-PA), and further fibrin destruction by plasmin [60]. In the blood, fibrinogen circulates surrounded by multiple macromolecules, non-covalently bound to plasma proteins and polymers, which affect the fibrin polymerization and the availability for its further fibrinolysis. A relation between fibrin structure and intensity of fibrinolysis processes is associated with the protein-polymer and its electrostatic charge. Negatively charged DNA promotes formation of densely packed networks of thick fibrin filaments, which are less accessible to plasmin [56]. Polyphosphate, another anionic polymer, leads to the formation of a heterogeneous clot structure, requiring less plasmin for lysis [61]. It has been shown that histones carrying negatively charged lysine and arginine interact with fibrinogen to increase its resistance to fibrinolysis [62]. The underlying mechanism of such effect is not fully understood. Histones also affect the organization of lateral protofibrils, resulting in higher resistance of fibrinogen to t-PA-mediated fibrinolysis [63].

NETs histones increase fibrin polymerization and strengthen thrombus structure even without direct binding to fibrin. The antifibrinolytic potential of histones increases when they bind to fibrin.

Another essential component of fibrin-related mechanical and biochemical stability is the factor XIII (FXIII), which binds to fibrinogen in the circulating blood [64]. Factor XIII is activated by thrombin in the presence of calcium ions with the formation of active transglutaminase (FXIIIa) which further promotes formation of covalent isopeptide bridges between glutamine and lysine in the α- and γ-chains of the inter-fibril fibrin monomers. Altogether, it leads to formation of γ–γ dimers and polymers as well as high-molecular weight α–α and γ–α, which increase the clot density and the number of erythrocytes retained in it [65]. Factor XIIIa also covalently binds other plasma proteins to fibrin, including antifibrinolytic molecules such as α_2-antiplasmin, plasminogen activator-2 inhibitor, and TAFI [64]. These effects render FXIIIa a fundamentally crucial for stabilizing the fibrin clot and a target for antithrombotic therapy [66]. Histones are enriched in lysine and can act as a source of amino groups in transglutamination reactions [65].

Thus, histones increase fibrin stabilization in a variety of ways. Histones that protect fibrin from destruction compete for plasmin as substrates. This effect is enhanced while histone binding to fibrin coupled to factor XIIIa transglutaminase. This effect is reversible by using factor XIIIa and LMWH inhibitors. Non-covalently bound histones increase lateral aggregation of protofibrils, leading to their thickening and increasing the fibrin mass-length index [67] (**Fig. 2**).

Suppression of plasmin by NETs histones / Подавление плазмина гистонами NETs

While small concentrations of histones bind to plasminogen, the synthesis of plasmin t-PA is stimulated, whereas high histone concentrations suppress t-PA-mediated plasmin synthesis.

Plasmin is a broadly specific serine protease that binds to arginine and lysine, and hence histones are considered as a candidate for plasmin targets. Competing with fibrin for plasmin binding sites, histones interfere with plasmin activity and fibrinolysis triggered by t-PA.

LMWH, NETs and fibrinolysis / НМГ, NETs и фибринолиз

LMWH interfere with the binding of NETs histones to fibrin and improve the processes of fibrinolysis. Cationic histones have a high affinity for negatively charged heparin [68]. This effect accounts in part of how LMWH interferes with the suppression of fibrinolysis by disrupting the binding of histones to fibrin. Therapeutic dose of LMWH prevents the binding of fibrin to histones without affecting the inter-protofibril binding [67]. Heparins have been shown to neutralize the damaging effect of histones in sepsis [68], thrombosis [25], thrombocytopenia [70], and platelet activation [35] (**Fig. 2**).

DNA contribution / Вклад ДНК

Studies have shown that DNA increases the formation of complexes between tissue plasminogen activator and plasminogen activator inhibitor-1 (PAI-1) [71], reduces the intensity of plasmin synthesis from plasminogen under the action of t-PA on the thrombus surface [51], binds proteins responsible for fibrin degradation and reduces their release by fibrin thrombi [63], as well as additionally penetrates into fibrin filaments and blocks plasmin-mediated thrombus lysis. Blood clot samples obtained from patients with strokes and heart attacks showed that *ex vivo* thrombolysis occurs more often in the presence of DNases combined with t-PA [51].

NETs and anticoagulants / NETs и антикоагулянты

Thrombosis is usually controlled by antithrombin III (AT-III), thrombomodulin, and tissue factor pathway inhibitor (TFPI).

Anticoagulant production during infectious process declines due to damage to the endothelium, and the mechanisms of anticoagulation are suppressed by neutrophil elastase [72]. Usually, intact endothelial cells exert anticoagulant properties. Glycosaminoglycans on the endothelial surface act as heparin-like cofactors that facilitate binding to antithrombin followed by production of

a potent thrombin inhibitor. Endothelial cells also express thrombomodulin, which, by binding thrombin, leads to decreased activation of protein C regulating coagulation via proteolysis of cofactors Va and VIIIa. Proinflammatory cytokines lead to endothelial damage and decreased level of surface glycosaminoglycans [73]. The third most crucial anticoagulant is TFPI, an inhibitor of the TF-FVIIa complex [74]. Neutrophil elastase secreted by NETs is involved in TFPI inactivation processes.

Antithrombin III is a glycoprotein synthesized in the liver and inactivating enzymes of both the external and internal coagulation pathways, including thrombin, factor Xa, and factor IXa [75]. Heparin enhances contacts between thrombin and antithrombin, thereby increasing its anticoagulant activity against AT-III. AT-III also has an anti-inflammatory effect mediated by its interaction with syndecan-4, the proteoglycan of heparan sulfate [76]. When AT-III binds to heparin, its affinity for syndecan-4 increases so that the anti-inflammatory effects of AT-III may be of great importance in treatment of patients with septic conditions [77].

It is known that the concentration of AT-III continuously decreases during sepsis; however, inactivation and degradation of antithrombin in thrombin–antithrombin complexes is not the main cause for this decline [78]. Increased permeability of endothelial cells plays an essential role in reducing antithrombin concentration [79]. Increasing endothelial permeability subsequently promotes neutrophil infiltration. AT-III prevents accumulation of neutrophils and reduces intensity of related NETs formation [79]. T. Iba et al. demonstrated that the administration of AT-III decreases the concentration of H3 histone and nucleosomes in animal models of inflammation [80] (**Fig. 2**).

NETs, von Willebrand factor and ADAMTS-13 / NETs, фактор фон Виллебранда и ADAMTS-13

Plasma glycoprotein of von Willebrand factor accounts for the platelet delivery to site of damaged vascular wall and promotes their subsequent activation and aggregation [81]. Activity of the vWF is determined by its size. Ultra-large vWF multimers (UL-vWF) released from endothelial cells can spontaneously activate circulating platelets and other blood cells, promoting thrombosis [82]. Metalloproteinase ADAMTS-13 specifically cleaves the multimer at Tyr1605–Met1606 regions in the A2 domain, thereby regulating the size and activity of vWF multimers and preventing thrombogenesis [83].

NETs result in decreased ADAMTS-13 activity. Both extracellular DNA and NETs histones can bind to vWF, leading to even greater recruitment of new neutrophils to the focus, enhancing the pro-inflammatory effect. In several conditions, e. g., in sepsis, coupled to increased vWF concentration, a decrease in ADAMTS-13 activity occurs. However, no definitive explanation has been found for this effect yet, which might also develop due to NETosis. During inflammation, activated neutrophils in NETosis release various cytokines, proteases, peptides, and reactive oxygen species such as H_2O_2, many of which promote secretion of large amounts of vWF multimers [84]. At the same time, proteolysis of ADAMTS-13 by NETs proteases occur [85]. In addition, J. Chen et al. showed that reactive oxygen species from NETs oxidize a site on the vWF A2 domain at Met1606 for binding to ADAMTS-13 that converts methionine to methionine sulfoxide subsequently hampering potential of ADAMTS-13 to break this vWF region [86]. The same reactive oxygen species oxidize methionine in the ADAMTS-13 by markedly reducing its activity [87]. Thus, NETosis promotes elevated circulation of vWF multimers and decreased ADAMTS-13 activity that enhance stronger platelet aggregation and vascular occlusion.

Peptidyl arginine deiminase 4 (PAD4), which converts positively charged histones arginine residues into neutral citruline [88] being required for chromatin decondensation is actively involved in NETosis. Recent studies by N. Sorvillo et al. showed that PAD4 citrullinates plasma ADAMTS-13 in the arginine motifs, thereby increasing its activity [89].

To sum up issues noted above, the components of NETs, on the one hand, significantly reduce the activity of ADAMTS-13 by oxidation, citrullination, and proteolysis. On the other hand, they competitively bind to vWF A2 domain, ultimately leading to elevated amount of vWF multimers with their prominent prothrombotic effect. The interactions between NETs and vWF turn in a vicious circle, wherein the NETs components contribute to decreased activity of ADAMTS-13, which leads to increased concentration of vWF multimers and further recruitment of new neutrophils, their activation, and NETosis, thereby enhancing the processes of thrombus inflammation. In this case, recombinant ADAMTS-13 and/or DNase 1 can be used to disrupt such pathogenic feedback loop.

Recently, NETs have been viewed as a cause of endothelial damage and immunothrombosis in COVID-19. In addition, vWF and ADAMTS-13 levels predict COVID-19 mortality (**Fig. 2**).

The role of NETs in thromboinflammation in COVID-19 patients / Место NETs в процессах тромбовоспаления у пациентов с COVID-19

Since the beginning of the global pandemic in early 2020, the 2019 coronavirus disease (COVID-19) has raised many questions for health service around the globe. COVID-19 is characterized by developing acute respiratory distress syndrome (ARDS) with acute pulmonary insufficiency, endothelial damage,

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immunothrombosis, as well as imbalanced coagulation and inflammation. Elucidating the pathophysiology is of paramount importance for proposing novel therapeutic strategies.

Hypercoagulability is always observed in severe COVID-19 [90]. Elevated levels of D-dimer, fibrinogen, and low concentration of antithrombin were noted in patient blood samples [91]. It has also been shown that patients hospitalized with COVID-19 had significantly increased level of NETs compared to the control group [92]. In particular, high levels of NETs were observed in the subgroup of COVID-19 patients who have been clinically diagnosed with at least one thrombotic case. L. Nicolai et al. found that in patients with COVID-19, NETs were found in microvascular blood clots in the lungs, kidneys, and heart [93]. B.J. Barnes et al. demonstrated prominent neutrophilic infiltration of the lung capillaries during the autopsy of patients who died from COVID-19 [94].

SARS-CoV-2 can directly trigger the synthesis of NETs by interacting with angiotensin converting enzyme 2 (ACE2) receptors via the ACE2-serine protease-TMPRSS2 dependent pathway [95]. The synthesis of NETs accompanies thrombosis in arteries, veins, and microcirculation, leading to the development of multiple organ failure [96]. Mechanistically, NETs DNA directly activates the external coagulation pathway [97], whereas NETs tissue factor initiates the internal pathway [98]. Serine proteases of NETs granules, such as elastase, promote coagulation by proteolysis of various inhibitors in the tissue factor pathway [99], which is accompanied by other mechanisms that SARS-CoV-2 virus may trigger and lead to micro- and macrovascular thrombosis. Among them are autoantibodies and cytokine-mediated activation of innate immune cells, including neutrophils and platelets, vasospasm under hypoxic conditions, and direct activation of endothelial cells by viral infection [100]. The three-way interactions between neutrophils, endothelial cells, and platelets may be critical for COVID-19-related thrombosis, as shown in other thrombotic-inflammatory disease models.

Along with NETs, ADAMTS-13 and vWF are also involved in the development of thrombotic conditions in COVID-19. Studies have shown a significant increase in plasma concentrations of vWF multimers and coagulation factor VIII secreted by activated damaged endotheliocytes, which is associated with hypercoagulability and a high risk of thromboembolism in patients with COVID-19 [101]. In addition, patients show a decreased ADAMTS-13 activity, which some researchers have proposed for using particularly as a marker of a high mortality risk [102].

Approaches to blocking NETs include the destruction of NETs by deoxyribonucleases and strategies that prevent the synthesis of NETs such as neutrophil elastase inhibitors, PAD4 inhibitors, and adenosine receptor agonists such as dipyridamole [103], antineutrophilic therapy may be part of individual therapy in COVID-19 therapy.

NETs as one of the markers of the systemic inflammatory response / NETs как один из маркеров системного воспалительного ответа

Sepsis-associated disseminated intravascular coagulation (DIC) results from the interaction of infection-induced inflammation and hypercoagulability in which neutrophils, platelets, and endothelial cells are involved [104, 105]. Activation of the coagulation system, weakening of the anticoagulant system, and suppressing the fibrinolytic system cause thrombotic complications, impaired microcirculation, and multiple organ failure in sepsis [106, 107]. An ideal system for assessing the severity of DIC and sepsis should include molecular biomarkers associated with DIC from endothelial cells, neutrophils, platelets, and traditional indicators. Currently, the thrombin–antithrombin, AT-III, prothrombin fragments 1+2 have already been introduced into the protocol for assessing the severity of septic complications.

The endothelial cell glycocalyx undergoes degradation during sepsis and the concentration of serum glycocalyx components such as syndecan-1 increases. It has been shown that its level is significantly associated with the mortality of septic patients [108]. In sepsis, vWF is expressed by endothelial cells, promotes platelet aggregation as well as adhesion and microthrombus formation. The activity and concentration of serum ADAMTS-13 metalloproteinase are reduced in sepsis, associated with an increased risk of mortality [109]. In addition, some studies showed that a specific marker of platelet activation (serum trigger receptor expressed on myeloid cells-like transcript-1) and P-selectin in platelets and endothelial cells are associated with sepsis-induced DIC [110]. P-selectin in platelets is also involved in the processes of NETosis [111]. Serum NETs lead to hemostasis dysregulation, and their amount in patients with sepsis-associated DIC is significantly increased [112].

NETs and pregnancy complications / NETs и осложнения беременности

Preeclampsia was the first complication of pregnancy in which NETs have been reported [113]. Pregnancy is characterized by a pro-inflammatory state, activation of immunetolerance, the failure of which can be one of the causes for developing preeclampsia [114, 115]. Initially, I. Sargent et al. suggested that the cause of the pro-inflammatory condition is the excretory function of the placenta, in which waste products are deposited particularly from the syncytiotrophoblast. The latter

builds up a continuous several-square-meter monolayer covering a villous tree being partially released into the maternal bloodstream and may exist in the form of microparticles (syncytiotrophoblast microparticles, STBM). Utilization of syncytiotrophoblast cells occurs typically due to apoptosis, but necrotic or aponecrotic processes may occur during preeclampsia, so that the contents acquire pro-inflammatory properties [116].

The activation of circulating neutrophils accompanies the pro-inflammatory state during pregnancy; in preeclampsia, a more pronounced activation with the formation of NETs, inflammation coupled to reactive oxygen species, and damage to the endothelium are noted [117]. Neutrophil activation is facilitated by the presence of STBM and its increased concentrations in preeclampsia. S. Giaglis et al. indicated that pregnancy is accompanied by inflammation and neutrophil activation [118, 119], suggesting that neutrophils synthesize excessive NETs under the control of granulocyte-macrophage colony-stimulating factor and/or sex hormones during pregnancy. Hormonal imbalances lead to increased production of NETs, local tissue damage, loss of pregnancy, or development of preeclampsia [118]. Studies have shown the presence of a large amount of extracellular DNA in preeclampsia [120] as well as the presence of NETs directly in the intervillous space in patients with preeclampsia [121]. The results of other studies indicate that placental interleukin-8 triggers NETosis [113], and also proved indeed the tissue factor from NETs initiated miscarriage, inducing a cascade of reactions involving reactive oxygen species [113]. Numerous studies on miscarriage suggest an essential role in balancing prooxidant factors (e. g., free radicals) and antioxidant factors in pregnancy maintenance and normal development [122–124]. Oxidative stress is a disorder caused by imbalanced production of reactive oxygen species and antioxidants. The former are released, among the others, by neutrophils due to the activity of NADPH-oxidase resulting in oxygen radicals (respiratory burst), which trigger the antibacterial defense mechanism [125, 126].

Antiphospholipid syndrome (APS) is a complex autoimmune disorder that leads to thrombosis and fetal loss in the presence of antiphospholipid antibodies. Several studies have shown that the C5a component of complement triggers tissue factor expression in neutrophils, thereby leading to trophoblast damage and fetal loss [127–129]. Such data indicate that neutrophils play an essential role in the pathogenesis of APS. In addition, antiphospholipid antibodies can stimulate NETosis, launching a new pathological thrombus formation pathway [130, 131]. NETs are detected in areas of necrosis in the placental basement membrane in late pregnancy, simultaneously with increased concentrations of serum extracellular DNA and thrombin–antithrombin complexes in animal model of pregnancy loss [122].

NETs and perinatal loss / NETs и перинатальные потери

Neutrophil hyperactivity and its role in neonatal sepsis have not been fully elucidated yet.

The interest of NETs in the context of neonatal sepsis is accounted for by the data that sepsis is a systemic inflammatory response to infection, and symptoms are elicited by host defense systems rather than by invading pathogens. The main hallmark of sepsis in newborns is an extremely rapid course of hyper-inflammatory immune response [132]. During endotoxemia, neonatal myeloid-derived cells skew to inflammatory phenotype, contributing to fatal septic course [133]. In addition, another important feature of sepsis contributing profoundly to its outcome is activation of coagulation with downregulated anticoagulant system and fibrinolysis, resulting in multisystem organ dysfunction [134, 135].

Neutrophils are the key players, providing the first line host defense [136]. However, severe sepsis can dysregulate neutrophil migration. Instead, neutrophils accumulate in vital organs, such as the lung, kidney, intestinal wall [137], which aggravates tissue damage and development of organ dysfunction.

Reports on the role of NETs in neonatal sepsis pathology are sparse. However, increasing evidence has been showing that NETs are implicated in the pathogenesis of organ dysfunction and targeting NETs represents a potential therapeutic option.

D.F. Colon et al. demonstrated that neonatal vs. adult C57BL/6 mice subjected to sepsis or LPS-induced endotoxemia produced significantly higher levels of NETs, and that such outcome was accompanied by increased organ injury and production of pro-inflammatory cytokines. The increased NETs level was associated with elevated expression of PAD4 and histone H3 citrullination in the neutrophils. Furthermore, treatment of infant septic mice with PAD4 inhibitor markedly attenuated sepsis. Importantly, the severity of neonatal sepsis was positively correlated with the level of NETs [138].

C.U. Stiel et al. evaluated markers of NETs formation in human umbilical cord blood and compared their predictive value to current early-onset sepsis (EOS) markers. However, no differences of the NETs markers were found between neonates that developed infection within 72 hours postpartum and control group [139]. C.C. Yost et al. reported that neutrophils of both premature and term born infants have very low neutrophil activity and fail to form NETs in response to inflammatory stimulation. This impairment of neonatal neutrophils was due to a neonatal NET-inhibitory factor (nNIF) expressed during the first 2–3 days of life. In fact, the authors suggest a tight control of perinatal NETs formation to prevent hyperinflammation, NETs-mediated vascular injury, and thrombosis [140].

Recent studies evaluating neonatal, and not umbilical cord blood, markers of NETs showed an association with

sepsis [141]: elevated circulating cell-free DNA levels in neonatal plasma have been associated with late-onset sepsis (LOS), as well as necrotizing enterocolitis (NEC). Neutrophils capable of releasing NETs have been also described as potential sepsis biomarkers in neonates. A consistent up-regulation of circulating cell-free DNA and neutrophil-associated proteins at or shortly before the onset of neonatal LOS and/or NEC in three different species (human, pig and mouse) was demonstrated. Elevated circulating cell-free DNA levels 1–6 days before NEC onset in preterm infants suggest that sub-clinical systemic inflammation at an early stage of NEC may stimulate neutrophils to release DNA in the circulation, which may add further inflammatory insults. Up-regulated fibrinogen chains and vWF were among circulating proteins involved in platelet activation and blood coagulation that differed between control as well as LOS and NEC patients. These data support the previously described evidence about strong interaction between NETs structures and activated platelets during endothelial injury and sepsis in adults [142]. Taken together, these results imply that targeting neutrophils may be a promising strategy to diagnose neonatal sepsis and predict prognosis in such patients.

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