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NETs production and citrullinated histone H3 level in children with tuberculosis

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ABSTRACT

Aim. To characterize parameters of neutrophil extracellular trap (NET) production in the leukocyte culture and citrullinated histone H3 (citH3) level in peripheral blood to assess the features of NETosis in children with tuberculosis.

Materials and methods. The study included 20 children with active pulmonary tuberculosis (TB group) and 20 clinically healthy children without signs of sensitization to *Mycobacterium tuberculosis* antigens (control group). The ability of neutrophils to form NETs under *ex vivo* exposure to a non-specific immune stimulant and the concentration of citH3 in peripheral blood were investigated.

Results. Neutrophils in children with tuberculosis formed filamentous NETs (*Me* = 21.0 and *Me* = 16.0, respectively; $p = 0.0474$) and cloud-like NETs (*Me* = 10.5 and *Me* = 4.0, respectively; $p = 0.0068$) more frequently than the controls. Filamentous NETs prevailed in both groups. However, cloud-like NETs were registered in all patients in the TB group (100%) and only in 15 of 20 children in the control group ($\chi^2 = 16.01$; $p < 0.0068$). The concentration of citH3 in the blood was 18.9 times higher in the TB group than in the control group (*Me* = 26.5 and *Me* = 1.4, respectively; $p = 0.0041$). A strong positive correlation was found between the citH3 concentration and the generation of filamentous ($r = 0.86$; $p = 0.0137$), but not cloud-like NETs ($r = 0.95$; $p = 0.0008$) in both groups.

Conclusion. The high level of citH3 in the TB group can reflect its NETosis-induced release and be caused by increased NETosis *in vivo*. This may be due to the previously formed potential of neutrophils to generate NETs (a proNETotic phenotype), which is consistent with our observation of an increased ability of isolated neutrophils to form extracellular traps *ex vivo* in children of the TB group.

Keywords: tuberculosis in children, neutrophil extracellular traps (NETs), NETosis-forming ability of neutrophils, citrullinated histone H3, neutrophil ability to form an extracellular trap

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Характеристика продукции нейтрофильных внеклеточных ловушек и концентрации цитруллинированного гистона H3 у детей, больных туберкулезом

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РЕЗЮМЕ

Цель исследования: охарактеризовать показатели продукции нейтрофильных внеклеточных ловушек (НВЛ) в культуре лейкоцитов и концентрацию цитруллинированного гистона H3 (citH3) периферической крови для оценки возможных особенностей реализации нетоза у детей, больных туберкулезом.

Материалы и методы. В исследование вошли 20 детей с активным туберкулезом органов дыхания (группа «Туберкулез») и 20 клинически здоровых детей без признаков сенсибилизации к антигенам *Mycobacterium tuberculosis* (группа «Контроль»). Выясняли способность нейтрофилов к формированию НВЛ при воздействии *ex vivo* на них неспецифического антигенного стимулятора и исследовали концентрацию цитруллинированного гистона H3 в периферической крови.

Результаты. Нейтрофилы больных туберкулезом детей чаще, чем детей в группе «Контроль», формировали нитевидные НВЛ (*Me* = 21,0 и *Me* =16,0 соответственно; $p = 0{,}0474$) и облаковидные НВЛ (*Me* = 10,5 и *Me* = 4,0 соответственно; $p = 0{,}0068$). В обеих группах преобладали нитевидные НВЛ. Однако облаковидные НВЛ были отмечены у всех представителей группы «Туберкулез» (100%), а в группе «Контроль» были выявлены только у 15 из 20 обследуемых ($\chi^2 = 16{,}01$; $p < 0{,}0068$). В группе «Туберкулез» медиана концентрации citH3 в крови была в 18,9 раза выше, чем в группе «Контроль» (*Me* = 26,5 и *Me* = 1,4 соответственно; $p = 0{,}0041$). Выявлена сильная положительная корреляционная взаимосвязь между концентрацией citH3 и генерацией нитевидных ($r = 0{,}86$; $p = 0{,}0137$), но необлаковидных форм НВЛ в группе «Туберкулез» и контрольной группе ($r = 0{,}95$; $p = 0{,}0008$).

Заключение. Высокий уровень citH3 в группе «Туберкулез» может отражать его нетоз-индуцированную продукцию и быть следствием возросшей активности данного процесса *in vivo*. Существенную роль в этом может играть ранее сформированная потенциальная готовность нейтрофилов к образованию НВЛ (пронетотический фенотип), что согласуется с нашим наблюдением о возрастании способности к формированию внеклеточных ловушек *ex vivo* изолированных нейтрофилов обследуемых лиц данной группы.

Ключевые слова: туберкулез у детей, нейтрофильные внеклеточные ловушки, нетозобразующая способность нейтрофилов, цитруллинированный гистон H3, способность нейтрофила к формированию внеклеточной ловушки

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Mycobacterium tuberculosis infection is accompanied by an increase in the number of Th1 and Th2 lymphocytes, inducing the development of an imbalance of immune responses in patients with pulmonary tuberculosis (TB) [1]. Regulation changes in the immune response are associated with the development of a special cytokine profile [2], stipulating emergency granulopoiesis, which eventually restructures the functional abilities of neutrophils [3].

The formation of neutrophil extracellular traps (NETs) or NETosis, discovered by V. Brinkmann and A. Zychlinsky in 2004, is a mechanism of non-specific protection provided by leukocytes in response to a wide-range of stimuli of both bacterial and non-bacterial origin [4]. NETs are supramolecular structures consisting of decondensed DNA – histone complexes with proteins fixed on their surface, released from neutrophil granules: neutrophil elastase, myeloperoxidase, and other specialized proteins [5]. This NET content contributes to the bactericidal function, which is undoubtedly essential in various infectious diseases, including TB. Impaired NETosis significantly impacts the development of a number of diseases and the risks of their complications [6].

Pronounced NETosis manifestations have been evidently observed in patients with severe forms of TB [7, 8]. Fluorescence microscopy is a routine method for studying NETosis [9]. Various intercalating DNA dyes and fluorescent labeling antibodies are applied to the components of neutrophil leukocytes in the test techniques [9]. It was demonstrated that the process of NETosis in response to antigenic stimuli was accompanied by morphologically different fluorescent microscopic phenomena observed in the sample [10, 11]. At the same time, both the so-called cloud-like and filamentous NET forms were detected, which may reflect different degrees of decondensation of chromatin that formed these forms of NETs. Therefore, the prevalence of a certain NET form (among all NETs observed in a sample) turned out to be associated with a TB course [12]. It is known that the process of chromatin decondensation in NETosis is ensured by histone modification disrupting the non-covalent interaction of histones with DNA.

It has been shown that citrullination of histones is observed in NETosis, including histone H3 with modification of the arginine residues R2, R8, and R17 [4, 13, 14]. Specifically, in neutrophils obtained from adults with TB, an immunofluorescent label to citrullinated histone H3 (citH3) was identified after stimulation with *M. tuberculosis*, whereas no labels were detected in healthy individuals [15]. In adults, citH3 concentration in the blood correlated with the severity of TB and the presence of cavities [8]. It has been demonstrated that citH3 can be determined in the blood serum, also as a marker of NETosis [4]. However, it remains unclear to what extent the citH3 level circulating in the blood can be related to the activity of post-translational enzymatic histone modification in neutrophils in TB patients in NETosis. Taking into consideration the fact that NETosis in children may be accompanied by excessive activation of neutrophils and more pronounced NETosis compared to adults [16], studying the role of NETosis in the infectious process in children with *M. tuberculosis* infection is relevant and significant from the point of view of medicine. No data on citH3 concentration in the peripheral blood in relation to the ability of neutrophils to form NETs in children with TB was found in the available literature.

The aim of the study was to characterize NET production parameters in leukocyte culture and the level of citH3 in the peripheral blood to assess the features of NETosis in children with TB.

MATERIALS AND METHODS

The study included 20 children with active respiratory tuberculosis (TB group) and 20 healthy individuals (control group). The TB group included 14 children diagnosed with primary tuberculous complex (PTC), 3 children with infiltrative pulmonary tuberculosis (IPT), and 3 children with intrathoracic lymph node tuberculosis with bronchopulmonary lesions who were treated at the Specialized Children's Tuberculosis Clinical Hospital, Omsk, in 2022–2023. Inclusion criteria for the TB group were as follows: age of 4–14 years; newly diagnosed active respiratory TB established by the Central Control Commission of the TB Hospital; prescribed chemotherapy for newly diagnosed tuberculosis; an informed consent by the parent (or legal guardian) for the child to be included in the study. Exclusion criteria were concomitant diseases and detected isolated extrapulmonary TB.

The control group included healthy children followed up at the Children's Department of Omsk City Polyclinic No. 10, who, according to the results of the regular annual checkup, had a negative reaction to tuberculin. The controls matched the children from the TB group in terms of gender and age, according

to the copy-pair method. Inclusion criteria for the control group were as follows: a negative reaction to the Mantoux test with 2 TE; no contacts with TB patients; an informed consent signed by the parent (or legal guardian) for the child to participate in the study. Exclusion criteria were immune-associated diseases and acute respiratory diseases within one month preceding the study.

The ability of isolated neutrophils to form NETs was assessed in the control and TB groups according to the method described in the patent [3]. After we isolated the neutrophil culture using Ficoll-Urografin density gradient centrifugation, the ability of neutrophils to form NETs was evaluated. Since specific sensitivity to antigens is atypical for neutrophils, the technique provoking neutrophils to form NETs was applied. Neutrophils were incubated for 30 minutes at 37 °C in a medium containing a non-specific antigenic stimulant (a mixture of *Lactobacillus reuteri*, *L. acidophilius*, *L. rhamnosus* and *Bifidobacterium longum*). Upon completion of the incubation, the number of luminescent-positive objects in the sample was counted using fluorescence microscopy: neutrophils (intact, activated, and hyperactivated), early NETosis cells, and NETs with differentiation of cloud-like and filamentous forms (Fig. 1).

The obtained values were expressed as a percentage of the total number of fluorescent objects (FO) in the sample. In addition, the NET capture coefficient was calculated as the ratio of objects captured by a trap to the total number of NETs. Negative control in studying the ability of a neutrophil to form NETs was carried out by incubating a neutrophil culture with 0.9% NaCl in a volume equal to the volume of the non-specific antigenic stimulant used in the main series of the study. The concentration of citH3 was determined in the peripheral blood serum samples in accordance with the instructions of the manufacturer using the Citrullinated Histone H3 (Clone 11D3) ELISA Kit test system (Cayman Chemical Company, USA).

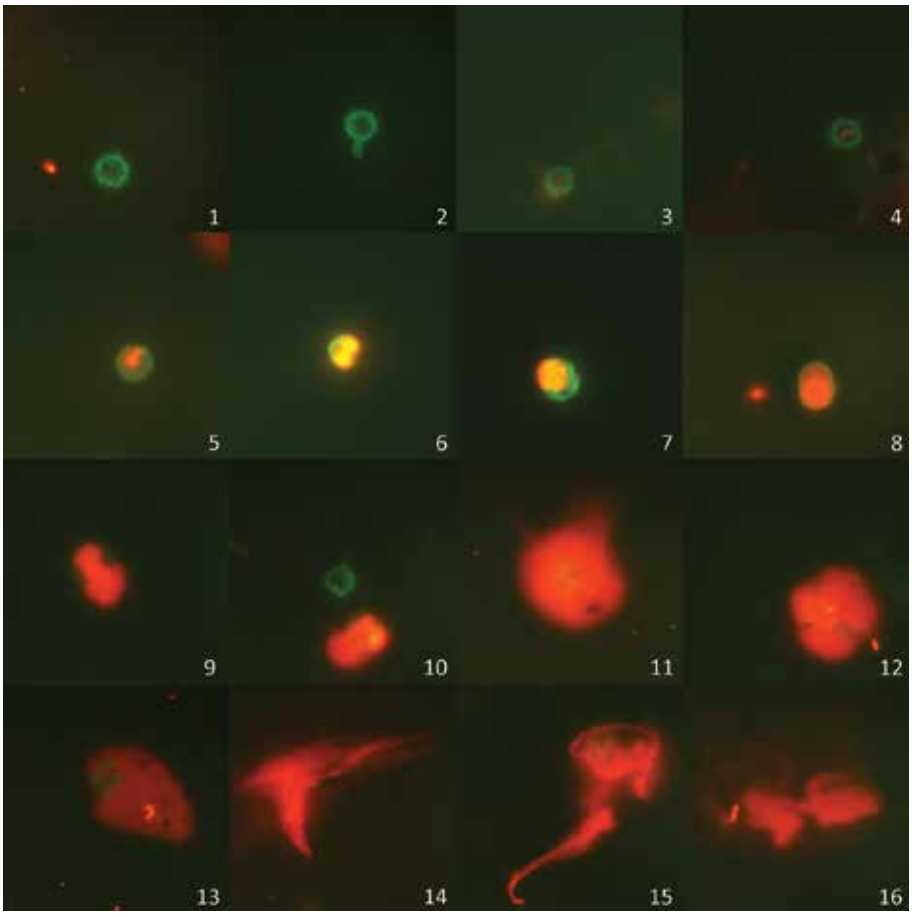

Fig. 1. Micrographs of the objects found in a sample of the isolated fraction of neutrophils: 1, 2 – intact neutrophils; 3, 4 – hypoactivated neutrophils; 5, 6 – activated neutrophils; 7, 8 – hyper-activated neutrophils; 9 – early NETosis cells; 10 – intact neutrophil (top), an early NETosis cell (bottom); 11 – a cloud-like neutrophil extracellular trap; 12, 13 – cloud-like neutrophil extracellular traps with a captured bacterium; 14, 15 – filamentous neutrophil extracellular traps; 16 – a filamentous neutrophil extracellular trap with a captured bacterium. Fluorescence microscopy, ×1,000.

The statistical analysis of the obtained data was carried out using the Statistica 8.0 software. The data distribution in the groups was assessed using the Shapiro – Wilk test. Since the data obtained had a non-normal distribution, the results were represented as the median and the interquartile range (Me (Q_1; Q_3)). The Mann – Whitney U-test was used to calculate the statistical significance of differences for independent samples. The Spearman's rank correlation coefficient was used to measure the association between two variables *ex vivo*, namely neutrophil ability to form NETs and citH3 concentration in the peripheral blood. Pearson's chi-squared test was applied to compare the forms of distribution for individual features of fluorescence microscopy images in the groups. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Neutrophils isolated from the peripheral blood in the TB group were more likely to form NETs during incubation in the presence of th antigenic stimulant (compared to the control group). The number of NETs from all FOs was 31.5 (26.4; 9.6) and 21.1 (19.3; 23.8) in the TB group and the control group, respectively, $p = 0.0087$ (Fig. 2). Neutrophils in the TB group formed predominantly filamentous NETs: 21.0 (19.2; 28.6) compared to the control group 16.0 (15.8; 19.2), $p = 0.0474$ (Fig. 3). Cloud-like NETs (% of all FOs) were detected less often in neutrophil samples in the control group than in the TB group: 4.0 (3.5; 6.2) vs. 10.5 (6.9; 12.3), respectively, $p = 0.0060$.

However, they were not found in neutrophil samples in 5 children of the control group, whereas cloud-like NETs were identified in all patients in the TB group ($\chi^2 = 16.01$; $p < 0.0068$). The NET capture rate (Fig. 4) in the control group was comparable with that in the TB group ($p > 0.05$). No NETs were detected in the negative control.

The median citH3 concentration in the peripheral blood (Fig. 4) was 18.9 times higher in the TB group than in the controls ($p = 0.0041$, the Mann – Whitney U-test). Median values were 1.4 pg / ml (0.9; 1.8) and 26.5 pg / ml (2.7; 33.6) in the control group and in the TB group, respectively.

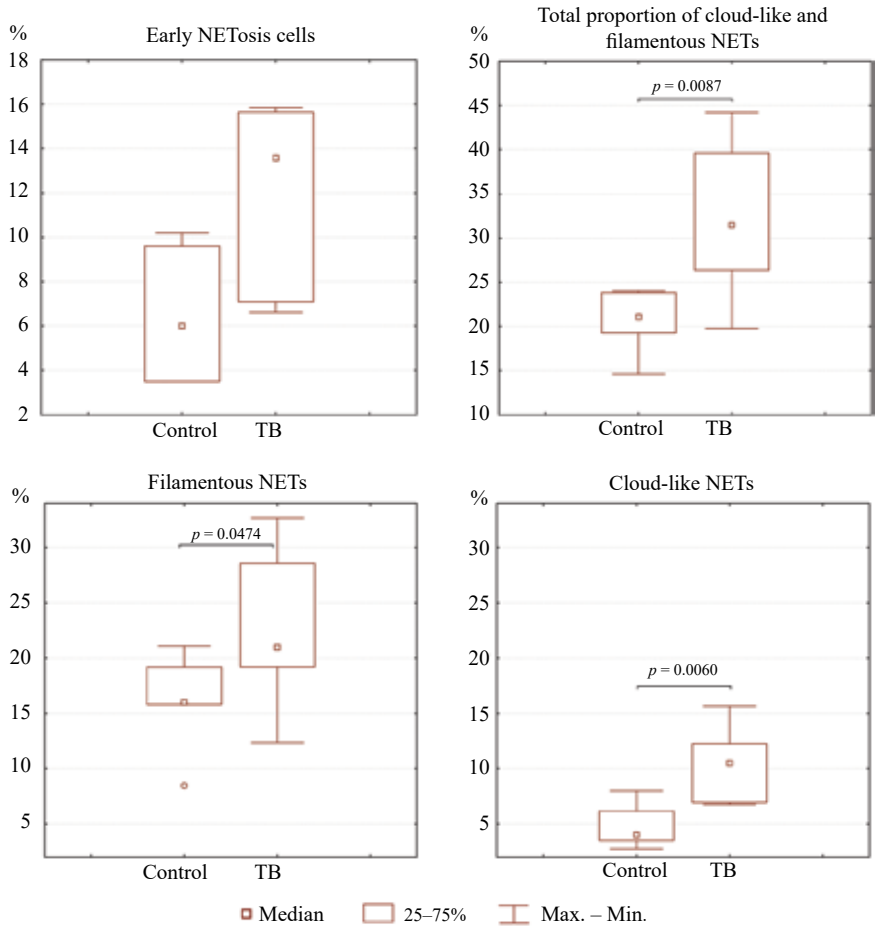

Fig. 2. Fluorescent objects in a sample of isolated neutrophils after stimulation: early NETosis cells, neutrophil extracellular traps

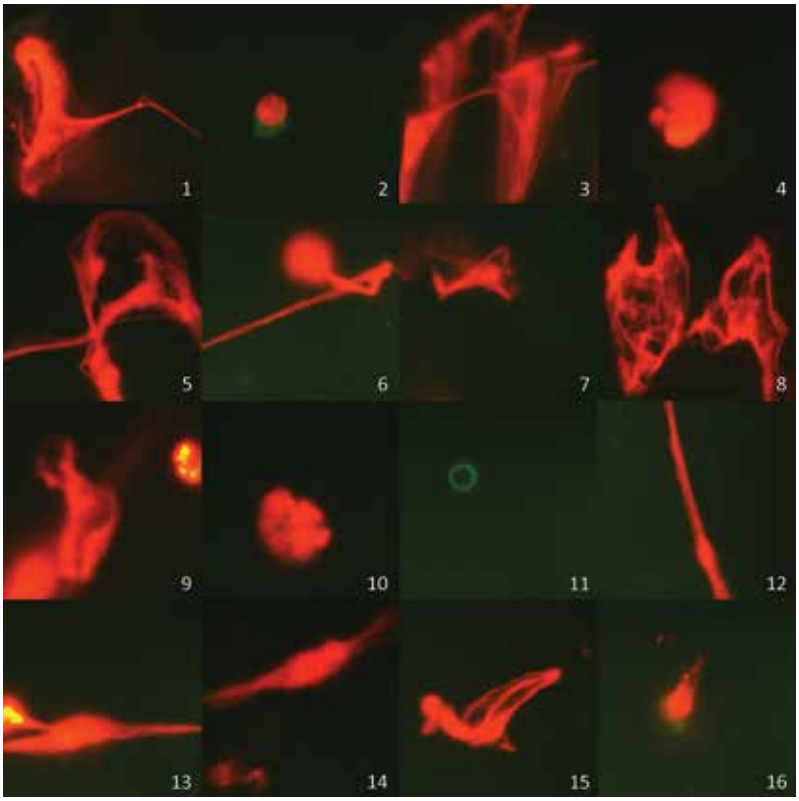

Fig. 3. Examples of fields of view in the sample of isolated neutrophils in children with tuberculosis after exposure to a non-specific antigenic stimulant: filamentous NETs (1, 3, 5–9, 12–15), cloud-like NETs (4, 10), early NETosis cell (16), hyperactivated neutrophil (2), intact neutrophil (11). Fluorescence microscopy, × 1,000

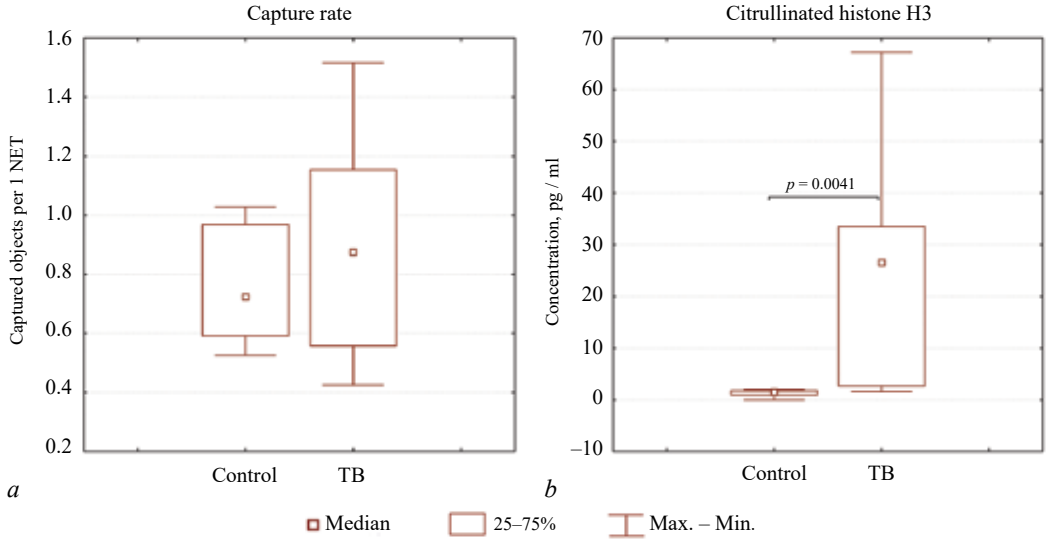

Fig. 4. Capture rate of neutrophil extracellular traps (*a*); citrullinated histone H3 concentration in the peripheral blood (*b*)

After incubation of neutrophils in the presence of the non-specific antigenic stimulant, an expected decrease in the number of intact neutrophils was noted in the sample as a likely consequence of their activation and NETosis. The number of intact neutrophils from the total FO number in the samples of the TB group was 34.6 (26.7; 40.7), while in the control group, this value was significantly higher, 54.0 (46.3; 61.4), p = 0.0040 (the Mann – Whitney U test). Moreover, in the TB group the percentage of hypoactivated neutrophils was slightly smaller than in the control group: 2.6 (1.2; 4.9) vs. 6.0 (4.8; 6.9); p = 0.0295. There were no statistically significant differences between the study groups in terms of the number of activated and hyperactivated neutrophils ($p > 0.05$).

The study of correlations in the control group revealed two strong correlations between the citH3 concentration in the blood and the proportion of

filamentous NETs ($r = 0.95$; $p = 0.0008$) and between citH3 in the blood and the proportion of early NETosis cells ($r = 0.77$; $p = 0.0408$). The study of correlations in the TB group revealed three strong correlations between the citH3 concentration in the blood and the proportion (in %) of filamentous NETs ($r = 0.86$; $p = 0.0137$) and between citH3 in the blood and the proportion of all NETs ($r = 0.89$; $p = 0.0068$). The correlation between citH3 in the blood and the percentage of hyperactivated neutrophils was negative ($r = –0.89$; $p = 0.0068$). No other statistically significant correlations were found between the citH3 concentration in the peripheral blood and the proportion of individual FOs.

DISCUSSION

The diversity of approaches and the lack of a laboratory mainstay in studying NETs lead to ambiguity in the interpretation of research findings. Prominent researchers in this field have failed to reach a consensus on many issues related to NET morphology, mechanisms, the sources of their formation, as well as the regulation of this process [18]. In most publications, the traps were represented by filamentous structures in micrographs obtained using fluorescent dyes to visualize NETs or their components [19]. However, some authors have pointed out that NETs can also take a cloud-like form [11, 18].

Unlike most researchers, Shida Yousefi et al. assume that the appearance of cloud-like structures, consisting predominantly of extracellular DNA, after exposure of neutrophils to certain agents is not the result of NETosis but is due to other forms of cell death associated with cytolysis. Thus, the authors do not classify cloud-like structures as true NETs, whereas they consider filamentous structures as NETs originating from mitochondrial DNA [20, 21]. These authors suppose that the formation of filamentous structures is a normal innate immune response, while the appearance of cloud-like structures is associated with the formation of the so-called vicious circle of inflammation followed by the development of autoimmunity [20, 21]. Regardless of the formation ways and mechanisms of supramolecular cloud-like structures, including nucleoproteins, the opinion expressed by Shida Yousefi et al. is consistent with the results obtained in this study.

A significantly larger number of cloud-like NETs were found in patients with TB compared to the control group, where such NETs were rare. Filamentous NETs were also detected in fairly large numbers in healthy children. Presumably, it is the presence of cloud-like NETs that is associated with the development of the autoimmune reaction observed in TB. Thus, in a number of studies, autoantibodies to various autoantigens were found in patients with TB [22].

The circulation of damage-associated molecular patterns (DAMPs) in the blood is one of the reasons for this response [22]. DAMPs are a rather heterogeneous group of molecules, including histones [23], which also have direct cytotoxicity [24]. Previously, while examining adult patients with TB, it was revealed that a higher level of citH3, whose formation is closely related to NETosis [4, 13, 14], is associated with an increased content of neutrophils in the blood, the presence of cavities in the lungs, and low efficiency of anti-TB treatment [8]. This is consistent with our results that demonstrated a significantly higher level of citH3 in the blood serum of children with TB compared to healthy children. In our previous study, citH3 level in children with TB was significantly higher than in adults with cavities, which was detected using identical test systems in both studies [8]. Probably, at the initial stages of TB, NETosis may play a more crucial role in the development of autoinflammation.

Moreover, in this study, the citH3 level in the blood serum was associated with the formation of filamentous NETs rather than cloud-like ones by peripheral blood leukocytes. At the same time, NETs formed in the site of inflammation may be a source of citH3 in the blood serum. The assessed ability of neutrophils to form NETs describes the pro-NETotic phenotype of neutrophils, which are more likely to form NETs in chronic inflammation [20]. The above correlation can probably be explained by comparing our results with the study by Florence Guillotin et al. [19].

These authors declare the presence of filamentous structures that contain DNA and are recognized by antibodies to myeloperoxidase and citH3 as morphologically similar to vital NETosis observed in fluorescence microscopy [19]. This approach is slightly contradictory because it is based on a set of hypotheses, and it is consistent with the opinion of Shida Yousefi et al. suggesting that the source of filamentous structures is mitochondrial DNA [20, 21]. However, it should be taken into account that mitochondrial DNA does not contain histones [25]. The equivalents of other NETosis types referred to in the article as non-vital NETosis are filamentous structures not containing citH3 [19]. The design of this study did not include the registration of cloud-like

structures. Apparently, suicidal and other NETosis types were simultaneously observed with the vital NETosis. At the same time, in the mentioned study, non-vital NETosis prevailed in the pathology group (pre-eclampsia) in comparison with the control group, and NETosis combined with histone modification prevailed in both groups [19].

In this regard, it is yet to be investigated whether cloud-like structures containing DNA that we observed are a type of suicidal NETosis or another type of cell death. However, they may reflect the process that does not require the regulation of chromatin decondensation mediated by enzymatic modification of histones. The emergence of this phenomenon may reflect the development of an autoimmune component of inflammation. At the same time, an increase in the concentration of citH3 in children with TB can demonstrate the efficacy of the innate immune response and be a normal compensatory adaptation process.

CONCLUSION

A higher level of citH3 was observed in the blood of children with TB compared to healthy controls. In the *ex vivo* study, the relative amount of filamentous and cloud-like NETs was greater in the neutrophil culture exposed to a non-specific antigenic stimulant in children with TB than in a similar experiment with neutrophils of healthy children. It was revealed that the number of filamentous NETs formed *ex vivo* in both groups was positively related to the citH3 level in the blood. However, a similar correlation was not registered between the number of cloud-like NETs and the level of citH3 in the blood. It is likely that the higher level of citH3, an enzymatic modification product, in the blood of children with TB reflects higher activity of NETosis *in vivo*, which may be due to an increased ability of neutrophils to form NETs.

The impact of numerous factors of the immune response in TB probably determines the formation of a unique pro-NETotic neutrophil phenotype. Given the non-specific nature of neutrophils, it can be assumed that in the situation of increased readiness for NETosis, the range of antigenic structures capable of triggering and stimulating NETosis may significantly expand. In particular, the antigens of normal human microbiota, including the antigens of bacterial gut symbionts, can also stimulate NETosis, which is probably indicated by the results of our study.

The data obtained in our study have shown that *in vivo* and *ex vivo* methodological approaches applied for NETosis investigation do not provide equal information content and can complement each other since they describe different aspects of the NETosis phenomena under study. In children with TB, the formation of cloud-like forms of NETs in the culture of peripheral blood neutrophils in response to the stimulation turned out to be notably more common. Researchers consider such NET forms abnormal [21]. Inefficacy of the NETosis reaction in the long-term course of the disease can significantly affect the development of predisposition to autoinflammation and immunopathology. Studying these issues may become the subject of a separate line of research.

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Novikov D.G., Zolotov A.N. – conception and design. Ptukhin A.O., Romanova M.A. – selection of the clinical base for analysis, clinical examination of patients. Romanova M.A. – interpretation of the spiral computed tomography results. Zolotov A.N. – conducting fluorescence microscopy. Kirichenko N.A. – pre-analytical stage of the study, conducting fluorescence microscopy. Novikov D.G. – conducting ELISA blood serum research. Novikov D.G., Zolotov A.N. – analysis and interpretation of the data. Indutny A.V. – justification of the manuscript, critical revision of the manuscript for important intellectual content. Mordyk A.V. – final approval of the manuscript for publication.

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